THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

DOMINANT NEGATIVE SUPPRESSION OF KV12 CHANNELS IN TRANSGENIC MICE

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SPRING 2013

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biology
with honors in Biology

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ABSTRACT

Neuronal hyperexcitability is a characteristic feature of many nervous system diseases, such as epilepsy. It is therefore critical to understand how excitability is regulated in the brain. Subthreshold voltage-gated K^+ channels (such as EAG family channels) provide a critical buffer against hyperexcitability, and knockout of these channels in the forebrain of mice leads to epilepsy. A dominant negative (DN) transgenic strategy was designed to inducibly knockout Kv12 potassium channels (which are known to limit excitability of pyramidal neurons) in individual neurons to understand how loss of these channels leads to hyperexcitability and epileptogenesis. Additionally, the transgenic mice will be used to test the role of Kv12 channels in learning and memory. Transgenic mice were created to couple DN suppression of Kv12 channels with a switch in fluorescent protein marker expression from green to red. The ability to compare the function of hyperexcitable (red) neurons to normal (green) neurons in the same animal will help us to better understand how epileptogenesis occurs.

The transgene has been successfully inserted into mice, and we have verified that DN expression correlates with a green to red color change in forebrain neurons upon induction of Cre-recombinase. These mice should allow for direct electrophysiological comparison of the contributions of neighboring wild type and hyperexcitable (DN) neurons to circuit function in living brain slices. Future experiments included a detailed characterization of the expression pattern and behavioral analysis of the mice in hope of answering the questions: Can one titrate induction? And if so what degree or pattern of DN expression leads to epileptogenesis?

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This undergraduate honors thesis was completed in Dr. Timothy Jegla's research lab. I would like to thank Dr. Jegla for his guidance and direction over the past three years. I would also like to thank Alham Saddat, Brooke Kanaskie, Xiaofan Li, and Hansai Liu for their various and valuable contributions to this project. Finally, I would like to thank my thesis reader Dr. Gong Chen.

Chapter 1: Introduction

Epilepsy is characterized by widespread hyperexcitability across large regions of the brain and many epilepsy mutations increase neuronal excitability[3]. However, it is not yet understood how hyperexcitability in individual neurons leads to epileptiform activity. Genetic studies of ion channels have yielded important clues, including the observation that subthreshold voltage-gated K^+ channels provide a critical buffer against hyperexcitability[4, 5]. For instance, knockout of subthreshold K^+ channels Kv7.2 and Kv12.2 in the forebrain of mice leads to epilepsy[6-9]. Standard gene knockout techniques are limiting, however, because all neurons have the same genotype[10]. This limitation makes it impossible to study the effects of individual hyperexcitable neurons on circuit function and prevents us from asking a key question: How many hyperexcitable neurons are required to cause disease? Answering this question is critical to understanding how diseases such as epilepsy arise. To do so, we must be able to manipulate genes in individual neurons and identify those live neurons with a fluorescent protein marker to allow for study[11].

The long term goal of this project is to investigate how hyperexcitablity is controlled in various neural circuits and how many hyperexcitable neurons are required to cause diseases, namely epilepsy. Epilepsy involves abnormal excitability in the nervous system, which exemplifies the importance of understating how excitability is controlled in neural circuits[12]. This research primarily concerns the knockout of the subthreshold K^+ channel gene family Kv12. We propose an inducible DN transgenic strategy to allow us to compare the function of wild type (Green) and hyperexcitable (Kv12 DN, Red) live

neurons in the same brain slice. We will be able to control the time and extent of DN expression, which will potentially allow us to determine the degree of hyperexcitability that leads to changes in seizure threshold and epileptogenesis. We chose to create a dominant negative transgenic strategy over a standard gene knockout because we wanted to add distinct fluorescence markers to label neurons before and after channel manipulation. This is potentially difficult to incorporate into the native locus without disrupting expression. In order to ensure native channel function prior to induction, we used a transgenic strategy to inducibly express a dominant negative subunit from a different locus. Through this research we hope to understand how different neurons influence circuit function, and determine the number and distribution of hyperexcitable neurons that lead to seizure threshold changes in mice. My role in this long-term project is to make and validate the Kv12 DN transgenic mouse model that will be used in future experiments for behavioral analysis and detailed characterization of the expression pattern in the forebrain of mice.

Epilepsy

The nervous system disease of interest in this research project is epilepsy. Epilepsy causes patients to suffer from uncontrollable seizures[3]. Seizures are a result of a sudden synchronous impulse of electrical activity in the brain. Patients who suffer from repeated seizure episodes (at least two) are diagnosed with epilepsy[13]. The spike waves that cause seizures can affect one's behavior, consciousness, breathing, and muscle tone[14]. Epilepsy affects approximately 20 to 40 million individuals worldwide[15].

The sudden spike in electrical activity is caused by disruption in the electrical communication between neurons allowing them to incessantly fire. A person's electrical activity can be analyzed via an electroencephalogram (EEG) and is a tool used to help diagnose epilepsy[16]. EEGs measure voltage changes induced by neuronal activity[16]. Epileptic patients have abnormal electrical neuronal firing resulting in ictal events (synchronized spiking)[3, 17]. Brain scans of normal humans indicate desynchronized firing resulting in the presence of a comparative "flat line," (no high amplitude spikes on an EEG) while seizures are characterized by high amplitude spikes[18].

There are two major divisions of seizures experienced by epilepsy patients. The most frequent type is a partial seizure[19]. Partial seizures are typically isolated to a specific region in one cerebral hemisphere, rarely lasting longer than two minutes[18]. The other type of seizure is a primary generalized seizure which involves both hemispheres of the brain and results in a loss of cognizance from a few seconds to several minutes[18].

The causes of epilepsy are grouped into two categories, idiopathic/cryptogenic and remote symptomatic[15, 20]. Researchers estimate idiopathic/cryptogenic causes account for nearly seventy percent of all epileptic cases[21]. Approximately fifty percent of genetically, idiopathic inherited epileptic cases are due to a mutation in ion channels that directly influence neuronal excitability[4]. Remote symptomatic cases can result from head trauma, fevers exceeding 105° Fahrenheit, and recreational drug use[21-24].

Epilepsy is the result an alteration in the firing pattern of neurons[3]. Most neurons have a resting membrane potential of -70 mV (hyperpolarized state) due to the unequal distribution of ions on either side of the membrane[25]. When the summation of electrical inputs from the dendrites cause enough voltage gated sodium channels to open and

depolarize the membrane past the threshold level, normally -55 to -30 mV, an action potential is produced[25]. Subthreshold potassium channels are activated below this threshold and therefore control the excitability of neurons[25]. By activating at hyperpolarized levels below threshold, subthreshold potassium channels control the summation of input, altering neuronal firing pattern. Epileptic patients have been shown to have mutations in subthreshold KCNQ2 or KCNQ3 (Kv7.2 channels)[26-30].

Over the years, researchers have shown that mice are good models for human epilepsy diseases[7]. Presently, every anticonvulsant drug used to treat epilepsy was first analyzed and investigated using rodent models. Human epilepsy mutations have been phenocopied to create mouse models. Dominant negative suppression of Kv7 channels in mice has resulted in a similar epileptic phenotype as in humans [7, 26-28, 30, 31]. Similarly, knockout of subthreshold Kv12.2 channels in the forebrain of mice results in hyperexcitable neuronal firing in the hippocampus, leading to the development of epilepsy[6].

Understanding how excitability is regulated in the brain is of vital importance[4]. Through our research, we hope to better understand the mechanisms behind epilepsy to allow for future development of pharmaceutical drugs. Current drugs target ion channels, but subthreshold potassium channels are not yet exploited as a target class. We want to learn how loss of Kv12 channels leads to epileptogenesis to see if gain of function could serve as new drug targets. Knockout data would be used to validate the channel is involved in the epilepsy pathway, but since Kv12 channels are activated at subthreshold levels, creating a buffer against hyperexcitability, drugs would need to be developed to increase the normal activity of these channels to help prevent seizures. There are many

patients that still are not treated with the current antiepileptic drugs[3, 32-34]. Therefore, there is great interest in the development of new drug targets.

Kv12 Channels

Voltage gated potassium channels have two major physiological functions. They control subthreshold excitability (altering spike probability and frequency) and are responsible for action potential repolarization. Potassium ions flow out of the cell due to the concentration gradient (higher concentration of potassium ions inside as compared to outside the cell)[25]. At typical physiological voltages, the flow of positive potassium ions out of the cell leads to hyperpolarization[25]. Therefore, potassium channels are used to counteract excitatory signals and repolarize action potentials[25, 35].

We are primarily concerned with the role Kv channels play at subthreshold levels. Kv12 channels dampen the excitability of neurons by activating at subthreshold levels, and therefore provide a critical buffer against hyperexcitability[2]. Since positive potassium ions flow out of the cell, in order produce an action potential, there has to be a net inward flow of ions to produce a depolarization current to offset the flow of potassium charge plus enough to reach threshold levels[25]. Subthreshold Kv channels produce a hyperpolarized current making it harder for depolarization of neurons to threshold levels[25].

Since epilepsy is characterized by widespread hyperexcitability across large regions of the brain[3] and many epilepsy mutations increase neuronal excitability, we proposed to investigate if loss of Kv12 channels in pyramidal neurons contributes to hyperexcitbality in epilepsy. A similar channel, Kv7.2 causes epileptic phenotype upon

dominant negative suppression[31]. Additionally, constitutive knockout of Kv12.2 channels also lead to epilepsy; however, these mice theoretically could have exhibited developmental defects upon knockout of Kv12.2 channels[6]. Seizure phenotypes are more severe in Kv7 DN mice if current suppression occurs during development[31]. One reason we propose to use an inducible dominant negative strategy is to investigate if knockout of Kv12 channels in adult brain of mice will produce the same epileptic phenotype. Through our strategy we can determine if Kv12 channels contribute to managing seizure thresholds or if the developmental defects in the constitutive knockout predisposed the mice to epilepsy. We want to figure out if other subthreshold targets similar to Kv7.2 are related to epilepsy and if so are they good targets? The first step is to better understand how Kv12.2 channels control the excitability of neurons, and how loss of Kv12.2 leads to seizures.

Kv12 channels are a part of the ether-a-go-go (EAG) superfamily channels (Kv10, 11, and 12)[12]. The EAG family comprises of three subfamilies, Eag, Erg, and Elk. The Elk family includes the Kv12 channels[12]. Kv12 channels have been genetically conserved since the metazoan lineage, indicating its relative biological importance in controlling intrinsic excitability of neurons[6]. Primary expression of Kv12 neurons is in the cortex and hippocampus regions of the brain[1, 36] (Figure 1).

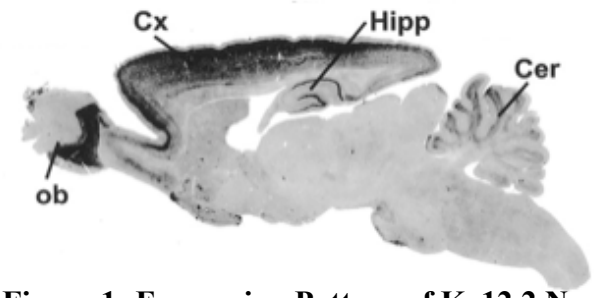


Figure 1: Expression Pattern of Kv12.2 Neurons in Mice[1]

Kv12.2 neurons are heavily expressed in the cortex and hippocampus where knockout causes hyperexcitabilty and epilepsy. To enable experiments, we are looking for a transgenic that overlaps DN expression with this pattern[1].

Voltage gated potassium channels are tetrameric. Each subunit consists of six membrane spanning alpha helices (S1-S6) which form a selectivity pore and four independent transmembrane voltage sensors[25, 37] (Figure 2). Similar EAG subfamily potassium channels can form heteromeric channels. The pore-forming subunits of Kv12.1 (KCNH8) and 12.2 (KCNH3) can form functional heteromeric channels[2]. This allows for a dominant negative strategy to suppress passage of potassium ions by disrupting one of the pore forming subunits of the channel (Figure 2 and 3).

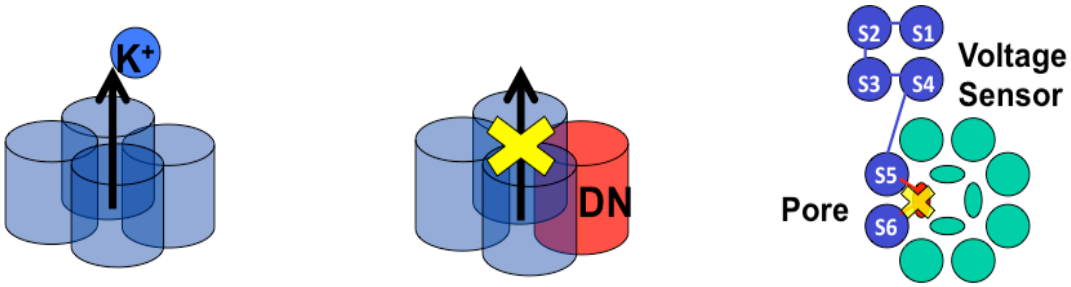


Figure 2: Dominant Negative Disruption of Kv12 Selectivity Filter

For these experiments, we are overexpressing a modified Kv12 subunit that has a disrupted selectivity filter, but can still coassemble with native channel subunits. Potassium channels function as tetramers which allows for the possibility of DN suppression[2]. Channels containing the DN subunit cannot conduct potassium and are therefore nonfunctional. The DN subunit specifically assembles only with other Kv12 family channels[2].

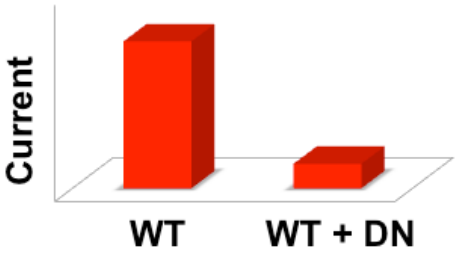


Figure 3: Schematic Depiction of the Change in Neuronal Kv12 Current

Upon expression of the DN subunit, neuronal Kv12 current will be significantly damped due to the prevention of the passage of potassium through the channel.

Thy-1 Dominant Negative Transgenic Construct

A novel Thy-1 dominant negative transgenic construct was designed to couple an inducible knockout of voltage gated Kv12 channels to changes in fluorescent protein color in the forebrain of mice (Figure 4). The construct is 14.628 kilobase pairs in size (See Appendix A).

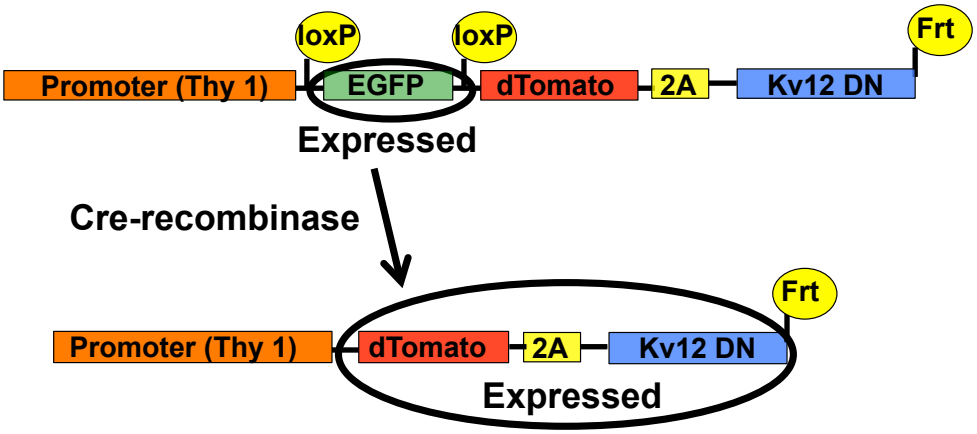


Figure 4: Induction of Cre recombinase Expression

The transgene is designed to express EGFP which will allow us to tell where the transgene is active. In this state, the red florescent protein (dTomato) and the Kv12 DN subunit are not active due to a polyadenylation sequence after EGFP. Upon expression of Cre recombinase, the EGFP is spliced out and both dTomato and the DN subunit are now expressed. The neurons will switch from green to red fluorescence. DTomato and the DN subunit are encoded on the same polypeptide, but are separated by a self-cleaving 2A peptide sequence, resulting in production of separate proteins.

We are using a Thy-1 promoter to drive expression because it provides a high level of expression in most types of neurons[38]. Thy-1 is the most abundant neuronal glycoprotein in mammals, and is bonded to the plasma membrane by a glycosyl-phosphatidylinositol[39, 40]. Thy-1 is specifically expressed in the areas of interest for this project: cortical and hippocampal pyramidal neurons that express Kv12 channels [44, 45].

The transgene is designed to express green florescent protein (EGFP) in order to visualize where the transgene is active. In this state, the red florescent protein (dTomato) and the Kv12 DN subunit are not expressed, so a wild type electrical phenotype is expected (Figure 4).

In the Thy-1 vector, upstream and downstream of the EGFP portion are loxP sites which are recognized by Cre recombinase. Cre recombinase can be used to knockout a gene in specific cell or label specific cells with a fluorescent protein marker[11, 41, 42]. A novel approach combining these techniques could allow functional characterization of genetically distinct neurons in brain circuits. LoxP sites are marked by two 13 base pair palindromic sequences with a random 8 base pair filler sequence between the repeats[43] (Figure 5). Cre recombinase splices out DNA located between the two loxP sites, leaving one single loxP site after recombination[11, 41, 42].

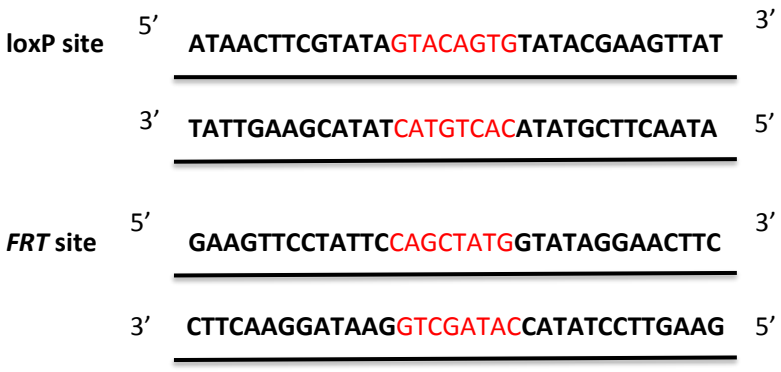


Figure 5: Example of loxP and FRT sites

LoxP and FRT sites consists of two 13 bp palindromic sequences. Indicated in red is a random 8 bp filler sequence between the two repeats. Cre recombinase recognizes the loxP sites, while Flp recombinase recognizes FRT sites, removing any DNA sequences between the two sites.

Upon expression of Cre-recombinase, the EGFP is spliced out and the normally inactive dTomato and Kv12 dominant negative subunit are now expressed. DTomato and the DN subunit are encoded on the same polypeptide, but are separated by a self-cleaving 2A peptide sequence, resulting in production of separate proteins (Figure 4). The transgene is dominant negative because it results in a gene product that can interfere with the function of normal gene products. Now the neurons will fluoresce red indicating the dominant negative subunit is incorporated along with the native subunits of Kv12 channels, preventing the flow of potassium[11, 41-43].

An additional site-specific recombination sequence is downstream of the Kv12 DN portion of the construct, a flippase recognition sequence (FRT) site. Flp recombinase recognizes the FRT sites to prevent multiple copies of the transgene from being inserted into the host's genome. If more than one copy of the Kv12 DN sequence is present, Flp recombinase will splice out the DNA sequence between any two FRT sites. Flp recombinase only works if there are two FRT sites. If there is only one FRT site then Flp recombinase does not splice any portion of the sequence. The mechanism for the Flp recombinase is the same as Cre recombinase although the two 13 base pair sequences that make up the recognition site are slightly altered[11] (Figure 5). In our construct, Cre and Flp recombinase were used for splicing purposes rather than recombination, insertion, or reversal of sequences[11, 43]. The purpose of using Flp recombinase allows us to minimize potential problems in expression of the transgene by reducing it to a single copy (Figure 6). Sometimes large rafts can be recognized as foreign DNA and get silenced. Additionally, there is a possibility we could get mixed expression off of multiple

expressing cassettes if Cre processing is incomplete, making it difficult to identify wild type and DN suppressed neurons.

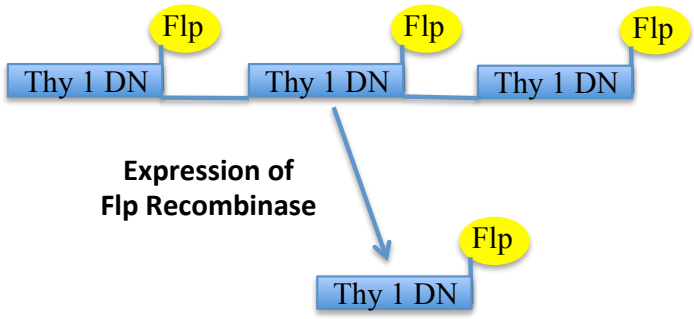


Figure 6: Mechanism of Action for Flp Recombinase

The purpose of using Flp recombinase is to eliminate multiple copies of the transgene from being expressed in the mice. Flp recombinase is designed to recognize FRT sites (a specific two 13 bp palindromic sequence separated by an 8 bp random sequence) and will splice sequences between any two FRT site, preventing multiple copies of the transgene from being expressed.

Chapter 2: Methods

Molecular Cloning

The Thy-1 DN plasmid was created by first setting up a series of polymerase chain reactions (PCR) using oligonucleotides listed in Results (Table 1), PFU as the DNA polymerase, 10x PFU reaction buffer, and 40 mM dNTP mix. The PCR products were purified by gel electrophoresis on a 1% agarose gel and a Qiagen QIAquick Gel Extraction kit was used to isolate the DNA. In order to ligate separate pieces of the plasmid together, the PCR products and the vector had to be digested with specific restriction endonucleases enzymes to produce the same DNA overhangs. For the restriction enzyme digestion reactions we used enzymes from New England BioLabs (NEB), 10X NEBuffer, and Bovine Serum Albumin (BSA). The proper buffer used for each digestion was determined using the double digest finder from NEB. Each digestion was incubated at 37°C for 2-3 hours. After gel purification, each DNA piece was ligated together using T4 ligase and 10X ligase buffer and incubated at room temperature (25°C) for 45 minutes.

The plasmid was transfected into 50 ul chemically competent E. *coli* cells (Invitrogen's One Shot TOP10 cells) and incubated on ice for 30 minutes. Upon a 30 second heat shock (42°C water bath), super optimum broth with catabolite repression (SOC media) was added to the reaction followed by an incubation at 37°C for one hour. The competent cells were plated on NZY agar plates with ampicillin and incubated at 37°C for 18 hours. The agar plates are made with 100X ampicillin; therefore, only bacteria colonies with an ampicillin resistant gene (contained in Brainbow Thy-1 vector

backbone, Figure 9) will be able to grow. The vector backbone has an ampicillin resistance gene. This minimizes the chance of contamination and allows for the selection of only bacteria colonies with the correct sequence[44].

Minipreps (using Qiagen QIAprep Spin MiniPrep kit) were performed in order to isolate DNA from each of the bacterial colonies. To verify the bacterial colonies contained the correct genomic DNA inserts, conformation digestion reactions were set up. Each miniprep was digested with the same enzymes initially used in ligation. Gel electrophoresis was used to identify minipreps with the correct banding pattern. Clones dropping the correct bands upon digestion were sent for Sanger DNA sequence analysis at the Genomics Core Facility (University Park, PA) to confirm proper construct assembly and absence of undesired mutations.

Cleaning of Transgene for Pronuclear Injection

Prior to pronuclear injection, the sequence confirmed Thy-1 DN plasmid miniprep was purified using a Qiagen EndoFree Plasmid Maxi kit. Eluted DNA was dialyzed against microinjection buffer twice with Millipore filter. The microinjection buffer consisted of 10 mM Tris, pH 7.4, 0.25 mM EDTA, 10 mM NaCl in sterile, DNase/RNase free water. The final concentration of the transgene DNA was diluted to 10ng/μL in microinjection buffer[45].

Isolation of DNA from Mouse Tail

In order to genotype transgenic mice, we isolated DNA from tail clippings, approximately 2 millimeters. Mice tail tissue was lysed and DNA was captured using Qiagen's Gentra Puregene Mouse Tail kit. Once the DNA was extracted from the tail clips, genotype PCR reactions were set up to identify transgene and Cre alleles (described in results). The PCR products were ran on a 1% agarose gel to determine if the mice contained the transgene.

Vascular Perfusion of Mice

Prior to removal of mouse brains, the mice had to be perfused with a fixative agent. First the mice were injected with Avertin (2, 2, 2-Tribromoethanol) (30 ml/kg) to fully anesthetize the mice without affecting circulatory functions[46, 47]. A scapula was used to open the chest cavity, mediastinum, and then the pericardial cavity, removing any connective tissue in order to completely expose the heart. A cannula was inserted into the left ventricle and aorta. A phosphate buffered saline (PBS) solution was pumped through the cannula for 3 minutes (10 ml/min) to exchange the mouse's blood. Then the cannula pumped paraformaldehyde, to fixate the brain, for 10 minutes at a rate of 10 ml/min. The mouse was then decapitated and the brain was removed. The brain was stored for 6 hours in 40 ml of paraformaldehyde solution. After 6 hours, the brain was transferred to a 40 ml solution of 20% sucrose in PBS and allowed to incubate until it settled to the bottom. The brain was then transferred to a 40 ml solution of 30% sucrose in PBS until it sank.

Once the brain hardened, we made 50 micron brain slices in regions of interest (hippocampus and cortex) using a microtome. Each brain slice was collected and placed in a 24 well container with PBS.

Antibody Staining

The brain slices were permeabilized with 1% Triton X-100 in PBS for 60 minutes. After 60 minutes, the solution was carefully aspirated and replaced with PBS for two washes each for 5 minutes. Next the specimens were incubated for 60 minutes in a Serum Block (anti-donkey). Following the Serum Block, another five minute PBS wash was performed. The brain slices were incubated overnight at 4°C with the primary antibody (1:1000 anti-GFP and anti-RFP). After incubation, the primary antibody was aspirated and the specimens were washed three times with PBS for 10 minutes each. The PBS was removed and replaced with the secondary antibody (1:500 Alexa Fluor 488 dye conjugated with anti-mouse antibody) for 60 minutes at room temperature. The plate was covered with aluminum foil to prevent light exposure. Three final PBS washes were performed each for 10 minutes. Each slice of brain tissue was mounted using ProLong Gold Antifade Reagent with DAPI onto slides and a transparent nail polish was used a sealer and stored at 4°C until future use.

Isolation of Brain RNA

First the mice were anesthetized via an injection of Avertin (.03 ml/gram). Cervical dislocation sacrifice was performed to remove the brains. The isolated brains

were approximately 50 mgs from the specific regions of interest, hippocampus and cortex, and placed in a 15ml tube and placed on ice. The tissue was frozen in the -80°C freezer for 30 minutes. After 30 minutes, the tissue was removed and transferred to a 15 ml Precision Tissue Grinder in a 15ml tube and ground on ice until the tissue was a thin liquid consistency. One ml of buffer RLT with BME (10 ul/ml) (to kill native RNase) was added to the tube and ground until it was a homogenous mixture. The mixture was transferred to a QIAshredder Mini Spin Column (in the RNeasy Plus Mini Kit) and spun for 2 minutes at 13.2 RPM. The protocol for the RNeasy Plus Mini Kit was followed in order to isolate RNA from the mice brain lysate.

We reverse transcribed the RNA using a reverse transcriptional polymerase chain reaction (RTPCR) to obtain cDNA. 10 ul (out of the 30 ul originally isolated RNA using the RNeasy Plus Mini Kit) was combined with 1ul of dT oligo primers, 1ul of 10 mM dNTP Mix, and 1ul of sterile, nuclease-free, H_2O from the Invitrogen SuperScript III Reverse Transcriptase Kit. The mixture was incubated at 65°C for five minutes in order to remove any RNA secondary structure. After five minutes, the mixture was placed on ice for a minute and centrifuged at low speed for 30 seconds. Next, 4ul of 5X First Strand Buffer, 1ul of 0.1 M DTT, 1ul of RNaseOut (40 U/ul), and 1 ul of SuperScript III RT (200 U/ul) were added to the mixture. The mixture was centrifuged for 30 seconds at low speed followed by an incubation of 60 minutes at 42°C. In order to inactivate the reverse transcription reaction, the mixture was incubated at 70°C for 15 minutes. Following the incubation, the reaction was placed on ice for two minutes and centrifuged for 30 seconds at low speed. 1 ul of RNase H (2U) was added to the mixture and incubated for 20 minutes at 37°C. Lastly, the reaction was centrifuged for 30 seconds at low speed. 1 ul of

the isolate RNA was used in each subsequent PCR to detect for the presence of the Kv12.1 DN subunit.

Chapter 3: Results

Construction of Transgene

The Thy-1 dominant negative construct was made by inserting mEGFP, dTomato, and mouse Kv12.1 portions into Brainbow Thy-1 1.1 vector[11] (Figure 7). Brainbow Thy-1 1.1 vector was chosen as the backbone for the construct because it is a proven vector that will allow us to incorporate our insert[11].

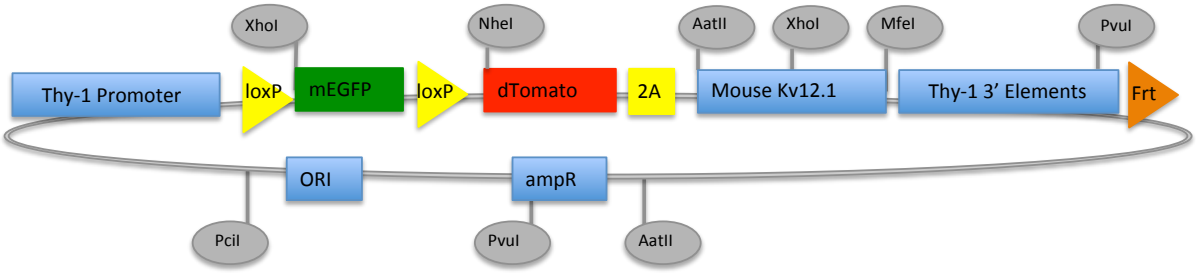


Figure 7: Thy-1 Dominant Negative Transgenic Construct Design with Cloning Enzymes

Brainbow Thy-1 1.1 vector was the backbone for the construct. The construct was created by PCR amplification of EGFP, dTomato, and mouse Kv12.1 DN subunit. Thy-1 is expressed widely in neurons and will drive expression of EGFP. Downstream of EGFP is a polyadenylation sequence, which will prevent expression of downstream elements. The upstream loxP site was in the Brainbow Thy-1 1.1 vector and we designed a second loxP site after the EGFP using specific primers (Table 1). This will allow for conditional knockout. Upon expression with Cre recombinase, the DNA sequence between the loxP sites is spliced out, leaving behind one loxP site. DTomato and mouse Kv12.1 will now be expressed. DTomato and the mouse Kv12.1 DN subunit are encoded on the same transcript, but the polypeptides are separated by a self-cleaving 2A peptide sequence, resulting in the production of separate proteins. Additionally, the Brainbow Thy-1 1.1 vector has an Frt site. If multiple copies of the transgene are inserted into the host's genome, Flp recombinase recognizes and splices between FRT sites. This allows us to make sure we are only working with a single copy of the transgene.

Membrane bound Green Fluorescent Protein (mEGFP) is a gene containing a sequence coding for a green fluorescent protein followed by a polyadenylation sequence to prevent the expression of downstream elements[11]. It is used in molecular biology to tag

a specific cell to know which are expressing the specific transgene. The purpose of using mEGFP in our construct is because neurons are complex and we want to outline them. The Thy-1 promoter will drive expression of mEGFP, and therefore if a cell expresses the transgene it will fluoresce green[11].

The Thy-1 DN plasmid utilizes an additional fluorescent protein, dTomato. DTomato has the same function as mEGFP, but fluoresces red. GFP and dTomato can be viewed using standard fluorescent imaging techniques.

A portion of the construct contains mouse Kv12.1 gene. This gene encodes a dominant negative subunit for the knockdown of Kv12.1 channels in mice. The DN subunit is a modified Kv12 subunit in neurons that has a disrupted potassium selectivity filter; therefore, channels containing the subunit cannot conduct potassium and are nonfunctional[1, 2]. The DN subunit specifically assembles with other Kv12 family channels and heteromeric channels are nonfunctional[1, 2]. *In vivo* expression will therefore reduce or eliminate native Kv12 currents.

The first step of the project involved PCR amplification of band 1A (EGFP cassette), band 1B (Tomato cassette), and band 1C (mKv12.1 DN) (Figure 8). The template DNA used for the amplification of band 1A (EGFP cassette) was pCDNA 3.1 HG plasmid, a homemade lab plasmid with EGFP and a polyadenylation signal. The template DNA used for amplification of band 1B (Tom cassette) was Brainbow 1.1[11] and the template DNA used for the amplification of band 1C (mKv12.1 DN) was mKv12.1 DN lentivirus construct existing in the lab. We designed specific primers to amplify each band (Table 1). For band 1A, the primers for the amplification for the sense and antisense strand were DN TG EGFP met and DN TG EGFP Stop, respectively. The sense primer

for band 1B was DN TG Tom Met and the antisense primer was dTomato 2A. DN TG Aat2 Met was used as the sense primer and DN TG DN Stop was used as the antisense primer for the PCR reaction of Band 1C (Table 1).

Table 1: List of Molecular Cloning Primers

DN TG EGFP Met and Stop were the sense and antisense primers used for the amplification of band 1A (EGFP cassette). The primers were designed to include a translation initiation sequence, Xho1 cloning site (highlighted yellow), MfeI cloning site (highlighted red), and NheI cloning site (highlighted green). The amplified EGFP piece off of the pCDNA 3.1 HG plasmid should be approximately 1 kb in size. DN TG Tom Met and dTomato 2A were the sense and antisense primers for the amplification of band 1B (Tomato cassette). The primers were designed to include a translation initiation sequence, NheI cloning site (highlighted green), the second loxP site (underlined), and AatII cloning site (highlighted blue). The amplified piece off of the Brainbow 1.1 vector should be approximately 800 bp in size. DN TG Aat2 Met and DN TG DN Stop were the sense and antisense primers for the amplification of band 1C (mKv12.1 DN). The primers were designed to include a translation initiation sequence, AatII cloning site (highlighted blue), and MfeI cloning site (highlighted red). The amplified piece off of the mKv12.1 DN lentivirus construct should be approximately 3.3 kb in size.

Construct Primers	Sequence	Expected Size
DN TG EGFP Met	AATACTCGAGCCACCATGCTGTGCTGCATCAGAAGA ACTAAACCGGTTGAGAAGAATGAAGAGGCCGATCAG GAGAGATCTATGGTGAGCAAGGGCGAGGA	1 Kilobase Pairs
DN TG EGFP Stop	AGAGCAATTGCAACGCTAGCCTGCTATTGTCTTCCC AATC	
DN TG Tom Met	GTGTGCTAGCATAACTTCGTATAGGATACTTTATAC GAAGTTATGCCACCATGCTGTGCTGCATCAGAAGAA CTAAACCGGTTGAGAAGAATGAAGAGGCCGATCAGG AGATGGTGAGCAAGGGCGAGGA	800 Base Pairs
dTomato 2A	CCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGC CCTCTCCACTGCCCTTGTACAGCTCGTCCA	
DN TG Aat2 Met	CGGTGACGTCGAGGAGAATCCTGGCCCAATGCCGGT TATGAAAGGAT	3.3 Kilobase Pairs
DN TG DN Stop	ACAACAATTGAAGTTCCTATACTTTCTAGAGAATAG GAACTTCGCGCGTGATCTAGAGTCGCGGTGATTATA CGTTTATGGCTTTGC	

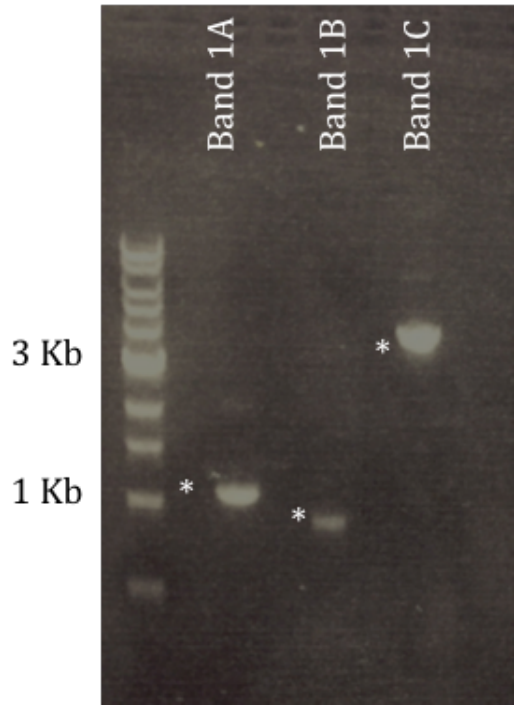


Figure 8: Agarose Gel of Band 1A, 1B, and 1C PCR Product

The PCR products for the amplification of band 1A, 1B, and 1C were ran on a 1% agarose gel. Each of the bands were the expected size: band 1A was 1 kb, band 1B was 800 bp, and band 1C was 3.3 kb. DNA was isolated from each band using a Qiagen QIAquick Gel Extraction kit.

The second step of the construct involved inserting band 1A into the Brainbow Thy-1 1.1 vector. In order to perform this step, both Band 1A and the Brainbow Thy-1 1.1 vector were digested with Xho1 and Mfe1 to produce complimentary DNA overhang sequences (Figure 9: A-B).

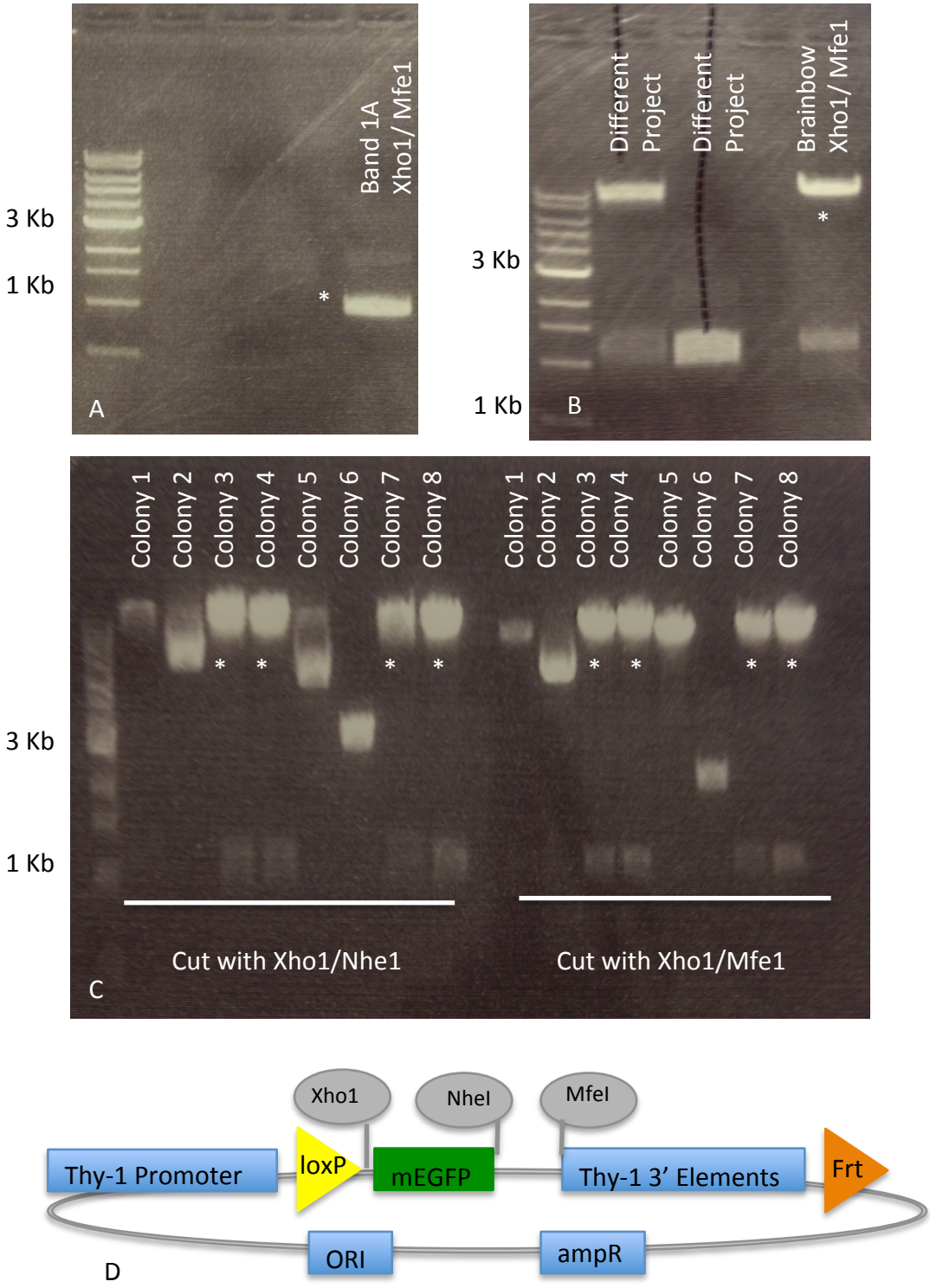

3 Kb
1 Kb
Band 1A
Xho1/ Mfe1
A
3 Kb
1 Kb
Different Project
Different Project
Brainbow
Xho1/ Mfe1
B
Colony 1
Colony 2
Colony 3
Colony 4
Colony 5
Colony 6
Colony 7
Colony 8
3 Kb
1 Kb
Cut with Xho1/Nhe1
Cut with Xho1/Mfe1
C
Xho1
NheI
MfeI
Thy-1 Promoter
loxP
mEGFP
Thy-1 3' Elements
Frt
ORI
ampR
D

Figure 9, A-D: Thy-1 DN Cloning Step 2

A- Band 1A was digested with Xho1 and Mfe1 and ran on a 1% agarose gel. Band 1A digestion yielded a band at the expected band length of 1 kb. The expected band length did not change after digestion because the restriction sites are at the ends of the piece and only altered the band length by a few base pairs.

B- Brainbow Thy-1 vector was digested with Xho1 and Mfe1 and ran on a 1% agarose gel to isolate the digested band of interest (indicated with asterisks). The digested Brainbow Thy-1 1.0L vector produced a band 9.3 kb in size and dropped an expected 1.7 kb piece.

C- To confirm the DNA minipreps had the correct insert (band 1A) in the Brainbow Thy-1 vector, each was digested with the original cloning enzymes Xho1 and Nhe1. Upon digestion, each miniprep should drop a 1kb piece (band 1A) from the Brainbow Thy-1 vector (9.3 kb piece). A secondary confirmation digest was set up with Xho1 and Mfe1. The reason for the Xho1/Mfe1 digestion was to make sure an Mfe1 site is in the construct because an Mfe1 site is necessary for the triple ligation in step 3 of the cloning project. Since the Mfe1 site is at the beginning of the Thy-1 3' element, each digestion was expected to drop a 1kb piece. Minipreps 3,4,7, and 8 each dropped the appropriate band.

D- Schematic of construct after ligation of band 1A into the Brainbow Thy-1 1.1 vector in step 2. Nhe1 and Mfe1 cloning sites will be used in the triple ligation in step 3 to insert dTomato and mKv12.1 DN subunit into the vector.

After the restriction fragments were gel isolated, Band 1A and Brainbow Thy-1 1.0L were ligated together (Figure 9: D). Two confirmation reactions using the enzymes Xho1/Nhe1 and Xho1/Mfe1 were set up to determine if band 1A was successfully inserted into the Brainbow Thy-1 1.0L vector. Upon confirmation digest, minipreps 3, 4, 7, and 8 were sequence analyzed using Thy-1 3' and Thy-1 5' sequence oligonucleotides (Table 2). Miniprep 3 properly contained band 1A inserted into the Brainbow Thy-1 vector with no mutations (Figure 9: D).

Table 2: Sequencing Primers for Cloning Steps 2 and 3

Thy-1 3' and 5' Seq were used to sequence confirm that step 2 clones contiain band 1A in the Thy-1 vector. To confirm the products of the triple ligation in the step 3 properly contain band 1A, 1B, and 1C in the Brainbow Thy-1 vector, mKV12.1 5A Seq, mKV12.1 5s Seq, EGFP 3UTR Seq, mKv12.1 1420A, mKv12.1 1210S, mKv12.1 2300A, and mKv12.1 2150S primers were designed. Each oligonucleotide primed various regions of the Thy-1 construct. Using several oligonucleotides allowed for the entire insert (Band 1A, Band 1B, and Band 1C) to be sequence confirmed, making sure there were no mutations in the construct.

Sequencing Primers	Sequence
Thy-1 3' Seq	CAGAGGAAGGACCTCGAATTAACG
Thy-1 5' Seq	GGAACTCTTGGCACCTAGAGGA
mKV12.1 5A Seq	CCAGGGACTTTTCTATCTGAAGC
mKV12.1 5s Seq	GATTACTGGCGCCACAGAACACCT
EGFP 3UTR Seq	CGCATTGTCTGAGTAGGTGTCATTC
mKv12.1 1420A	CCATCTAGAGTACATTCTCTGTATG
mKv12.1 1210S	GGAATCTCCATATTATGGCAAC
mKv12.1 2300A	GGTGGGTCAGCTTCCTGCTTGGT
mKv12.1 2150S	CACAAGGGGATCCTCTGTCTCACA

The third step in the cloning of the Thy-1 DN construct involved a triple ligation of band 1B (Tomato cassette), Band 1C (mKv12.1 DN), and sequence confirmed miniprep 3 from step two (Figure 10: A). Band 1B was digested with enzymes Nhe1 and Aat2. Band 1C was digested with Aat2 and Mfe1, and miniprep 3 was digested with Nhe1 and Mfe1 (Figure 10: B). Band 1B and Band 1C need to be joined at the Aat2 site but since there is also an Aat2 site in the Thy-1 vector backbone, a triple ligation is necessary. By ligating all three pieces together at once, the two inserts (band 1B and 1C) have to ligate together before inserting into the vector. Band 1B and 1C will ligate with miniprep 3 because both have an Nhe1 and Mfe1 cloning sites (Figure 10: A).

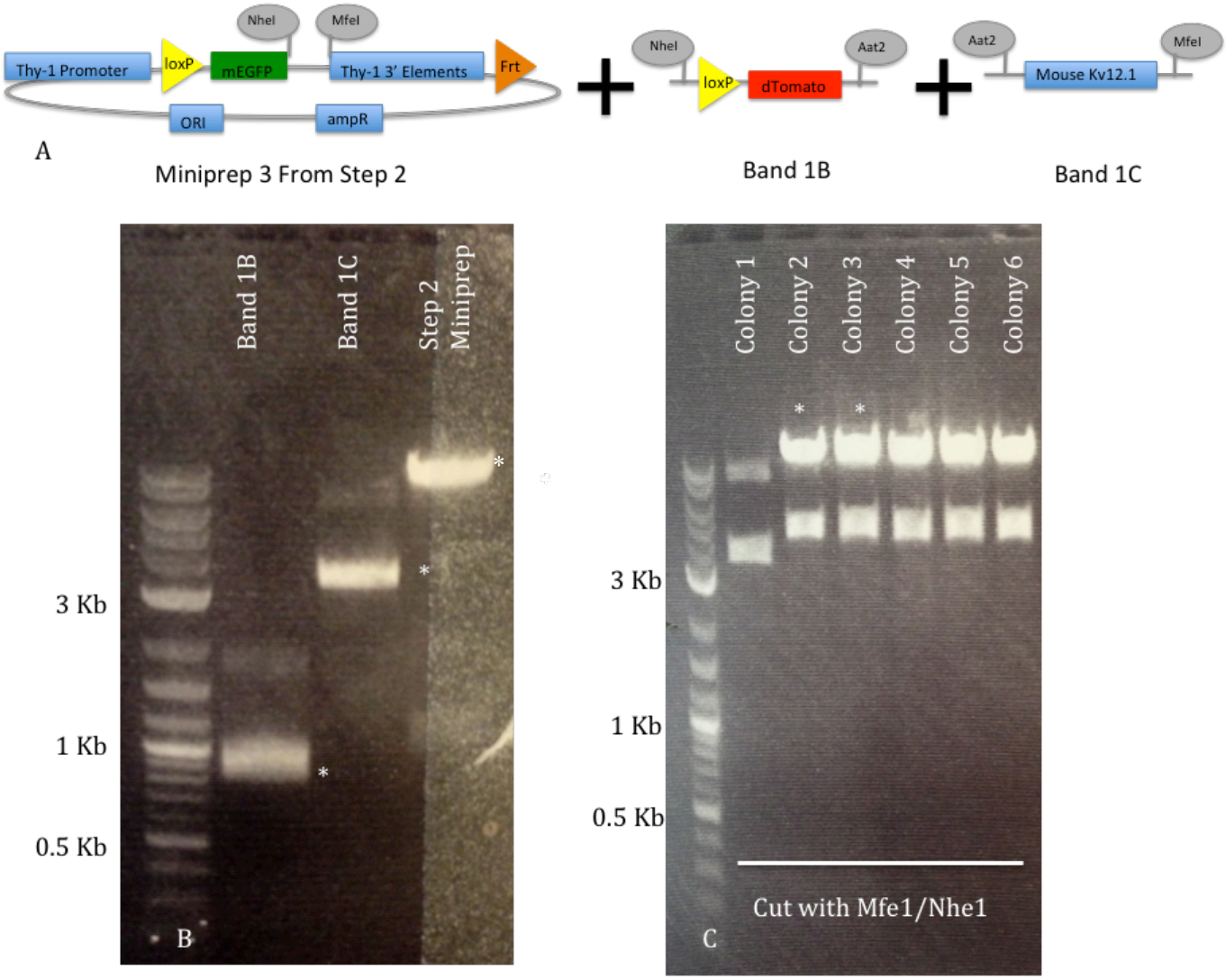


Figure 10, A-C: Thy-1 DN Cloning Step 3

A- Schematic depicting the vector (Miniprep 3 from step 2) and the two inserts (Band 1B and 1C) that will be ligated together in step 3. The enzyme cloning sites are indicated to show how each piece fits together in the triple ligation.

B- Band 1B was digested with Nhe1/Aat2, Band 1C was digested with Aat2/Mfe1, and step 2 miniprep was digested with Nhe1/Mfe1. The digested bands of interest are indicated with asterisks and were gel isolated. The expected sizes of band 1B was 800 bp, band 1C was the expected size of 3.3 kb, and step 2 miniprep was 10.3 kb after digestion. Each piece remained relatively the same size after digestion because the enzyme cloning sites are at the ends of each piece.

C- A confirmation digest was set up to analyze if the triple ligation worked. Each miniprep was cut with Mfe1/Nhe1. Miniprep 2-6 dropped the expected 4.2 kb piece (Band 1B and 1C) leaving the Brainbow Thy-1 vector with band 1A insert (10.3 Kb piece).

After each piece was digested, a triple ligation was set up using bands 1B and 1C as the inserts and step 2 miniprep as the vector (Figure 10: A). The triple ligation did not work the first time, but the solution was to clone band 1B (Tom cassette) and band 1C (mKv12.1 DN) separately into TOPO vectors (using Invitrogen's TOPO TA Cloning Kit with One Shot TOP10 Competent Cells) to produce more DNA insert than we had originally.

A conformation digestion reaction using Mfe1/Nhe1 was set up to confirm the triple ligation (Figure 10: C). Minipreps 2-6 showed the correct banding pattern and were sent for sequencing analysis using primers: Thy-1 3' seq, EGFP 3UTR seq, mKV12.1 5a SEQ, mKV 12.1 5s SEQ, mKv12.1 1420A, mKv12.1 1210S, mKv2300A, and mKv12.1 2150S (Table 2). Sequencing confirmed that minipreps 2 and 3 properly had band 1A, 1B, and 1C in the Brainbow Thy-1 vector with no mutations.

In order to use the final construct for pronuclear injection, the enzymes PvuI and PciI were used to cut off the plasmid backbone. The ampicillin resistance and origin of replication were removed from the vector (Figure 11).

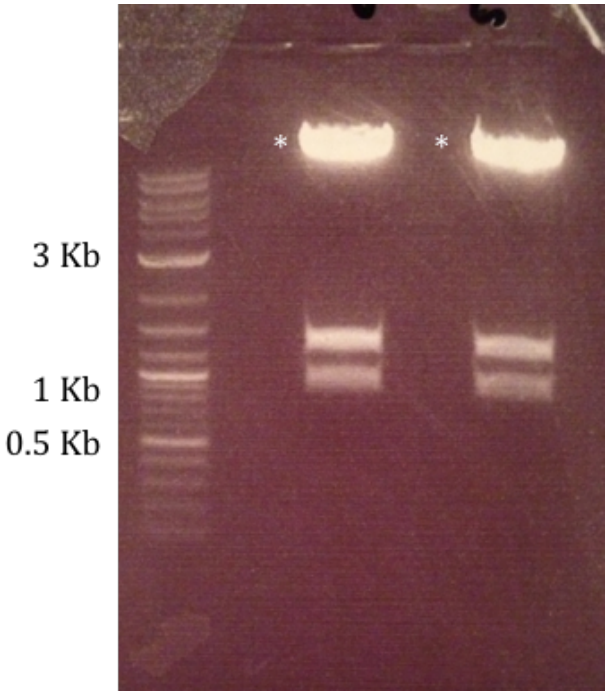


Figure 11: Thy-1 DN Construct Digested with PvuI/PciI

Before the Thy-1 DN construct could be used for pronuclear injection, the origin of replication (ORI) and ampicillin resistance portions of the vector had to be removed using the enzymes PvuI and PciI. The Thy-1 DN construct dropped two bands of the appropriate size (900 and 1300 bp) after digested with PvuI and PciI. To increase the concentration of the Thy-1 DN vector for pronuclear injection, two simultaneous digestions were set up and the bands of interest (labeled with asterisks) were gel isolated and combined. The vector was cleaned using a Qiagen EndoFree Plasmid Maxi kit.

Pronuclear Injection

The Thy-1 DN plasmid was sent to the Pennsylvania State University Transgenic Mouse Facility (B124 Life Science Building University Park, Pennsylvania) for pronuclear injection into mice[45]. The transgene was injected into pronuclei of fertilized eggs. The fertilized eggs were implanted into a pseudopregnant female strain C57B1/6 "Black 6" mouse who gave birth to potential transgenic pups[48]. The pups with the transgene are of interest to us.

Genotyping Kv12 DN Transgenic Mice

On June 23. 2011, we received thirty-four offspring from the pronuclear injection. Each of the offspring needed to be genotyped for presence of the transgene. The primers used for the genotyping reactions were LG 3S/LG 5A, LG3S/LG5A, and SG3S/SG3A (Table 3 and Figure 12: A).

Table 3: Genotyping Primers

The table lists the specific sequence for the primers used to genotype Kv12.1 DN transgenic mice. LG 5A and LG 3S detect the presence of the Kv12.1 DN subunit. PCR reaction using these two primers should yield a band 743 bp in length. LG 5A and LG 3S will not detect the native Kv12.1 allele because it has large introns that will not produce a PCR product. SG 5A and SG 5S detect the presence of the Thy-1 promoter and EGFP portion of the construct. The genotyping reaction using these two primers will produce a 1021 bp piece. SG 3S and SG 3A prime the ends of the Kv12.1 DN subunit and Thy-1 3' Elements and will produce a 1041 bp piece. Cre A and Cre S are primers used to detect the presence of the loxP site for which Cre recombinase recognizes and will splice out upon expression. A genotyping reaction using Cre A and Cre S primers will produce a 100 bp piece.

Primer	Sequence	Recognized Allele	Expected Size
LG 5A	CCCCCAGCGTGTTGTTGCCATAAT	Kv12.1 DN	743 Base Pairs
LG 3S	GAGCCGAGCCGTCCTTTATCACA		
SG 5A	GCTCCTCGCCCTTGCTCACCATAG	Thy-1 And EGFP	1021 Base Pairs
SG 5S	TGAGGCCAACCCAAGAAACATAGT		
SG 3S	CTCACCAGCCTTGCCTACATTTG	Kv12.1 DN and Thy-1 3' Elements	1041 Base Pairs
SG 3A	ATCCTGCCTCCCCTACCCACCATAC		
Cre A	GTGAAACAGCATTGCTGTCACTT	LoxP Site Post Cre Expression	100 Base Pairs
Cre S	GCGGTCTGGCAGTAAAAACTATC		

Wild type mice will not present a band upon gel electrophoresis because the genotyping primers each recognize a specific DNA sequence only present in the transgene and not in the mice genome (Figure 12: A).

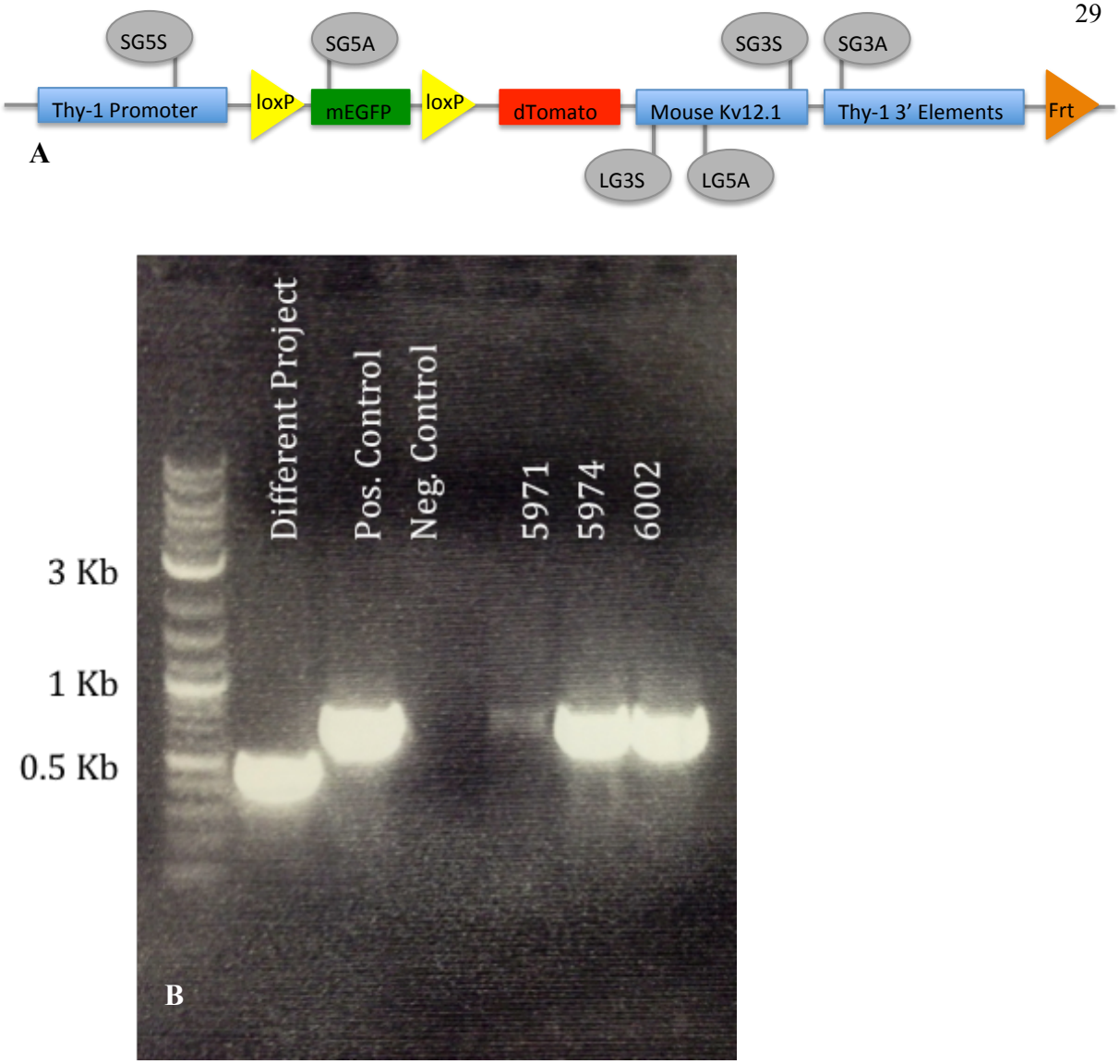


Figure 12, A-B: Genotyping Founder Mice

A- Schematic depicting the location of the genotyping primers used to detect the presence of the transgene in the genome of the mice. SG 5S and SG 5A prime in opposite directions and detect the presence of the Thy-1 promoter and EGFP portion of the transgene. LG 3S and LG 5A both prime portions of the Kv12.1 DN subunit, and SG 3S and SG 3A overlap and detect the presence of the Kv12.1 DN subunit and Thy-1 3' Elements of the original Brainbow vector.

B- Gel agarose picture of genotyping reactions for the three founder mice (5971, 5974, and 6002) using the primers LG 3S and LG 5A. Each of the founder mice produced a band approximately 800 bp, indicating the presence of the transgene. We choose these to be breeders for future lines based on brightness of band fluorescence. In this gel founder 5971 had low band fluorescence, but offspring from this line showed a consistent bright level of fluorescence. This gel is an example of the type of results we obtain from the genotyping reactions. Subsequent genotyping gels were not shown in this paper.

The founder mice that produced the brightest bands (5971, 5974, 5980, and 6002) were chosen to be breeders for future transgenic lines (Figure 12: B). Founder mouse 5980 died prior to breeding.

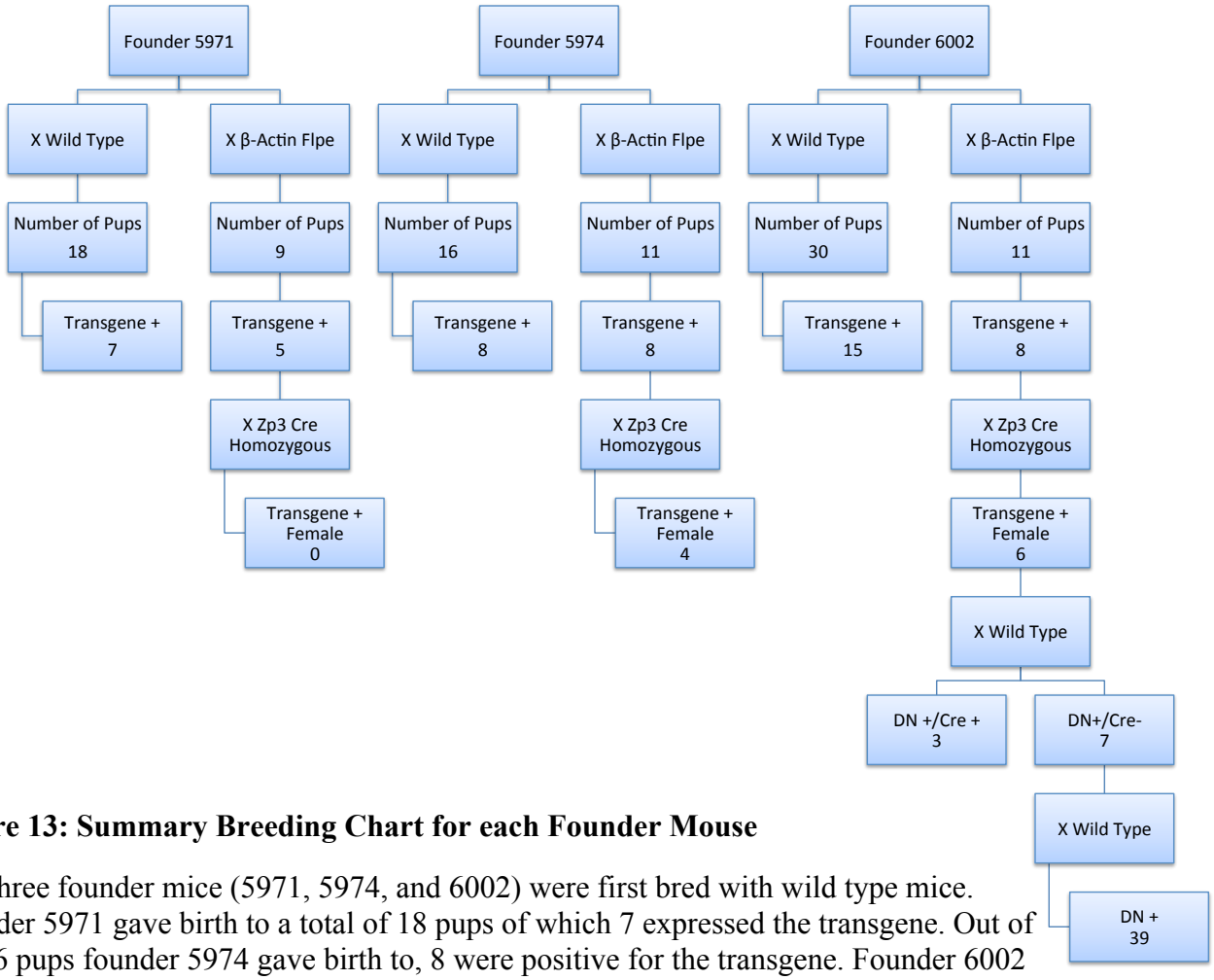


Figure 13: Summary Breeding Chart for each Founder Mouse

The three founder mice (5971, 5974, and 6002) were first bred with wild type mice. Founder 5971 gave birth to a total of 18 pups of which 7 expressed the transgene. Out of the 16 pups founder 5974 gave birth to, 8 were positive for the transgene. Founder 6002 gave birth to 15 pups positive for the transgene. Each founder also was bred to a mouse homozygous for a beta-actin driven Flpe transgene. Beta-actin drives expression of Flp recombinase, guaranteeing only one copy of the transgene allele is inherited by each offspring. Founder 5971, 5974, and 6002 gave birth to 5, 8, and 8 pups positive for the transgene respectively. The transgene positive pups from the breeding between the founder and beta-actin Flpe mice were bred with mice homozygous for Zp3 Cre allele. From this breeding, transgene positive females were of interest because the Zp3 cre allele is expressed in the oocytes. Founder 6002 line had 6 transgene positive females. Theses females were bred with wild type male mice. Since the female mother expresses Cre recombinase in the oocyte, offspring from this breeding will have a processed DN transgene. The EGFP is spliced out, and the Kv12.1 DN subunit and dTomato are now expressed. Mice positive for the Kv12.1 DN subunit and no Cre recombinase expression were bred with wild type mice to produce pups for future behavioral experiments.

Each of the founder mice (5971, 5974, and 6002) were set up with wild type mice to increase the number of transgenic mice (Figure 13).

Additionally, the founder mice were bred with mice homozygous for an allele coding for Flp recombinase (Figure 13). Since the insertion of the transgene is random, we can only detect the presence of the allele but cannot count the number of copies. Flp recombinase allows us to make sure we are only working with one copy of the transgene. Usually transgenics have a single insertion, but the transgene can go in tandem repeats at one locus. Beta-actin is the promoter of Flp recombinase and is widely expressed, guaranteeing germ line deletion. Therefore, offspring will inherit a transgene allele with a single copy. A single copy off of a strong promoter should be enough to drive high transgene expression. Additionally, we do not want a full knockout at a site we do not completely understand. This is important because we want to observe a high fidelity color switch, and multiple copies of the transgene could result in the expression of various colors. The mice were tested for the transgene by genotyping with the primers LG 3S/LG 5A, LG3S/LG5A, and SG3S/SG3A (Table 3).

Once enough founder mice were bred with Flpe mice, a constitutive DN knockout breeding scheme was set up (Figure 13 and 14). The reason we choose to perform a constitutive DN breeding strategy first is because it is a fast and easy way to determine if the DN transgene is expressed in neurons and if a change from green to red fluorescence is observed upon expression of Cre. Additionally, the constitutive DN breeding serves as an important behavioral control for maximal spatial expression of the DN transgene. We can determine if we have the potential to induce epilepsy by observing if these transgenic

mice exhibit a hyperexcitable phenotype by DN suppression of hippocampal and cortical pyramidal neurons.

Transgene positive mice from the Flp breeding were crossed with mice carrying a zona pellucida protein 3 (Zp3) cre allele for expression of Cre-recombinase in the germ line[49]. Only female heterozygous mice in the first generation were selected for further breeding because the Zp3-cre allele is only expressed in the oocyte. These females were breed with wild type C57B1/6 "Black 6" mice. The offspring from this breeding should have a processed transgene. EGFP should be excised in the entire animal and we expect all targeted neurons to fluoresce red and express the Kv12 DN transgene (Figure 14).

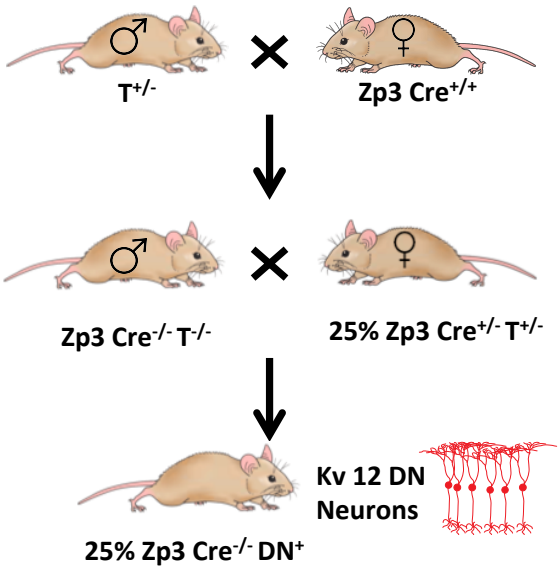


Figure 14: Constitutive DN Transgene Expression Scheme

Female transgene positive mice were bred with male homozygous dominant for Zp3 Cre allele. Based on Mendelian genetics, fifty percent of the first generation offspring should be hemizygous for the transgene and heterozygous for the Zp3-cre allele. From this breeding, female mice hemizygous for the transgene and heterozygous for the Zp3 Cre allele were chosen for a second round of breeding. Females were selected because Zp3 Cre is expressed in oocytes. These females were bred with wild type (Zp3 Cre -/- transgene -/-) mice. Since Cre recombinase is expressed in the oocyte, EGFP will be spliced out and dTomato and Kv12.1 DN subunit will be expressed in the offspring. There is a 25% chance the offspring will be hemizygous for the Kv12.1 DN subunit and have no copies of the Zp3 Cre allele. Kv12 neurons in these mice will fluoresce red and express the dominant negative subunit, preventing the passage of potassium.

LG3A/LG5A and SG3S/SG5A primers were used to test for the transgene in the second generation of pups in the constitutive KO breeding scheme. The SG5A/SG5S primers set could no longer be used to test for the transgene because it amplifies DNA coding for EGFP, which has been excised in the germ line of these mice. We designed special primers to test for the presence of allele coding for Cre using the primers Cre A/Cre S (Table 3).

Preliminary Analysis of Transgene Expression Pattern

Preliminary histology analysis was performed to identify the transgene expression pattern in the forebrain of the Kv12 founder mice (Figure 15). The images were produced using a confocal microscope. EGFP antibody staining was required because the images are from fixed tissue preps. Upon excising the brain, there is a significant reduction of fluorescent protein activity. The EGFP label in the construct is used to identify bright living neurons during experiments. Areas fluorescing green are where the transgene is being expressed and upon expression of Cre recombinase, will change to red. The primary areas of fluorescence were the hippocampus, specifically the dentate gyrus. We are working on viewing the expression pattern of the transgene in the cortex, but we believe we will get similar results.

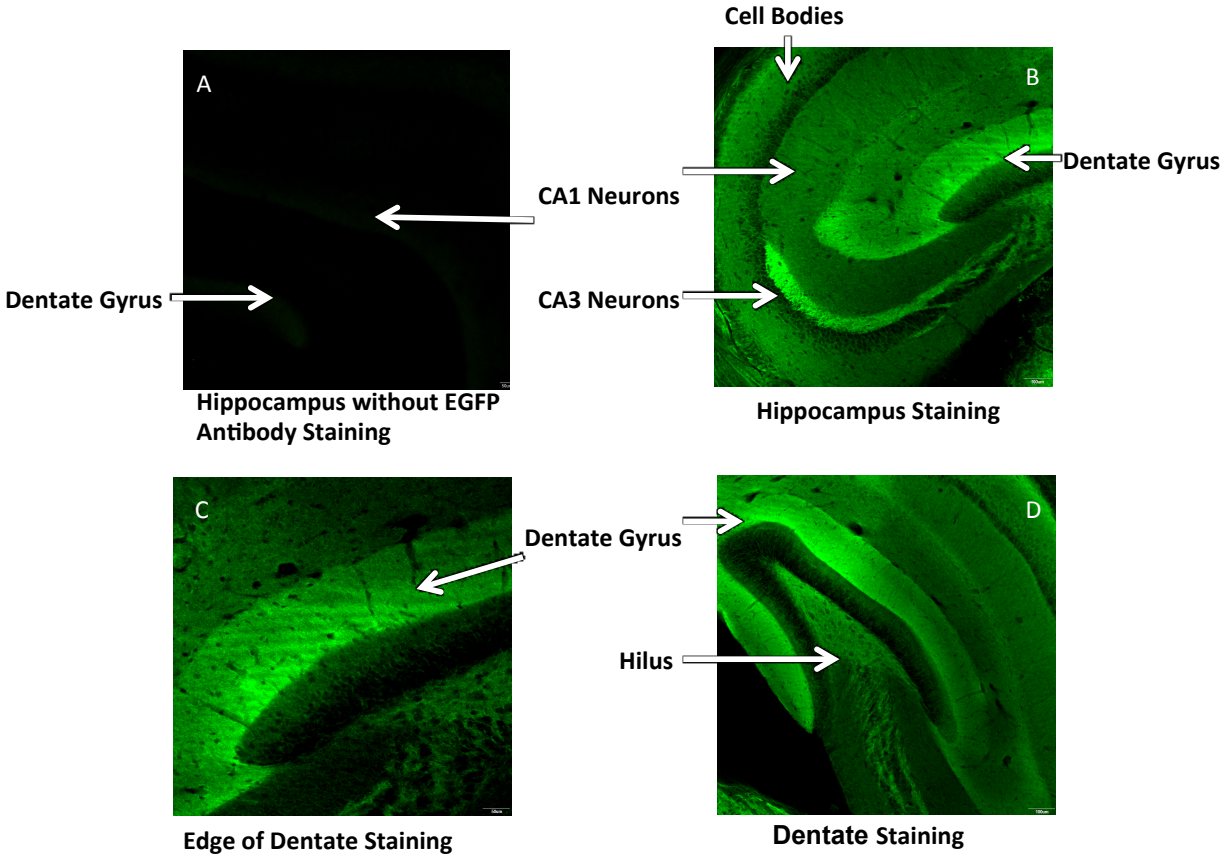


Figure 15, A-D: Transgene Expression in Hippocampus using Confocal Microscopy

A- Control image of the hippocampus with no EGFP antibody staining. Allows for the observation of native fluorescence.

B- Hippocampus with EGFP antibody staining. Areas fluorescing green indicate expression of the transgene. There is high expression in the dentate gyrus and CA3 neurons of the hippocampus.

C- Magnified view of the edge of the dentate gyrus stained with EGFP antibody.

D- Alternate view of the hippocampus showing transgene expression in the dentate gyrus as well as the hilus.

Isolation of Brain RNA

In order to test for the expression of the Kv12.1 DN subunit, RNA was isolated from the forebrains of the transgenic mice. For the experiment we used two mice, both

second-generation offspring from the constitutive breeding scheme. These mice expressed Cre recombinase, which should have excised the EGFP and allow for the expression of the Kv12.1 DN subunit. A separate mouse not expressing Cre recombinase was chosen as a comparison.

Using cDNA from the hippocampus and cortex, a PCR reaction was set up in order to test for the presence of the Kv12.1 DN subunit (Figure 16, A-C). For each mouse, two PCR reactions were set up using either DN RT 5'S/DN RT 5'A or DN RT 3'S/DN RT 3'A primers (Table 4).

Table 4: PCR Primers to Test for Presence of Kv12.1 DN Subunit

There were two primer sets used to detect the presence of the Kv12.1 DN subunit. DN RT 5'S and DN RT 5'A tested for the presence of the 5 prime end of the subunit. Primer set DN RT 3'S / DN RT 3'A detects the 3 prime end of the Kv12.1 DN subunit. The expected molecular band size of the PCR using either primer set is 300 bp.

Primer	Sequence	Recognized Allele	Expected Size
DN RT 5'S	GTGCAACTGCCCGGCTACTACTACG	5' End of Kv12.1 DN	300 Base Pairs
DN RT 5'A	ATTGGCCAGGATGAAGTTGCTATGTGT		
DN RT 3'S	CCCCCATTCAGATACCACA	3' End of Kv12.1 DN	300 Base Pairs
DN RT 3'A	TCTAGAGTCGCGGTGATTAT		

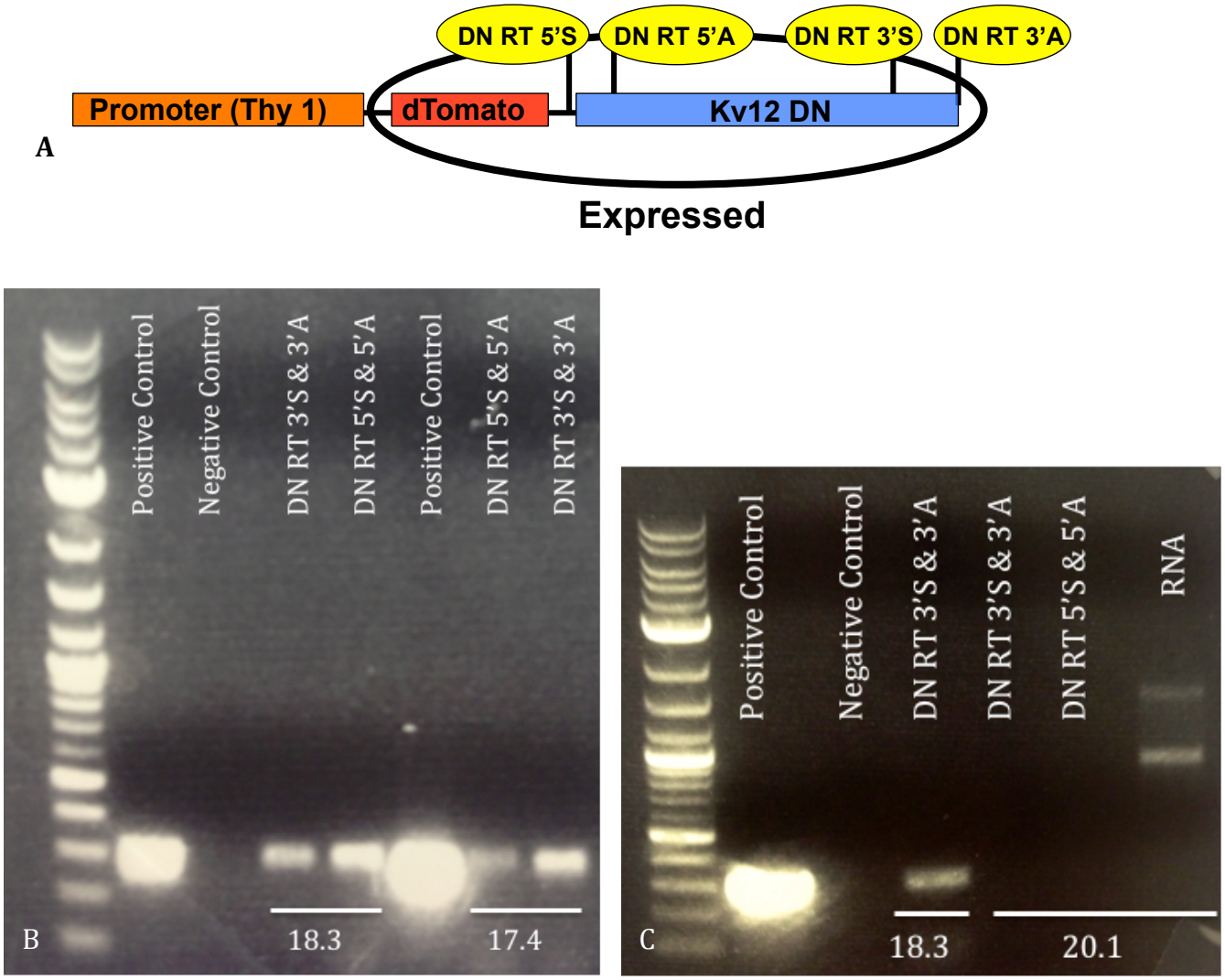


Figure 16, A-C: Detection of Kv12.1 DN Subunit from Isolated RNA

A- Schematic depiction showing where each primer binds in post Cre mice. Primer sets, DN RT 5'S / DN RT 5'A and DN RT 3'S / DN RT 3'A detect the five-prime and three prime ends of the Kv12.1 DN subunit, respectively.

B, C- The PCR reactions were ran on a 1% agarose gel. The positive control was the original Kv12.1 plasmid (diluted 1:3000) set up with each PCR primer set. The expected band was 300 bp for both primer sets. Mice 18.3 and 17.4 both expressed Cre recombinase in the germ line, splicing out EGFP and expressing dTomato and Kv12.1 DN subunit. Therefore, since each of these mice was expressing the Kv12.1 DN subunit, both were expected to produce a 300 bp band. Mouse 20.1 was from the first generation of constructive breeding scheme and does not express Cre recombinase. In this mouse, Kv12.1 DN subunit is not expressed and therefore should not produce a 300 bp band. Additionally, RNA from mouse 20.1 was ran on the gel to determine that the absence of a band was not due a lack of isolated RNA.

The mice expressing Cre recombinase properly produced a band, while the mouse with no expression did not yield a band.

Summary

The Thy-1 DN construct was successfully cloned and used for pronuclear injection to create founder mice expressing the transgene. Preliminary histological analysis indicates the transgene is expressed in one area of interest (hippocampal pyramidal neurons); however, we are performing ongoing histological studies to determine the transgene expression in cortical pyramidal neurons. Additionally, excision of Cre does activate the Kv12.1 DN subunit. We have not yet extensively characterized the expression of red fluorescent protein in the transgenic mice.

Chapter 4: Discussion

Future of Current Studies

Detailed characterization analysis will be performed to determine if the transgene is expressed in layer 2/3 of cortical pyramidal neurons that express a significant number of Kv12.2 channels[6]. We also want to determine if the transgene is expressed in all hippocampal, dentate, and cortical cells or only in a subset of these neurons. We believe seizure phenotypes will arise in these key areas due to the high expression of Kv12 channels[6].

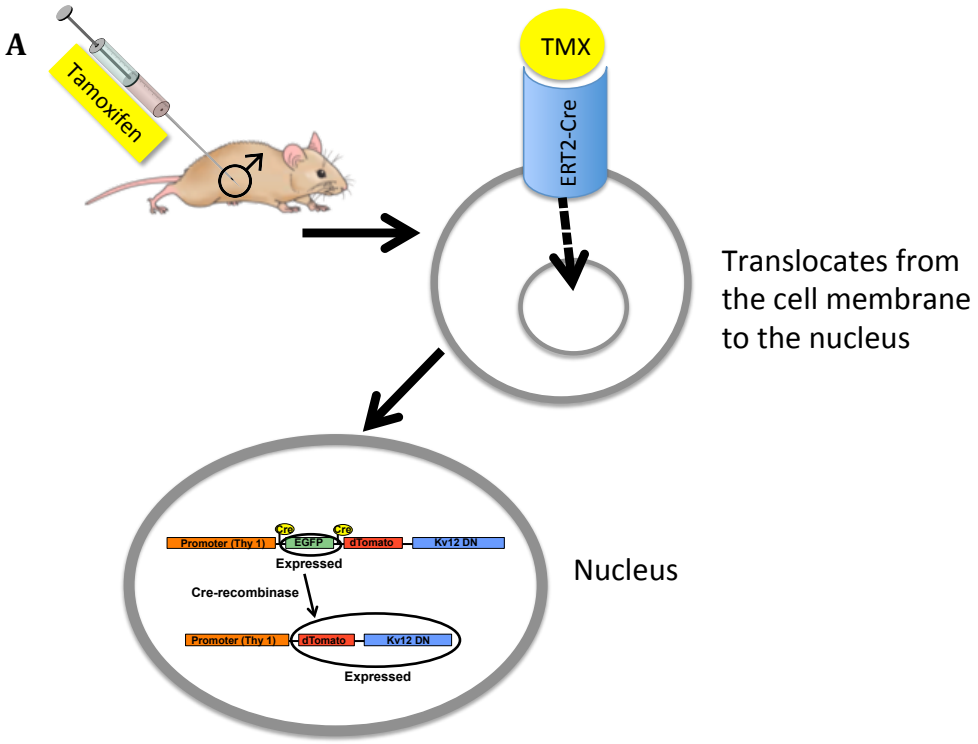
Additionally, we want to determine if the level of DN expression is sufficient to knockdown Kv12 channels, and if so by how much. Electrophysiological recordings from cortical or hippocampal pyramidal neurons of wild type and DN mice will be used to determine if DN mice are hyperexcitable in response to current injection[6] and have smaller subthreshold potassium currents, due to the elimination of Kv12 current.

In the future, we also want to determine if constitutive DN mice have a low seizure threshold. This can be determined by a pentylenetetrazole (PTZ) chemoconvulsant test. The constitutive DN mice should be hyper-responsive and have stronger and faster seizures upon injection of the PTZ-chemoconvulsant[6]. Additionally, the constitutive DN mice can be EEG monitored for the observation of spontaneous seizures. If either the PTZ-chemoconvulsant test or EEG monitoring shows that the constitutive DN mice are hyperexcitable and have a low seizure threshold, an inducible DN transgenic breeding scheme will be set up.

Inducible DN Transgene Expression

An inducible DN breeding strategy will allow us to compare function of wild type (green) and hyperexcitable (red) live neurons in the same brain slice to directly compare their ability to respond to different stimuli. In this breeding scheme, Cre expression is under the control of a modified estrogen receptor (ERT2-Cre) and can be induced by tamoxifen administration[50, 51] (Figure 17).

Estrogen receptors are a nuclear hormone receptor that assembles on the surface of cells. The ERT2-Cre (protein fusion) receptor has been engineered to only bind exogenous tamoxifen instead of native estrogen. Once tamoxifen is bound to the ERT2-Cre receptor, the complex translocates from the cell membrane to the nucleus. Cre-recombinase is now expressed in the nucleus, where the transgene is located. Cre-recombinase splices out the EGRP, resulting in the expressing of dTomato and the DN subunit (Figure 17).


A
Tamoxifen
TMX
ERT2-Cre
Translocates from the cell membrane to the nucleus
Promoter (Thy 1)
EGFP
dTomato
Kv12 DN
Expressed
Cre-recombinase
Promoter (Thy 1)
dTomato
Kv12 DN
Expressed
Nucleus

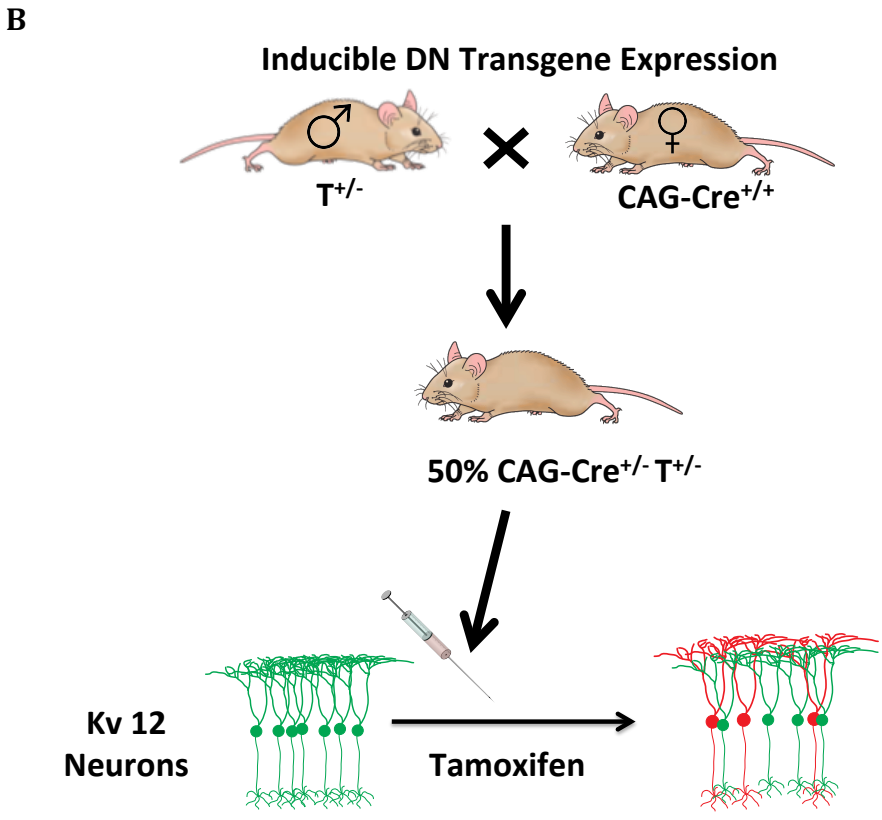

B
Inducible DN Transgene Expression
T+/-
CAG-Cre+/+
50% CAG-Cre+/- T+/-
Kv 12 Neurons
Tamoxifen

Figure 17, A-B: Inducible DN Transgenic Strategy

A – Schematic depiction of the mechanism of action for the modified estrogen receptor, ERT2-Cre. Tamoxifen, which is normally an estrogen receptor antagonist, binds to the ERT2-Cre membrane bound receptor. The tamoxifen ERT2-Cre complex translocates from the cell membrane to the nucleus. Cre recombinase is now expressed in the nucleus and excises the EGFP portion of the Thy-1 DN transgene. The neurons will now express the Kv12.1 DN subunit and fluoresce red.

B - The breeding scheme for the inducible DN transgene line begins with a male mouse hemizygous for the transgene bred with a female homozygous for the CAG-Cre allele. The CAG-Cre allele codes for the membrane bound CAG-Cre receptor. Based on Mendelian genetics, 50% of the offspring should be heterozygous for the CAG-Cre allele and hemizygous for the transgene. Kv12 neurons should fluoresce green where the transgene is expressed and upon injection of tamoxifen will allow for inducible DN suppression of Kv12 neurons.

These mice can be inducibly knocked out with varying amounts of tamoxifen administration. This will allow us to control of time and extent of DN expression with tamoxifen injection, and will potentially allow us to determine the degree of hyperexcitability that leads to changes in seizure threshold and epileptogenesis. We can determine if DN suppression in adult animals leads to seizures by varying the number of hyperexcitable neurons.

Appendix A

Thy-1 DN Construct DNA Sense Strand Sequence

GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAG

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CGA

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EDUCATION

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B.S. in Biology, Neuroscience Option, Business Concentration
Honors in Biology
Expected May 2013

SALISBURY TOWNSHIP HIGH SCHOOL Allentown, PA
Fall 2005 – Spring 2009

RESEARCH / EXTERNSHIPS

PENN STATE INSTITUTE OF THE NEUROSCIENCES University Park, PA
Undergraduate Research Assistant. Supervised by Dr. Timothy Jegla Fall 2010-Present
Investigating how voltage gated potassium channels influences circuit function and the number and distribution of hyperexcitable neurons influences circuit function, leading to seizure threshold changes in transgenic mice.

PENN STATE UNIVERSITY SUMMER EXTERNSHIP Phoenixville, PA
Phoenixville Hospital. Supervised by Dr. Joshua Goldberg Summer 2011
Mentored by Dr. Goldberg in the fields of otorhinolaryngology, head and neck surgery, facial plastic surgery, and robotic surgery.

PENN STATE ENGLISH DEPARTMENT, HONORS ENGLISH 30 University Park, PA
Sony Electronic Reader Research Project. Supervised by Dr. Diana Gruendler Spring 2010
Piloted Sony e-readers throughout the semester. We designed a new version of the e-reader called "The Sony Scholar," specifically geared towards college students. At the end of the semester, we presented our ideas to Sony Electronics executives.

HONORS AND AWARDS

The Pennsylvania State Undergraduate Research Award, 2010 & 2011 - Received grants of $1,000 each year to help fund my portion of the research in Dr. Timothy Jegla's Research Lab

Academic Excellence Scholarship, Schreyer Honors College, 2009 – Present

Phi Beta Kappa, Academic Honor Society, Spring 2013

Phi Kappa Phi, National Honor Society, Spring 2012 - Inducted as a junior because I was in the top 7.5% of my academic class.

Honorary A+ in Chemistry 113: Experimental Chemistry II Lab, Spring 2010

CLASSY Awards: Top 10 Finalist, Summer 2011 – The Penn State Free Wheelchair Mission was a top 10 finalist amongst thousands of philanthropy clubs in the category of "Most Influential College Organization."

LEADERSHIP / ACTIVITIES

ALPHA EPSILON DELTA, THE PREMEDICAL HONOR SOCEITY, Fall 2009 – Present

Distinguished Membership. National Honors Membership Spring 2011. Help organize and run various health related events throughout the year.

PENN STATE FREE WHEELCHAIR MISSION, Fall 2010 – Present

Treasurer. A nonprofit organization that focuses on giving the gift of mobility to those less fortunate around the world. The first college based chapter of Free Wheelchair Mission.

SALISBURY HIGH SCHOOL SOCCER PROGRAM, Fall 2007 – Present

Coach and Mentor. Develop high school girls and boys soccer skills on and off the field through various drills and exercises including: scrimmages, weight training, and yoga. During the summer of 2011 and 2012, I coached the Salisbury High School girl's varsity summer league team at Lehigh University.

PENN STATE INTRAMURAL SOCCER PROGRAM, Spring 2010 – Present

Player. Residential Hall Soccer League. Every spring, I participate in an intramural soccer league at Penn State.

LEHIGH UNIVERSITY SUMMER SOCCER LEAGUE, Fall 2009 – Present

Player. F.C. Gore. Every summer since high school, I participate in an intramural soccer league with Salisbury High School alumni.

ATLAS THON, Fall 2009 – Spring 2010

Helped raised money and awareness for pediatric cancer.

RESEARCH PRESENTATIONS

Ackerman C, Saadat A, Jegla T. Dominant Negative Suppression of Kv12 Channels In Transgenic Mice.
Penn State University Undergraduate Research Exhibition. University Park, PA, April 11, 2012.

Ackerman C, Saadat A, Liu H, Jegla T. Dominant Negative Suppression of Kv12 Channels In Transgenic Mice.
Thomas Jefferson University Sigma Xi Research Day 2013. Philadelphia, PA, April 17, 2013.