

**The Mechanisms of GABAergic Synapse Loss in Neurons Lacking Functional
Ankyrin-G**

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A thesis submitted in partial fulfillment of the Degree of Bachelor of Science in Neuroscience
with Honors

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Abstract:

While bipolar disorder remains one of the most debilitating and costly neuropsychiatric diseases, the mechanisms underlying the disease etiology remain poorly understood. Multiple genome wide association studies (GWAS) demonstrated that the *ANK3* gene, which encodes the ankyrin-G protein, is one of the most significant genes linked to bipolar disorder. A critical tryptophan residue, W1989, located within the giant exon of ankyrin-G, is necessary for the interaction between the GABA_A-receptor-associated protein (GABARAP) and ankyrin-G, which stabilizes GABAergic inhibitory synapses. Here, we use a knock-in mouse model expressing the *ANK3*-W1989R human variant, which completely abolished the interaction between ankyrin-G and GABARAP. We see a reduction in overall GABA_A-receptor levels at the somatodendritic domain and AIS in W1989R cultured hippocampal neurons compared to WT neurons. We hypothesize that in the W1989R loss-of-function ankyrin-G model, clathrin heavy chain (CHC) directly interacts with GABARAP to promote GABA_A-receptor endocytosis. Preliminary data using immunoprecipitation and protein purification suggest binding between CHC and GABARAP. Further, we attempt to rescue GABA_A-receptor levels in W1989R neurons using a potential dominant-negative CHC-eGFP construct. Inhibiting the interaction between CHC and GABARAP may be a potential therapeutic target to restore GABAergic synapses in patients with neuropsychiatric disease.

Table of Contents

<u>Abstract</u>	ii
<u>Acknowledgments</u>	iv
<u>Summary</u>	1
<u>Introduction</u>	6
<u>Materials & Methods</u>	17
<u>Results</u>	24
<u>Discussion</u>	33
<u>References</u>	39

Scientific Acknowledgements

I would like to acknowledge Andrew Nelson's work in this honors thesis. Figure 9 is a reproduction of his work, included with his permission.

Personal Acknowledgements

I would like to thank my PI and my mentor, Dr. Paul Jenkins, for his continued guidance and support for the past 2.5 years that I have been a part of the Jenkins Lab. It has been a privilege for me to be a part of his lab and he has been a role model for the kind of scientist I hope to become.

I would also like to thank Andrew Nelson, a PhD candidate in the Jenkins Lab. Andrew has been my mentor since I joined the lab. His advice and knowledge have been invaluable resources. Working on this project with him has been my great pleasure. He has not only made me a better scientist, but also instilled in me a passion and dedication to the field of neuroscience. I thank him for his continued patience, motivation, and enthusiasm throughout the experimentation and the thesis writing process. I would also like to thank the rest of the Jenkins Lab for their support and assistance throughout this process. I would like to thank Dr. Sara Aton for co-sponsoring my work and Dr. Richard Hume for reading my thesis.

Summary

This thesis is within the field of neuroscience, which is the study of the nervous system and, most notably, the brain. Some cells in the brain are called neurons. Neurons, like all cells, are composed of mostly fluid which contains structures that are enclosed by a thin membrane. The fluid is water, but the structures within the fluid could be proteins, ions, or other molecules. Intracellular refers to components within the cell membrane. Extracellular refers to components on the outside of the cell. A cell is a place where many vital life processes can occur. While the membrane protects the cell from the extracellular medium, it is not completely insulated. The membrane is selectively permeable, meaning it allows some components into the cell and blocks the entrance of others. Some molecules are so small, they can pass right through the membrane. Other molecules are necessary for cell function, but they need facilitation into the cell. Their admittance is possible because of components that do not reside inside or outside the cell, but are actually on the membrane itself. These components can be receptors or channels (to name a few). They tend to be very specific for a particular molecule. For example, an ion channel for sodium ions will generally only allow sodium ions to pass through. Ions are electrically charged atoms or molecules that cannot pass through the cell membrane without help because their electric charge is repelled by the membrane. This configuration is true for all cells; however, neurons do have very unique properties that allow them to function to interpret the world. Some of these unique properties have to do with how neurons communicate.

Neurons communicate using electrical signaling and are said to be electrically excitable. Excitability refers to the fact that neurons have an electrical potential, but it is transient and changes over time. These changes happen very quickly and are instrumental in electrical signaling. Electrical charges move when there is a difference of potential between two points; when electrical

charges move, it is called a current. The same model exists in a battery. In any battery, a car battery or a calculator battery, there are two terminals: positive and negative. Across these terminals is the “potential” or the difference in charge. Once the battery is hooked to a conductor, the current is generated. The current is the flow of ions. Conductors are any sort of medium in which charges can move. Metal, for example, is a good conductor and wood is not. Salty water is also a good conductor and this allows conductance in the cell. The ions of the cell are free to move around and create current. There are many important ions in the brain, and intracellular and extracellular solutions of all organisms have ions; sodium, potassium, and calcium are some of the most important ions. Neurons, like most cells, have a potential across their membrane. The inside of the cell is slightly lower (more negative) than the outside of the cell. Normally not much current can flow because the membrane doesn't usually let electrical charges (in the form of ions) get across it. However, the potential can change quickly and dramatically. In fact, the potential can change very considerably over the course of a few milliseconds and then return to its previous state. This is the meaning of electrically excitable: a sudden and dramatic increase in membrane permeability to ions (more precisely, to particular ions, mostly but not exclusively sodium and potassium). When this happens, there is an influx and then an outflow of ions; in other words, charges are moving and thus we have a current. These electrical events are called action potentials. Action potentials across the brain (from the same neuron and different neurons) look the same. This is because the processes which generate an action potential are the same. An action potential, once triggered, will persist to its end. There are no “half” action potentials. In this way, a neuron is thought to binary. A 1 would mean action potential firing and a 0 would mean no firing.

Neurons have an interesting shape, or morphology, which facilitates the propagation of an action potential. Neurons usually have an oval body or soma, like many other types of cells, but

they also have dendrites and axons, which project from the body like branches of a tree. The dendrites and axons are very important to a neuron's function. Action potentials are usually triggered near the neuron's body, close to where the axon is. The action potential then "travels" down the axon until it reaches the end of the axon, or axon terminal. This is where a synapse is formed. A synapse is a place where part of one neuron's axon terminal comes close to the membrane of another neuron (usually a patch of membrane on that neuron's dendrite, but also sometimes on the cell body, or even occasionally on its axon). The neuron which contributes the axon terminal is called the presynaptic neuron and the other neuron is the postsynaptic neuron. The action potential travels until it reaches the axon terminal where it essentially runs out of membrane and voltage potential which generate it. Before the electrical signaling ends, however, the action potential sets into motion a process by which molecules, called neurotransmitters, from the presynaptic neuron are released into the extracellular medium. These molecules are packaged into small membrane-bound spheres called synaptic vesicles, and the action potential causes the vesicles to spill out of the presynaptic neuronal membrane and drift to the postsynaptic neuronal membrane. This membrane is very close; the space in between the neurons is called the synaptic cleft and it is a very small gap. Thus, the electrical action potential is translated into a chemical event. The neurotransmitters from the presynaptic cell interact with the postsynaptic cell through receptors on the membrane, which are specific to particular neurotransmitters as mentioned earlier. The neurotransmitter can bind to its specific receptor which can then cause a conformational change in the receptor (it changes its shape). These receptors often contain ion channels and when the receptor changes shape, the ion channel is closed or opened. Receptors also may be coupled, or connected to pathways that eventually close or open ion channels. When ion channels are opened to cause a net influx of positive ions (like sodium or potassium), there is a change in ion

flow which can then reach a threshold to trigger an all or none action potential. This action is said to be excitatory. Conversely, when negative ions are allowed to flow through, like chloride, which prevents an action potential, since the inside of the cell is already negative. This action is called inhibitory. Some presynaptic neurons release neurotransmitters that excite action potentials and are thereby called excitatory neurons. An example of such a neurotransmitter glutamate, which is an amino acid. Some presynaptic neurons release neurotransmitters that inhibit action potentials and are thereby called inhibitory neurons. An example of such a neurotransmitter is *gamma*-Aminobutyric acid, or GABA. These neurotransmitters do not inherently have excitatory or inhibitory properties, but the receptors that they bind to cause excitatory or inhibitory effects.

There is an order to neuronal connections and communication. Some parts of the brain deal exclusively with one aspect of sensory processing and another region of your brain processes a different stimulus. For example, there are neurons that can specifically translate light into an electrical impulse which allows us to process that light input and have vision. Other neurons translate molecules that contribute to taste. The brain can therefore be thought of as an organization of neuronal networks. These networks consist of many neurons and their synapses. There can be neuronal networks for vision, hearing, taste, touch etc. It is also important that these networks work together, so you can see what you touch, for example. Or hear what you are seeing. Electrical potential changes are essentially generating our interpretation of our environment. Small electrical changes may seem unimportant, but receptors on cells transduce environmental stimuli into graded potential changes that then initiate a neural representation of the world. This completely informs our actions within that world. With this in mind, it is easy to see how dysfunction, or impairment, in any of the steps involved in this process can lead to debilitating issues.

Diseases that deal with nervous system are termed neuropsychiatric diseases. These diseases can manifest in a variety of behavioral changes. Depression, for example, demonstrates as lack of interest and motivation, and changes in sleep and appetite. Schizophrenia is characterized by hallucinations, delusions, loss of initiative and cognitive function. Bipolar disorder has alternating symptoms of depression (described earlier) and mania, which can manifest as delusions, euphoria (happiness and excitement) and/or psychosis (or loss of touch with reality). Most neuropsychiatric diseases increase suicidal ideations. Many treatments for neuropsychiatric disorders only attempt to treat the symptoms. The behavioral output is so debilitating that it is reasonable to attempt to mitigate those adverse and incapacitating actions. However, as it has been explained, behavior is completely a result of underlying neuronal connections and signaling. If there is a severe disruption in normal behavior, then there is an issue somewhere in the fundamental components as well. It is therefore imperative that we seek to treat neuropsychiatric disease from its ultimate components, not just as a mood disorder. It is through this approach that many affected individuals will be able to find relief and it is this goal that motivates the following thesis.

Introduction

Genetic Association with Bipolar Disorder

Bipolar disorder is a neuropsychiatric disease that is characterized by episodes of mania and depression. These behaviors can affect social, emotional, and cognitive behavior (Muhleisen et al., 2014). This disorder is characterized not only by high rates of attempted suicide and premature death, but also by a substantial economic burden. It is one of the top ten most disabling diseases (days lost/year) (Kleine-Budde et al., 2014; Moreira et al., 2017). Some of the most commonly used medications for bipolar disorder, such as lithium and valproate, do not show significant differences in drug vs. placebo effects in many patients (Bartoli et al., 2018). Therefore, it is imperative that the mechanisms underlying the pathophysiology of bipolar disorder are uncovered to identify novel therapeutic targets for the treatment of bipolar disorder as well as related neuropsychiatric diseases (Bartoli et al., 2018).

Bipolar disorder is highly heritable, meaning it has a strong genetic component to its inheritance and maintenance in the population. The heritability estimates can range from 60-80% for bipolar disorder (Muhleisen et al., 2014). Genome-wide association studies (GWAS) are a common approach to identify genetic variations associated with a particular disease, such as bipolar disorder, by scanning markers across the complete sets of DNA, or genomes, of many people. These genetic variations, referred to as single nucleotide polymorphisms (SNPs), are variations in single base pairs of DNA found in the patient genomes as compared to a standard reference sequence. Most SNPs encompass neutral variation. However, occasionally, a SNP that correlates to a particular disease builds up in the population through genetic means (it is passed on through parental lineage). Recently, GWAS studies uncovered number of risk loci associated with the development of bipolar disorder, including *CACNA1C*, *ADCY2*, *ZNF804A*, *ERBB2*, *SYNE1*,

ODZ4, *DGKH*, and *ANK3* (Ferreira et al., 2008; Craddock and Sklar, 2013; Cardno and Owen, 2014; Chen et al., 2014; Muhleisen et al., 2014; Tao et al., 2014; Hou et al., 2016). One of the largest and most recent GWASs evaluating 9,747 bipolar patients/ 14,278 neurotypical controls showed that the *ANK3* gene had the greatest number of statistically significant SNPs within the bipolar patient cohort (Muhleisen et al., 2014). *ANK3* is among the most consistent and significant genes linked to bipolar disorder (Schulze et al., 2009; Hatzimanolis et al., 2012; Leussis et al., 2012; Harrison et al., 2018); however, the mechanisms by which *ANK3* variants alter brain circuitry and contribute to bipolar disorder is poorly understood. While neuropsychiatric diseases are highly heritability, the odds ratio for SNPs associated with disease through GWAS are often low. For example, the odds ratio for the *ANK3* locus is approximately 1.4 for bipolar disorder, albeit *ANK3* has some of the highest significance and largest effect sizes by GWAS (Ferreira et al., 2008; Schulze et al., 2009; Hatzimanolis et al., 2012; Leussis et al., 2012; Roussos et al., 2012; Chen et al., 2014; Muhleisen et al., 2014). Since common genetic variants, such as the SNPs identified by GWAS, have a small impact on disease susceptibility, it is important to understand how rare genetic variants in *ANK3* impact brain function and contribute to the pathophysiology of bipolar disorder.

Ankyrin-G as the Master Organizer of Plasma Membrane Proteins

ANK3 encodes a protein called ankyrin-G, a membrane-associated protein that is essential for the spatial organization of other proteins at the plasma membrane. Ankyrin-G has three main isoforms; the 190kDa isoform, the 270kDa isoform, and the giant 480kDa isoform that arise from alternative splicing of the *ANK3* gene (**Fig. 1**). The 190kDa splice variant is expressed in most tissues and cell types throughout the body including the brain, heart, skeletal muscle, kidney, and

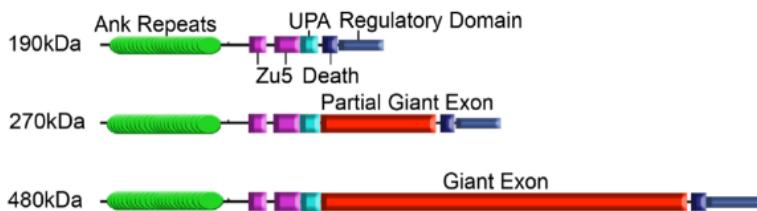


Figure 1: The three main splice variants of ankyrin-G in the nervous system.

retina (Bennett, 1979; Bennett and Davis, 1981; Davis and Bennett, 1984; Peters et al., 1995). The 270kDa and 480kDa isoforms of ankyrin-G are primarily expressed in the

nervous system, which consists of the brain and spinal cord, as well as the nerves that propagate signals throughout the body. In neurons, recent studies have identified the 480kDa ankyrin-G as the master organizer of the axon initial segment (AIS) and the nodes of Ranvier, where it clusters a large number of ion channels, transporters, and cell-adhesion molecules, including voltage-gated sodium channels, KCNQ2/3 potassium channels, the cell adhesion molecule neurofascin, and the cytoskeletal protein β IV-spectrin, which links these excitable domains to the underlying actin cytoskeleton (Pan et al., 2006; Jenkins et al., 2015b). The AIS is the site of action potential initiation and the node of Ranvier are gaps in the myelin sheath that allow for rapid, saltatory action potential propagation. Action potentials are the fast signaling of neurons that lead to chemical signaling which allows for communication between neurons. The communication site in neurons is called a synapse, which releases chemical information from one neuron to a neuron downstream. The AIS and the closely related nodes of Ranvier of myelinated axons are the basis of the evolution of fast signaling in vertebrates and are the best understood ankyrin–spectrin based membrane structures (Bennett and Lorenzo, 2013). Spectrin works in concert with ankyrin to organize the membrane spanning proteins associated with action potentials (Bennett and Lorenzo, 2013). Mutations in *ANK3* have been linked to bipolar disorder, and to a lesser degree schizophrenia, as well as other neurological diseases such as epilepsy, autism and severe cognitive dysfunction

(Ferreira et al., 2008; Schulze et al., 2009; Hatzimanolis et al., 2012; Leussis et al., 2012; Roussos et al., 2012; Chen et al., 2014; Muhleisen et al., 2014). Thus, it remains important to understand how disease-associated variants in *ANK3* affect ankyrin-G function and contribute to disease pathology.

The 480kDa Splice Variant of ankyrin-G Stabilizes GABAergic Synapses

Mouse models with genetic deletions of *ANK3* were used to further understand the effects of ankyrin-G loss-of-function on neuronal development and function. An ankyrin-G knockout mouse, which lacks all three of the main isoforms of ankyrin-G, dies in the late embryonic/early postnatal stage (Jenkins et al., 2013). Specific deletion of the exon encoding the 270 and 480kDa isoforms of ankyrin-G in mice causes a loss of AIS component clustering, including voltage-gated sodium and potassium channels, βIV-spectrin, and neurofascin (Jenkins et al., 2015b). These 270 and 480kDa knockouts also exhibit a >80% reduction in nodes of Ranvier (Jenkins et al., 2015b). Although the organization of the AIS and nodes of Ranvier was a well-known function for ankyrin-G, surprisingly, these mice also exhibited a loss of GABAergic synapses on the AIS and soma of cortical and hippocampal neurons (Tseng et al., 2015). This loss of GABAergic synapses resulted in decreased frequency and amplitude of GABA dependent currents as well as decreases in gamma oscillations, which are a measure of network synchronicity (Tseng et al., 2015). The giant 480kDa ankyrin-G thus is critical for the stabilization of GABAergic synapses and the generation of normal gamma oscillations. Cortical networks comprise two main classes of neurons: excitatory glutamatergic neurons and inhibitory GABAergic interneurons. GABAergic synapses are characterized by their ability to release the neurotransmitter GABA, which inhibits action potential firing of the neuron they synapse onto. There are many functions of these inhibitory synapses, one

of which is feedback inhibition, in which an excitatory neuron activates a hippocampal pyramidal neuron which then in turn activates the inhibitory GABA neuron which can then inhibit excitatory neurons (Benes and Berretta, 2001) Feedback inhibition is crucial in regulating excitatory neuron firing and the lack of this inhibition can lead to constitutive hyperexcitability (Marin, 2012).

GABAergic inhibitory synapses are also crucial for the proper synchronization and function of neuronal networks, which are important for normal cognition, mood, and behavior. In particular, the activity of fast-spiking parvalbumin-positive (PV+) GABAergic interneurons have been shown to be important for generating gamma oscillations due to their high-rate and rhythmic firing patterns (Sohal, 2012; Hu et al., 2014). Fast oscillations in the gamma range (30–100Hz) function as an organizer of temporal encoding of new information and organize the storage and recall of previously stored information (Sohal, 2012). Abnormalities in GABAergic interneuron circuitry and decreased gamma oscillations have been found in patients with neuropsychiatric diseases, including bipolar disorder (Benes and Berretta, 2001; Torrey et al., 2005; Benes, 2010; Konradi et al., 2011; Sohal, 2012; Ozerdema et al., 2013; Lazarus et al., 2015; Schubert et al., 2015). Recent studies suggest that gamma oscillations could act as potential biomarkers for diagnosing disease as well as tracking treatment response in bipolar disorder (Benes and Berretta, 2001). Further, studies that analyzed postmortem brain tissue from patients with bipolar disorder identified decreases in PV+ interneurons as a potential cause for the reduced gamma oscillations (Torrey et al., 2005), which could potentially inform a mechanism of the disease etiology. Reduction in PV+ interneurons (but not pyramidal neurons) could also account for a slight reduction in hippocampal volume, which has historically not been seen in bipolar patients but may be beyond the resolution of previous imaging (Konradi et al., 2011). Reduction in hippocampal volume has been observed in other neuropsychiatric disorders such as depression and

schizophrenia (Konradi et al., 2011). Thus, abnormalities in GABAergic interneurons have been linked to neuropsychiatric diseases, such as bipolar disorder and schizophrenia (Konradi et al., 2011); however, the mechanisms that contribute to disease pathology remain poorly understood

W1989 as Critical Residue for Ankyrin-G/GABARAP Binding

Decreased expression of GABAergic markers including the GABA-synthesizing enzyme GAD67, parvalbumin (PV), the GABA transporter GAT1, and various GABA_A-receptor subunits has been seen in bipolar patients (Konradi et al., 2011; Chattopadhyaya and Cristo, 2012; Lazarus et al., 2015). These changes were predominantly observed in layers II/III of the prefrontal cortex and CA2/3 regions of the hippocampus, brain regions associated with the cognitive (learning and memory), emotional, and mood related behaviors disrupted in bipolar patients (Benes and Berretta, 2001). In addition, altered GABA_A-receptor mediated currents from

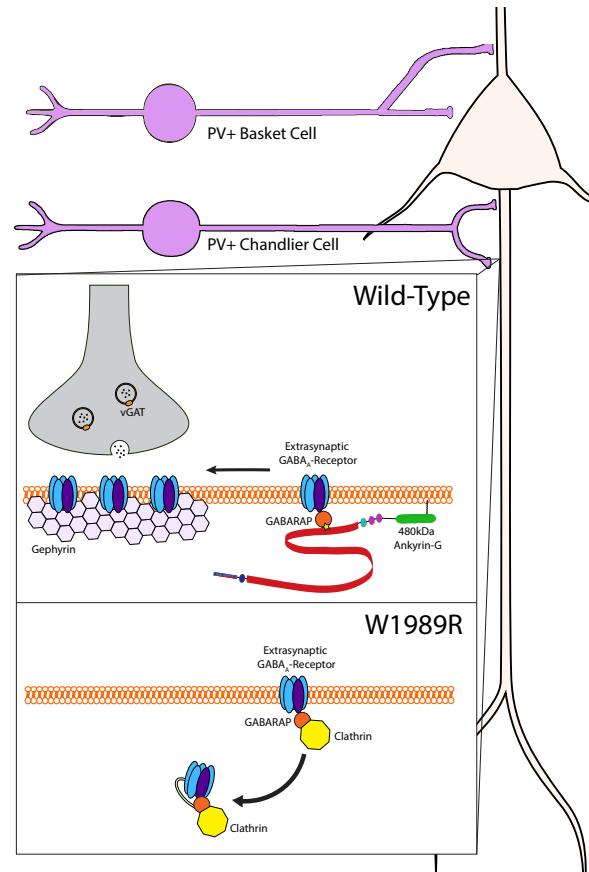


Figure 2: Model of proposed changes in Ank3 W1989R mice and Clathrin/Ankyrin-G opposing actions.

PV+ cells appear to be a primary source of abnormal cortical network synchronization, which is characteristic of bipolar disorder. The giant, 480kDa isoform of ankyrin-G has been shown to stabilize GABAergic synapses on the soma and AIS of glutamatergic neurons by clustering GABA_A-receptors to the postsynaptic membrane (Tseng et al., 2015) (Fig. 2). The GABA_A-

receptor-associated protein (GABARAP) has been shown to interact with the intracellular domain of the GABA_A-receptor gamma subunit *in vivo* and *in vitro* and facilitate GABA_A-receptor trafficking between the cell surface and intracellular compartments (Jacob et al., 2008). Yeast two-hybrid screens, an assay used to uncover protein-protein interactions, identified GABARAP as a binding partner with ankyrin-G. In a proximity ligation assay, with antibodies against ankyrin-G and GABARAP, puncta indicating interaction between the two target proteins were shown on the surface of somatodendritic and AIS membranes in wild type neurons (Tseng et al., 2015). GABARAP is a member of the LC3 family of microtubule-associated proteins. An LC3-interacting motif within the giant exon of the 480kDa ankyrin-G contains a critical tryptophan residue (W1989) necessary for binding to GABARAP (Tseng et al., 2015), Isothermal titration calorimetry data, which is used to assay the binding of small molecules based on thermodynamic parameters, revealed the binding affinity between ankyrin-G and GABARAP is approximately 15 nM (Tseng et al., 2015), the strongest affinity binding partner of the LC3 protein family. Mutation of the W1989 residue to arginine (W1989R) completely abolished the binding between ankyrin-G and GABARAP (Tseng et al., 2015). Since this mutation is found within the giant exon of ankyrin-G, mutation of this residue only directly affects the 270 and 480kDa isoforms. Expression of the W1989R mutant in cultured neurons failed to rescue surface GABA_A-receptors (Tseng et al., 2015), demonstrating ankyrin-G binding to GABARAP is necessary to stabilize GABAergic synapses. Interestingly, W1989R 480kDa ankyrin-G associates with the somatodendritic membrane and rescued the AIS localization of voltage-gated sodium channels, neurofascin, and βIV-spectrin (Tseng et al., 2015). This indicates that outside the interaction with GABARAP, this mutant version of ankyrin-G is fully functional. We therefore hypothesized that the association

between GABARAP and the giant exon-encoded domain of 480kDa ankyrin-G is essential for stabilizing GABAergic inhibitory synapses (Tseng et al., 2015).

W1989R Loss of Function ankyrin-G Mouse as a Model for GABAergic Synapse Loss

Understanding the mechanisms underlying disrupted GABAergic interneuron connectivity is critical for developing new therapeutic strategies for patients with bipolar disorder. Studying the specific effects of loss-of-function ankyrin-G at GABAergic synapses *in vivo* has thus far not been possible since available animal models either die early in development or lack ankyrin-G at other neuronal domains such as the AIS and nodes of Ranvier. Paul Jenkins created a knock-in mutation of the W1989R mutation in *Ank3*. Andrew Nelson of the Jenkins lab immunostained brain sections for the AIS marker ankyrin-G as well as the paranodal marker Caspr and the nodal marker βIV-spectrin. The data showed ankyrin-G is still capable of clustering the AIS in the cortex of W1989R mice and the mice also have normal morphology and a normal number of nodes of Ranvier. Since the AIS and nodes of Ranvier are maintained in the W1989R mice, this knock in mouse provides a model to specifically study the loss-of-function ankyrin-G at GABAergic synapses. These mice also live well into adulthood (>P350), in contrast with the total ankyrin-G knockout mouse, and have no observable behavioral seizure activity. To assess the effect of the W1989R mutation on GABA synapses, coronal brain sections of the homozygous W1989R mice and WT littermates were immunostained with the presynaptic inhibitory marker, vesicular GABA transporter (vGAT). Preliminary data show the loss of inhibitory synapses on the soma and the AIS of glutamatergic neurons in layer II/III of the somatosensory cortex. Furthermore, the cultured hippocampal neurons from the homozygous W1989R mice have reduced postsynaptic GABA_A-receptor clustering on the cell surface compared to the clustering seen on the WT neurons. Whole-cell patch-clamp

recordings in whole brain slices showed a reduction in both the frequency and the amplitude of the GABA-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) in the homozygous W1989R mice compared to WT littermates. Intriguingly, coronal brain sections that were immunostained with PV revealed no change in the number of inhibitory PV+ interneurons in the homozygous W1989R mouse compared to WT (WT: 33.6 ± 3 , W1989R: 30.5 ± 3.3 cells/ $500\mu\text{m}^2$, $P=0.50$) and the mutant mice PV+ cells display normal fast-spiking electrophysiological properties when evoked. PV+ cells synapse on the soma, proximal dendrites and the AIS of excitatory glutamatergic neurons (Martin et al., 1983; Gilbert, 1994; Kawaguchi and Kubota, 1997). The data

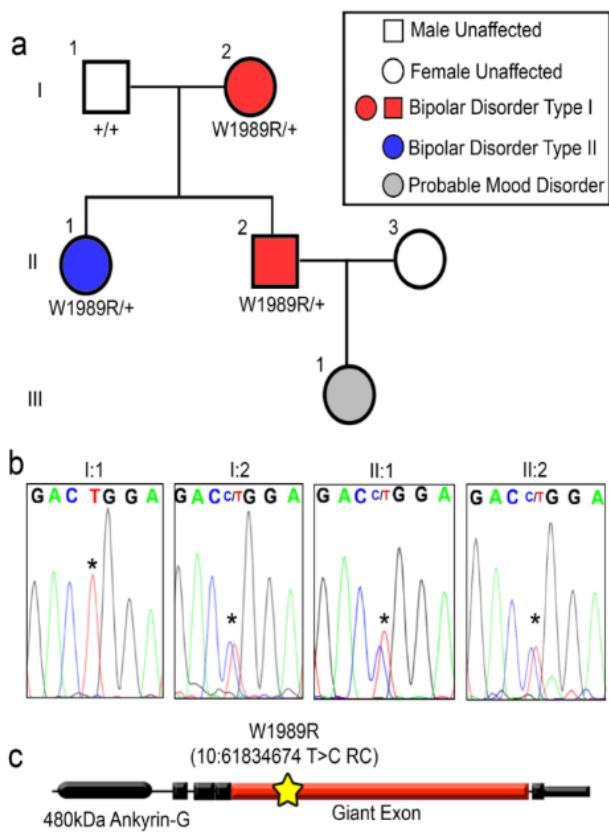


Figure 3. Identification of ANK3 W1989R in a family with bipolar disorder.

Family pedigree with individuals heterozygous for W1989R mutation ankyrin-G. With sequencing confirming the mutation in the mother and the proband.

suggest that the PV+ interneurons lose their connections to the AIS and the soma in the W1989R mutant mice. Understanding the mechanism of the GABA synapse loss may provide insight into novel therapeutic targets that restore the connection between PV+ interneurons and the glutamatergic cells.

W1989R Mutation found in Family with Bipolar Disorder

The Jenkins lab recently identified a family with bipolar disorder carrying the *ANK3* W1989R variant in a collaboration with the Heinz C. Prechter Bipolar Research Program at the University of Michigan. (**Fig. 3**) This variant

is found in ~1:10,000 unrelated European Americans, according to the most recent data from the gnomAD project. The proband, or starting point for the genetic study, (II:2, age 45) is a Caucasian male diagnosed with type I bipolar disorder, which is characterized by recurrent mania and depression, and is successfully treated with lithium. His mother (I:2, age 73) was diagnosed with bipolar disorder in her mid-30's and is currently diagnosed with chronic depression and irritability consistent with hypomania. His sister (II: 1, age 42) is diagnosed with BD type II with hypomania and mixed affective features. The proband's daughter (III:1) has been recently been diagnosed with major depression. Lastly, the father (I:1, age 72) has no history of any psychiatric disorder. Both the proband and the mother are heterozygous for the W1989R mutation. While preliminary data reported has been done in homozygote W1989R mice, future studies using heterozygotes will enhance our understanding of this human *ANK3* variant on neuronal circuitry. This will be valuable in identifying novel therapeutic targets to treat bipolar disorder. This patient data further emphasizes the translational importance of understanding the effects of the loss-of-function W1989R *ANK3* variant, which will have a broader on patients with variants in *ANK3* that lead to reduced mRNA expression.

Clathrin Mediated GABA_A-receptor Endocytosis

Ankyrin-G opposes the endocytosis of plasma membrane receptors such as its binding partners at the AIS and the GABA_A-receptor at the AIS and somatodendritic domains (Fache et al., 2004; Tseng et al., 2015). The GABA_A-receptor is dynamic in its location and changes between synaptic and extrasynaptic localization, as well as in intracellular compartments (Tseng et al., 2015). Recent studies have shown that in neurons lacking the 480kDa ankyrin-G, GABA_A-receptors undergo increased rates of endocytosis, or internalization, resulting in the loss of both

presynaptic and postsynaptic markers such as GABA_A receptors, gephyrin, and vGAT (Tseng et al., 2015). Attempts to rescue endocytosis were made using dynasore, a non-specific endocytosis inhibitor. Treatment with dynasore successfully rescued the loss of membrane GABA_A-receptor localization (Tseng et al., 2015). The mechanisms of endocytosis need to be elucidated, particularly in the W1989R model, to uncover a target to restore GABAergic synapse loss in W1989R mice. We hypothesized that following loss-of-function ankyrin-G, as seen in the W1989R mice, clathrin heavy chain (CHC) is able to directly interact with GABARAP to promote GABA_A-receptor endocytosis. (Kittler et al., 2000; Mohrluder et al., 2007). In polarized epithelial cells, the 190kDa isoform of ankyrin-G directly opposes CHC-mediated endocytosis to promote the localization of E-Cadherin (Jenkins et al., 2013) and the proper formation of the epithelial lateral membrane (Jenkins et al., 2015a). Clathrin mediates postsynaptic GABA_A-receptor endocytosis through clathrin-coated vesicles (Mohrluder et al., 2007). Furthermore, CHC has recently been shown to directly interact with GABARAP in nuclear magnetic resonance spectroscopy (Mohrluder et al., 2007). The proposed binding site of CHC and GABARAP are residues 512- 516 of the CHC (Mohrluder et al., 2007). This corresponds to amino acid sequence DWDFL, which overlap very well against the homologous GABARAP residues. The proposed binding site was tested *in vitro*, through the use of residues 510-522 of CHC. This synthetic polypeptide bound directly with recombinant GABARAP *in vitro* (Mohrluder et al., 2007). While this interaction suggests a potential antagonistic relationship between CHC and ankyrin-G, the functional role of this interaction has not been studied. Elucidating the mechanism of GABAergic synapse loss through CHC mediated endocytosis and mapping a binding site between CHC and GABARAP could provide a potential pharmacological target. If CHC directly binds to GABARAP to promote GABA_A-receptor endocytosis, developing a therapy specific to GABARAP that blocks

CHC's ability to bind could potentially restore GABAergic transmission in bipolar disorder patients. This would create a molecular phenocopy of a fully functional ankyrin-G system, in which ankyrin-G opposes CHC endocytosis.

Here, we begin to uncover the mechanism of GABA_A-receptor endocytosis by evaluating direct binding between CHC and GABARAP. We initially determined the effect of the W1989R mutation on GABA_A-receptor localization in cultured hippocampal neurons by immunostaining for the GABA_A-receptor and ankyrin-G and performing confocal microscopy. We expect to see a loss of GABA_A-receptor clustering in W1989R mutant neurons compared to WT neurons. We also attempt to co-immunoprecipitate CHC by overexpressing tagged GABARAP-V5 constructs in cultured NIH3T3 cells. Lastly, we tested a dominant-negative that contains the proposed binding site of CHC to GABARAP (Mohrluder et al., 2007), in W1989R neuronal cultures to oppose endogenous CHC binding to GABARAP and prevent GABA_A-receptor endocytosis in neurons lacking functional 480kDa ankyrin-G.

Materials and Methods

Constructs

The coding sequence of the GABARAP (UniProt: Q9DCD6) construct was PCR amplified from a mouse brain cDNA library. Gateway cloning was done with full length GABARAP-pENTR plasmids and destination vectors tagged with -GFP, pMal-6xHis, pGex-6xHis and -V5 using a ThermoFisher Scientific Gateway LR Clonase Enzyme kit. Destination vectors were selected for with ThermoFisher Scientific One Shot *ccdB* Survival 2 T1^R Chemically Competent Cells. Constructs for *in vitro* protein purification were cloned into destination vectors pMal-6xHis and

pGEX-6xHis. CHC pENTR had to undergo mutagenesis to remove a stop codon before use (Agilent Technologies QuikChange II Site-Directed Mutagenesis Kit). Confirmation of proper mutagenesis was done by sequencing the PCR product of the mutagenesis reaction. Constructs that used for IP analysis were cloned into a destination vector pcDNA6.2/N-EmGFP DEST or pLenti6-V5 DEST vector with AMP/CHLOR resistance. Digestion of -V5 clones was done with AscI (ThermoFisher Scientific) and AcII (Anza ThermoFisher Scientific) restriction enzymes to confirm GABARAP positive clones. Digestion of -6xHis clones was done with SpeI and ApaI (Anza ThermoFisher Scientific). 1% TAE gel electrophoresis was done with 100 ml 1X TAE buffer (48.4 g Tris base, 11.42 mL acetic acid, 20 mL 0.5M EDTA free acid pH 8.0; adjust final volume to 10 L) and 1 g Agarose, 7 μ L Radian GelGlow PreStain was used to visualize bands. Gold Biotechnology DNA ladder was used. Imaging was done using UVP Multi-User Imager. Clones were amplified in ThermoFisher Scientific TOP10 Chemically Competent Cells (*E. Coli*) and positively selected for using ampicillin plates. Qiagen Maxi Prep Kit was used for DNA isolation. Vector -pENTR, pLenti6 -V5 DEST, pMal-6xHis, and pGex-6xHis tagged vectors were constructed by my PI, Paul Jenkins. Vector pcDNA6.2/N-EmGFP DEST was a gift from Dr. Jeremy C. McIntyre of University of Florida. Truncated Clathrin fragment was designed based on the proposed GABARAP/CHC binding site, residues 512-516 of CHC (Mohrluder et al., 2007). Fragment was ligated into peGFP -N1 vector and amplified in ThermoFisher Scientific TOP10 Chemically Competent Cells (*E. Coli*). Sequencing done by the University of Michigan Sequencing Core confirmed successful cloning.

Neuronal Culture and Transfection

Hippocampi were dissected from postnatal day 0 (P0) mice, treated with 0.25% trypsin and 100 μ g/ml DNase in 2mL HBSS with 10mM HEPES, and then gently triturated through a glass pipette with a fire-polished tip. The dissociated neurons were then plated on poly-D-lysine and laminin-coated 35mm MatTek dishes in 0.5mL of Neurobasal-A medium containing 10% (vol/vol) FBS, B27 supplement, 2 mM glutamine, and penicillin/streptomycin. On the following day, 2.5 mL of fresh Neurobasal-A medium containing 1% FBS, B27, glutamine, and penicillin/streptomycin was added to the dish. AraC was added at 1:1000 to protect against glial and fibroblast overgrowth. Plates were returned to incubation at 37°C until experimentation. Cells were transfected at 21 days, and fixed at day 23. Antibodies against the GABA_A-receptor (ms, 1:1000) and ankyrin-G (rb, 1:1000) were used. To fill the cells with soluble GFP, the dissociated hippocampal cultures were transfected with an empty-eGFPN1 plasmid. Briefly, 3 μ g empty-eGFPN1 plasmid was added to 100 μ l of Neurobasal-A and, in a second tube, 3 μ l of Lipofectamine 2000 was added to 100 μ L of Neurobasal-A. 3 μ g truncated CHC fragment that contains the proposed CHC/GABARAP binding site in viral vector was added to 100 μ l of Neurobasal-A and, in a second tube, 3 μ l of Lipofectamine 2000 was added to 100 μ L of Neurobasal-A. The two tubes were mixed and incubated for 15 min at room temperature. The neuronal growth media was then aspirated from the dishes and saved, the transfection was added dropwise to 14 DIV neurons, and the transfected cells were incubated at 37°C for 1 hr. The transfection mixture was aspirated and the original neuronal growth media was added. The cells were maintained in culture until 21 DIV and fixed for immunofluorescence as described below.

Immunofluorescence of Cultured Neurons

Dissociated hippocampal neurons were fixed for 15 min at room-temperature with 4% paraformaldehyde, followed by methanol for 10 min at -20°C, and blocked with blocking buffer (5% BSA, 0.2% Tween 20 in PBS). Primary antibodies were diluted in blocking buffer and incubated at 4°C overnight. The following day cells were washed 3 x 15 min with PBS containing 0.2% Tween 20, incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature, washed 3 x 15 min, and mounted with Prolong Gold.

Confocal Microscopy

Samples were imaged on a Zeiss LSM 880 with a 60X NA1.4 Oil/DIC Plan-APOCHROMAT objective and excitation was accomplished using 405, 488, 561, and 633 nm lasers.

Western Blot

Untransfected and transfected NIH3T3 fibroblast cells were washed 3 times with PBS. 500 μ L of 5x PAGE buffer (5% (wt/vol) SDS, 25% (wt/vol) sucrose, 50 mM Tris, pH 8, 5 mM EDTA, bromophenol blue) was added to each 10 cm plate of cells. Lysates were sonicated for 10 1 second pulses with pulse amplitude set to 50%. The lysates were heated at 65°C for 10 minutes.

The lysates can be stored at -80°C indefinitely. The samples (10 μ L-volume) were ran on a 3.5-17% gradient gel in 1X Tris buffer, pH 7.4 (40 mM Tris, 20 mM NaOAc, and 2 mM NaEDTA) with 0.2% SDS. Transfer to nitrocellulose was performed overnight at 300 mA at 4°C in 0.5X Tris buffer with 0.01% SDS. Membranes were blocked with 5% BSA in TBS-T and incubated overnight at 4°C with primary antibodies (ms CHC/ms V5 1:1,000) diluted in blocking buffer. Membranes were washed 3 x 15 min with TBS-T and incubated for 1 hour with LiCor fluorescent

secondaries (1:5,000) in blocking buffer. Membrane were then washed 3 x 15 min in TBS-T, 1 x 5 min TBS, and 1 x 5 min in ddH₂O before being imaged on LiCor Odyssey Clx imager.

Antibodies and Reagents

The following antibodies and dilutions were used: rabbit anti-total ankyrin-G (1:1000, lab-generated), mouse anti-GABA_A receptor β2-3 (1:1000, Sigma, MAB341), mouse anti-Clathrin (1:1000, Fisher Scientific, MA1-065), mouse anti-GFP (1:1000, BioLegend 1GFP63), mouse anti-V5 (1:1000 Invitrogen R960-25). Fluorescently conjugated secondary antibodies Alexa Fluor 488, 568, or 647 (1:250, Life Technologies) and Alexa Fluor 594-Streptavidin (1:1000, Jackson ImmunoResearch 016-580-084). The following reagents were used: FBS, Poly-D-lysine, Laminin, Paraformaldehyde, DNase, Urea, and N-ethylmaleimide were from Sigma-Aldrich. B27 supplement, GlutaMAX, Penicillin-Streptomycin, Neurobasal-A, Hank's Balanced Salt Solution, Trypsin, Hepes, Lipofectamine 2000 and Prolong Gold Antifade Reagent were from Life Technologies. Bovine serum albumin was from Gemini Bioproducts. Tween 20 was from Calbiochem.

Protein Purification

Glycerol stocks were grown up in a 25 mL culture overnight. The culture was then diluted 1:50 in a 250 mL conical. The culture was grown until the OD recorded was roughly 0.800 (grown for about 1 hour). The 250 mL culture was induced using 500 μL 0.1 M IPTG. Induced cultures were shaken for 5 hours at 30°C. OD was recorded every 1 hour to ensure induction was taking place. Culture samples were taken at every hour. After 5 hours, hourly samples (1-2 mL) were pelleted for 30 seconds, 4°C at full speed using table top centrifuge. The pelleted was lysed with 50 μL 5×

PAGE buffer with DTT. Lysates were sonicated and heated at 65°C for 10 minutes. The samples (10 μ L volume) were ran on a 3.5-17% gradient gel in 1X Tris buffer, pH 7.4 (40 mM Tris, 20 mM NaOAc, and 2 mM NaEDTA) with 0.2% SDS. Gel was fixed with gel fix for 30 minutes and stained with Coomassie blue for one hour. Gel was washed 3x with DI water and destained overnight with gel destain and charcoal paper at 4°C with rocking. Imaged using UVP Multi-Use imager. The binding assay was performed by diluting overnight culture 1:20 in 250 mL and growing for ~1 hour until OD reached 0.800. 500 μ L 0.1 M IPTG was used for induction. Based on Coomassie gel, 4 hours was determined sufficient for proper induction. Induced culture was grown for 4 hours at 30°C. Pelleted 1-2 mL of culture, 30 s, 4°C full speed (table top centrifuge). The supernatant was aspirated and the pellet was washed 2x in 100 μ L STE buffer (100 mM NaCl; 10 mM Tris-Cl, pH 8.0). Washed pellet was resuspended in 100 μ L lysozyme in STE buffer on ice for 15 minutes. 15 μ L 10% sarkosyl (1.5 % final concentration) was added, vortexed for 5 seconds. Lysates were sonicated on ice in a bath sonicator, on full power. Debris were pelleted in a microfuge for 5 minutes at full speed, 4°C. Supernatant was transferred into a new 1.5 mL tube and 30 mL of Triton 10% x-100 (Sigma Aldrich) was added; vortexed and keep on ice. This supernatant, theoretically containing protein, was used for the binding assay.

Glutathione and nickel beads were washed 3x with STE buffer. Beads were pelleted for 30s on table top centrifuge, full speed, 4°C. 30 μ L of nickel beads was added to tubes containing protein bound to a maltose binding protein (MBP) from the original vector construct and a 6xHis tag. The histidine tag could bind to the nickel and the MBP enhances the solubility of the protein of interest. 30 μ L of glutathione beads was added to tubes containing protein with pGEX 6xHis because these constructs contain glutathione S-transferase (GST) gene fusion proteins which can bind to glutathione beads. 0.1 mM Imidazole, 500 μ L, was added to CHC/GABARAP pMal

6xHis—NI (nickel beads) to elute the protein from the resin. Rotated at 4°C for 10 minutes. Beads were pelleted for 5 mins on table top centrifuge, full speed, 4°C. Beads and supernatant were separated, supernatant contains purified protein eluted from nickel beads. CHC supernatant was added over GABARAP-pGEX6xHis—Glu beads; GABARAP supernatant was added over CHC-pGEX6xHis—Glu beads. (Supernatant still contained 6xHis tag and MBP because those factors were not cleaved away). Rotated at 4°C for 1 hour. Beads were washed 3x with STE buffer and 50 µL 5× PAGE buffer was added. Lysate was pelleted and samples (10 µL-volume) were ran on a 3.5-17% gradient gel in 1X Tris buffer for western blot analysis as described above. Two western blots were ran, one probed for CHC and one probed for GABARAP (ms CHC/GABARAP 1:1000).

Immunoprecipitation

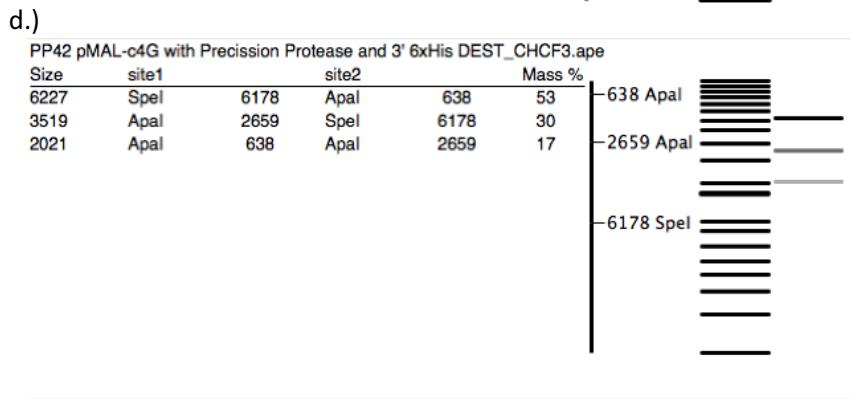
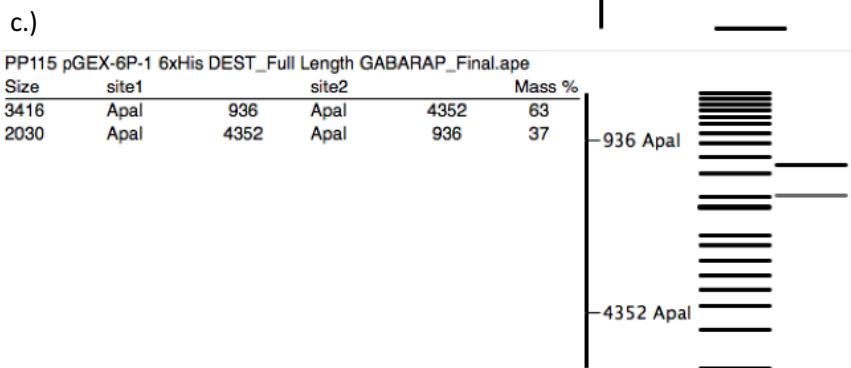
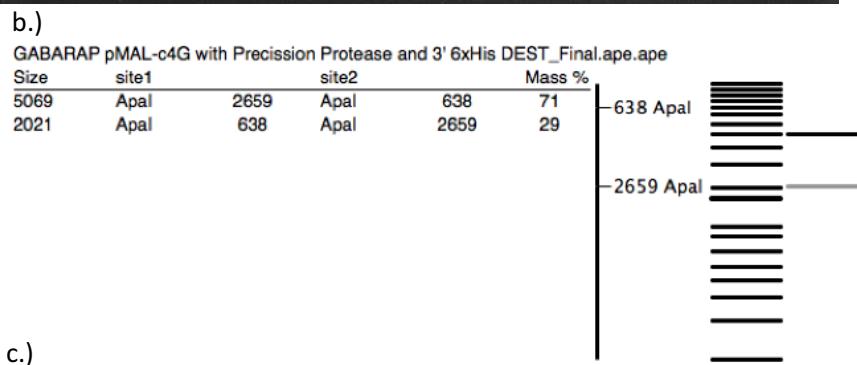
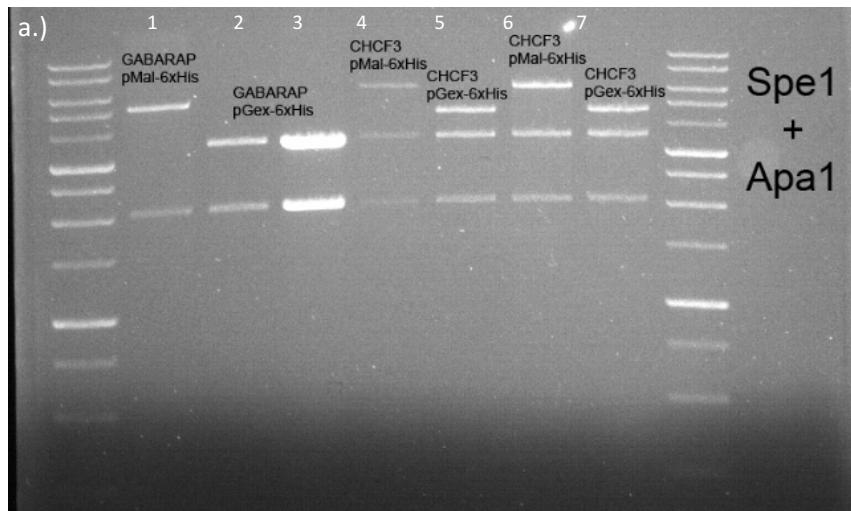
NIH3T3 cells were initially transfected with the GABARAP-V5 plasmid. Briefly, 5 µg of the GABARAP-V5 plasmid was added to 500 µl of Optimem and, in a second tube, 12 µl of Lipofectamine 2000 was added to 500 µl of Optimem. The two tubes were mixed and incubated for 15 min at room temperature. The media (1X DMEM, 10% Fetal Bovine Serum, 1% Pen-Strep) was then aspirated from the dishes, the transfection was added dropwise to cells in 10 cm TC treated dish, and the transfected cells were incubated at 37°C for 4 hr. The transfection mixture was aspirated and prewarmed media was added back. The cells were maintained in culture for 48 hours before aspirating media and washing cells 3x with PBS. Transfection efficiency was >90%. 500 µl FAK buffer (1% IPEGAL, 10% Glycerol, 1X Tris pH 8, 5M NaCl) with protease inhibitors was added to dishes and dishes were incubated on ice for 5 minutes before lysate was collected. Lysates were incubated on ice for 10 minutes. Lysate was passed through .5 mL syringe 5-7 times. 2 µl of anti-V5 antibody was added to lysate and nutated for 1 hour at 4°C. 50 µl of magnetic

protein-G beads (BioLabs) were added to the mixture and nutated overnight at 4°C. Mixture was placed on a magnet and supernatant was aspirated. Beads were washed 5x with PBS and 50 µl 5x PAGE buffer (5% (wt/vol) SDS, 25% (wt/vol) sucrose, 50 mM Tris, pH 8, 5 mM EDTA, bromophenol blue) was added. Lysates were heated at 65°C for 10 minutes. Sonication (10 1 second pulses, amplitude at 50%) was done if needed. 10 µl of lysate was loaded, using a magnet, onto 17% gradient gel in 1X Tris buffer, pH 7.4 for western blot analysis as described above. One blot was probed for CHC and another for GABARAP (ms CHC/GABARAP 1:1000). IP using GABARAP pLenti6 V5 DEST was repeated 3 times, IP using GABARAP pcDNA6.2/N-EmGFP DEST was repeated once.

Results

Identification of correct full length CHC and GABARAP pMal-6xHis/pGEX-6xHis Clones

To determine the interaction between CHC and GABARAP, we attempted to purify full length CHC and GABARAP *in vitro*. To do this, we made constructs that could be used in a binding assay after pure protein was isolated. CHC and GABARAP were first cloned into pMal-6xHis and pGex-6xHix tagged destination vectors using gateway cloning. The entry clone used for gateway cloning was GABARAP-pENTR. The restriction enzyme digest shows correct band length for GABARAP pMal 6xHis (fig. 4a) and correct band lengths for GABARAP pGEX 6xHis. The gel also revealed correct identification of CHC pMal 6xHis and CHC pGEX 6xHis (fig. 4a). Confirmation of bands was done by designing digestion on ApE plasmid editor software. (Fig. 4b-e).



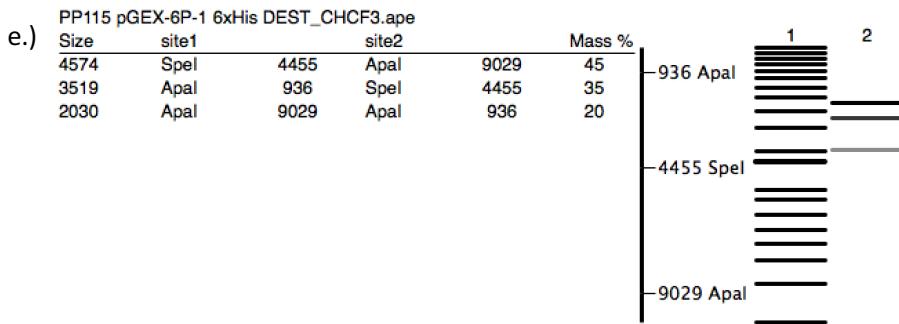


Figure 4: Confirmation of correct clones through restriction enzyme digest, correct band lengths observed post gateway cloning reaction utilizing GABARAP/CHC pENTR dTOPO as entry and pGEX/pMal 6xHis as destination vector. a.) Identification of clones using DNA electrophoresis. Clones were digested with SpeI and ApaI. GABARAP pENTR clone was used in the gateway cloning as the entry clone. b.) Correct band lengths for GABARAP pMal 6xHIS, 5069 kb and 2021 kb c.) Correct band lengths for GABARAP pGEX 6xHIS, 3416 kb and 2030 kb d.) Correct band lengths for CHC pMal 6xHis, 6227 kb, 3519 kb, and 2021 kb e.) Correct band lengths for CHC pGEX 6xHIS, 4574 kb, 3519 kb, and 2030 kb

High levels of CHC and GABARAP protein expression at following IPTG induction

Constructs GABARAP pMal-6xHis, GABARAP pGEX-6xHis, CHC pMal-6xHis, and CHC pGEX-6xHis were used in the *in vitro* protein purification assay. Full length GABARAP and CHC were grown in bacterial culture by inducing expression using 0.1 M IPTG (500 μ L). We used IPTG because it triggers transcription of the *lac* operon and can be used to induce protein expression when the gene is under the control of the lac operator. Coomassie stain allowed us to visualize the protein expression in the gel. One gel showed induction at time points 1-3hr (fig. 5a) and a second showed induction at time points 3-5hr (fig. 5b). Induction of protein at correct molecular weight (with fusion protein weight taken into account) was seen at all time points (fig. 5a, b). Hour 4 was determined to be sufficient to induce proper expression of protein. We then induced protein expression for 4 hours and purified protein for the *in vitro* binding assay.

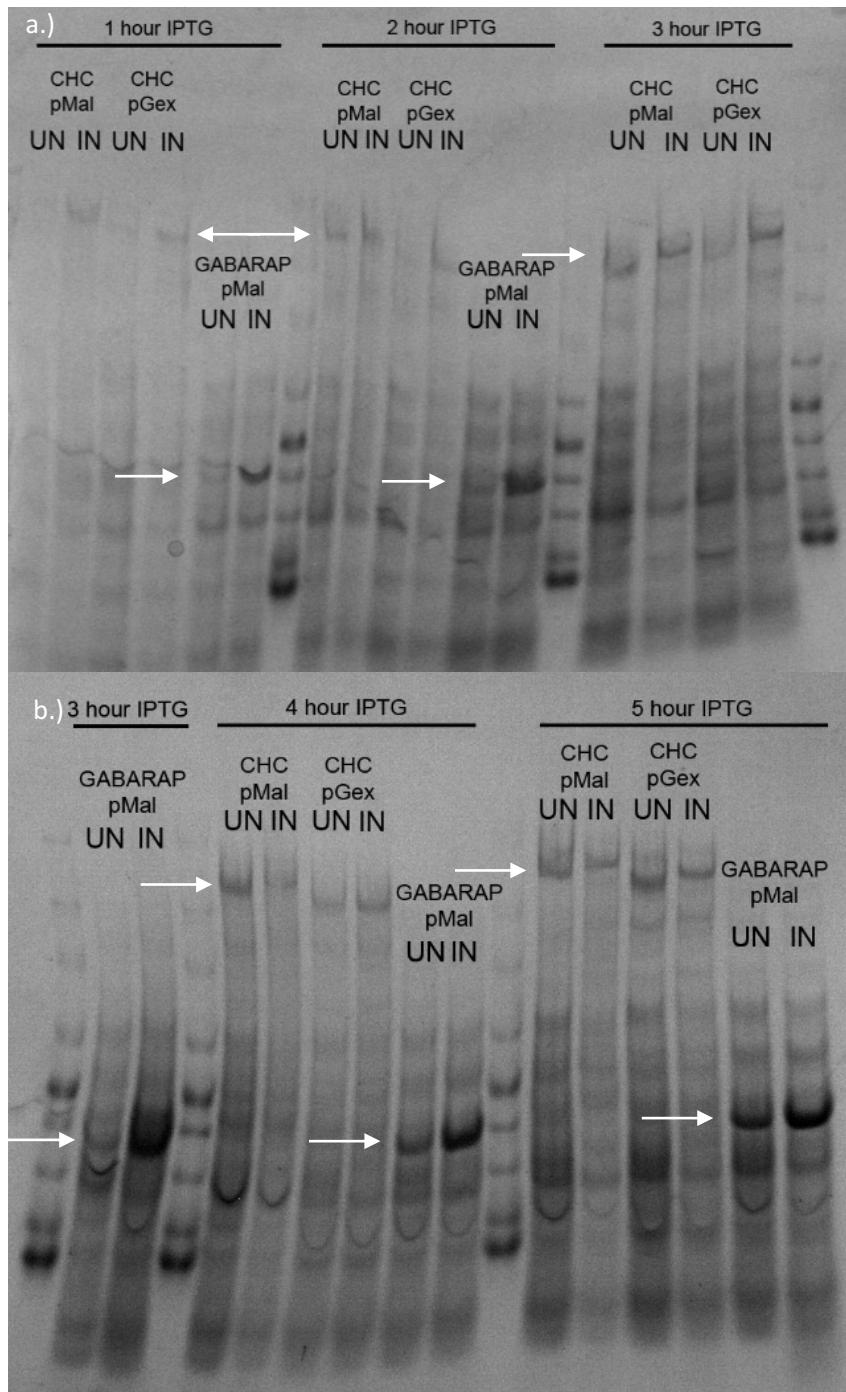


Figure 5: Protein purification yields high expression of GABARAP and CHC at 1 hour post induction continuing to 5 hours post induction. a.) GABARAP pMal 6xHis, GABARAP pGEX 6xHis, CHC pMal 6xHis, and CHC pGEX 6xHis protein purification at hours 1-3. Bands of interest noted with arrows. b.) GABARAP pMal 6xHis, GABARAP pGEX 6xHis, CHC pMal 6xHis, and CHC pGEX 6xHis protein purification at hours 3-5. Bands of interested noted with arrows.

Western Blot Analysis of Binding Assay from Purified Proteins

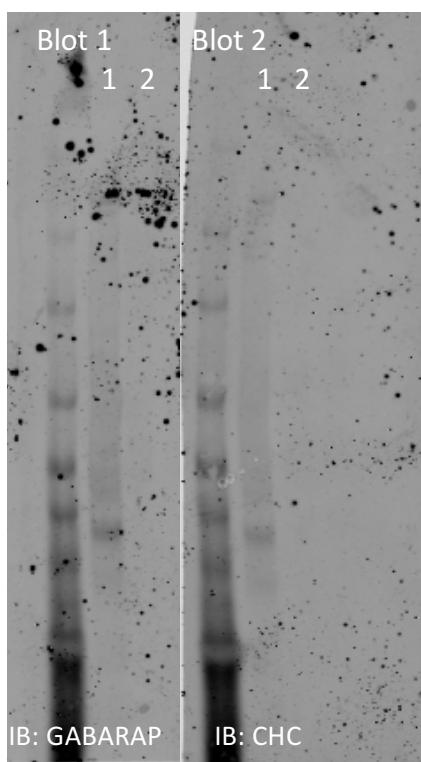


Figure 6: Western blot reveals lack of protein purification and lack of co-isolation of CHC and GABARAP.

After completing the binding assay and performing a western blot to probe for the presence of both GABARAP and CHC, we found that there was no detectable protein on the glutathione resin (fig. 6). Even if the two proteins did not bind, we should still see expression of the protein that was bound to the glutathione beads because the glutathione beads were still in the sample used to run the western blot. In other words, even if we ran one pure protein supernatant over the other pure protein bound to beads and they did not bind, or the pure protein supernatant did not contain the isolated pure protein, then the protein on the beads should be the only result visualized in the western blot. Since we did not see this, this means that during the assay we prematurely washed away our protein of interest. If GABARAP and CHC are

able bind, then the pure protein in the supernatant will remain on the glutathione beads even after final washes. The assay needs to be repeated. But before it is, the methods also need to be improved upon to obtain an accurate result. For example, an issue with how it was originally done is that we did not cleave the MBP from the protein. The MBP is a large 42.5kDa protein that could interfere with CHC/GABARAP interaction. There are also other caveats to this method of experimentation. Lack of CHC/GABARAP interaction using purified proteins could be due to lack of post-translational modifications that are necessary for their ability to bind, or necessary adaptor proteins that facilitate the interaction *in vivo*. In general, we could not form any conclusions based on this assay alone because there are many ways it could render a false negative. Thus, we wanted to

perform immunoprecipitation experiments in mammalian cells to detect binding between CHC and GABARAP because in this assay we are able to more reliably express GABARAP protein and therefore more reliably test binding interactions.

Identification of correct full length GABARAP-V5 Clones

For the IP, we used GABARAP-V5 as well as a GABARAP-GFP fusion proteins. Gateway cloning was used to clone GABARAP into V5 and GFP tagged destination vectors. Restriction enzyme digest revealed proper cloning of GABARAP pLenti6 V5 DEST (Fig. 7a). Lane 5 contained an additional band, suggesting incomplete digestion or alternative product, so this construct was not used. Appropriate band intensity was also observed. GABARAP pLenti6 V5 DEST was later used in immunoprecipitation.

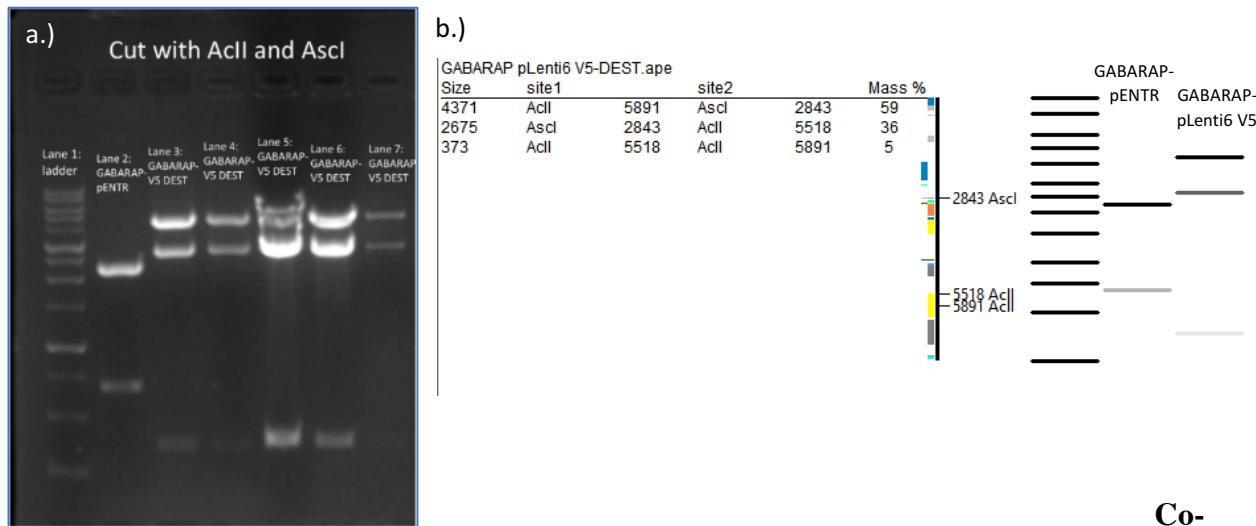


Figure 7: Confirmation of correct clones through restriction enzyme digest, correct band lengths observed post gateway cloning reaction utilizing GABARAP pENTR dTOPO as entry and pLenti6 V5 DEST as destination vector. a.) Identification of clones using DNA electrophoresis. Clones were digested and compared against Previously confirmed GABARAP pENTR clone. GABARAP pENTR clone was used in the gateway cloning as the entry clone. b.) Confirmation of bands was done by designing digestion on ApE plasmid editor software. AsII and AscII cut sites shown.

Immunoprecipitation of GABARAP and Clathrin Heavy Chain

Immunoprecipitation (IP) of overexpressed full length GABARAP in NIH3T3 cells was performed to determine whether GABARAP and CHC are capable of interacting in mammalian cells. Western blot revealed that GABARAP-GFP transfected NIH3T3 cells undergo IP, CHC is faintly co-precipitated, whereas CHC is seen in untransfected cells with IP with greater intensity (Fig. 8d). Positive control (non-IP lysate) confirms the presence of CHC and absence of GABARAP in untransfected cells (Fig. 8d). Blot probed for chicken anti-GFP confirms the presence of GABARAP in transfected cells, with or without IP (Fig. 8c). Given this, we moved to using the GABARAP-V5 as the addition of a 27 kDa -GFP tag to GABARAP (13kDa) may affect binding with CHC. In contrast, V5 is a small epitope that would be less likely to affect protein-protein interactions. Western blot revealed that GABARAP-V5 transfected NIH3T3 cells undergo IP, CHC is precipitated, whereas untransfected cells that undergo IP do not show CHC with similar intensity (Fig. 8b). Positive control (non-IP lysate) confirms the presence of CHC and absence of GABARAP in untransfected cells (fig. 8b), however, in the blot stained for V5, presence of GABARAP was not seen in transfected cells (Fig. 8a). We are currently repeating this experiment with more developed procedures to obtain a more conclusive result.

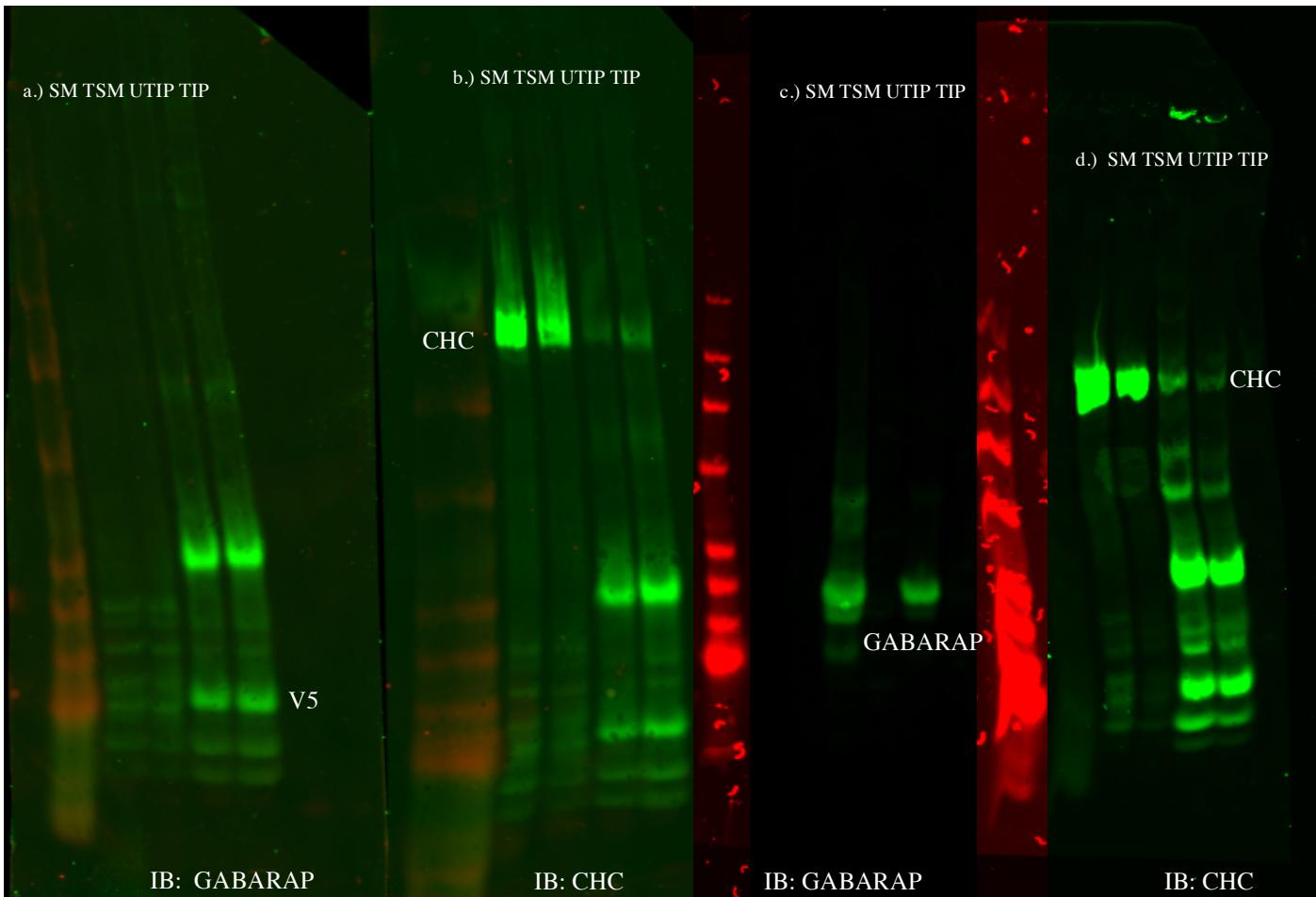


Figure 8: Western Blot of NIH3T3 cells expressing GABARAP after IP was done to observe interaction between GABARAP and CHC. Determination of correct band weight was done with destination vector weight added. SM: Starting material; TSM: transfected starting material; UTIP: IP performed on untransfected cells; TIP: IP performed on transfected cells

- a.) NIT3T3 cells transfected with GABARAP pLenti6 V5 DEST underwent IP to detect CHC precipitation. Blot was probed with mouse anti-V5.
- b.) NIT3T3 cells transfected with GABARAP pLenti6 V5 DEST. Blot was probed with mouse anti-Clathrin. CHC seen at correct molecular weight.
- c.) NIT3T3 cells transfected with GABARAP cDNA6.2/N-EmGFP DEST. GABARAP seen at correct molecular weight.
- d.) NIT3T3 cells transfected with GABARAP cDNA6.2/N-EmGFP DEST. CHC seen at correct molecular weight.

W1989R Cultured Hippocampal Neurons Show decreased GABA_A-Receptor expression.

Dissociated W1989R hippocampal neurons show decreased localization of the GABA_A-receptor at the somatodendritic domain and AIS (labeled with AnkG) as compared to wild-type neurons. In addition, there was no change in W1989R ankyrin-G localization compared to WT ankyrin-G (fig. 9). Immunofluorescence revealed that the truncated fragment partially restores the GABA_A-receptor expression at the somatodendritic and AIS membranes, suggesting that the clone could serve as a dominant-negative.

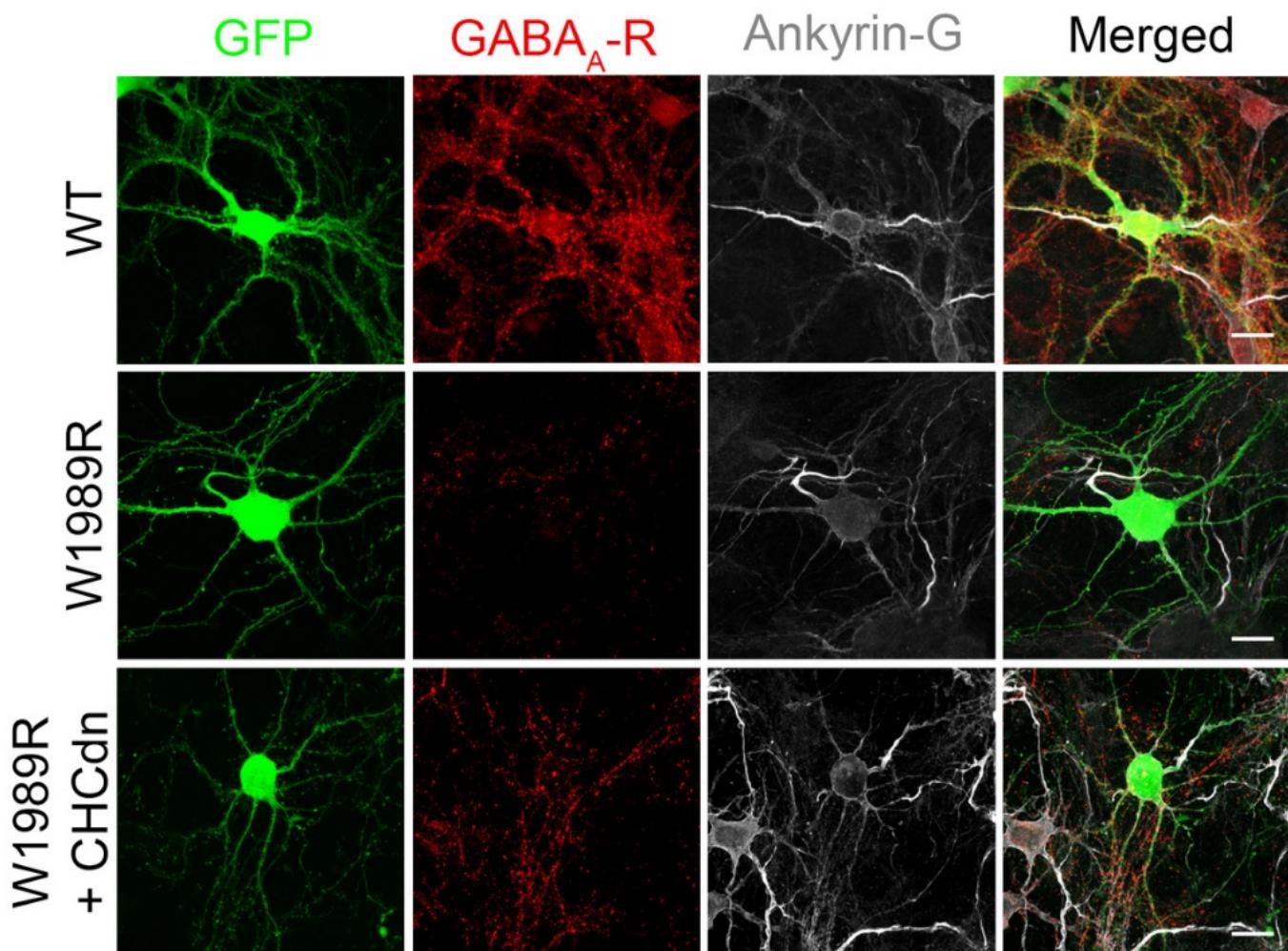


Figure 9: Hippocampal neurons from WT, W1989R, and W1989R transfected with CHC proposed dominant-negative (CHCdn). Immunostained for GABA_A-receptor and ankyrin-G. Scale bar 20 μ M.

Discussion

Significant progress has been made in recent years uncovering the underlying mechanisms of bipolar disorder; however, progress has been delayed due to many biases in the neuropsychiatric research. For example, Emil Kraepelin hypothesized that some neuropsychiatric disorders, such as schizophrenia, are structural brain disorders, bipolar disorder has no neural substrate (Konradi et al., 2011). Despite this theory, an increasing number of studies have shown that bipolar disorder is a result of altered neuronal circuit function and intracellular signaling (Harrison et al., 2018). Bipolar disorder is highly heritable, meaning it carries a strong genetic component. In fact, a child with an affected parent has about a ten-fold increase in susceptibility to the disease, and twin studies show heritability estimates of between 0.7-0.8 (Harrison et al., 2018). However, the importance of susceptible loci, SNPs with high correlation with disease, have been shown to be more and more important in the inheritance of the disease. It is therefore equally important to understand the functional effects of human variants on the brain, both molecularly and functionally (Schulze et al., 2009; Hatzimanolis et al., 2012; Leussis et al., 2012; Harrison et al., 2018). *ANK3* is implicated in many neuropsychiatric diseases, however much more work needs to be done in order to understand its implications with regards to bipolar disorder. The presence of the W1989R variant in a family with bipolar disorder demonstrates the importance of understanding how this loss-of-function mutation contribute to the pathophysiology of bipolar disorder. Abnormalities in GABAergic inhibitory circuitry have also been implicated in a variety of neurological disorders. Thus, understanding the relationship between ankyrin-G and CHC and how they interact with the GABA_A-receptor could provide a better understanding of the abnormalities in GABAergic synapses in bipolar patients and could uncover novel therapeutic targets to rescue these affects.

Here we saw a reduction in the GABA_A-receptor levels in neurons with the W1989R mutation as compared to WT neurons. In order to investigate the mechanisms of GABA_A-receptor loss and to ultimately create a means to restore receptor loss, I purified CHC and GABARAP proteins to determine if they bind through western blot analysis. I was able to induce large amounts of protein to use in this analysis. However, after performing a binding assay using glutathione and nickel beads, I did not observe any protein. This suggests that the proteins were washed away during some point in the assay because even the proteins that were supposedly anchored to beads in the lysate used for the western blot are not seen, meaning that the lysate sample used contained no protein of interest. While the blot probed for CHC and the blot probed for GABARAP showed lack of binding, this is not indicative of what is occurring endogenously in mammalian cells. Other than potential for the proteins to have been prematurely washed away, there are alternative explanations for this result. Purified proteins were grown in bacteria cells, which synthesize and package proteins in a manner different from eukaryotes. This means the protein is free of other endogenous factors and post-translational modifications that may be necessary for binding *in vivo*, such as adaptor proteins, kinases, palmitoylation enzymes, etc. The vector was also tagged with MBP, which is a large component that can interfere with protein-protein interactions. The large tag could interfere with the ability of the proteins to bind, especially considering the binding site consists of a small number of residues. If the method is repeated in the future, we should collect samples at “check point” steps and run western blots to isolate in which steps we lose protein. We therefore moved onto co-IP. In the co-IP experiments, I transfected the constructs containing GABARAP into cells that already possess endogenous CHC. NIH3T3 cells do not contain endogenous GABARAP, but contain the potential necessary factors (adaptor proteins, kinases etc.) that would enable binding of CHC and GABARAP. It was in this assay that we do see evidence

of CHC and GABARAP binding, as I was able to co-precipitate CHC from cells with overexpressed GABARAP (fig. 8b). While there appears to be an interaction, some CHC remains in an untransfected cell elution, even after IP is done. This was also seen in previously published results (Mohrluder et al., 2007) and could indicate the protein's "stickiness" or nonspecific binding to the protein G beads that results in a faint signal. A confounding result is that in cells transfected, GABARAP was not shown (fig. 8a). However, GABARAP was precipitated in the IP done with the GFP tagged vector, though very faint expression of CHC was seen (fig. 8c, d). CHC may not be able to co-precipitate because of the obstructive effects of the large GFP protein that prevent CHC, bound to the GFP vector, from binding appropriately. Possible ways of improving upon technique would be increasing washes to ensure that the endogenous cell products are not included in the final elution, as well as preclearing the lysate. Preclearing the lysate before adding antibody to the lysate would insure that nonspecific binding to protein-G beads is controlled for. Protein-G beads should be added to the lysate before the antibody to allow for nonspecific compounds to bind. The supernatant should be separated from the beads and the precleared supernatant should subsequently be used for the IP. This would ensure a reliable result from the IP and enhance the evidence of a CHC/GABARAP interaction.

Possible future directions after consistent IP could be mutating the binding site of CHC/GABARAP. The possible binding site contains CHC residues 512-516 (Mohrluder et al., 2007). However, confirmation of this finding can be done through yeast two hybrid screens. Mutating this binding site by single amino acid changes could serve to create a more effective dominant-negative that could oppose CHC mediated endocytosis. Mutagenesis could be used to create dominant-negative mutants and IP could be used to demonstrate nullified CHC/GABARAP binding and rescue of GABA_A-receptor membrane localization *in vitro*.

We tested the possibility of a dominant-negative mutant by cloning a short fragment of CHC that contains the proposed binding site into a -GFP tagged vector. This potential dominant-negative was transfected into W1989R neurons to observe whether we could restore GABA_A-receptor clustering similar to WT levels. We found that the short fragment did not fully restore the GABA_A-receptor plasma membrane localization in W1989R cells, but there is evidence or restoration. More imaging as well as quantification of these results needs to be done to determine the clone's efficacy as a dominant-negative. We also want to improve proposed dominant-negative so that it functions more suitably in the cells. The particular fragment we used was inserted into a -GFP tagged vector. -GFP is large protein with a molecular mass of 27kDa. This is especially large compared to the small fragment which contains 10 amino acid residues. This could affect the ability of the short fragment to bind to GABARAP in culture and therefore it would be unable to act as a dominant-negative. To mitigate this, we can use a vector with a smaller tag, such as a FLAG-tag which is only 8 amino acids. A way to overcome this issue completely would be to use a vector with a T2A "self-cleaving" peptide. This T2A sequence would be in between the CHC fragment and the vector tag, such as GFP. Therefore, when the clones with the CHC fragment and the T2A are expressed in culture, expression of the T2A will cleave the CHC fragment away from the GFP allowing it to function as a dominant-negative, irrespective of the large GFP tag. The GFP tag, however, could function to allow visualization of transfected cells. This would allow us to assess the efficacy of the dominant-negative alone, without confounding factors related to the vector vehicle.

Going forward, there are many avenues to understanding the GABAergic synapse loss in loss of function ankyrin-G neurons. However, understanding the relationship between CHC and GABARAP, as well as ankyrin-G and GABARAP would underlie future study. CHC may not

bind to GABARAP, it may bind directly to the GABA_A-receptor. If CHC does bind not bind to GABARAP, or alternatively, it selectively binds to one isoform of GABARAP, then mutating CHC at the binding site will be more efficacious in preventing receptor endocytosis. It will also be advantageous to study CHC adapter protein interactions. Adapter proteins may mediate the binding of CHC and GABARAP to allow for endocytosis. Mutating these adaptor proteins may also serve as an efficacious target for prevent endocytosis. CHC/GABARAP interactions would need to be fully elucidated to create a rescue mechanism. Fully mapping the binding site between these proteins is of the utmost importance. Possible experimentation that could accomplish this could include proximity ligation experiments. Proximity ligation (PLA) is a technology that allows for more specific analysis on protein interaction than traditional immunoassays allow. The technique uses one pair of oligonucleotide labeled secondary antibodies that bind when they are in close proximity, which is if the proteins the antibodies recognize are in close proximity. A single primary antibody is used, just as in traditional immunofluorescent assays, and the secondary antibodies equipped with oligonucleotides then hybridize and create a DNA circle. The DNA circle is amplified, and fluorescent probes against the DNA can be added to view this amplification under the microscope. Fluorescent can therefore only be achieved one the secondary antibodies are in close enough proximity to allow for DNA circle amplification. This indicates protein proximity of 10-15 nm, which can signify binding.

The ultimate goal of these approaches is to create a more scientifically informed or evidence based approach to treating, diagnosing and categorizing bipolar disorder. Bipolar disorder is another example of a neuropsychiatric disease involving mood, emotion and behavior that has been excluded from neurobiological study (Harrison et al., 2018). Better interventions that take into account genetics should be the future of bipolar disorder clinical treatment.

Understanding the role ankyrin-G in neuronal function will also prove productive in understanding other neurological disorders, such as schizophrenia and autism. *ANK3* has been seen as a genetic risk factor for these diseases as well. While the etiology of bipolar disorder remains unsettled, the work presented here represents some of the first steps in elucidating an underlying physiological/neurological component to the disorder. GABAergic synapse reduction is an increasingly strong candidate for this component. Future work with the W1989R mutation and CHC/GABARAP could inform a more effective pharmacological agent in treating bipolar disorder, that works to restore this synapse loss.

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