Viral-Mediated Overexpression of Neuroligin2 in the Adult Hippocampus Leads to Enhanced Synaptic Inhibition

by

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Abstract:

In this thesis, I explore the effect of using a viral vector AAV to overexpress the inhibitory synaptic protein neuroligin2 (NLGN2) in the hippocampi of adult mice. I hypothesize that NLGN2 is necessary and sufficient to drive the formation of GABAergic synapses in the mammalian adult hippocampus. The goal of my experiments was to use western blot analysis to determine whether AAV-mediated viral delivery of NLGN2 led to concomitant increases in the expression of other proteins localized to the GABAergic synapse. I measured the change in total protein concentration of important inhibitory pre and post-synaptic marker proteins and found that AAV significantly increases expression of NLGN2 in the hippocampus. Moreover, this change in NLGN2 expression is coupled with comparable increases in other inhibitory synaptic proteins. VGAT, an important pre-synaptic inhibitory protein was significantly increased, and gephyrin, an important post-synaptic inhibitory protein was also increased.
Acknowledgements:

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Part 1. Introduction

Introduction:

The nervous system maintains an important balance of inhibitory and excitatory signals in order to function properly. The underlying molecular and cellular mechanisms are not fully understood (Michael Okun and Lampl 2009). In the mammalian forebrain, the majority of inhibitory synapses are formed by GABAergic interneurons. These synapses use the signaling molecule GABA, which binds to GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the post-synaptic cell to create an inhibitory post-synaptic current (IPSC), preventing the cell from sending a signal of its own. Co-activation of inhibition and excitation is a basic functional principle in many neurological activities in the cortex (JS Isaacson and Scanziani 2011),(N.C. de Lanerolle, J.H. Kim et al. 1989). Loss or dysfunction of inhibitory GABAergic interneurons has been linked to a number of neurological disorders, including autism and temporal lobe epilepsy (TLE) (Damasio and Maurer 1978). It has been found that increased excitability of granule cells (GCs) in the dentate gyrus of the hippocampus is partially responsible for epileptogenesis in TLE (Dudek and Staley 2012). To better understand these disorders, and how the brain functions as a whole, it is important to understand the development and regulation of the inhibitory synapse, something that is still not well understood.

To understand synaptic inhibition, a better understanding of the molecular components of GABAergic synapses is necessary. It is not known how the formation of inhibitory synapses is regulated, or whether an alteration in neurogenic proteins is capable of altering inhibitory synapse number, size or location. Neuroligin2
(NLGN2) has been identified as a key cell adhesion protein involved in GABAergic synapse formation and stabilization (Craig and Kang 2007). The virus AAV provides a safe and efficient method for delivering the NLGN2 gene to the hippocampus of adult mice in order to overexpress NLGN2. Western blot analysis effectively quantifies the synaptic change in protein expression caused by NLGN2 overexpression, elucidating the role of NLGN2 in the development of the inhibitory synapse.

**Temporal Lobe Epilepsy and Seizure Disorders:**

Epilepsy is characterized by a surge of electrical activity in the brain, usually directly caused by an onset of excitatory firing. This massive spike in neural activity will almost always cause a change in behavior, often a spasm or series of spasms. These incidences can lead to significant cognitive defects, even excluding the external damage it is possible to sustain during an episode. However, a single seizure does not constitute a disorder. The disease is characterized as epilepsy only when an individual suffers from spontaneous, recurrent seizures (French, Williamson et al. 1993). The most common form of epilepsy is TLE, which arises from an excitatory synaptic imbalance in the temporal lobes. The imbalance is created by malformation in the brain during a period of epileptogenesis, generally after the brain suffers an insult, like physical trauma (Dudek and Staley 2012).
Seizures can cause aberrant neurogenesis of newborn excitatory granule cells. These cells can be characterized by aberrant basal dendrites and enhanced spine structures (Sebastian Jessberger, Chunmei Zhao et al. 2007). Aberrant neurogenesis has been shown to be a root cause of the disorder, as it has been previously demonstrated that inhibitors of adult neurogenesis, like rapamycin, suppress seizures in mouse models (Ljungberg, Sunnen et al. 2009). Loss of inhibitory interneurons in CA1 and CA3 of the hippocampus, coupled with altered gene expression and physiological changes leads to a new, hyperexcitable environment for the GCs, allowing for epileptic seizures and associated cognitive deficits (Tang and Loke 2010). One possible way to treat the root causes of TLE is to increase synaptic inhibition in the hippocampus in order to fix the imbalance that resulted in the condition.

**The Molecular Composition of GABAergic Synapses:**

GABAergic interneurons maintain much of the balance of excitation and inhibition in the mammalian forebrain, and can form long-reaching axons to inhibit multiple cells, such as GCs. The GABAergic synapse includes a plethora of proteins, and is based around the signaling molecule, GABA. There are two types of GABA receptors. The first is the ionotropic GABA_A receptor, which opens a chlorine channel upon binding of GABA, directly hyperpolarizing the cell. The second is the metabotropic GABA_B receptor, which is linked by G-proteins to potassium channels to hyperpolarize the cell after an action potential. Through these mechanisms, both
receptors serve to inhibit the future firing of action potentials by the post-synaptic cell in the presence of GABA. GABA is released from the pre-synaptic GABAergic interneuron via vesicles by the vesicular GABA transport protein (VGAT). The GABA receptors are found on the post-synaptic cell as a part of a scaffold, which is mostly organized by a key scaffolding protein gephyrin, which is regulated by a guanine nucleotide exchange factor collybistin. There are many proteins involved in this scaffold, however postsynaptic neuroligins and the presynaptic neurexins they bind to have been identified as particularly important for the stabilization of the inhibitory synapse (Craig and Kang 2007)(See figure 1).
Figure 1: Components of the GABAergic Synapse

Figure 1: NLGN2 guides membrane tethering of the inhibitory postsynaptic scaffold by binding the scaffolding protein gephyrin as well as the gephyrin regulator collybistin (Varoqueaux, Zhang et Al. 2009). NLGN2 also stabilizes inhibitory synapses by binding with a presynaptic protein neurexin, tethering the synaptic membranes together. On the pre-synaptic side, VGAT mediates release of GABA, which binds to GABA_A receptors on the post-synaptic side, initiating the IPSC.

(Adapted from Pizarelli and Cherubini, 2011)
**The Neuroligin Family of Synaptic Proteins:**

NLGN2, the key protein in this study, is one of 5 protein isoforms of neuroligins: NLGN1, NLGN2, NLGN3, NLGN4X and NLGN4Y. All but NLGN4X have been found in the rodent brain. NLGN4X has been identified as a human-specific protein (Kreuger, Tuffy et al. 2012). These proteins are all genetic paralogues of each other, but are found in different cell types across the nervous system. Other than expression, which differs by cell type, the main differences between them are the additions or deletions of specific domains. They all maintain a membrane-bound portion and an end for protein binding (van der Kooij, Fantin et al. 2014) (See figure 2).

**Figure 2: Structure of NLGN2 and NLGN1**

![Figure 2](image)

Figure 2: The structures of NLGN2 (a) and NLGN1 (b) are very similar for the bulk of the protein in domain 1 (shown in green), which binds to the post-synaptic membrane and neurexin. The amino acid sequence differences are shown in (c). The two paralogues differ mainly in an added domain for NLGN1, shown in gray in panel (b) and in orange in panel (c).

Figure adapted from van der Kooij, M. A., et al. (2014). "Impaired Hippocampal Neuroligin-2 Function by Chronic Stress or Synthetic Peptide Treatment is Linked to Social Deficits and Increased Aggression." Neuropsychopharmacology 39(5): 1148-1158. Adapted with permission.
The NLGN family is composed entirely of cell adhesion proteins, which all have pre-synaptic neurexins as binding partners. Taken together, these protein pairs serve to physically hold synapses together, like molecular glue (Camin Dean and Dresbach 2005). NLGN2 is the main neuroligin found GABAergic synapses, but NLGN3 is present in GABAergic as well as glutamatergic synapses (Budreck and Scheiffele 2007). Throughout the nervous system, the other neuroligins also serve to hold synapses together. Various studies on NLGNs have determined them to play important roles in synaptic development, but NLGN2 stands out as the main NLGN required for GABAergic synapses.

In in vitro studies, NLGN2 has been found to induce synapse formation at points of contact between axons of adjacent neurons. These cultured neurons also displayed increases in synaptic density with NLGN2 overexpression, suggesting it plays an important role in synaptic development (Sheiffele, Fan et al. 2000). Previous in vivo overexpression studies in adult mice showed drastic increases in inhibitory synaptic contact size in the frontal cortex. Moreover, EEG analysis demonstrated that this synaptic increase was linked to increased inhibition in these transgenic mice (Hines, Wu et al. 2008). When NLGN2 is knocked out genetically, inhibitory synapses are still able to form, but electrophysiological data demonstrated that there were fewer IPSCs in KO adult mice. They had malformed inhibitory synapses that led to an imbalance of excitation and inhibition. This was likely due to the lack of NLGN2 needed to maintain and stabilize the inhibitory synapses (Pouloupolos, Arumuni et al. 2009). Further studies in NLGN2 deficient mice found fewer GABA receptors and other vital post-synaptic proteins in GCs.
They also found drastically increased excitation of those GCs, due to a lack of inhibition. This further suggests that NLGN2 plays a major role in proper formation and maintenance of inhibitory synapses (Jedlicka, Hoon et al. 2011). Overall, studies suggest that NLGN2 is a key protein in maintaining synaptic inhibition in the mammalian brain. However, its role in development has not been well understood. It is not yet known which proteins NLGN2 interacts with in the inhibitory synapse. One way to better understand the role of NLGN2 in the inhibitory synapse is by analyzing the protein changes in inhibitory synapses when NLGN2 is overexpressed.

**The AAV Vector as a Method for Gene Delivery:**

An effective method of determining whether it is possible to alter inhibitory synapse size, location or number through changes in single neurogenic protein is to analyze the effects of altered genetic expression. For this thesis, my lab overexpressed NLGN2 in the hippocampus of adult mice. To affect gene expression in a targeted location, we injected an adeno-associated virus (AAV) into the hippocampus of adult mice. AAV has been used therapeutically, and as an effective research tool to alter neuronal activity and study the formation of synapses in the cortex (Fekete, Chiou et al. 2015). It is an excellent method for synaptic gene delivery, due to its ability to sustain expression long-term in neurons and infect a variety of tissues by integrating directly into the genome of targeted cells. Moreover, the immune response to AAV is relatively mild compared to other vectors. It is replication-defective, so it is unable to form all of the necessary viral particles for
viral genome replication or synthesis, rendering it relatively safe (Zaiss, Liu et al. 2002).

There are many different genetic isoforms of the AAV virus, but for this thesis, the recombinant isoform AAV-DJ was used. It is a hybrid virus made from the genetic recombination of several different wild-type AAV isoforms: AAV avian, bovine, goat, 2, 4, 5, 7 and 9. By combining viral cap sequences from fragments of multiple different pre-existing viruses, AAV-DJ has an enhanced ability to transduce into multiple cell types, allowing it to infect many different species and tissues. This versatility makes AAV-DJ an excellent vector for research (D. Grimm, J. S. Lee et al. 2008).

The main downside to using AAV as compared to other gene delivery systems is its size. It will hold less than 5 kb, severely limiting the number and size of genes it is able to deliver (Kurylo 2009). For this research, all that the virus needs to deliver is a CMV promoter to maintain overexpression, a fluorescent reporter gene (mCherry) and the gene for NLGN2 itself. This set of genetic information fits within the limits of the AAV vector, making it a reliable and safe way to generate the NLGN2 overexpression in mice necessary for this project (See figure 3).
Western blotting:

To determine the effect of NLGN2 overexpression on the synaptic composition of the hippocampus in adult mice, I quantified the changes in protein expression. One way to observe the changes is by analyzing the expression of various synaptic marker proteins other than NLGN2. By comparing the amounts of inhibitory synaptic marker proteins (like pre-synaptic VGAT or post-synaptic gephyrin) or excitatory synaptic marker proteins (like pre-synaptic Vglut or post-synaptic PSD95) in mice with NLGN2 overexpression and control mice, it is possible to visualize the synaptic changes brought about by NLGN2 overexpression. Western
blots are a well-established method for quantifying the ratios of different proteins in a given sample.

Western blotting is a method for the electrophoretic transfer of proteins from a polyacrylamide gel to a nitrocellulose membrane. On the gel, electrophoresis can separate the proteins by weight, and on the membrane, they can be identified through antibody targeting. Before being run on a gel, the sample concentrations have to be standardized. To do this, a BCA assay needs to be done on the samples. A BCA assay uses a protein dye to mark proteins in a sample, and then measures the total absorbance in the sample (see figures 4A and 4B). Compared to a set of samples with known concentrations, the protein concentration in a sample can be determined based on the measured absorbance. This allows approximately the same amount of total protein from each sample to be analyzed on the western blot.

Once their concentration is standardized, proteins from a sample can be run on a polyacrylamide gel through protein electrophoresis, and then transferred from the gel to the much sturdier PVDF membrane. Attached to the membrane, the proteins are left accessible to antibody targeting in a way that is impossible in the gel. Through this technique, specific proteins can be easily identified from the sample (Towbin, Staehelin et al. 1979). After primary antibodies have attached to the proteins of interest, HRP-conjugated secondary antibodies can bind to the primary antibodies to allow for identification.

Horseradish peroxidase (HRP) is an enzyme that can cleave specific light-emitting substrates, such as a femto or pico reagent. The light emitted from the cleaving of these substrates can be measured quantitatively as an optical density,
and is directly proportional to the amount of protein targeted by the primary antibodies. This allows the amount of a given protein in a sample to be measured. To control for varying amounts of protein loading in each lane of the gel, a control protein needs to be measured as well. Proteins that are easy to image and expressed in most cell types are used as loading controls. Examples include: actin, tubulin and GAPDH. By standardizing optical densities across every lane and every gel, the relative amounts of specific proteins in a sample can be elucidated. Due to its ability to quantifiably determine the relative amounts of different proteins in a tissue sample, western blot analysis is an ideal tool for understanding the synaptic effect of NLGN2 overexpression (See figure 4).
Figure 4: An Overview of Western Blotting

(A) An example BCA assay with protein and protein dye. The dye on its own looks green, but the more protein in a given well the more purple appears, which can be easily measured using spectroscopy. (B) A concentration curve generated by protein standards is used to calculate the concentration in a given sample. The ‘y’ in the equation is the absorbance of a sample, and it can be solved for ‘x’ to determine the protein concentration. (C) The gel electrophoresis machine separates proteins by weight. (D) After running proteins through a gradient gel, it is possible to see the protein dye in the blue at the bottom, and the protein ladder on the sides. (E) The PVDF membrane has visible proteins after a ponceau red stain. The ladder is used to determine correct molecular weights, and the lines represent where the membrane is cut in order to target specific molecular weights with the correct antibodies. The proteins analyzed in this experiment were gephyrin at 93 kDa, NLGN2 at 90 kDa (both on the 75-150 kDa strip), VGAT at 57 kDa (on the 50-75 kDa strip), actin at 42 kDa (on the 37-50 kDa strip) and mCherry at 29 kDa (on the 20-37 kDa strip).
Part 2. Methods and Materials

Animals:

Eight male transgenic VGAT-ChR2(H134R)-EYFP mice were used for these studies (Jackson Labs). They were kept in separate cages, and were raised to ages ranging from 8-10 weeks prior to injection, to weights of at least 25 grams. The Wesleyan University Institutional Animal Care and Use Committee approved all protocols used in the treatment of these mice.

Stereotaxic Surgery:

Stereotaxic injections of the viral vector AAV/DJ-CMV-mCherry-2a-mNLGN2 were used to overexpress NLGN2 in the left hippocampus of these mice. CMV is a ubiquitous promoter that was used to express pAAV-cDNA6-mCherry-NLGN2 (Addgene). The plasmid was used to both overexpress NLGN2 and a marker gene, the red fluorescent protein mCherry. As an internal control, each mouse had a separate vector injected into the right hippocampus. The right hippocampus was injected with AAV/DJ-CMV-mCherry, to allow CMV to ubiquitously express the marker protein without overexpressing NLGN2 (See figure 2). Before AAV injection, .3mL of medicam (Henry Schein) was injected subcutaneously. The mice were then anesthetized via inhaled isoflurane gas, and remained under through nose-cone delivery for the entirety of the surgery (David Kopf Instruments, Vet Equip, Harvard Apparatus).

A 5µl glass Hamilton syringe with a 30° bevel-tip needle was used to inject the virus at three separate locations per hemisphere. The mice were given injections
into CA1 of the hippocampus and into each blade of the dentate gyrus at the coordinates AP 1.9 mm, ML±1.25 mm, DV 2.5, 2.3 and 1.4 mm. The lower blade of the dentate gyrus was injected first, and then the needle was subsequently withdrawn to the other two shallower sites. Each site was given .5µl of AAV over the course of 5 minutes. After the injections were finished, the needle remained in place for an additional 5 minutes, and then Vetbond tissue adhesive was used to close the incision sites. The mice were put on heating pads to wake up from the isoflurane before being put back into their cages. Meghan van Zandt performed all stereotaxic surgeries uses for this thesis.

**Western blot analysis of adult mouse hippocampi:**

Eight AAV-infected adult mice were euthanized 4-6 weeks post viral injection using a cervical dislocation. The left and right hippocampi were extracted via craniotomy, put in Eppendorf tubes on dry ice, and then frozen at -80°C. The membrane cellular fractions were obtained by homogenizing the tissue with a mortar and pestle in 320mM sucrose (Fluka Analytic, SZBD0500V) TEVP, made with 1M tris-buffered saline at pH 7.4 (RPI, T60050-1000.0), 100mM sodium orthovanadate (Aldrich, 450243-10G), 1M NaF (Sigma, S7920), .5M EDTA (Fischer Biotech , BP120-500), .25M EGTA (Amresco, 0732-10G), and protease inhibitor (Roche Diagnostics, 11873580001). The homogenates were centrifuged for 10 minutes at 2500rpm in a Sorvall centrifuge, and the supernatant was extracted. The supernatant was then spun for 15 minutes at 10000rpm and the remaining P2 pellets were resuspended in TEVP. The P2 fractions were sonicated using a sonic
dismembrator (Fisher Scientific FB460) and frozen at -80°C for storage. The protein concentration in each sample was calculated using a BCA assay kit (Thermo Scientific 23227), with protein standards at 2,000 µg/mL, 1,500 µg/mL, 1,000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL and 0 µg/mL.

Western blotting was done using and SDS-Page electrophoresis system (Bio-Rad 525BR) with 4-20% precast gradient gels (Bio-Rad 4561091S). The samples were diluted to 20µg of protein with sample buffer. The sample buffer was made with .1M Trizma base at pH 6.8 (Sigma, T6066-1KG), .1mM SDS (Fischer Scientific, BP166-500), .2M BME (Sigma, 36696LK), .5M glycerol (Sigma, G2025) and 1mM bromophenol blue (Fischer Biotech, BP115-25). 25 µl of sample was loaded into each well. All gels were run at a constant 125V for 90 minutes. The proteins were transferred onto a .45 µm thick PVDF membrane (Bio-Rad, 1620264) using a blot-transfer apparatus (Bio-Rad 153BR) at 115V for 75 minutes. The proteins were visualized using a 10x ponceau S stain (Sigma-Aldrich, P3504-10G), and then the membranes were cut at 150, 75, 50 and 37 kDa based on molecular weight standards, to have each important protein on a different strip. (See figure 4E)

Antibodies:

The strips of PVDF membranes were probed with various antibodies at different dilutions with a 20-hour incubation, including: mouse anti-actin (American International PLC, N350, 1:2000), mouse anti-gephyrin (Synaptic Systems 147011, 1:1000), rabbit anti-mCherry (Thermo Fisher PA5-34974, 1:1500), mouse anti-
PSD95 (Pierce MA1-046, 1:32000), rabbit anti-NLGN2 (Synaptic Systems 129203, 1:4000), and mouse anti VGAT (Synaptic Systems 131011, 1:8000). After three 10-minute washes in 10x tris-buffered saline (Research Product International Corp., T60050-1000.0) with tween 20 (Wesleyan University, 34766), also known as TBST, the strips were probed with secondary antibodies with a 1-hour incubation. The secondary antibodies used were HRP-conjugated goat anti-mouse (Cell Signaling Technology 7076S, 1:1500) and anti-rabbit secondary (Cell Signaling Technology 7074S, 1:1500). All antibodies were added in a 5% bovine serum albumin solution in 10x TBST. The antibodies were detected using a maximum signal femto substrate (Thermo Scientific 34096) with a 3-minute incubation and then imaged with a 30 second exposure time in a gel box (Syngene G:Box Chemi XX6) using GeneSeq software.

**Optical density analyses for Quantification of Western Blots:**

The images of the blots were inverted and adjusted to avoid saturation in the GeneSeq software. They were enhanced using the Photoshop auto-contrast function to reduce background noise. The images were then opened in ImageJ for optical density analysis. The optical darkness was measured using the densitometry tool to get optical density curves. By hand, the line tool was placed from the first bend of the major curve to the beginning of the second curve to separate out the background intensity of the image. ImageJ then integrated under the curve and over the line to obtain an optical density value for each protein in each sample (see figure 5). All values were controlled for loading by normalization to actin using the
corresponding lanes. Statistical analysis was done on Microsoft Excel using the Student’s T-Test function, with two tails and assuming variable standard error, with an average of 2-4 replicates for each sample.

**Figure 5: ImageJ Optical Density Analysis of Western Blot Images**

![ImageJ Optical Density Analysis of Western Blot Images](image)

Figure 5: (A) is an example western blot looking at the key protein NLGN2. The leftmost lane is an overexpression trial, and the lanes then alternate between overexpression and control trials. The yellow boxes are the areas that ImageJ is doing optical density analysis on. (B) displays the optical density curves generated by ImageJ. Each curve has a corresponding band in the western blot image. Left to right is the optical density from the top of the box to the bottom. The lines are made by hand and used to cut out the background from the curve. ImageJ can integrate under these curves in order to generate the optical density values used to identify relative amounts of proteins in a sample.
Part 3. Results

It is not known whether increased genetic expression of a single synaptic protein can alter synapse size, location or number. NLGN2 has been shown to be a key protein in inhibitory synapse development and maintenance, but its role is still unknown. I hypothesize that NLGN2 is necessary and sufficient to drive the formation of GABAergic synapses in the hippocampus of adult mice. To test whether NLGN2 overexpression can increase the size or number of inhibitory synapses in the mouse hippocampus, I performed western blot analysis on AAV-treated mice, comparing the hippocampus with the overexpression vector to a control.

To determine whether viral-mediated overexpression of the NLGN2 gene would lead to an increase in expression of the NLGN2 protein, I examined hippocampal P2 fractions containing the AAV overexpression vector and compared them to hippocampal P2 fractions containing the control vector. Eight mice were used as their own internal controls, with the left hippocampus given the NLGN2 overexpression vector and the right hippocampus the control vector. The red fluorescent protein mCherry was used for quantifying viral infection in each mouse. Sufficient material was obtained to compare up to four different gels for western blot analysis. On each blot, I probed for five different synaptic or control proteins and quantified levels of expression.

To control for different amounts of tissue loaded in each lane of the gel, I measured the amount of actin present on each blot. Actin is a protein found in all cells, and is unlikely to vary among samples. Therefore, differences in actin concentration between samples are representative of the total differences in the
amount of protein present in each lane. By dividing the optical density generated for each other protein by the optical density of the corresponding actin curve, the actual ratios of protein expression can be elucidated. The antibody for actin was very robust, and the actin bands formed very reliable optical density curves, demonstrating its ability to account for variation in gel loading and protein concentration in different samples and trials. There was very little recognizable variance in actin between samples (see figure 6).
Figure 6: (Left) This figure displays a representative western blot band from each trial, comparing overexpression to control for every protein. Each image is from the same mouse, mouse 2, comparing its left and right hippocampi. It is possible by eye to see the optical density differences between the overexpression band and the control band for all proteins except for actin. (Right) ImageJ optical density curves each corresponding to the bands seen on the left. Again, it is possible to see the size differences in the curves between the different trials of the same proteins.
To test whether genetic delivery of the NLGN2 gene via AAV actually increased expression of the NLGN2 protein, I measured the amount of NLGN2 expression in all samples. Viral infection of the NLGN2 overexpression vector led to an increase in the expression of NLGN2 of approximately 1.5-fold compared to control hippocampal tissue infected with the empty viral vector, after normalization to actin expression (p<.02) (see figures 7A and 8A). Moreover, there was an overall low variance in NLGN2 expression, with a standard error of .129 in hippocampal tissue with the overexpression vector and only .088 in tissue with the control vector (see table 1). This demonstrates that the AAV vector was successful at reliably increasing the protein expression of NLGN2, so the changes found in other proteins can likely be attributed to this cause.

To determine whether increased NLGN2 expression has effects on the pre-synaptic membrane, I measured the expression levels of the inhibitory pre-synaptic protein VGAT in P2 hippocampal fractions containing the overexpression vector compared to P2 hippocampal fractions given the empty control vector. The protein was imaged reliably, and had approximately a 1.6-fold increase in protein expression in samples that were given the NLGN2 overexpression vector compared to samples given the control vector after normalization with actin (p<.05) (see figures 7B and 8B). Like NLGN2, VGAT had a relatively small variance, with a standard error of .173 in overexpression hippocampal tissue and .129 in controls (see table 1). This demonstrates that overexpression of NLGN2 causes a significant increase in VGAT levels in mouse hippocampi.
To see what changes NLGN2 overexpression causes in protein expression on the post-synaptic membrane, I measured the protein expression of inhibitory post-synaptic scaffolding protein gephyrin in overexpression hippocampal tissue compared to control tissue. Gephyrin was not imaged as reliably as the other proteins (see figure 6), and therefore I collected fewer replicates. Despite this, the variance was low, with a standard error of .182 in overexpression hippocampi and .113 in controls (see table 1). It did exhibit the smallest measured increase of all the proteins, with the NLGN2 overexpression vector only generating a 1.38-fold increase of gephyrin expression compared to the control vector after actin normalization. This suggests a trend of gephyrin increasing with NLGN2 overexpression, but it did not reach significance (p>.1) (see figures 7C and 8C).

To make sure that the AAV virus was being adequately expressed in enough cells in the mouse hippocampus, I measured the amount of mCherry protein present in hippocampal tissue given the overexpression vector and the amount of mCherry in control tissue, with normalization to actin. Both vectors had the mCherry red fluorescent reporter gene, so amount of mCherry protein expression was representative of the amount of viral expression in each sample. The protein had a high background and was found over a wider range of masses than the other protein bands. It had a high variance overall, with a standard error of .276 in overexpression hippocampi and a .347 in controls (see table 1 and figures 7D and 8D). So, despite the lack of statistical significance (p>.05), it demonstrated a strong trend towards increased expression in the control hippocampi compared to the overexpression ones. It demonstrated the largest increase between overexpression and control
hippocampal tissue. Control tissue exhibited a 1.66-fold increase in mean mCherry expression compared to overexpression trials.

Figure 7: Box and Whisker Plots of Optical Density Ratio

Figure 7: Box and whisker plots of the optical densities for each protein in overexpression samples and controls. These values have all been corrected for loading by normalization to actin. All significance tests were done using a Student T-Test. (A) Plot of NLGN2, with a significance difference p<.02 (B) Plot of VGAT, with a significant difference of p<.05. (C) Plot of gephyrin, which fails to reach a significant difference due to variance, p>.1. (D) Plot of mCherry, which also fails to reach significance due to variance, p>.2.
Figure 8: Scatter plots of protein optical density normalized to actin in overexpression and control samples. This data is the same information seen in figure 7, but displays a data point for each animal tested. Every data point seen here is the mean optical density for a given mouse hippocampus. (A)
Optical densities of NLGN2 p<.02. (B) Optical densities of VGAT p<.05. (C) Optical densities of gephyrin p>.1. (D) Optical densities of mCherry p>.2.

Table 1: Western blot results

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<th>Protein</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>N</th>
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<th>Over/normal</th>
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Table 1: The mean normalized optical densities for each protein in overexpression and control hippocampi, and the variance between them. The table shows the standard deviation, standard error, and the sample size for each group. This table also displays the p-values from the two-tailed Student T-Test and the ratio of the means to demonstrate the fold change between hippocampi given the overexpression vector and hippocampi given the control vector.

Part 4. Discussion

Analysis of Results:

I hypothesized that NLGN2 is necessary and sufficient to drive the formation of GABAergic synapses in the mammalian adult hippocampus. The goal of my experiments was to use western blot analysis to determine whether AAV-mediated viral delivery of NLGN2 led to concomitant increases in the expression of other proteins localized to the GABAergic synapse. To achieve this, first NLGN2 had to be
successfully overexpressed in experimental hippocampi. AAV proved to be a powerful tool for increasing cellular NLGN2, as the overexpression hippocampal tissue had significantly larger amounts of NLGN2 compared to control tissue. This is highly important, because it demonstrates that NLGN2 was successfully increased in this project, and will be in subsequent projects that analyze the effect of NLGN2 overexpression using AAV. Moreover, the effect on individual cells must be much greater than even what appeared in the western blots. Western blots analyze total protein concentration in an entire tissue sample, which in this case is a whole mouse hippocampus. AAV is a virus, which despite being injected directly into the hippocampus, is highly unlikely to be expressed in every cell that the western blots measured. Significant increases (~1.5 fold) in the total NLGN2 expression necessarily means a much larger increase in the cells that are actually infected.

To get an understanding of the degree of viral expression, mCherry expression was also measured. The AAV construct for NLGN2 overexpression partnered mCherry and AAV under the same promoter, so every instance of extra NLGN2 in tissue infected with the overexpression virus should be coupled with a red fluorescent protein. Measuring the amount of mCherry in a sample correlates with the amount of viral expression in that sample, and therefore the total excess NLGN2 for the experimental tissue. Interestingly, mCherry was found to be notably higher in control tissue compared to experimental tissue (see figure 7D and 8D). It was not significant, due to the highly variable nature of viral expression. In a given cell, a virus can integrate anywhere from one to dozens of times (Robert M. Kotin, Menninger et al. 1990). However, the trend is definitely there, with hippocampal
tissue injected with the control vector demonstrating more viral expression than the hippocampal tissue injected with the overexpression vector.

AAV is a relatively small delivery system, with a maximum load of 5kb (Kurylo 2009). The overexpression construct is fairly large, with a 1.5kb reporter gene and a 835 amino acid protein, totaling approximately 4kb (2016). The control virus is considerably smaller without the added load of having to code for NLGN2, at only 1.5kb. The discrepancy in viral expression between overexpression and control brains is likely due to this size difference. There is a multitude of ways that AAV expression could be limited by its size. It is possible that it integrates more effectively when smaller, so that the control hippocampi were working with more viral integration sites and, therefore, able to make more mCherry. Another possibility is that under the CMV promoter, the change in gene length increases the transcription and translation times enough to reduce the proteins generated. It would have to be a significant time increase to be visualized by western blotting, but it is possible. This effect could be tested more in the future by looking at varying construct lengths in AAV under the CMV promoter.

Whether or not the construct length affected viral integration directly, the fact that there is a visible difference in viral expression between the experimental and control groups demonstrates that the virus necessarily infected a subset of the total cells measured. An approximately 1.5-fold increase was seen in mCherry between the two groups, so even if the control virus infected every single cell that was measured, the overexpression virus at most infected 2/3 of the cells. This shows that in individual cells, the effect of the virus on NLGN2 and all measured
proteins is going to be notably more significant than the western blots are able to visualize.

Gephyrin is a scaffolding protein on the post-synaptic membrane alongside NLGN2, so if NLGN2 has the ability to recruit other synaptic proteins, gephyrin is a logical choice. Gephyrin did not have a significant change (p>.1), but it did have a definite trend towards increased expression in the overexpression tissue (see figures 7C and 8C). This suggests that gephyrin may be recruited as part of the synapse, but there are several possibilities as to why it did not reach significance. For one, it was a more difficult protein to image on westerns (see figure 6) and, therefore, there were fewer available data points to look at for statistical analysis. As a scaffolding protein, gephyrin already exists in fairly large amounts on the post-synaptic membrane, and so a slight increase by NLGN2 in a few select cells may be small enough to fail to reach significance across the entire hippocampus. It is also entirely possible that the trend seen here is just an artifact of the blots, and that NLGN2 does not recruit gephyrin as part of its role in developing the inhibitory synapse. This could be due to gephyrin’s role as a scaffold, since it is more peripherally related to inhibitory IPSC’s than say, VGAT or GABA receptors. More western blots analyzing gephyrin would be necessary to conclusively say how NLGN2 interacts with gephyrin on the post-synaptic membrane.

To see the effect of NLGN2 on the inhibitory pre-synaptic membrane, VGAT levels were analyzed through western blots. An increase in VGAT is especially important, because it demonstrates the ability of NLGN2 to have an effect on proteins across the synapse. If increasing NLGN2 can cause recruitment of pre-
synaptic proteins, it suggests that NLGN2 not only takes part in the building of the post-synapse, but that it plays a major role in the development of the entire synapse. NLGN2 overexpression increased VGAT levels ~1.5 fold, with a significance of p<.02, which is comparable to the changes seen in NLGN2 itself (see figures 7B and 8B). These results demonstrate that VGAT increases with NLGN2 at a ratio of almost one-to-one. This strongly suggests that NLGN2 recruits VGAT in the formation of the inhibitory synapse. The mechanism for this is still unclear, but it likely has to do with the recruitment of NLGN2’s presynaptic binding partner neurexin. If NLGN2 can recruit important pre-synaptic proteins, like the GABA transporter, it is likely the major protein in the formation of the inhibitory synapse and has effects ranging across both sides of the synapse.

Through the NLGN2 and mCherry data, this project demonstrates that AAV can effectively increase NLGN2 protein expression in the hippocampi of adult mice. However, more profoundly, through the gephyrin and VGAT data, this project demonstrates the effects of NLGN2 overexpression on the inhibitory synapse. If increasing NLGN2 likely increases both VGAT and gephyrin, it suggests a causal effect. NLGN2 may be a protein that is necessary and sufficient for the formation of the inhibitory synapse, causing increased synaptic protein expression on both pre and post-synaptic membranes.

**Points of error:**

For this project, a new western blot protocol had to be developed. During many of the first runs, specific incubation times and dilutions were still being
worked out. Many images were too bright, or not bright enough (see figure 9) and had to be discarded. Some of the gels were not run for enough time, so the molecular weights of the key proteins were too close together to visualize. This led to protein bands that had to be analyzed across multiple strips. The majority of those data points were unusable, but a few blots were still quantifiable and generated legitimate data. Unfortunately, not all of the hippocampi have an equal number of data points to draw from (see tables 2-5). That means that the final optical density value generated for some trials are more validated than others. The overall sample size of eight samples per group is already small, and with limited replicates there is more noise in the data. It is likely that this noise is the cause of the lack of significance in the gephyrin and the mCherry, but that it is unavoidable. Mouse hippocampi are small, and many of the samples were used up by the end of the experiment. To achieve significance, more mice need to be analyzed with those same proteins.
Figure 9: Western Blot Issues

Figure 9: Images of representative issues encountered during western blotting. (A) A demonstration of incomplete antibody incubation seen a few times with different proteins. In this image, it is possible to tell that only the middle of the membrane was able to be properly targeted by antibodies, and that the ends were not given adequate incubation. (B) Example of a strip that suffered from contamination during the washing steps of western blotting. The PVDF membrane is incredibly sticky, and is able to pick up any amount of dirt or other proteins that it comes in contact with during the process. (C) Example of two strips generated from a gel that was not run for a long enough time. The different protein sizes were not separated enough along the gradient gel, so when they were cut, the VGAT band was found on two different membranes, leading to a blot that has a cut down the middle. (D) A burned electrophoresis apparatus that was connected to a faulty power supply. The power supply sent too high a current through the gel and caught fire, burning the apparatus and destroying the gel and the tissue within it.

Future directions:

This project demonstrates that NLGN2 can be successfully overexpressed using the AAV virus, and that this manipulation leads to increased expression of both pre and post-synaptic proteins, localized to GABAergic synapses. NLGN2 therefore likely plays an important role in the development of these synapses. The mechanism for this is still unclear, but my data suggests that NLGN2 is sufficient to facilitate synapse development.

The mechanism that allows NGLN2 to facilitate synapse development is still unknown. Cells on both sides of the synapse may use NLGN2 as an upstream genetic
regulator for how to express synaptic proteins. Perhaps the amount of NLGN2 present at any given synapse is used to moderate the number of synaptic proteins that cells on both sides of the synapse produce. Conversely, NLGN2 could play a role in recruitment of other key inhibitory proteins. NLGN2 is part of a post-synaptic scaffold (Craig and Kang 2007), and it may be the protein that the rest of the synaptic scaffold is built around (see figure 1). A way to test whether NLGN2 is regulating expression of other synaptic proteins would be to compare the levels of mRNA transcripts of inhibitory synaptic markers like VGAT and gephyrin. If they were increased in mice that were given the NLGN2 overexpression vector, it would suggest a genetic regulation of protein expression. If not, then NLGN2 is more likely to play a role in localizing other proteins to the synapse. Both of these mechanisms would only directly affect the post-synaptic membrane. However, the pre-synaptic neurexin may also be recruited by NLGN2 because of its role as a binding partner, which could lead to recruitment of pre-synaptic proteins as well.

This thesis demonstrates that through genetic delivery of a single synaptic protein, it is possible to generate measurable changes in the synapses of an adult mammal. The significant increase in synaptic proteins across the both sides of the synapse in adult mice demonstrates a large degree of change in these mice. In a few weeks, the amount of inhibitory synaptic expression increased by at least 1.5-fold. This suggests a remarkable plasticity in the hippocampus, as a significant amount of mature neuronal development was mediated by NLGN2 overexpression. The exact nature of this NLGN2-mediated increased protein expression is still unclear, and so the effect of NLGN2 on hippocampal inhibition requires more study. If this increased
protein expression is representative of new synapses being generated by the AAV-infected cells, it would suggest a remarkable degree of plasticity for fully mature neurons. However, it is also possible that these proteins represent an increase in synaptic size. Regulation of synaptic size would demonstrate less neuronal plasticity, but would show a large degree of continual maintenance of synapses. It is also possible that synapse size and number both increase with the genetic delivery of NLGN2. Future densitometry measurements of synapse size and number on adult mouse hippocampal tissue infected with the AAV overexpression vector will help elucidate the mechanism through which NLGN2 generates synaptic growth.

The increase in VGAT and gephyrin shown here strongly suggests that NLGN2 plays a key causal role in the development of the inhibitory synapse. However, the electrophysiological changes following experimental elevation of synaptic proteins are not yet known. To fully understand NLGN2’s role in directing the formation of inhibitory synapses, electrophysiological studies need to be done on the inhibitory synapses that are made in the presence of the NLGN2 overexpression vector. It is possible that increased expression of synaptic proteins does not correlate with a stronger inhibitory signal. The proteins measured by western blotting may not actually correlate with proteins in the synapse. They were all P2 fractions, which means that they are on the cell membrane, but not necessarily in synapses. To conclusively show that NLGN2 increases synaptic inhibition in the adult hippocampus, the IPSC’s on cells with NLGN2 overexpression need to be compared to those on normal cells. If the increased expression of all of these synaptic markers is also followed with an increased electrophysiological
response in the post-synaptic cell, it will prove that the increased protein expression is actually causing an increased neuronal signal. Electrophysiological testing will significantly help demonstrate the inhibitory effect generated by NLGN2.

One aspect of the hippocampus that this project did not explore was the effect of NLGN2 overexpression on the excitatory synapses of the brain. Considering NLGN2’s ability to affect cells across the synapse, it is possible that it could have unforeseen effects on the excitatory synapses as well. A simple continuation of this research would be to replicate the experiments using excitatory pre and post-synaptic markers. It would be interesting to see whether the pre-synaptic vesicle glutamaterigic transporter (VGlut), would be increased or decreased in the highly inhibitory environment created by NLGN2 overexpression. It would also be important to examine the expression of other excitatory post-synaptic markers, including components of glutamate receptors and post-synaptic density protein 95 (PSD95) (Morgan Sheng and Kim 2011).

To further validate my findings, it will be important in future work to examine more inhibitory synaptic proteins and increase the sample size of mice. This experiment was done with only 8 mice and 16 hippocampi, and a larger data set would be more sensitive to the effect. Gephyrin was not found to be significantly increased, but the trend is there, so likely with more western blots, a statistically significant finding could be attained. It is also likely that NLGN2 can recruit more inhibitory proteins in addition to VGAT and gephyrin. Collybistin is an important post-synaptic regulator of the gephyrin scaffold, and if it were to be significantly increased, there would be more evidence to support that the effect seen here,
NLGN2 overexpression on the size of the post-synaptic membrane, is real. Lastly, a highly important protein that was not evaluated in this paper is the NMDA receptor, the post-synaptic receptor of GABA. To fully demonstrate the effect of NLGN2, NMDAR needs to be evaluated. Demonstrating that the GABA receptors as well as the transporters are recruited by NLGN2 would show that NLGN2 is solely capable of forming all major parts of the inhibitory synapse.

One assumption that this paper relies on is that mice can work as their own internal controls. For all mice tested, the left hippocampus was given the overexpression vector and the right one was given a control vector. This works, unless there is a difference in protein expression between the two hemispheres in adult mice. It is unlikely that this would be the case, but one way to tie up all loose ends would be to compare protein levels in the two hippocampi of wild-type mice, just to be sure that there is no inherent bias in protein expression masquerading as results.

There are several different cell types that have been documented as crossing the hippocampal suture, which could cause a transfer of effects from one side of the hippocampus to the other. The main hippocampal contralateral connections are pyramidal cells from CA3, but there are also pyramidal cells from CA1 and even various other cell types from both CA3 and CA1, including: aspiny stellate cells, pyramidal basket cells, polygonal basket cells, horizontal basket cells, and stellate cells (Walter K. Schwerdtfeger and Buhl 1986). The AAV vectors were injected into the dentate gyrus and CA1 of the hippocampus, so it is possible that some efferent neurons from the CA1 of the left hippocampus infected with the NLGN2
overexpression vector could connect to the right hippocampus. Stellate cells can be GABAergic interneurons, which could increase inhibition on the right hippocampus from the left side. Luckily, this would only reduce the difference seen between the experimental and control samples, and since a strong effect was seen, there was likely either no viral transfer across the hippocampal suture, or it was negligible.

Understanding the development of the inhibitory synapse is also important in many medical issues, such as TLE and autism. TLE, for example, is most commonly caused by a lack of proper inhibition in the hippocampus. One way to treat TLE is by transplanting GABAergic precursor stem cells to allow for growth of new inhibitory interneurons in the hippocampus. It is possible that increasing NLGN2 expression may be a way to increase the effectiveness of those transplants.

Comparing the effectiveness of GABAergic transplants in treating epilepsy in NLGN2 overexpression and normal mice would be an incredibly interesting and important way to continue this research and gain an increased understanding of inhibitory synapses, TLE, adult neurogenesis, and stem cells.

The data in this thesis demonstrates high levels of increased synaptic inhibition and the effect in individual cells is likely significantly larger than the 1.5-fold increase seen across the entire hippocampus. It is possible that NLGN2 overexpression could be used as a treatment for epilepsy even without the use of transplants. It would be that much easier and efficient to treat TLE through genetic delivery of a single gene than having to deal with cellular transplants of progenitors. Without transplants or chemicals, NLGN2 delivery could prove to be a highly effective and safe way to treat TLE and other diseases caused by a lack of inhibition.
Lastly, NLGN2 is one of many other proteins in the NLGN family. If the role of NLGN2 is so important in the formation of inhibitory synapses, it is possible that other NLGN’s will be found to be important in their respective synapses. Perhaps NLGN1 will be found to increase expression of excitatory synaptic markers on both sides of the synapse. By employing the same strategy used here, a much more cohesive understanding of all synapse formation may be in sight by identifying other key proteins of the NLGN family.

Part 5 References:


Part 6. Appendix:

Table 2: All raw data for NLGN2

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Table 2: All raw data used in this paper for optical densities of NLGN2. Each trial was a separate western blot ran on the same tissue, probed for NLGN2 and actin and then normalized after ImageJ analysis. The right four trials are the overexpression left hippocampi for the eight mice, and the left
four trials are the control right hippocampi. Some data points were excluded due to the incredibly high variance they displayed relative to the data from the other blots.

Table 3: All raw data VGAT

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Table 3: All raw data used in this paper for optical densities of VGAT. Each trial was a separate western blot ran on the same tissue, probed for VGAT and actin and then normalized after ImageJ analysis. The right four trials are the overexpression left hippocampi for the eight mice, and the left four trials are the control right hippocampi. Some data points were excluded due to the incredibly high variance they displayed relative to the data from the other blots.

Table 4: All raw data Gephyrin

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Table 4: All raw data used in this paper for optical densities of gephyrin. Each trial was a separate western blot ran on the same tissue, probed for gephyrin and actin and then normalized after ImageJ analysis. The right two trials are the overexpression left hippocampi for the eight mice, and the left two trials are the control right hippocampi. No data points had to be excluded, but gephyrin had fewer data points available than the other proteins due to probing issues during the western blotting.

Table 5: All raw data mCherry

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Table 5: All raw data used in this paper for optical densities of mCherry. Each trial was a separate western blot ran on the same tissue, probed for mCherry and actin and then normalized after ImageJ analysis. The right four trials are the overexpression left hippocampi for the eight mice, and the left four trials are the control right hippocampi. Some data points were excluded due to incredibly high variance relative to the other trials.
Figure 10: Western Blot Analysis Without the Use of Photoshop

Figure 10: In the protocol I followed for western blotting, I used the Photoshop auto-contrast function to make the images clearer for optical density analysis. To test that this image enhancing did not alter the results, I did an optical density analysis of an unaltered western blot image of NLGN2 seen in (A). Like all the blots, the lanes alternate between left and right hippocampi. (B) The optical density curves for each lane seen in (A). Compared to figure 6, it is clear that there is significantly more noise in the optical density curves without enhancement. (C) A table of statistical analyses of the optical density curves generated in (B). The data shows, with a two-tailed Student’s T-Test p<.01, that the two groups are still significantly different, and the ratio was even found to be more significant than the overall data (See table 1). This is likely an artifact of the noise seen in the unenhanced blot.