Balancing Act: Investigating the Role of Neuroligin 2 in GABAergic Synapse Development and Stabilization

By
Samantha Mory Maisel
Faculty Advisor: Dr. Janice Naegele

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"To know the brain...is equivalent to ascertaining the material course of thought and will, to discovering the intimate history of life in its perpetual duel with external forces."

-Santiago Ramón y Cajal
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ABSTRACT

The mechanisms responsible for guiding the formation of inhibitory synapses are not well understood. Alterations of GABAergic circuits contribute to the pathophysiology of various brain diseases, including epilepsy, autism spectrum-related disorders, and schizophrenia (Marin, 2012). Research on both excitatory and inhibitory synaptogenesis in the developing and adult brain has shed light on synaptogenic molecules that are central to the process. Studying the synaptic mechanisms of the GABAergic connections in a healthy brain allows a deeper understanding of hippocampal circuitry, and possibly novel molecular sites for therapeutic intervention in systems that need repair. The molecular mechanisms regulating GABAergic interneuron synapse formation are not well understood, however, research suggests that the postsynaptic scaffolding molecule neuroligin 2 (NLGN2) is a vital part of the process; it is crucial for forming and stabilizing inhibitory synapses. To test the role of NLGN2 in the developing and adult cerebral cortex and hippocampus, I used AAV-mediated gene delivery to manipulate the expression of NLGN2 in transgenic mice expressing channel rhodopsin2 and eYFP under the control of the promoter for the vesicular GABA transporter (VGAT). Immunohistochemical and confocal analyses suggest higher densities of NLGN2 clusters in neurons infected with the overexpression vector as well as an increase in postsynaptic gephyrin clusters and GABAergic synapses onto these neurons. My preliminary findings suggest that in both the developing and adult forebrain, GABAergic synapses retain neuroplasticity that can be manipulated with an AAV-mediated approach.
CHAPTER 1: INHIBITORY CIRCUITS IN THE HIPPOCAMPUS AND MECHANISMS OF GABAERGIC SYNAPSE FORMATION

I. INTRODUCTION

Inhibitory synapses throughout the brain are critical in maintaining excitatory and inhibitory synaptic balance that underlies proper neuronal circuit activity. The majority of inhibitory neurotransmission in the mammalian forebrain occurs at GABAergic synapses, mediated by GABA which acts through ionotropic and metabotropic GABA_A and GABA_B receptors, respectively. Impairments in GABAergic circuits at the cellular and synaptic level underlie a number of neurological disorders and are one of the pathological hallmarks of Severe Temporal Lobe Epilepsy (TLE).

Stem-cell transplantation is a powerful approach to replenish lost populations of GABAergic interneurons and stimulate circuit repair. Prior studies showed that, in the mouse pilocarpine model of TLE, GABAergic progenitor transplants into the dentate gyrus (DG) integrated synaptically and ameliorated seizures (Henderson, Gupta et al., 2014; Cunningham et al., 2014; Hunt et al., 2013). The molecular mechanisms regulating GABAergic interneuron synapse formation in endogenous circuits and following stem cell transplantation are not well understood, however, research suggests that the postsynaptic scaffolding molecule, neuroligin 2 (NLGN2), plays a vital role in the process. NLGN2 is thought to play an essential role in synapse formation and stabilization via interactions with its transynaptic binding
partner neurexin (NRXN) as well as other postsynaptic proteins localized at GABAergic synapses.

NLGN2 most likely plays a key role in mediating the synaptic integration of transplanted GABAergic interneurons. My research investigates the role of NLGN2 in interneuron synapse formation in the hippocampus by examining the effects of NLGN2 overexpression on GABAergic synapse formation in the adult and developing hippocampal and cortical regions. Research suggests that NLGN2 functions differently at axo-dendritic versus perisomatic sites of synapse formation, and is particularly important in regulating perisomatic synapses originating from paralbumin (PV)-expressing GABAergic interneurons (Gibson et al., 2009; Poulopoulos et al., 2009). I hypothesized that increasing NLGN2 expression would lead to an increase in gephyrin clusters and GABAergic innervation at perisomatic sites.

Most in-vivo studies looking at the role of NLGN2 in synapse formation used overexpression or knockout animal models to alter NLGN2 expression (Blundell et al., 2009; Poulopoulos et al., 2009), and few have utilized adeno-associated virus (AAV)-mediated gene delivery (Kohl et al., 2013, 2015). The properties of AAV make it a very suitable research tool for investigating and manipulating the structure and function of neural circuits. The use of AAV allows for spatio-temporal manipulation of expression of specific genes to study the effects on neural circuits in particular brain regions or cell types. In this way, AAV offers an efficient means of gene delivery to study molecular mechanisms of synapse formation in vitro and in
**vivo** in healthy and diseased cells/tissue. This approach can be used to manipulate endogenous cells and perhaps stimulate circuit repair in neurodegenerative diseases or conditions characterized by neuronal injury such as traumatic brain injury, stroke, or intractable epilepsy.

**II. EPILEPSY**

*Epilepsia* in Latin means to seize, to be taken hold, owing to the pre-Hippocratic view that something sacred, either of the gods or demonic, was taking hold of a person and causing the uncontrollable movements characteristic of some seizures. The “sacred disease”, as it became known, was treated by purification and incantations until around 400 BCE, whereupon Hippocrates denounced the sanctification of the disease, suggesting it to be a disorder of the brain. Epilepsy remained quite enigmatic and was referred to up until the 20th century as “the falling sickness”, a name alluding to the complete loss of posture and ultimate falling exhibited in people who suffered from grand mal seizures (Novarino et al., 2013). Since the late 20th century our understanding of seizure disorders and the underlying pathologies that are potential targets for novel therapies has increased vastly.

The healthy human brain exhibits remarkable plasticity, while maintaining highly regulated and precisely wired neuronal circuitry that sustains an appropriate balance between neuronal excitation and inhibition. This balance is delicate and vulnerable to neuronal injury; events that disturb normal patterns of neuronal activity,
like stroke or head trauma have potential to give rise to seizure activity in the brain, and perhaps resulting phenotype, or the development of epilepsy in severe cases. Epileptic insults are generally characterized by abnormal electrical activity in the brain, loss of consciousness, and/or loss of bodily control. An epileptic insult, a seizure, does not constitute epilepsy. Rather, epilepsy is a group of disorders characterized both by a more permanent tendency to generate epileptic seizures, as well as the resulting pathophysiological, cognitive, and behavioral changes (Fisher, 2014). Epilepsy is often linked to cognitive impairments and behavioral disorders. For example, autism spectrum disorders, mental retardation, migraine, learning disabilities, memory impairments, anxiety, depression, and conduct disorders are all significantly overrepresented in patients with epilepsy (Kleen et al., 2012; Pellock, 2004; Hermann, 2008; Tuchman et al., 2010). The cognitive and behavioral deficits and abnormalities are some of the most common and severe consequences of seizures and can complicate epilepsy management and shorten lifespan.

Seizures broadly fall into one of two categories: focal or generalized. Focal seizures, also known as partial seizures, arise in one part of the brain and don’t spread across the corpus callosum unless they turn into generalized seizures. Some focal seizures, like those characterizing temporal lobe epilepsy (TLE), are often associated with developmental defects or head trauma and can present with symptoms of nausea, Déjà vu, or altered states of consciousness if the seizure spreads to greater regions of the cortex (NIH, 2015). Generalized seizures, on the other hand, result from abnormal neuronal activity in both hemispheres of the brain and often present
with more severe behavioral phenotypes, like muscle spasms, that tend to be associated with a seizure (Lerche et al., 2001). Generalized seizures can cause brain damage and are sometimes associated with some form of genetic disorder, for example, mutations that may affect ion channels critical in neuronal firing behavior. Although there are many functional complexities and causes of different types of epilepsies, the general process of seizure generation is a product of a change in firing patterns of many neurons; neurons that normally exhibit asynchronous firing patterns begin to fire together in large waves of synchronous activity that can and often do spread to other parts of the brain.

Temporal lobe epilepsy (TLE) is one of the most common forms of focal epilepsies, characterized by spontaneous, recurrent seizures, thought to arise from an imbalance of excitation and inhibition in the temporal lobes. This imbalance often develops during a period of epileptogenesis (known as the latent period) following initial insult in the brain (Dudek and Staley, 2012). This process of epileptogenesis links the brain injury to a subsequent, gradual emergence of epilepsy; it encompasses collective cellular and large systems-level circuit changes whereby spontaneous recurrent seizures develop (Dudek and Sutula, 2007).

Extensive research in human and animal models of TLE have shown that changes in gene expression, loss of GABAergic interneurons, and a multitude of neuroplastic changes resulting from repeated and sustained seizures, work in conjunction to foster a hyperexcitable environment conducive to further epileptic insults and onset of cognitive deficits (Dudek and Sutula, 2007; de Lanerolle 1989,
Tang and Loke 2010). Alterations in GABAergic (inhibitory) and glutamatergic (excitatory) circuitry in the hippocampus contribute to epileptogenesis and the development of recurrent seizures in TLE. These alterations include degeneration of GABAergic populations throughout the hippocampus, most notably in the hilus, as well as compensatory sprouting mechanisms by surviving inhibitory interneurons, which aren’t effective in preventing seizure activity or epileptogenesis (de Lanerolle et al, 1989; Zhang et al., 2009; Thind et al., 2010). Changes in the principal cells of the DG of the hippocampus also contribute to the subsequent loss of inhibition on the excitatory dentate granule cells (DGCs) of the hippocampus. In epileptic models, the DGCs exhibit changes in composition of their GABA_A receptors as well as synaptic proteins that are critical in functional synaptic activity (Fang et al, 2011; Cohen et al., 2003).

DGCs are renewed throughout life in a process known as adult neurogenesis, and, in epileptic animals, many newborn DGCs exhibit altered migration and dendritic growth (Parent et al., 2006; Ribak et al 2000; Kron et al, 2010). Studies in animal models of TLE have revealed that DGCs born into an epileptic environment have abnormal morphologies that contribute to hyperexcitability, including hilar-basal dendrites, mossy fiber sprouting, and ectopic somas (Houser 1990; Parent et al, 1997, 2005; Ribak et al, 2000; Buckmaster et al, 2002; de Lanerolle et al, 2003). Though the actual mechanisms for these abnormalities and how they contribute to epileptogenesis are not known with any certainty, there is general consensus that these
processes contribute to hyperexcitability, and thus may be important targets for therapeutic interventions.

To date, there are no good cures for TLE. The least invasive treatments include drugs that increase GABAergic transmission. Often, anticonvulsant drugs don’t work for extended periods of time and tend to have adverse effects. Seizures are hard to control with anticonvulsant medications and about 1/3 of the patients with TLE suffer intractable seizures that are unresponsive to medications. For these patients the medical recourse may be surgical removal of epileptic foci (P. Robb, 1975; Cosgrove and Cole, 2005). However, for patients severely inflicted in both hippocampi, removal of surgical foci would result in severe inability to form declarative memories, among other things.

As one important contributing factor in acquired TLE is a deficit in GABAergic neurotransmission, circuit repair to balance excitation and inhibition in TLE is an idea that has gained traction. Rather than medicating, methods of GABAergic transplantation aim to stimulate circuit repair. Previous studies have examined long-term effects of transplanting GABAergic progenitors into the DG in a murine model of TLE (Henderson et al., 2014). These studies showed that GABAergic progenitor transplants lead to significant attenuation of spontaneous seizures, beginning by about 3-4 weeks after transplantation (Henderson et al., 2014). Optogenetic and post-mortem immunohistochemical experiments following the EEG recordings suggested that transplanted GABAergic cells had synaptically integrated into the host brain where they provided synaptic inhibition onto endogenous GCs.
The grafts reduced seizures by approximately 40%, formed synapses onto GCs, and increased spontaneous IPSCs. The strong seizure suppression in this study became transient after about 80 days post-transplant. The exact mechanisms of transplant-mediated seizure suppression are unknown, and so too are the mechanisms that underlie this drop off in seizure suppression. The survival and function of neural stem cell grafts definitely depends upon their proper synaptic integration into host circuitry. One current idea about why the effects of the transplants wear off, is that the transplanted cells mature and form stable synaptic connections that cannot innervate the hyperexcitable DGCs born at later times. It is possible as well that the transplanted cells form more transient synapses that do not remain stable over long periods of time. A better understanding of the molecular mechanisms regulating adult hippocampal GABAergic synapse formation could inform these transplant studies and perhaps support other means of stimulating repair. To further understand the synaptic and molecular changes that encompass epileptic circuit reorganization, it is necessary to understand the structure and circuit organization of the hippocampus in a healthy brain.

III. STRUCTURAL AND CELLULAR ORGANIZATION OF THE HIPPOCAMPUS

The hippocampal formation is a folded structure in the medial temporal lobe that is critical in learning, declarative memory, and spatial navigation, and has been shown to exhibit structural plasticity throughout life. The definition of the term
“hippocampal formation” often encompasses the structure comprised of six regions linked by large and unique unidirectional projections: the dentate gyrus (DG), the hippocampus proper (CA3, CA2, CA1), the subiculum, presubiculum, parasubiculum, and entorhinal cortex (EC). There are various major cortical and subcortical inputs and outputs of the hippocampal formation but the perforant pathway- the excitatory projection formed by cells in the EC to the DG- is one of the best-studied.

The DG is comprised of the molecular layer (MOL), granule cell layer (GCL) and polymorphic layer, or the hilus. Closest to the hippocampal fissure, the MOL contains comparatively few cell bodies and is often split categorically into the outer (OML), middle (MML), and inner (IML) molecular layer. Deep within this layer lies the GCL, a cell layer densely packed with excitatory dentate granule cells (DGCs) that send unipolar dendrites up into the MOL. These cells are continuously added to the region throughout life at a slow rate in a process termed adult neurogenesis (Bayer, 1982).

Adult neurogenesis occurs in an area at the base of the GCL called the subgranular zone (SGZ) that fosters a microenvironment containing the chemical and molecular components necessary to maintain a neural stem cell population. In a timespan of about 2-3 weeks newborn neurons from the SGZ migrate into the GCL and form functional synaptic connections (Zhao et al., 2008). For this first two weeks, these cells receive GABA-mediated excitatory input, owing to the transient expression of the sodium-potassium chloride importer (NKCC1), after which point GABAergic input becomes hyperpolarizing and the dendrites of adult born GCs...
extend into the MOL and receive glutamatergic input. In a healthy brain the cells are incorporated into existing dentate circuitry after about two months. As mentioned previously, DGCs born into an epileptic environment will exhibit abnormal morphologies that contribute to hyperexcitability, or tend to migrate aberrantly into the hilus or sprout axons back up near the dendrites of other GCs in a manner that facilitates hyperexcitation of neighboring GCs (Houser 1990; Parent et al, 1997, 2005; Ribak et al, 2000; Buckmaster et al, 2002; de Lanerolle et al, 2003).

The GCL exhibits a “U” or “V” shape (depending on the plane of section) with a suprapyramidal blade (adjacent to CA1) and infrapyramidal blade. The DG and MOL enclose the hilus, which harbors a variety of cell types, mostly glutamatergic mossy cells and GABAergic interneurons. Cornu ammonis 1-3, Latin for Ammon’s horn (CA1-3), contains densely packed cell layers comprised of the principal cells, or CA pyramidal neurons. CA3 cells are closet to the DG and project to CA2 and CA1 regions.

The major cortical input to the hippocampus comes from axonal projections from the entorhinal cortex (EC), with minor projections arising in the presubiculum and parasubiculum, basal forebrain, brainstem and supramammillary nucleus (Kohler, 1985; Amaral and Kurz, 1985; Magloczky et al., 1994; Amaral et al., 2007). The EC separates sensory information from various modalities and sends projections to the hippocampus and other subfields of the hippocampal formation via the perforant pathway (PP) (Menno, 2007). Efferents from the EC to the DG are highly complex and well conserved, and the most common model for understanding principal
hippocampal circuitry is the canonical trisynaptic loop, which includes the projections from the EC to the DG. The trisynaptic loop EC and hippocampal circuits are shown in Figure 1.

Figure 1. Hippocampus-entorhinal cortex circuitry.
This transverse slice through the rodent hippocampus illustrates entorhinal cortex (EC)-hippocampal projections. Polymodal sensory input is carried to the hippocampus via direct and indirect paths from layer III and II neurons of the entorhinal cortex (EC), respectively. The direct pathway is characterized by a projection from layer III cells of the EC to the apical dendrites of CA1 pyramidal neurons. The indirect pathway, which represents the first connection in the canonical trisynaptic loop, begins with axonal projections from the lateral and medial entorhinal cortices to the outer and middle third of the molecular layer, respectively. The distinction between the medial and lateral EC projections to the MOL is not illustrated here but the projection is known as the perforant path, whereby these axons
innervate the dendrites of dentate granule cells (DGCs). The DGCs send their axons, via the mossy fiber pathway, to the apical dendrites of CA3 pyramidal cells, which then send their axons to the more proximal dendrites of CA1 pyramidal cells through Schaffer collaterals, and back to the deep layer of the EC, influencing projections back to the hippocampus in an excitatory loop.

After exiting the EC and entering underlying white matter and angular bundle, many PP fibers cross the subiculum and hippocampal fissure, to project to the DG and other hippocampal regions. These PP projections from layer III and II cells of the EC split and form direct and indirect projections to the hippocampus, respectively. These pathways are large unidirectional projections that mainly form excitatory synapses onto excitatory cells (Menno, 2007; Nafstad, 1967). The direct pathway is characterized by a projection from layer III cells of the EC to the apical dendrites of CA1 pyramidal neurons, which then sends information out of the hippocampus. The indirect pathway begins with axonal projections from layer II of the EC innervating the outer and inner third of the molecular layer, where the dendrites of DGCs arborize. These GCs give rise to axons called mossy fibers, which collateralize significantly in the hilus and terminate there as well as the stratum lucidum of the CA3 region. This projection constitutes the mossy fiber pathway, named so because the GC axons sport distinct synaptic boutons that look like moss. These are the axons that sprout in an epileptic brain; they sprout axon collaterals in the IML of the DG and form recurrent excitatory connections, a likely contribution to recurrent excitation and enhanced susceptibility to seizures (Sutula and Dudek, 2007). The CA3 pyramidal cells have highly collateralized axons that give rise to the Schaffer collaterals, which innervate CA1 pyramidal cell proximal dendrites. These collaterals then send signals back to
deep layers of the EC and subiculum, as well as to more superficial layers of the EC, completing a circuit loop (Witter, 2010; Amaral, 1993).

The connections between the EC, the DG, and Ammon’s horn principal cells is known as the excitatory trisynaptic loop. Granule cells are situated in such a way that they receive massive amounts of excitatory input from the EC, yet, in a healthy brain, excitatory propagation of signals is kept at bay and a very delicate balance of excitation and inhibition is maintained. This tight regulation occurs through primary mechanisms including the intrinsic properties of GCs, as well as feedforward and feedback inhibition from GABAergic interneurons throughout the hippocampus. The death of interneurons throughout the limbic circuit in epilepsy is one factor that facilitates propagation of activity through the hippocampus.

Despite their positioning to send and receive extensive excitatory input, GCs in a healthy brain exhibit reluctance to fire, and rarely do so synchronously. They are equipped with both synaptic and extrasynaptic GABA_A receptors (GABA_AR) that bear distinct properties critical to preventing runaway excitation in these cells. Extrasynaptic GABA_ARs have a high affinity for GABA and do not desensitize, resulting in a tonic GABA current activated by the extrasynaptic GABA. This decreases the propensity of GCs to fire an action potential (AP) (Coulter and Carlson, 2007; Hsu, 2007; Farrant and Nusser, 2005). Both of these receptors undergo structural changes in TLE that disrupt the tight control on excitation in GCs.

In addition to the principal cells of the trisynaptic loop, the hippocampus is home to a variety of GABAergic interneurons that show heterogeneous neuropeptide
and calcium binding protein expression, distinctive electrophysiological properties, and differences in synaptic targets. Together, these populations provide powerful feedforward and feedback GABAergic inhibition to the principal cells.

IV. GABAERGIC INTERNEURONS OF THE HIPPOCAMPUS

Cortical and hippocampal GABAergic interneurons are born in the ventral telencephalon ganglion eminences and migrate extensively to reach their destinations, where they mature into functional and distinct subtypes. They serve to maintain a delicate balance of excitation and inhibition in the hippocampus and play critical roles in population oscillations and synaptic plasticity (Buzaki et al., 1996). Interneurons throughout the hippocampus are abundant, especially in the hilus, where they send distinct and local axonal projections to principal cells and to each other. Although GABA is the classical neurotransmitter expressed by these cells, they are extremely complex and heterogeneous in terms of co-expression of other neuroactive molecules and calcium binding proteins (Freund and Buzaki, 1996; Amaral, 1978; Jinno and Kosaka, 2006). There are various means of classifying interneurons, but commonly they are classified according to their axonal targets or neurochemical identities based on the expression of calcium binding proteins and neuropeptides (Houser, 2007). However, there really is no single best way to classify interneurons because they show significant electrophysiology and morphological diversity, even within subtypes of GABAergic interneurons identified by the same neurochemicals (and sometimes these neurochemicals are co-expressed) (Pawelzik et al., 2002; Jinno and Kosaka 2006) (Fig. 2).
Figure 2. The Hippocampus is characterized by principal cells with unique properties and a variety of interneurons with distinct electrophysiological properties, axonal projections, and neurochemical markers. Principal, excitatory cells are red and GABAergic interneurons are in green. Outward arrows and closed circles at the axon terminals represent excitatory and inhibitory synapses, respectively. Below the name of each cell type is a common neurochemical marker for their immunoreactivity. Densely packed dentate granule cells (DGCs) make up the granule cell layer (GCL) and exhibit prototypical, unipolar, spiny apical dendrites that extend into the molecular layer (MOL). Their axons collateralize in the hilus, innervating GABAergic interneurons and mossy cells, eventually terminating on apical dendrites of CA3 pyramidal cells. These axons have characteristically large boutons that form en passant synapses with their target cells. Newborn DGCs arise from the subgranular zone (SGZ) of the DG. The principal cells of CA1 and CA3 are pyramidal cells, situated in a densely packed cell body layers of like cells as well as...
different GABAergic interneurons, namely basket cells. **Mossy cells** are glutamatergic cells situated in the hilus. They have large cell bodies with characteristic thorny excrescences and axonal projections to the IML and also form synapses onto GABAergic interneurons in the hilus. **Basket cells** are GABAergic interneurons typically located at the base of the GCL and display apical and basal dendrites and axon collaterals throughout the GCL that form synapses with the cell bodies of multiple GCs and other basket cells. Hilar perforant path–associated cells (HIPP) are GABAergic interneurons with cell bodies in the hilus and axonal projections to the outer 2/3 of the MOL. Hilar commissural-association pathway-related cells (HICAP) are GABAergic interneurons with cell bodies in the hilus and axonal projections to inner 2/3 of the MOL. Molecular perforant path-associated cells (MOPP) are GABAergic interneurons with cell bodies in the molecular layer and axonal projections that target the OML. Connections exist between these groups of interneurons in the dentate gyrus, though the axon collaterals of the MOPP cells for the most part don’t overlap with those of the HIPP cells or the mossy cell axonal projections (Morgan et al., 2007). *This is an extremely limited representation of the hippocampal interneurons and their excitatory axonal targets. Not represented here at all are the synaptic connections between interneurons.*

The GABAergic interneurons that form perisomatic synapses are the basket and axoaxonic cell populations. Basket cells encompass five morphological types that have different dendritic arborizations and somal positions (Ribak, 1992). Most notably dentate basket cells have cell bodies at the base of the GCL, with apical and basal dendrites. Their axons originate from the apical dendrites and send axon collaterals throughout the GCL, forming synapses onto cell bodies of multiple GCLs and other basket cells. Due to the location of these cells, they receive a lot of input onto their somas and apical and basal dendrites. The significant input they receive from mossy fibers facilitates strong feedback inhibition to GCs and, because their apical dendrites are also targets of the perforant path axons, they create strong feedforward inhibition (Ribak, 1992). The calcium binding protein parvalbumin (PV) is present in all basket cells, though less than half of the basket cell terminals are
immunoreactive for PV. PV aids in the firing properties of basket cells. Specifically, they exhibit large and fast hyperpolarizations, allowing them to fire non-adapting trains of action potentials (APs) at high frequencies (Hua et al., 2014). Basket cells are often referred to as fast spiking interneurons for this reason. Their fast spiking property may be particularly important in regulating thalamocortical oscillations, and they tend to be connected via gap junctions (Freund and Katona, 2007; Kawaguchi, et all 1987). These cells are located throughout the hippocampus and the percentage of GABAergic interneurons that express PV is higher in CA3 and CA1 pyramidal regions than in the DG. Many DG basket cells are also immunoreactive for Cholecystokinin (CCK) and can form inhibitory synapses onto each other (Houser, 2007).

A xoaxonic cells are a second type of hippocampal interneuron whose somas are located near the GCL or within the hilus as well as CA1 and CA3. This type of interneuron has characteristic axons that branch extensively and form synapses mostly onto the axon initial segments of granule cells. Many of these cells are immunoreactive for PV, in addition to the basket cells. The proximal synaptic locations of axoaxonic and basket cells allow them to exert significant control over the output of DGCs.

Another central class of interneurons in the DG terminate onto the dendrites of their target cells, and are known as hilar interneurons associated with the perforant path (HIPP). Their cell bodies are in the hilus and their axons project to the outer 2/3 of the MOL, where they terminate in the outer molecular layer (OML) and co-mingle
with excitatory input from the EC onto DGC apical dendrites. These synapses onto GC dendrites may shunt EPSPs at or near the synaptic site (Coulter, 2007; Freund and Buzsaki, 1996). In this way they provide powerful feedback inhibition and are crucial to keeping excitation at bay. Located in the hilus, most of these cells are immunoreactive for somatostatin (SOM) or neuropeptide Y (NPY) and have dendrites that extend across the hilus (Houser, 2007). This population of HIPP cells have been shown to be particularly vulnerable to excitatory damage and are often lost in animal and human models of TLE, impairing this gating of excitation from the EC (Buckmaster and Dudek, 2006; Dudek, 2010). Interestingly, some of the surviving hilar SOM⁺ interneurons enlarge and sprout their axons in the MOL of DG, forming new synapses onto dendrites of GCs (Peng et al., 2013; Zhang et al., 2009). Also important for feedback inhibition are hilar Commissural-association pathway-related cells (HICAP). With a cell body in hilus these cells send axonal projection to inner 2/3 MOL, and many are immunoreactive for CCK. HICAP and HIPP cells form synapses onto basket cells and have significant effects on the activity of their target interneurons (Savanthrapadian, 2014). Other interneurons in the hilus that tend to form synapses onto interneurons often express the calcium binding protein calretinin and frequently coexpress other neuromodulators (Miles et al. 1996). One other class of interneurons that terminate onto the dendrites of target cells are responsible for feedforward inhibition to GCL dendrites. These cells, known as molecular perforant path-associated cells (MOPP), have cell a body in the MOL and, like HIPP cells, innervate the OML, the site of many excitatory synapses onto distal dendrites of GCs.
V. SYNAPTogenesis AND FUNDAMENTAL REGULATORY MOLECULES OF GABAergic SYNAPSE FORMATION

Synaptogenesis and the postsynaptic density

Most inhibitory neurotransmission in the mammalian forebrain is mediated by GABA acting through ionotropic GABA_A and metabotropic, GABA_B receptors. GABAergic synapses, thus, are crucial for proper functionality of neuronal connectivity and maintaining a balance of neuronal excitation and inhibition. A better understanding of the molecular mechanisms regulating GABAergic synapse formation can help to inform research aiming at circuit repair by identifying key molecules involved in maintaining a balance of neuronal excitation and inhibition.

The ability for humans to think, move, behave in any way really, depends on a nervous system that fosters functional neural networks, into which upwards of 86 billion neurons and 85 billion nonneuronal cells are interconnected and wired into complex circuits (Azevedo et al., 2009). This tightly regulated and highly plastic communication is contingent upon proper formation and stabilization of synapses, specialized junctions at which a neuron can communicate with a target cell. The two general categories of synapses are electrical and chemical, which differ fundamentally in their means of transmission. My research focuses on chemical synapses. They are unique to neurons and allow unidirectional communication of discontinuous neurons via highly regulated release of neurotransmitters.
Synaptic contacts are generally formed between the presynaptic axon terminal and the postsynaptic dendrite, soma, or axon of the cell that will receive and transduce the signal. The process of synapse formation is highly complex but consists of characteristic steps that facilitate and mediate the alignment of pre and postsynaptic partners. The steps include the recognition by an arriving growth cone of its appropriate partner cell and the consequential recruitment of specialized pre-and postsynaptic proteins that will form and stabilize a functional synapse (Colón-Ramos, 2009; Brose, 1999). The initial pairing of pre-and postsynaptic partners in the first stage of synapse formation is highly specific and requires proper implementation of developmental stages, such as cell fate specification, migration, axon guidance, dendritic growth, and synaptic target selection. In the initial stages of synaptogenesis, the axonal growth cone acts as a pathfinder, facilitating synapse formation with specific synaptic partners by responding to extracellular cues that mark correct pathways and prevent wrong ones. These axonal guidance cues vary and come from different receptor ligand families such as cadherins, ephrins, semaphorins, netrins, and other cell adhesion molecules (CAMs)(Huber et al., 2003). Upon contacting its appropriate partner, the growth cone transforms from a growing, moving, path-finding organelle into a presynaptic terminal. In apposition to this presynaptic membrane, and almost simultaneously, the neuronal plasma membrane of the postsynaptic cell undergoes differentiation, recruiting various proteins that will allow for aggregation of neurotransmitter receptors (NTRs) or NTR-associated proteins, making the cell highly specialized to receive the neurotransmitter-mediated signal
from the presynaptic axon terminal, and transduce that into biochemical and electrical changes inside that postsynaptic cell.

Proper synaptic maturation and function is contingent upon appropriate differentiation of the postsynaptic membrane. One fundamental feature of chemical synapses is the postsynaptic density (PSD), a generic term that has its roots in the ultrastructural electron-dense appearance of the postsynaptic membrane. The PSD faces the presynaptic active zone and consists of membrane-bound receptors embedded in a network of proteins that stabilize the apposition between pre and postsynaptic membrane. PSDs also cluster postsynaptic receptors and help to couple the activation of these receptors to the biochemical signaling events in the postsynaptic neuron. As a result the PSD contains a high concentration of ligand-gated ion channels, helping to ensure the generation of sharp signals following neurotransmitter release (Tyagarajan and Fritchy, 2012; Sheng and Kim, 2011).

Excitatory synapses are characterized by a large, electron-dense PSD, resulting from the contact initiation of glutamatergic axon terminals with postsynaptic dendritic spines containing glutamate receptors, principally N-methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (Sheng & Kim, 2011). Colloquially in the field “PSD” as a term often refers to excitatory synapses, owing to the fact that most inhibitory synapses are characterized by a very thin PSD, similar in size actually to the presynaptic active zone. For the sake of this review the term PSD is not wed to any one type of synapse.
The inhibitory PSD is mostly found on dendritic shafts, cell bodies, and axon initial segments of postsynaptic cells (Sheng & Kim, 2011). It serves the same overlying purpose as the excitatory PSD, though functions quite differently on a molecular basis. Advancements in techniques for studying synaptogenic molecular machinery support the notion that, despite its less distinct ultrastructural appearance, the inhibitory PSD is quite complex and serves as the basis for regulation of GABAergic synaptogenesis and transmission. Understanding the complexities of the excitatory and inhibitory PSD is critical to understanding how these synapses form, mature, and stabilize properly. Research on various molecules of the inhibitory PSD has gained traction in recent years, as alterations in the expression or function of PSD scaffolding molecules have been significantly associated with neuropathologies (Sudhof, 2008; Lin and Koleske, 2010).

**Key molecules at the postsynaptic site of GABAergic signals**

Many components of excitatory and inhibitory PSDs are subject to heterogeneous post-translational regulation that give synapses dynamic and plastic activity-dependent processes. In recent years a growing number of inhibitory postsynaptic molecules have been identified, and it is well noted that most are organized around gephyrin, a key protein in the scaffolding system of GABAergic synapses. Many proteins are responsible for assembling CNS inhibitory synapses (Fig.3), but research suggests that neuroligins (NLGNs) and their transsynaptic binding partners neurexins (NRXNs) are particularly important regulatory molecules
at GABAergic synapses and act to stabilize pre and post synaptic proteins across the synaptic cleft (Craig & Kang, 2007).
Figure 3. Postsynaptic Protein scaffold at GABAergic synapses.

Gephyrin trimers are thought to form a submembranous, hexagonal lattice that aggregates and anchors inhibitory neurotransmitter receptors (GlyRs and GABA_ARs), which are embedded in the postsynaptic membrane. Gephyrin and GABA_AR clustering are dependent on each other to an extent. Neuroligin2 (NLGN2) transynaptically binds to alpha and beta neurexins and facilitates proper postsynaptic differentiation in precise apposition to the presynaptic side by interacting directly and obliquely with proteins like gephyrin and collybistin (CB). CB is critical for stabilizing intracellular gephyrin and tethering it to the postsynaptic membrane. Mechanisms for gephyrin clustering at the GABAergic synapse are still largely under debate, though proper tethering of gephyrin to the postsynaptic membrane is thought to require the binding of complexes containing gephyrin, NLGN2, and CB.
Gephyrin

Named after the Greek word for bridge, the multi-domain protein gephyrin is expressed almost ubiquitously at inhibitory synapses and it is the principal scaffolding molecule of the inhibitory PSD, crucial for clustering GABA<sub>A</sub> and glycine receptors, the key mediators of synaptic inhibition (Prior et al. 1992; Kirch et al. 1993; Craig et al., 1996; Feng et al., 1998; Essrich et al. 1998; Su et al., 2007; Jacob et al. 2005; Tyagarajan and Fritchy, 2014; Specht and Triller, 2008). The inhibitory PSDs are characterized by dense clusters of gephyrin and, although gephyrin lacks the canonical PDZ domain (the intracellular interaction domain that plays important roles in organizing excitatory scaffolding proteins), it maintains the ability, via its E domain, to bind to an amino acid stretch on the intracellular tail present on the gephyrin’s binding partner NLGN, and is thought to form aggregates by spontaneous oligomerization (Fig. 4; Pouloupolos et al., 2009; Colin et al., 1996; Sheng and Sala, 2001; Kneussel & H. Betz, 2000; Garner et al., 2000). These oligomerization features suggest a model of GABA<sub>A</sub>R cluster formation whereby gephyrin forms a hexagonal lattice underneath the synaptic membrane that provides both stability to postsynaptic GABA<sub>A</sub> and glycine receptors in front of the presynaptic releasing sites, as well as a high number of binding sites for these receptors (Kneussel and Betz, 2000; Schwarz et al., 2001; Sola et al., 2001, 2004; Xiang et al., 2001; Maric et al., 2011; Kowalczyk et al., 2013).
(Dumoulin et al., 2010; Papadopoulos and Soykan, 2011).
Figure 4. Gephyrin regulates lateral mobility of GABA$_A$ and Glycine neurotransmitter receptors (A) Gephyrin comprises three structural domains, C, G, and E. The C domain links the G and E domains and is often subject to extensive post-translational modifications, like phosphorylation, that affect the oligomerization properties of gephyrin and thus its receptor-binding ability. Shown here in the C domain are sequences for important binding sites for Pin1 and collybistin, both of which are critical in regulating GABAergic signaling. (B) Gephyrin molecules retain the ability to trimerize and dimerize at G and E domains, respectively, allowing them to form a submembranous hexagonal lattice. Several subunits of GABA$_A$(α1–3 and β2–3) and glycine receptors are anchored by this lattice by binding the cytoplasmic loop of their beta subunit with the E-domain of gephyrin. It is thought that there is just one binding site per gephyrin E domain. The E domain of gephyrin is crucial as well for interactions with NLGN 2 and collybistin.

Gephyrin is critical in clustering both GlyR and GABA$_A$Rs, members of the superfamily of cys-loop ligand-gated ion channels; these receptors are integral ion channels that mediate fast synaptic transmission and exhibit a pentameric homo or heteromeric subunit structure (Tyagarajan and Fritchy, 2014). GABA$_A$Rs show extensive subunit heterogeneity, and research has shown that only a subset of GABA$_A$R subtypes, those containing the α1-, α2- or α3-subunit along with the γ2-subunit, are localized postsynaptically with gephyrin (Farrant and Nusser 2005; Tyagarajan and Fritchy, 2014). The exact role of gephyrin in the clustering of GABA$_A$Rs, or the means by which it does so is not yet fully understood, though data suggests that clustering of gephyrin and GABA$_A$Rs are to an extent mutually dependent on each other (Essruch 1998). This relationship is undoubtedly important and, in diseases such as epilepsy, both gephyrin and GABA$_A$R clustering are deregulated (Fang et al., 2011; Houser et al., 2012).

Gephyrin’s role in GABA$_A$R trafficking to synapses is thought to involve the formation of a complex of kinesin family motor protein 5 (KIF5) with GABA$_A$R-
associated protein and gephyrin. An alternative theory is that GABA_{A}R trafficking is mediated by KIF5 in complex with huntingtin-associated protein (Tyagarajan and Fritchy, 2014; Twelvetrees, A. et al., 2010). Recent evidence suggests that gephyrin phosphorylation plays a key role in gephyrin-dependent GABA_{A}Rs clustering as well (Zacchi et al., 2014; Antonelli et al., 2014).

Gephyrin is not a static scaffolding molecule; it is extremely dynamic in its interaction with a variety of signaling molecules. Extensive post-translational modifications, such as phosphorylation, alter the critical clustering properties of the postsynaptic scaffold and the plasticity and stability of GABAergic synapses. (Tyagarajan and Fritschy, 2014; Deprez et al., 2015; Antonelli et al., 2014)

Taken together, much of this research highlights the importance of regulation of the gephyrin scaffold at inhibitory synapses, for proper inhibitory neurotransmission. Gephyrin is key, but the ability of gephyrin to execute its functional and clustering properties is contingent upon its recruitment to the GABAergic synapse. Mobilization of gephyrin depends significantly on the contribution of guanine nucleotide exchange factor collybistin (CB) and neuroligin2 (NLGN2) (Südhof, 2008; Kins et al., 2000). Different models have been posited about complexes of gephyrin, CB, and NLGN2, as sufficient to induce clustering of inhibitory neurotransmitters receptors (Poulopoulos et al., 2009). One highly supported theory is that NLGN2, interacting extracellularly with presynaptic neurexins at GABAergic axon terminals, recruits postsynaptic gephyrin intracellularly but also gephyrin-bound CB. This is thought to aid in CB activation.
and to thus enhance the tethering of gephyrin to the postsynaptic membrane followed by recruitment of GABA\_\textsubscript{A}Rs and other proteins associated with the gephyrin scaffold (Varoqueaux et al. 2006; Poulopoulos et al. 2009). This theory is debated but it is clear that CB isoforms contribute significantly to the stabilization of intracellular gephyrin by tethering it to the postsynaptic membrane (Tyagarajan and Fritchy, 2014). Although many synaptic cell adhesion molecules are crucial to synapse formation, NLGN2 is the only one exclusively located at GABAergic synapses that interacts directly with gephyrin, and has been shown to be critical in the function of GABAergic synapses. The ability to direct and stabilize key molecules of the PSD make NLGNs prime candidates as central organizing molecules that stabilize complexes of and pre and post synaptic proteins across the synaptic cleft (Craig and Kang, 2007).

VI. NEUROLIGINS IN SYNAPSE FORMATION, MATURATION, AND FUNCTION

Neuroligin 2 (NLGN2) is a member of the neuroligin (NLGN) family of cell adhesion molecules (CAMs). Research on NLGNs has gained footing because of their central roles in functional synaptic organization and the mounting evidence supporting crucial roles for NLGNs and neurexins (NRXNs) in psychiatric illnesses; genetic alterations of these CAMs are implicated in autism spectrum disorders, schizophrenia, and other cognitive illnesses (Sudhof 2008, Jamain et al., 2003; Bourgeron, 2009;).
NLGNs are a family of postsynaptic CAM proteins that transsynaptically bind with presynaptic NRXNs to mediate precise signaling between the presynaptic axon and the postsynaptic target (Siddiqui and Craig, 2011). These transmembrane proteins contain three major domains that regulate their function: a cholinesterase-like domain (CLD), an O-glycosylation cassette, and a short C-terminal tail (Ichtchenko et al., 1995). The homologous extracellular acetylcholinesterase-like domains bridge the synaptic cleft and bind beta neurexins, while the intracellular c-terminal motifs bind to PDZ domains of several postsynaptic proteins (Fig. 5).
Adapted from Li et al., 2007
**Figure 5. Neuroligin 2 binds transynaptically with neurexin**

(A) The three major domains that regulate neuroligin (NLGN) function are the cholinesterase-like domain (CLD), an O-glycosylation cassette (O-Glyc), and a short C-terminal tail. (B) The homologous extracellular CLDs bridge the synaptic cleft and bind beta neurexins (NRXNs), while the intracellular c-terminal motifs bind to PDZ domains of several postsynaptic proteins. NLGNs and NRXNs are differentially located at excitatory and inhibitory synapses, owing in part to their extracellular domains that are modified by alternative splicing. Alternative splicing at position 4(S4) of beta NRXNs changes its binding specificity for its postsynaptic NLGN partner, thus changing it ability to form glutamatergic or GABAergic synapses. Each NRXN isoform only pairs with certain NLGN isoforms; inclusion of alternative exon at S4 position in beta NRXN (+S4) creates a high affinity for NLGN2, and triggers clustering of NLGN2 as well as gephyrin. NLGN2 binds gephyrin through a conserved cytoplasmic motif known as the gephyrin-binding motif.

For the most part, the transynaptic binding partners of NLGNs, NRXNs, are localized presynaptically and their distribution at excitatory and inhibitory synapses is regulated by alternative splicing patterns (Krueger et al., 2012). NLGNs are key mediators of synapse differentiation and formation, but it is the transynaptic interaction with NRXNs that underlies the ability of NLGNs to induce functional synapses (Futai et al. 2013).

Seminal in vitro studies of these molecules showed that the expression of NLGN 1 or 2 on the surface of non-neuronal cells was sufficient to induce morphological and functional presynaptic differentiation at contact sites in contacting axons of co-cultured neurons (Scheiffele et al. 2000). This ability was specific to NLGNs and complimentary studies showed that heterologous expression of NRXNs in non-neuronal cells induces formation of synapse-like contacts with the cultured neurons (Graf et al., 2004). Moreover, overexpression of NLGNs in cultured neurons has been shown to increase synapse density (Scheiffele et al., 2000; Prange et al., 2004).
Although NLGNs can induce these artificial synapses *in-vitro*, they aren’t necessary for synapse formation and function *in-vivo*. NLGN knockout studies suggest that NLGNs are more important for stabilizing nascent synaptic sites rather than the initial formation of synaptic contacts, though they are definitely required for proper and functional synapse maturation and brain function (Varoqueaux et al., 2006; Chubykin et al., 2007; Arstikaitis et al., 2011; Chen et al., 2010). It has been well documented that NLGNs control the formation and balance of excitatory and inhibitory synapses, though they function heterogeneously (Chih et al, 2005).

There are 5 known isoforms of NLGNS known in humans encoded by distinct genes (NL1,NL2,NL3,NL4x,NL4Y), 4 of which have been identified in the rodent brain, and shown to localize at different types of synapses (Krueger et al., 2012). NLGN1 is present at glutamatergic synapses (Song et al., 1999), and NLGN2 has shown to be localized to GABAergic synapses and, as recent research shows, postsynaptic sites of cholinergic synapses (Varoqueaux et al., 2004; Takács, Freund, and Nyiri, 2013). NLGN3 is present at GABAergic and glutamatergic synapses (Budrek and scheiffle 2007), while NLGN4 has been shown to localize at glycineric synapses (Hoon et al 2011). NLGN2 and NLGN1 are the only known NLGNS to be brain-specific proteins, as transcripts of NLGN3 and NLGN4 are detected in peripheral tissue (Bollinger et al., 2001; Philibert et al., 2000). These various isoforms are differentially enriched in postsynaptic specializations, suggesting their unique ability to affect synaptic transmission and the recruitment of synaptogenic proteins at different types of synapses.
NLGN2 is a prime candidate for organizing and maintaining GABAergic synapses. In addition to its specific location at these synapses in the CNS, it directly binds Gephyrin and CB, and can induce the recruitment of gephyrin to cluster sites when exogenously clustered at the surface of cells (Poulopulos, 2009; Graf et al., 2004). Consistent with the location of NLGN2 to inhibitory synapses, NLGN2 KO mice showed impairments in inhibitory transmission (Blundell et al., 2009; Gibson et al., 2009; Poulopoulos et al., 2009; Jedlicka et al. 2011), whereas NLGN over-expression in animals increased inhibition (Hines et al., 2008). NLGN2 knockout studies in adult mice have a decrease in gephyrin puncta at perisomatic sites and electrophysiological data suggest fewer inhibitory postsynaptic events (Poulopoulos, 2009). Moreover, deletion of NLGN2 selectively impairs synaptic transmission mediated by fast spiking PV interneurons, but not GABAergic synapses formed by somatostatin interneurons (Gibson et al., 2009).

In vivo studies of NLGN2 deficient mice showed a strong increase in DGC excitability and impaired network inhibition strongly associated with reduced GABA_AR-mediated IPSCs recorded from the GCs (Jedlicka et al. 2011). In this study, the number of GABA_A and Gephyrin clusters on the somata of GCs was reduced, supporting the idea that NLGN2 is critical for the proper formation of perisomatic synapses.

At the behavioral level, manipulations of NLGN2 expression yields behavioral phenotypes consistent with a decrease in inhibitory transmission or overall alterations in hippocampal excitation/inhibition balance. A loss of NLGN2 increased
anxiety like behavior, whereas NLGN2 overexpression reduced aggression and led to
significant changes in behavior as well as composition of inhibitory pre and post
synaptic proteins (Blundell et al., 2009; Wöhr et al., 2013; Kohl, 2013, 2014).
Acting through the recruitment of gephyrin, NLGN2 is clearly important in
maturation and differentiation events at postsynaptic sites, particularly the
perisomatic synaptic sites.

Few studies have examined NLGN2 in diseased states, but a detailed analysis
of synaptic properties in mice post-SE showed that the levels of NGLN2 were
reduced in both newborn and adult GCs (Jackson et al., 2012). Taken together,
NLGN2 may be a key regulatory molecule of GABAergic synapse formation and
stabilization.

VII. AAV-MEDIATED GENE DELIVERY AS A TOOL TO INVESTIGATE
SYNAPSE FORMATION

The ability to transfet nucleic acids into cells has become crucial in neuronal
cell biology, facilitating studies of specific gene and protein function within the larger,
highly controlled systems at work in the brain. Some widely used methods to
transfect mammalian neural precursors and postmitotic neurons include electrical
transfection methods like electroporation and nucleofection, chemical transfection
methods such as Ca\(^{2+}\)-phosphate/DNA coprecipitation, lipofection, and various virus-
based transfection methods, including lentivirus, adenovirus, and adeno-associated
virus (Karra and Dahm, 2010).
With the advent of new, high expression vectors containing promoters linked to the gene(s) of interest (GOI), viral-mediated gene delivery has become more common. These vectors offer a means of temporally and spatially controlling expression of a GOI, and thus are important avenues for studying cell behavior in healthy systems, and possibly being able to repair circuits in broken ones. The transfection methodology central to my research utilizes adeno-associated virus (AAV), which has been used therapeutically in clinical trials, and as a research tool to study synapse formation, underlying molecular properties of behavior, and mapping and manipulating neuronal circuitry and activity (Fekete et al., 2015; Tsang and Young, 2014; Kohl et al., 2013; Betley and Sternson, 2011). The key features of AAV that make it an attractive gene delivery system are its lack of pathogenicity, ability to transduce a wide variety of cells regardless of stage in life cycle, and long term gene expression in the CNS (Büning et al., 2008; Kaplitt et al., 1994; Lo et al., 1999).

In 1965, the first AAV was discovered as a contaminant of adenovirus preparations, and dubbed “defective virus particles,” as they were small DNA-containing particles unable to replicate unless simultaneously inoculated with adenovirus (Atchison et al., 1966). These “defective virus particles” have gained considerable attention in recent years, owing to their potential as vectors for gene therapy and research tools. AAV are small single stranded DNA (ssDNA) viruses (parvoviruses) and, because they normally require co-infection of helper virus for productive infection to occur, AAV serotypes are ascribed to a separate genus of the
parvoviridae family, dependovirus (Goncalves, 2005). The replication deficiency underlies the apparent lack of AAV pathogenicity, and is largely the appeal of using these vectors in research and therapeutic models (Goncalves, 2005). AAV also maintains the ability to integrate into the host cell genome at a specific site on the 19th chromosome of humans, where it remains latent until the cell is infected with a helper virus that would allow for replication of AAV and shedding of infectious particles. In terms of working with AAV as a potential therapeutic model, this makes it extremely reliable and predictable when it comes to inserting a gene into the host cell’s genome. This is distinct from other popular viral-based transfection methods like retroviruses, which are unpredictable in this regard and subject to insertional mutagenesis (Weitzman et al., 1994).

*Structure and function of AAV*

One of the smallest viruses with a non-enveloped capsid, the AAV genome (ssDNA) consists of three open reading frames (ORF), flanked by two inverted terminal repeats (ITRs). The principal two ORFs, *rep* and *cap*, produce both non structural proteins responsible for replication and packaging of the viral genome, and structural proteins for producing viral capsid proteins, respectively (Carter and Samulski, 2000). A third ORF exists as an alternative reading frame in the *cap* gene and encodes protein that helps localize capsid proteins to the nucleolus and aids in capsid assembly (Sonntag et al., 2010). The ITRs flanking the *rep* and *cap* genes are required for the primer independent synthesis of the second strand of DNA, and do so
by forming hairpins that contribute to self-priming (Koczot et al., 1973). The AAV itself does not encode a polymerase, but instead relies on the host cell’s polymerase activities to replicate its DNA and become double-stranded though DNA synthesis, after which it can integrate into the chromosome of the host cell (Ni et al., 1998).

Though the processes that regulates trafficking of AAV particles into the nucleus of the host cell are still not fully understood, quite a bit about the AAV lifecycle is. AAV infection begins by binding to a host cell, which is achieved through the ubiquitous heparan sulphate proteoglycan structures on the host cell’s surface, followed by co-receptor-mediated internalization (Summerford and Samulski, 1998, 1999). It is thought that this utilization of such a ubiquitous docking site explains partially the broad tropism of the virus. Once the AAV enters into host cell’s nucleus it can follow the lytic or lysogenic pathway of its life cycle, occurring if the cell is infected with a helper virus, or when helper virus is absent, respectively. Upon infecting a human cell alone, AAV latency is ensued by preferential integration of the virus genome into a specific region of human chromosome 19 (designated AAVS1). This site-specific integration involves the AAV Inverted Terminal Repeats (ITRs) and Rep proteins (Rep 78 & Rep 68) (Kotin et al., 1991). Following integration into the host cell’s DNA, AAV can produce RNA and protein. It is thought that once the ssDNA to double-stranded DNA(ddDNA) conversion occurs, recombination at the ITRs forms a duplex rAAV genome that can either exist in a circular form (or linear concatemers) thought to be responsible for vector persistence and long-term transgene expression (Nakai et al., 2000). The descriptions above characterize most
wildtype AAV, though the AAV vectors ordinarily used in research and gene therapy are some form of recombinant AAV (rAAV).

Recombinant AAV

As mentioned previously, the AAV genome consists of two principal ORFs flanked by two inverted terminal repeats (ITRs). Recombinant forms of AAV are created by inserting a GOI between the ITRs in place of the ORFs, rep and cap. Rep and cap then are supplied in trans in packaging constructs along with adenoviral helper genes that are needed for replication (Collaco et al., 1999). This leads to production of a vector unable to replicate, even in the presence of a helper.

In using rAAV as a research tool it is critical not to overlook the variability of AAV serotypes, which are variations within a species of virus classified based on cell surface antigens (Baron, 1996). AAV serotypes differ in their capsule surface proteins and infection is contingent upon the specific interactions between these surface proteins and the infected cell. AAV2 is the most widely used AAV serotype for in-vitro and in-vivo gene delivery. In fact, most rAAV production involves co-transfecting an AAV2-derived gene expression vector with plasmids containing cap genes derived from different AAV serotypes, thus generating types of rAAVs that infect different cell types with varied efficiencies (Goncalves et al, 2005, Aschauer et al., 2013). Moreover only the cap sequence of AAV2 has to be replaced by the serotype specific cap- the ITRs as well as the rep ORF are typically derived from AAV2. Although AAV2 is the most extensively studied serotype, for some cell types
it has a rather low transduction efficiency, and so the isolation of different AAV serotypes has been really important in ensuring with which cell types the virus best interacts, and how efficiently it does so. In murine brain studies, for example, transduction efficiency and tropism of six AAV serotypes (1, 2, 5, 6, 8 & 9) varies significantly, highlighting the importance of picking the rAAV vector best suited to a particular study, taking into account species tissue, and region of interest (ROI) (Aschauer et al. 2013).

Recent research has attempted to merge some of the desirable qualities of multiple natural AAV isolates, the resulting vector termed AAV-DJ. This hybrid capsid was engineered via a mechanism known as DNA family shuffling technology which utilizes fragment reassembly to randomly combine cap sequences from 8 wildtype AAV serotypes, including AAV2, 4, 5, 7, 9, avian, bovine and goat (Grimm et al., 2008; Bartel et al., 2011). These Recombinant AAV-DJ vectors have been shown to mediate superior transduction efficiencies in cell types from different species and tissues in comparison with any other wildtype serotypes (Grimm et al., 2008). This makes AAV-DJ an attractive choice of recombinant vector for research purposes.

*Advantages and drawbacks of AAV*

Overall it seems that AAV is a safe and efficient vehicle for delivering DNA to various tissue targets, offering a nontoxic transduction of postmitotic cells and long-term gene expression in neurons. Much of this owes to its replication-defective
nature and the fact that it elicits very mild immune response compared with other viral vectors such as adenovirus (Zaiss et al., 2002).

Although AAV is superior in its good safety profile and potential for long-term gene expression, it does have several limitations. The principal limitation of rAAV vectors is the small packaging capacity of AAV particles (about 4.7 kb), as it becomes limiting in picking specific promoter types, reporter, and GOI. Adenoviruses, for example, can carry roughly twice as much material and retroviruses usually hold between 8-10kbp. The maximum insert size for rAAV is about 3.0 KB, though with the addition of basic tags such as GFP or RFP, the allowed size is even shorter, about 1.5 kb. While AAV is unable to carry large genes, steps are being taken to discover ways to expand the carrying capacity of AAV vectors by annealing the two ITRs (Kurylo, 2010). Some other drawbacks include slow onset to expression and inability to transduce some cell types (Day et al., 2014). Next generation AAV vectors are in the works to address these drawbacks. Overall, AAV seems to be an efficient, safe technique to study synapse formation and neural circuit dynamics.

**AAV and models of circuit repair**

Much of the ongoing research in the Naegele lab is aimed at stimulating circuit repair in a mouse model of epilepsy. One approach is to transplant GABAergic interneuron progenitors into the hippocampi of mice that have epilepsy, in order to replace lost interneurons. Prior studies show that fetal mouse GABAergic interneuron grafts into the dentate gyrus synaptically integrate and form widespread axonal arbors
Moreover, following synapse formation, murine or human GABAergic interneuron grafts appear to mediate seizure suppression in several different models of epilepsy (Henderson et al. 2014; Hunt et al. 2013; Cunnigham et al., 2014).

AAV-mediated gene delivery is a powerful approach for examining the molecular mechanisms responsible for stem cell-mediated circuit repair. Additionally, once a better understanding is achieved, it may become possible to augment or even replace cell-based therapies with gene therapy to stimulate repair and normalize circuit functions.

CHAPTER 2: AAV-MEDIATED OVEREXPRESSSION OF NLGN2 IN THE CEREBRAL CORTEX AND HIPPOCAMPUS OF ADULT AND DEVELOPING MICE

I. INTRODUCTION

GABAergic synapse number, location, and transmission critically uphold and regulate a balance between excitation and inhibition in the brain. Fewer studies have examined the molecular basis for GABAergic synapse formation, compared with the large scientific literature describing molecular aspects of glutamatergic synapses (Sheng and Kim, 2011). A number of disorders including autism, schizophrenia, and some forms of the epilepsies are characterized by an imbalance in neuronal excitation, due to GABAergic synaptic pathologies. Progress in identifying and characterizing
the families of genes regulating inhibitory synapse formation and stabilization have shown members of the NLGN family to be key regulatory molecules in the process. NLGN2 is localized chiefly at GABAergic synapses and aids in synapse formation and maintenance by interacting with gephyrin and other proteins to drive post synaptic differentiation and stabilization at nascent synaptic sites; research suggests that NLGN2 acts upstream of the protein scaffold organization in the pathway leading to postsynaptic differentiation at inhibitory synapses. (Varoqueaux et al., 2004; Poulouplous, 2009; Graf et al., 2004; Chich, 2007; Gibson et al., 2009; Jedlicka et al. 2011). It is clear that NLGN2 is critical for proper functioning of GABAergic synapse, and it has also been shown to heterogeneously function at different types of GABAergic synapses. That is, NGLN2 is vital to perisomatic synaptic transmission originating from PV-expressing interneurons but not necessarily transmission from SOM-expressing interneurons (Gibson et al., 2009; Pouloplous et al., 2009). The goal of this study was to investigate whether AAV-mediated overexpression of NLGN2 alters GABAergic synapses.

Viral vectors have become important tools for analyzing the molecular components required for proper formation and stabilization of synapses (Marie, 2013). rAAV is especially well suited for in-vivo gene delivery due to low pathogenicity, potential for long-term gene transfer, and high titer with a broad range of tropisms in both dividing and non-dividing cells. Importantly, rAAV shows promise as a mechanism for stimulating circuit repair (Hellström and Harvey, 2011).
Studying the role of NLGN2 in stem cell-mediated GABAergic synapse formation requires a better understanding of the effects of NLGN2 overexpression in-vivo in naïve mice at different ages. As a first step toward viral-mediated manipulation of GABAergic synapse formation, I investigated the effects of NLGN2 overexpression on gephyrin clustering and GABAergic innervation in the adult and developing mouse hippocampus and cerebral cortex.

II. EXPERIMENTAL METHODS

AAV-mediated Gene delivery

Throughout the course of these experiments I used three different rAAV vectors, produced by Vector Biolabs. All three vectors contained AAV2 ITR sequences with either CAG or CMV promoter, and a GFP or mCherry reporter, respectively (Figure 5). The AAV stocks were produced through co-transfecting HEK293 cells with the plasmid of interest (e.g. pAAV-cDNA6-mCherry-mNLGN2 plasmid) plus other needed helper-plasmid DNAs. Two days after transfections, cell pellets were harvested, and viruses were released through 3x cycles of freeze/thaw. Viruses were purified through CsCl-gradient ultra-centrifugation, followed by desalting. Viral titer (GC/ml - genome copies/ml) was determined through real-time PCR and sequencing verification was performed before packaging AAV.
Figure 6. AAV/DJ construct maps. (A) AAV/DJ-CAG-eGFP expresses a GFP reporter under control of a CAG promoter, a hybrid of the cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter, used to drive high levels of gene expression in mammalian expression vectors. The construct contains capsid proteins from AAV-DJ and ITRs derived from AAV2 (Titer: 1.0 x 10^{13} GC/ml). (B) AAV/JD-CMV-mCherry expresses mCherry under the ubiquitous CMV promoter and contains capsid proteins from AAV-DJ and ITRs derived from AAV2 (Titer: 1.8 x 10^{13} GC/ml). (C) AAV/DJ-CMV-mCherry-2A-mNLGN2 expresses the NLGN2 gene of interest (GOI) and mCherry under the CMV promoter and contains capsid proteins from AAV-DJ and ITRs derived from AAV2 (Titer: 6.4 x 10^{12} GC/ml). Tav2A is the peptide linker between mCherry and GOI ensuring co-expression at the same time. The 2.5kb ORF for mouse NLGN2 was PCR cloned into the BglII/XbaI sites pAAV-cDNA6-mCherry-T2A vector.

Animals

For experiments performed in adult mice:

Wildtype (Harlan Laboratories), VGAT-Venus (Line no. 39; Wang et al., 2009), and VGAT-ChR2(H134R)-EYFP mice from Jackson Laboratories (Zhao et al., 2011), all on the C57BL/6N background, were used for experimental procedures once they reached adulthood (at least six weeks of age). Wildtype mice were used in the initial time point analysis experiments, and VGAT-Venus and VGAT-ChR2-EYFP mice were used in all subsequent experiments that utilized the AAV/DJ-mCherry vector(s). All mice were bred, raised, and euthanized according to the Wesleyan Animal Care and Use Committee guidelines.

For experiments performed in neonatal mice:

Wildtype female mice (C57BL/6N, Harlan Laboratories) were bred with VGAT-ChR2-EYFP males (on the C57BL/6N background) and successful pregnancies were closely monitored. Before pups were born, baby powder was
sprinkled on the bedding to familiarize the mother with the smell; all pups handled later were rubbed with this same powder to help mask human scent and minimalize cannibalism of the pups. At P=0, prior to any experimentation, neonatal mice were left to nurse for a couple of hours after which they were removed to be screened for transgene. *All of the experiments performed on neonatal mice were done with or by Swechhya Shrestha.

*Stereotaxic AAV injections in adult mice*

Transgenic adult male C57BL/6 transgenic VGAT-ChR2 (Jackson Labs), adult male and female VGAT-Venus mice (Line no. 39, ;Wang et al., 2009), and adult male wildtype C57BL/6 mice (Harlan) were used for the study. Each mouse received an injection (i.p., 0.3 ml) of the non-steroidal anti-inflammatory Meloxicam (Mobic) 15-30 minutes prior to surgery. The mice were anesthetized for the duration of stereotaxic surgery by isoflurane gas inhalation (David Kopf Instruments, VetEquip, Harvard Apparatus). Stereotaxic locations were targeted using a stereotaxic device (Kopf), equipped with a Quintessential injector (Stoelting). For hippocampal CA1 injections, the AAV vector was stereotaxically injected at X, Y, Z= -1.4 mm relative to the cortical surface and a total volume of 0.5µl of control or overexpression virus solution (VectorBiolabs; see Fig.6 for titers) was injected at a rate of 0.2µl /minute. The needle was kept in position for 1 additional minute, and slowly withdrawn. Each mouse received bilateral injections. For dentate GCL injections, the needle was lowered to a depth of X, Y, Z=-2.5, relative to the cortical
surface, and 0.5µl of virus was injected at a rate of 0.2µl/minute. The needle was kept in position for 1 additional minute after injection, and then raised to a depth of z=-2.3mm, and another 0.5µl of virus was injected (Figure 7). A total of 1µl between the two hemispheres was injected in mice that received CA1 injections, and 2µl for mice that received GCL injections. The surgical scalp incision was sealed with 3M Vetbond. Mice were kept on a heating pad until they recovered from anesthesia and became mobile, and were then transferred to their home cages. All AAV waste was disregarded in accordance with the institutional Biosafety Committee requirements for BSL-2 containment and safety practices for work involving AAV.
**Figure 7. Stereotaxic injections into the hippocampal CA1 and GCL regions in adult mice.** For each injection 0.5µl of virus was injected at a rate of 0.2µl/minute. All mice received bilateral injections of either the same virus (experimental or control vector) and same location in each hemisphere, different location in each hemisphere with the same virus, or the same location each hemisphere with experimental AAV construct in one hemisphere, and the control in the other. **(A)** Injection site for CA1 injection (relative to cortical surface x=+1.25mm and -1.25mm, y= -1.9 mm, z= -1.4 mm). **(B)** Injection site for GCL injection (relative to cortical surface x=+1.25mm and -1.25mm, y= -1.9 mm, z= -2.5, -2.3).

*Intracerebroventricular AAV injections in neonatal mice*

Each P0 transgenic pup was anesthetized via cryoanesthesia followed by freehand injections of AAV into the cerebral lateral ventricles (figure 8). *AAV injections in neonatal mice were performed with or by Swechhya Shrestha.*

(Figure by Swechhya Shrestha, 2015; adapted from Kim et al., 2014, 2013).
Figure 8. Freehand AAV injection into the cerebral lateral ventricles of neonatal mice. Each pup was anesthetized via cryoanesthesia and, once tail pinch response was absent, the pup was transferred to a chilled surgery stage. All injections performed were freehand. To locate the injection site, Bregma and Lambda sutures were identified and a point 2/5 of distance from the Lambda suture to the eye was marked with ink. 1 µl of either control or experimental AAV vector diluted in 0.05% Trypan blue was slowly dispensed into each ventricle (3mm ventral from the skull) using a 32-gauge needle (Hamilton, 7803-04, RN NDL 6/PK). After sparse labeling from preliminary surgeries Trypan blue was no longer used to dilute the virus. Post-injection, the pup was transferred to a heating pad for recovery, whereupon baby powder was rubbed onto its body. Following normalization of the pup’s body temperature the pup was transferred back to the mother’s cage for nursing.

Tissue Processing

Mice were euthanized with an overdose of Fatal Plus (Henry Schein, 100 mg/kg, i.p.). The nervous system was fixed by transcardial perfusion with a rinse buffer (0.4M PO₄ buffer, sucrose, heparin(1mL/L), and ddH₂O, pH 7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The brains were removed, post-fixed in the same buffer containing fixative for 24 hours, blocked, and equilibrated in 10%, 20%, and 30% sucrose in 0.1M phosphate buffer (pH 7.4), then embedded and frozen in Tissue Freezing Medium (TBS). Cryostat sections were cut at 60µm and 12µm thicknesses and stored in antifreeze media or mounted directly onto SuperFrost Plus glass slides (Fisherbrand), respectively.

Immunohistochemistry and Antibodies

All antibodies used in these studies are described in Table 1.
The specificity of each antibody was verified by either Western blot analyses and/or immunostaining on brain sections as described by the manufacturer. For thick section immunohistochemistry, 60µm sections were stained free-floating using either Immunofluorescence (IF) or immunoperoxidase staining to visualize the AAV injection sites and morphologies of infected cells. Sections were permeabilized for 10 minutes in 0.3% Triton X (Sigma) in .01M PBS, pH 7.4, followed by incubation in blocking buffer (BB; 5% NGS; Vector labs, 2% BSA; Sigma Aldrich in 0.1% Triton-

<table>
<thead>
<tr>
<th>Primary Antibody (company; dilution)</th>
<th>Secondary Antibody (company; dilution)</th>
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<tr>
<td>rabbit anti-mCherry polyclonal (Thermo Scientific; 1:1000)</td>
<td>Goat anti-Rabbit IgG 568 (Molecular probes; 1:1000) Or Goat anti-Rabbit IgG biotinylated (Vector Labs; 1:1000)</td>
</tr>
<tr>
<td>chicken anti-GFP polyclonal (Aves; 1:1000)</td>
<td>Goat anti-Chicken IgY 488 (Aves; 1:1000)</td>
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<tr>
<td>Mouse anti-mCherry monoclonal (Clontech; 1:1000)</td>
<td>Goat anti-Mouse IgG 568 (Molecular probes; 1:1000)</td>
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<td>mouse anti-PROX1 monoclonal (EMD Millipore; 1:100)</td>
<td>Goat anti-Mouse IgG 647 (Molecular probes; 1:1000)</td>
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<td>mouse anti-CAMK11 monoclonal (EMD Millipore; 1:300)</td>
<td>Goat anti-Rabbit IgG 568 (Molecular probes; 1:1000)</td>
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<td>guinea pig anti-DCX polyclonal (EMD Millipore; 1:500)</td>
<td>Goat anti-Guinea pig IgG 647 (Molecular probes; 1:1000)</td>
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<td>rabbit anti-NLGN2 polyclonal (Synaptic Systems; 1:1000)</td>
<td>Goat anti-Rabbit IgG 647 (Molecular probes; 1:1000) Or Goat anti-Rabbit IgG biotinylated (Vector labs; 1:1000)</td>
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<td>mouse anti-Gephyrin monoclonal (Synaptic Systems; 1:300)</td>
<td>Goat anti-Mouse IgG 647 (Molecular probes; 1:1000)</td>
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PBS) for 1 hour. Primary antibodies were made in the same buffer and incubated with sections overnight at 4°C. Primary antibodies were detected with the appropriate species-specific conjugated secondary antibody. For IF, sections were incubated for two hours (room temperature) with a fluorescently tagged secondary antibody diluted in 0.1M PBS, pH 7.4, washed, and mounted using Prolong Gold or Prolong Diamond antifade reagent (Life Technologies). For triple labeling of gephyrin, mCherry, and GFP, 12µm sections were incubated in blocking buffer (BB; 5% NGS, 2% BSA in 1% Igepal; Sigma- Aldrich) for one hour. Primary and secondary antibodies were diluted in blocking buffer and sections were incubated at RT for 24 hours. For optimization of the NLGN2 and gephyrin staining, in place of Igepal I used Triton-X PBS in the blocking buffer at an increased concentration of 1.0% Triton-X PBS. Primary and secondary antibodies were made in this blocking buffer and all washes in between were done with the 1.0% Triton-X PBS.

For the immunoperoxidase staining, sections were washed with 1xPBS after incubation in primary antibody and incubated for three minutes in 0.3% H2O2 (to block endogenous peroxidase activity), and then incubated in biotinylated secondary antibody (90 minutes for GFP staining, 120 minutes for NLGN2 labeling). Sections were then washed in 1X PBS or 1% Triton-X PBS for the GFP stain and NLGN2 stain, respectively, and incubated in horseradish peroxidase conjugated Avidin-Biotin-Complex (ABC) (Vectastain ABC Kit, Vector Laboratories, USA), followed by 3 washes in PBS. Substrate reaction was carried out in 3,3′-diaminobenzidine HRP substrate (ImmPACT DAB substrate kit for peroxidase; Vector laboratories). Sections
were washed 3 times in 1xPBS. Free floating sections (60µm) were mounted on slides in Sta-On tissue adhesive (10mL/L; Leica Biosystems). Both 60 and 12µm sections were dried overnight on a slide warmer, dehydrated in ascending ethanols (25%, 70%, 90%, 95%, 100%) and cleared in Histoclear (National Diagnostics), and mounted in DPX (Sigma).

**Imaging and cellular analysis**

Photomicrographs showing the AAV injection sites and NLGN2 immunoreactivity, were taken of immunoperoxidase-stained sections on a Zeiss Axioplan microscope with a TUCSEN H Series 10 MP camera, using IS capture software. Images were taken with 5x, 20x, 40x, and 100x (oil immersion) objectives for morphological analysis of cell types. NLGN2 and Gephyrin analysis was performed using a Zeiss confocal microscope (Zeiss LSM 510). Fluorescent images and z-stacks were acquired with interval steps of 0.5µm using Zeiss Zen software at 63X (1.5 or 2 zoom water immersion). Hippocampal regions for imaging were chosen based on staining of the AAV reporter, either mCherry or GFP, and the pinhole setting was kept under 2AU. For gephyrin cluster analyses, z-stack images were collected at 63X (1.5 zoom water immersion). From the stack, three adjacent images (.5µm apart) were selected and analyzed (Zeiss Zen software). Maximum intensity projections were exported to be later analyzed with NIH Image J, using the puncta analyzer (Image J add-on; Bary Wark).
III. RESULTS

AAV/DJ-CAG-eGFP drives high expression in hippocampal and cortical neurons

AAV transduction efficiency differs significantly depending on region of infection and AAV serotype (Aschauer et al., 2013). Therefore, I first aimed to generate viral expression in my regions of interest with a control AAV/DJ vector, so that I could later drive efficient brain region-specific gene expression with an overexpression vector. In addition to verifying infection and stereotaxic coordinates I analyzed the relative time post infection (PI) required for maximal reporter expression in different hippocampal regions (Fig. 9). I performed a small-scale analysis of AAV/DJ-CAG-eGFP expression in wildtype (WT) mice by staining for the GFP reporter in mice that received GCL or CA1 injections at 2, 3, and 4 weeks PI.

I found that I was able to target the principal cell layers of the hippocampus, and that the AAV/DJ vector drives high levels of expression in the mouse brain starting as early as 2 weeks PI. The dentate granule cells (DGCs) 2 weeks PI exhibit some labeling throughout the GCL. A few DGCs were brightly labeled from the soma out to the dendrites but this was rare (Fig. 9 A). Some cells in the hilus exhibited high GFP expression and dense axonal labeling in the inner molecular layer (IML). As early as 2 weeks PI, AAV-mediated GFP expression in CA1 pyramidal neurons was evident. The AAV-GFP expression in pyramidal cells persisted and showed no decrement in expression from 2-4 weeks (Fig. 9 B,D,F). I observed that cortical cells,
most likely infected from syringe backflow, strongly expressed AAV-GFP throughout the somas and dendrites by 2 weeks PI (Fig. 9 G, H). DGC labeling seems to improve with time; GCs show the strongest GFP expression in cell bodies and dendrites 3 to 4 weeks PI.

Based on my results, the AAV/DJ shows a strong tropism towards hippocampal neurons, and the maximum GFP expression requires 2 weeks for pyramidal cells, and 3-4 weeks for DGCs.
**Figure 9. Time course of expression in GCs and CA1 pyramidal neurons infected with AAV/DJ-GFP.** (A) At two weeks post injection (PI) many axons and some cell bodies express GFP in the GCL. GFP+ processes line the inner molecular layer (IML), likely from HICAP cells. White arrowhead points to a very characteristic looking GC. This cell and its apical dendrites are clearly labeled, though this was not common at 2 weeks PI (B) Strong onset of GFP expression in CA1 pyramidal neurons as early as 2 weeks PI persisted and showed no decrement in expression from 2 to 4 weeks (D, F). (C) Many cell bodies expressing GFP are visible in the GCL, though without distinct processes labeled. Stronger expression of GFP is apparent in the infected hilar cells. (E) At 4 weeks PI GCs show the strongest and clearest GFP expression in cell bodies and dendrites. (G,H) Spiny, cortical pyramidal neurons, presumably a layer 5 pyramidal cell (G) and a layer 2/3 pyramidal cell (H), strongly express GFP throughout the entire cell at just 2 weeks PI. Scale bars for images A-F equal 100µm. Scale bars G and H equal 100 µm and 20µm, respectively.

**AAV/DJ infects heterogeneous populations of principal hippocampal neurons and drives high expression in GABAergic interneurons with negligible glial infection**

In the densely packed cell layers of the hippocampus GFP immunofluorescent expression is extremely high, making it hard to distinguish the heterogeneity of the infected cells. To get a better understanding of the morphological makeup of the cells I infected, I stained one of these sections 4 weeks PI for GFP and visualized the infected cells using the HRP substrate Diaminobenzidine (DAB). At 4 weeks PI, AAV/DJ-GFP expression was strong in a variety of spiny and aspinous hippocampal cells that exhibited distinct morphologies (Fig. 10). Larger pyramidal cells stained more darkly and extensively throughout their processes. The AAV/DJ construct infects heterogeneous hippocampal cell types and a negligible amount of glia as seen from morphological analysis.
Figure 10. AAV/DJ infects a variety of hippocampal cells with different morphologies at 4 weeks PI. Figures A-D are stained for GFP, the AAV/DJ reporter. (A) AAV labels cells throughout the densely packed GCL, though expression levels of GFP vary. (B) Suspected hilar interneurons are labeled, as well as cells in the GCL with characteristic granule cell morphology. Dark specks around some of the GCs are most likely axon terminals from basket cells infected with the AAV/DJ. (C,D) Some cells are labeled with clear pyramidal cell morphology, others more closely resemble mossy cells, though it is established that CA3 pyramidal cells often have morphology that is not pyramidal-like, but are not interneurons because of their dense spines and physiology (Scharfman and Myers, 2013). The cells display many dendritic spines on their apical and basal dendrites, suggesting they are excitatory. (E) Aspinous cell infected on the left next to a cell with archetypal pyramidal morphology. Scale bar in first panel equals 100µm. For all other images, A-E, scale bar equals 10µm.

To verify infection of principal hippocampal neurons, I immunostained AAV-infected brain tissue to detect the co-expression of the AAV with PROX1 (DGCs), DCX (immature DGCs and migrating neurons), and CAMKII (mature pyramidal neurons) (fig. 11). AAV DJ successfully infected principal cells in the hippocampus (DGCs and pyramidal neurons). Qualitative observations show that, compared to Mature DGCs and CA1 pyramidal cells, fewer newborn GCs were stained.

Previous experiments showed putative GABAergic interneurons with cell bodies in the hilus and extensive axonal labeling in the IML (Fig. 9A). To verify that these cells were GABAergic interneurons, I analyzed VGAT-ChR2-EYFP mice injected with AAV/JD-CMV-mCherry (fig. 11 D). The transgenic mice constitutively express YFP under the vesicular GABA transporter (VGAT), and the AAV drives mCherry expression under a ubiquitous CMV promoter. The AAV/DJ drives high mCherry expression in many hilar GABAergic interneurons as well as putative basket cells located at the base of the GCL(Fig. 11D).
Figure 11. AAV/DJ infects excitatory and inhibitory cells of the hippocampus

(A) Extensive colabeling of GFP and PROX1 shows that AAV/DJ-GFP infects many dentate granule cells (DGCs). (B) Colabeling of GFP and DCX shows that AAV/DJ-GFP infects immature granule cells. (C) AAV/DJ-GFP infects CA1 pyramidal cell as can be seen by extensive colabeling of GFP and CAMKII. (D) AAV/DJ-mCherry infects GABAergic interneurons of the dentate gyrus. This tissue was taken from transgenic mice that constitutively express YFP under the vesicular GABA transporter (VGAT), and the AAV used here drives mCherry expression under a ubiquitous CMV promoter. All scale bars equal 50µm.

Localizing NLGN2 and Gephyrin in adult, VGAT-Venus and VGAT-ChR2-EYFP animals

To study the effects of NLGN2 overexpression on GABAergic synapse formation in the principal cells of the hippocampus and cerebral cortex, the cell bodies and synaptic terminals of GABAergic interneurons were distinguished from excitatory cells and synapses using transgenic fluorescent reports driven by the vesicular GABA transporter (VGAT-Venus or VGAT-ChR2-EYFP mice). In all GABAergic interneurons VGAT-Venus mice express Venus protein, and VGAT-ChR2-EYFP mice, express eYFP and Channelrhodopsin(Wang et al., 2009; Zhao et al., 2011). The channelrhodopsin protein, is trafficked in the cell to the plasma membranes, enabling detailed visualization of YFP+ axon terminals (Fig. 12C).

Previous studies in our lab using transplanted medial ganglionic eminence (MGE) VGAT-Venus or VGAT-ChR2-EYFP transgenic embryos into the DG of adult mice, or in a co-culture system with astrocytes and hippocampal cells, revealed distinct morphological characteristics of the axons of transplanted cells; axonal arbors of transplanted ChR2-EYFP or Venus-expressing GABAergic interneurons branched extensively and their synaptic terminals exhibited very distinct boutons,
many of which were clearly apposed to large postsynaptic clusters of gephyrin, indicating a mature inhibitory synaptic complex (Henderson et al., 2014). In both VGAT-Venus and ChR2-EYFP adult mice, Venus or YFP are expressed throughout the axon, making visible large distribution of basket cell axons that demarcate the GCL, and cortical cells (Fig. 12A,B). Dense GABAergic innervation onto the somas and AIS profile the cell bodies of granule cells, CA3, and CA1 and cortical pyramidal cells(Fig. 12A,B). There is a clear distinction of GABAergic interneurons versus excitatory cells that are receiving GABAergic innervation (Fig. 12B,C). In adult ChR2-EYFP mice, en passant boutons characteristic of GABAergic synapses are distinctive, allowing for localization of synaptogenic proteins in pre- and postsynaptic terminals (Fig. 12C).

To visualize the synaptic proteins gephyrin and NLGN2, I examined the colocalization of gephyrin puncta with YFP+ axon terminals (Fig. 12C-E) as well as NLGN2 with gephyrin and Venus+ axon terminals (Fig. 12F-I, ). Confocal images from a z-stack are shown, and sites marked by crosshairs illustrate the apposition in x-, y-, and z- axes between presynaptic Venus or EYFP+ GABAergic axons and a cluster of postsynaptic gephyrin or gephyrin/NLGN2 clusters (Fig. 12J-K).

In summary, these experiments demonstrate that the methods I have used allow sufficient sensitivity to detect NLGN2 and gephyrin puncta in the confocal microscope and VGAT ChR2-EYFP mice show superior synaptic terminal labeling in comparison to VGAT-Venus mice.
Figure 12. Visualizing gephyrin and neuroligin2 at putative GABAergic synaptic sites in Adult, VGAT-Venus and VGAT-ChR2-EYFP mice.

(A) Confocal micrograph showing the dentate gyrus from an adult VGAT-ChR2-eYFP mouse immunolabeled for YFP. YFP+ axon terminals, most likely a large distribution of basket cell axons, outline cell bodies in the GCL and CA3. Cells in the hilus and SGZ that appear green across membrane are GABAergic interneurons. (B) confocal micrograph showing a segment of the cortex in a VGAT-Venus mouse labeled with DAPI and an antibody against GFP to visualize cell nuclei and GABAergic interneurons, respectively. (C) High magnification image from a segment of the VGAT-ChR2-EYFP cortex illustrates GABAergic interneurons as well as axon terminals around the somas of neighboring cortical cells. The axons exhibit distinct swellings characteristic of en passant GABAergic synapses. Cortical pyramidal cells appear as dark circles (B). (D) Gephyrin clusters of various sizes surround cell bodies of cortical neurons. (E) The majority of gephyrin puncta are localized around cell bodies of cortical pyramidal cells, and clustered along the YFP+ swellings, indicating that the gephyrin clusters are located postsynaptically. (J) Confocal image from a z-stack shows GABAergic synaptic varicosities in close apposition to postsynaptic gephyrin puncta surrounding the somas of cortical GABAergic interneurons and pyramidal cells. One of these sites marked by crosshairs illustrates the apposition in x-,y-, and z- axes between presynaptic eYFP+ GABAergic axon(s) and a cluster of postsynaptic gephyrin. (F-I) illustrate NLGN2 and Gephyrin localized to GABAergic axon terminals in a section from the CA3 region of a VGAT-Venus mouse. (K) Confocal images from a z-stack show NLGN2 and Gephyrin colocalized onto GABAergic axon terminals in VGAT-Venus mouse. One of these sites marked by crosshairs illustrates the apposition in x-,y-, and z- axes between presynaptic Venus+ GABAergic axons and clusters of postsynaptic gephyrin and NLGN2. Scale bar in A equals 50µm. Scale bar in B equals 100µm. Scale bar C-I equal 10µm. C-E are the same scale and F-I are the same scale. Z-stack images J, and K are from the same photos in E and I, respectively.
Extensive labeling of cells infected with AAV-DJ CMV mCherry show long-range targets and retrograde and anterograde labeling by AAV.

Synaptic analyses of AAV-infected cells in VGAT-ChR2-eYFP mice required control and overexpression AAV constructs with a mCherry reporter. Due to the small packaging capacity of AAV, inserting a GOI for the overexpression construct and a promoter required a promoter smaller than CAG. For consistency, I used control and overexpression vectors with a CMV promoter and mCherry reporter. Although the AAV was the same synthetic serotype (AAV/DJ), the new reporter could have affected viral expression. Therefore, I analyzed tissue 4 weeks PI to verify AAV expression.

I observed mCherry expression in cell bodies and axons throughout the hippocampus (Fig. 13). DGCs in the infrapyramidal blade exhibited high levels of mCherry expression and large axonal projections to CA3, characteristic of the mossy fiber pathway (Fig. 13A,B,D). Furthermore, axon terminals from the DGCs had distinct boutons characteristic of mossy fibers (Fig. 13B). I also observed a large projection originating from the base of the GCL and the MOL of the DG, projecting through the CA3 and CA1 field stratum radiatum (SR)(Fig. 13 A). The SR terminals were a lot smoother than the mossy fibers (Fig. 13C) and are likely Schaffer collaterals arising from AAV-DJ labeling of cell bodies in the CA3 and CA1 hippocampal regions in more anterior sections of the hippocampus, (Fig 13. H, I). In addition to the extensive hippocampal labeling, I observed axonal labeling in
dorsal and ventral parts of the Retrosplenial cortex (RSC) (Fig. 13G).
Figure 13. Extensive labeling of cells infected with AAV-DJ CMV mCherry show long-range targets of infected cells and suggests retrograde and anterograde labeling of AAV. (A) Representative image from the hippocampus of an adult VGAT-ChR2-eYFP mouse injected with AAV/JD-CMV-mCherry shows virally labeled GCs and interneurons in the DG as well as large axonal projections originating from the base of the GCL and the MOL, projecting through the CA3 and CA1 field stratum radiatum (SR). (B) Mossy fiber path terminals show characteristic mossy boutons. (C) Axonal projections from the base of the GCL to CA1 are smoother and most likely Schaffer Collaterals. (E,F) Anterior sections show labeling of cell bodies in the CA1 and CA3 hippocampal regions. (G) Axonal labeling is evident in the dorsal and ventral parts of the Retrosplenial cortex (RSC). Scale bar in A equals 100µm. B-G are of the same scale and scale bar equals 20µm.

Cells infected with AAV/DJ-CMV-mCherry-2A-mNLGN2 show increased NLGN2 expression

To determine whether the AAV/DJ-CMV-mCherry-2A-mNLGN2 virus led to an increase in the expression of NLGN2, DAB immunohistochemistry for nlgn2 was performed and the results are shown in Figure 14. Lower magnification images show faint endogenous NLGN2 staining throughout both hippocampi, but NLGN2 immunostaining throughout the hilus and MOL was darker in comparison to a mouse injected with the control vector (Fig. 14 A-B), suggesting that NLGN2 expression was higher.

Furthermore, cortical fields of view with AAV/DJ-CMV-mCherry-2A-mNLGN2-infected cells that exhibit varying mCherry expression show that density and brightness of NLGN2 in corresponding regions is greater in the cells or cellular processes with higher mCherry expression (Fig. 14C-H).

In line with previous data from the two different control AAV/DJ vectors (Fig. 9, 11), the overexpression vector drives mCherry expression most efficiently in
GABAergic interneurons (Fig. 14 I-K). Fig. 14 I-K shows a cortical GABAergic interneuron with high mCherry expression, surrounded by infected pyramidal cells and processes expressing mCherry less intensely. The corresponding NLGN2 stain shows a large increase in NLGN2 puncta density corresponding to the cell with high mCherry expression (Fig. 14 J). Nearby pyramidal cells with lower mCherry expression exhibit distinct and clear NLGN2 puncta as well. This is similar patterning as seen in Fig. 14 C,D,F,G. The cortical GABAergic interneuron with high levels of mCherry expression and its neighboring infected cells, are surrounded by robust YFP⁺ GABAergic axon terminals (Fig. 14K). These GABAergic axon terminals exhibit very large swellings in comparison with the cortical tissue from a naive VGAT-Chr mouse (Figure 14. C).

Taken together, these data suggest that neuronal labeling with AAV/DJ-CMV-mCherry-2A-mNLGN2 leads to increased NLGN2 expression. Moreover, NLGN2 puncta density was correlated with intensity of promoter expression, likely due to the peptide linker between mCherry and the NLGN2 gene, which ensures co-expression at the same time (Fig. 6 C).
Figure 14. Cells infected with AAV/DJ-CMV-mCherry-2A-mNLGN2 show increased NLGN2 expression. (A-B) show DAB immunohistochemistry for NLGN2 in coronal sections from mice that received the overexpression AAV vector (A) and control (B). Both sections were stained concurrently for NLGN2 and endogenous NLGN2 is labeled faintly throughout the hippocampi. The mouse that received the overexpression vector shows increased NLGN2 labeling throughout the hilus and MOL, suggesting that NLGN2 expression was higher (A). Figures C, D, and E show 3 cortical fields of view with AAV/DJ-CMV-mCherry-2A-mNLGN2-infected cells that exhibit varying mCherry expression. The corresponding fields of view (F, G, H) show NLGN2 expression in the virally labeled cells. Density and brightness of NLGN2 is greater in regions with higher mCherry expression. A cortical GABAergic interneuron with high mCherry expression, shows high NLGN2 expression as well as dense innervation from surrounding GABAergic axon terminals (I-K). Scale bar for A, B equals 60µm. C-G are of the same scale and scale bar equals 10 µm. E, H are of the same scale and scale bar equals 10µm. I-K are of the same scale and scale bar equals 20µm.

Gephyrin expression in AAV-expressing principal cells of the hippocampus

To ultimately test whether an increase in NLGN2 was accompanied by an increase in the GABAergic postsynaptic marker gephyrin, I identified gephyrin puncta in the CA1, CA3, and GCL regions of the hippocampus infected with the control vector. Gephyrin puncta were largely distributed around the somas and proximal dendrites of principal cells, with some regional variation in their size and distribution (Fig. 15 A-C). Punctate staining is clearest, most distinct, and easily quantifiable on the proximal dendrites and cell bodies in the CA1 and CA3 regions of the hippocampus (Fig. 15A-B). Gephyrin puncta in the GCL, however, are very fine an extremely dense, precluding determination of puncta numbers in the same manner as CA1 and CA3 (Fig. 15C). Puncta surrounding the proximal dendrites of DGCs in the IML of the DG more closely resemble those in the CA1 and CA3 region (Fig. 15C). Despite the clear labeling of gephyrin puncta in the CA3 region, there are
higher numbers of intracellular gephyrin aggregates, indicative of nonsynaptic gephyrin clusters.

These experiments show that Gephyrin puncta throughout the hippocampus are largely distributed around the cell bodies and proximal dendrites of principal cells and may have to be differentially quantified in various regions of the hippocampus. I also observed that AAV-DJ CMV mCherry drives relatively high expression in cell bodies throughout the hippocampus, but does not extensively label distal processes (Figure 15 A-C).
Figure 15. Gephyrin puncta distribution in AAV-DJ CMV mcherry-infected cells varies in different regions of the hippocampus. A-C show distributions of gephyrin puncta in the CA1, CA3, and GCL regions of the hippocampus infected with the control vector. Not shown here is the GFP channel that shows GABAergic interneurons and their axonal distributions. Bright specks apparent throughout somas of mCherry-expressing cells may be from excess mCherry protein taken up into the lysosomes of cells. All images, A-C are of the same scale and scale bar equals 20µm.

Pyramidal cells infected with the overexpression vector exhibit distinct gephyrin puncta and robust GABAergic innervation.

To address how the apparent increase in NLGN2 (Fig. 14) might affect GABAergic synapse strength and stabilization, I investigated gephyrin puncta expression in cells infected with the overexpression vector. Labeling from the overexpression vector was sparse and largely located in the cortex, limiting preliminary analyses to cortical pyramidal cells (Fig. 16A). Despite sparse labeling, cortical pyramidal cell showed strong mCherry expression in the soma and more proximal apical and basal dendrites (Fig. 16B). Gephyrin clusters and puncta were clear throughout the field of view but I observed distinct larger and brighter gephyrin puncta and clusters in regions of high mCherry expression in the apical and basal dendrites of the pyramidal cell, as well as processes of surrounding cells (Fig. 16C). Furthermore, this cell was heavily innervated by YFP+ GABAergic axon terminals with large axonal swellings. These results strongly suggest that NLGN2 overexpression in individual neurons can increase the size and density of postsynaptic gephyrin clusters and presynaptic GABAergic axon terminals.
Figure 16. Pyramidal cells infected with the overexpression vector exhibit distinct gephyrin puncta and robust GABAergic innervation.

(A) Photomicrograph showing sparse labeling of cells infected with the overexpression vector in a cortical region. Cell bodies and processes exhibit varying mCherry expression throughout the field of view. (B) One cortical pyramidal cell shows particularly strong mCherry expression from the overexpression vector throughout the soma and more proximal apical and basal dendrites. Neighboring is another cell with low mCherry expression, as well as numerous mCherry-expressing processes from various cells. (C) Large and distinct gephyrin clusters are detectable in regions of high mCherry expression (D) The NLGN2-overexpressing pyramidal cell is heavily innervated by GABAergic axon terminals. Density of GABAergic innervation appears greater in regions with increased gephyrin clusters. Scale bar in A equals 50µm. B-D are of the same scale and scale bar equals 20µm.
Robust GABAergic innervation onto AAV-infected cell in the neonatal mouse brain

Time did not allow for analysis of many neonatal brains infected with the overexpression and control vector. However, if the overexpression vector in adult brains elicits postsynaptic changes in the form of an increase in NLGN2 and gephyrin puncta, then perhaps in a developing brain this would translate into increased presynaptic GABAergic input. Therefore, I investigated changes in GABAergic innervation following NLGN2 overexpression in the developing brain. These experiments were performed with or by Swechhya Shrestha.

Previously I showed that the methods I used allow sufficient sensitivity to detect NLGN2 puncta in adult VGAT-ChR2-eYFP mice (Fig. 12). I verified that I could specifically visualize NLGN2 in neonatal pup tissue by examining the colocalization of NLGN2 puncta with venus+ axon terminals (Fig. 17A-D). The majority of NLGN2 puncta around cell bodies of cortical GABAergic interneurons and pyramidal cells clustered along the Venus+ swellings, indicating postsynaptic location. Confocal images from a z-stack illustrate the apposition in x-, y-, and z- axes between presynaptic Venus+ axons and a cluster of postsynaptic NLGN2 (Fig. 17D). Next, I looked at cortical regions from neonatal mice injected with control and overexpression AAV vectors, and their corresponding fields of YFP and NLGN2 expression (Fig. 17 E-L). NLGN2 puncta were slightly denser and more brightly labeled in cells with high levels of control AAV expression compared to the
overexpression vector. Moreover, cells that exhibited high expression of the overexpression vector showed denser and larger GABAergic innervations and axonal boutons, respectively (Fig. 17J,L). These results suggest an increase in GABAergic synaptic input on cells overexpressing NLGN2.
17. AAV-mediated NLGN2 overexpression in the developing mouse brain correlates with robust GABAergic innervation onto AAV-infected cells
(A-D) Confocal micrographs from a cortical region of a developing VGAT-Venus mouse show that GABAergic axon terminals and synaptic NLGN2 can be visualized in this tissue. The majority of NLGN2 puncta are localized around cell bodies of cortical pyramidal cells, and clustered along the Venus+ swellings, indicating that the NLGN2 clusters are located postsynaptically. (D) Confocal image from a z-stack shows GABAergic synaptic varicosities in close apposition to postsynaptic NLGN2 puncta surrounding the somas of cortical GABAergic interneurons and pyramidal cells. One of these sites marked by crosshairs illustrates the apposition in x-, y-, and z-axes between presynaptic Venus+ GABAergic axons and a cluster of postsynaptic NLGN2. E, I show cortical regions from a mouse injected with the control and overexpression AAV vector, respectively. Asterisks mark two cells of interest with high levels of mCherry expression. F-G, J-K show the corresponding YFP and NLGN2 in that region, and what all three look like merged (H, I). E-L are the same scale. Scale bar equals 10µm. A-C are the same scale and scale bar equals 10µm. D is an orthogonal view from the z-stack that is the maximum intensity projection in A-C.
In summary, I used AAV-mediated gene delivery to manipulate the expression of NLGN2 in mice expressing channel rhodopsin2 and eYFP under the control of promoter for the vesicular GABA transporter. Qualitative observations show higher densities of NLGN2 puncta in neurons that drive high expression of the overexpression vector as well as an increase in the size of gephyrin puncta in the adult brain, and increased GABAergic synaptic inputs onto these infected neurons in the developing brain.

IV. DISCUSSION

TLE and a number of autism spectrum disorders are characterized by dysfunctional GABAergic signaling and imbalance of neuronal excitation and inhibition. In Severe TLE, for example, the hippocampus undergoes hyperexcitable, neuroplastic changes that include degeneration of somatostatin (SOM)$^+$ and some neuropeptide Y (NPY)$^+$ GABAergic interneurons after SE in models of TLE. In addition to hyperexcitable circuitry changes post SE, populations of surviving hilar GABAergic interneurons exhibit compensatory neuroplastic changes to restore inhibition. Surviving hilar SOM$^+$ interneurons enlarge and sprout their axons in the DG, forming new circuits by innervating abnormal locations (Peng et al., 2013; Zhang et al., 2009). Despite such compensatory sprouting mechanisms, rodent models of TLE show diminished hippocampal inhibition and ongoing seizure activity (Kumar and Buckmaster, 2006; Zhang et al., 2009; Thind et al., 2010; Peng et al., 2013).
It is evident that in TLE the endogenous circuit repair mechanisms for restoring the balance of excitation and inhibition are not sufficient to prevent seizure activity. Stem cell transplantation models for TLE treatment aim to stimulate endogenous circuit repair and reverse aberrant phenotypes (Raedtt et al, 2007). Previous studies in the Naegele lab using optogenetics, long-term EEG recordings, and immunohistochemistry, suggest that fetal GABAergic interneuron grafts may suppress pharmacoresistant seizures by enhancing synaptic inhibition in DG circuits (Henderson, Gupta et al., 2014). This transplant project aims at better manipulating the timing and location of putative, restorative synapses that most likely underlie the seizure attenuation following the fetal GABAergic interneuron grafts. Knowledge about the mechanisms by which these transplanted cells functionally integrate into host circuitry is limited and underlies important questions, like why transplanted interneuron progenitors may be more effective at seizure suppression than endogenous sprouting mechanisms.

The molecular mechanisms regulating GABAergic interneuron synapse formation following transplantation are not well understood, though likely the postsynaptic scaffolding protein NLGN2 plays a key role. Research suggests that NLGN2 is essential for functional GABAergic synapse formation and stabilization. Most current knowledge about synaptic roles of NLGN2 is derived from manipulating NLGN2 levels in neuronal cultures or in-vivo using knockout (KO) or overexpression (OE) animals, though there are significant differences in results.
obtained between the different experimental systems (Hines et al. 2008; Chih et al., 2005; Kohl et al., 2013).

To better understand the role of NLGN2 in GABAergic synapse formation, I used AAV to locally manipulate NLGN2 expression and examine the effects of NLGN2 overexpression in-vivo in naïve mice at different ages. The preliminary findings suggest increased NLGN2 puncta in cells infected with an overexpression vector and increased postsynaptic gephyrin and presynaptic GABAergic inputs in regions with high levels of NLGN2 expression.

**Characteristics of hippocampal AAV/DJ infection**

AAV/DJ showed a strong tropism towards hippocampal and cortical neurons, and drove differential high reporter and transgene expression in heterogeneous cell populations starting two weeks PI (Fig. 9-11, 15). Less colabeling of GFP and DCX suggests that, compared to other hippocampal cell types, AAV/DJ infects newborn GCs less extensively (Fig. 11B). However, the animals were perfused 4 weeks PI, and neuronal daughter cells begin to downregulate DCX after around 2 weeks as they mature. It is possible that AAV did infect the cells born before or at time of injection, maybe even preferentially, but that DCX expression was largely diminished at the time the animal was perfused. Co-injecting AAV and a retrovirus would allow for better analysis of AAV transduction in populations of newborn GCs.

The three different AAV/DJ vectors largely infected cells throughout the hippocampus, though adjacent cells of the same population exhibited high variability.
in expression (Fig. 9A, 10A,B). This phenomenon is common in several AAV serotypes and thought to result from varying transgene promoter activity in different cell types (Watakabe et al., 2014). Furthermore, AAV/DJ consistently and preferentially drove high expression in pyramidal cells and GABAergic interneuron cell bodies and axonal plexus (Fig. 9, 11, 15, 16). The cortical and CA3 pyramidal cells with larger somas and extensive, spiny processes exhibited highest reporter expression throughout the cell (Fig. 9 G,H, 10C,D). It is thought that larger cells may allow more viral entry through widespread dendritic fields (Watakabe et al., 2014), and the consistent, high reporter expression in GABAergic interneurons may be correlated with their high metabolic activity. The variability of mCherry or GFP expression translates to variability in transgene expression, which is a potential issue for analyzing NLGN2 overexpression-mediated synaptic changes. For synaptic analysis on the single neuron level, this variability may be negligible if cells chosen for analysis are of similar expression intensities, as determined subjectively by the researcher, or by fluorescent intensity measurements.

Neurons infected with AAV/DJ vectors throughout the hippocampus showed long-range targets and evidence of retrograde labeling by AAV (Fig. 13). An animal that received AAV injections into the GCL exhibited labeling of GCs, Mossy fibers, Schaffer Collaterals, CA3 and CA1 pyramidal cell bodies, and axon terminals in the RSC (Fig. 13). Labeled CA3 and CA1 pyramidal cells could be due to a more lateral injection or leakage from the syringe upon removing it, respectively, as well as anterograde or retrograde AAV labeling. The labeled fibers in the SR are likely
Schaffer collaterals arising from AAV-DJ labeling of cell bodies in the CA3 and CA1 hippocampal regions in more anterior sections of the hippocampus (Fig. 13E-F). Transport of the virus from the cell body to the synapse (anterograde labeling) underlies transduction of brain regions far from the injection site, perhaps making focal gene expression a little more difficult. Research has shown serotype and cell type–specific retrograde labeling of AAV (viral transport from synapse to soma) (Aschauer et al., 2013; Castle et al., 2014). This mode of transport is highly selective, though it has been seen in the hippocampus using AAV5 (Aschauer et al., 2013).

AAV injections into the GCL resulted as well in axonal labeling in the retrosplenial cortex (RSC), suggesting a long-range projection originating from cells infected elsewhere. The RSC plays a critical role in spatial cognition and memory, and is located between limbic areas that are critical for memory formation, like the hippocampus. The RSC projects to various fields of the hippocampal formation and is connected reciprocally to the hippocampus via sparse projections from CA1, the postsubiculum, and the EC (Wyss and Van Groen, 1992). mCherry-expressing CA1 neurons in this animal (Fig. 13E) strongly suggests that the apparent axon terminals in the RSC originated from the labeled CA1 pyramidal cells. Both anterograde and retrograde axonal transport may be occurring in the adult and developing mouse tissue, as well as transynaptic labeling. In a model of local gene manipulation it is important to take into account the short and long-range transport of virus in cells.
Effects of NLGN2 overexpression on presynaptic GABAergic innervation and postsynaptic gephyrin clustering

Current knowledge on the role of NLGN2 in GABAergic synapse formation and stabilization is largely derived from manipulating NLGN2 levels in different experimental systems and animals, and yields conflicting results (Hines et al. 2008; Chih et al., 2005; Kohl et al., 2013). My experiments showed that cells infected with the overexpression AAV vector in the adult and developing cerebral cortex exhibited an increase in NLGN2 density, size, and brightness in a manner highly correlated with mCherry expression (Fig. 14,17). Surprisingly, the increase in NLGN2 puncta in these cells was not as distinct in the pup brain (Fig. 17). This could be due to lower levels of AAV expression than presented in the GABAergic interneuron in Fig. 14I-K. Other possible explanations are smaller NLGN2 puncta due to the young age of the animal, as well as variation in immunohistochemistry due to variability during the tissue processing stages. More cases need to be analyzed to properly address this finding.

Experiments in both the developing and adult brain, showed that cells with high mCherry expression from the overexpression vector were heavily innervated by GABAergic axon terminals with distinct and large synaptic boutons. In comparison with GABAergic axon terminals from an adult, naïve VGAT-ChR2-eYFP mouse, as well as terminals surrounding cells infected with control vector (Fig. 12C, 17H), the GABAergic axon terminals in regions infected with the overexpression vector were very dense and showed larger en passant swelllings, perhaps indicating greater
GABAergic innervation or just an increase in presynaptic vesicles (Fig. 14K, 16D). In a cortical region from an adult mouse injected with the overexpression vector, higher mCherry expression correlated with an increase in the cluster density and size of gephyrin puncta; gephyrin clusters are visible throughout the region, but are larger and brighter in regions of high mCherry expression (Fig. 16C). The apparent increase in gephyrin puncta clustering around proximal dendrites follows a similar pattern as the increase in NLGN2 expression in the processes with high mCherry expression in fig 15 E,H. This pattern of gephyrin is not consistent throughout the cell, and is most distinct in regions of large and dense GABAergic axon innervation, suggesting that NLGN2 overexpression increases gephyrin clustering at selective sites of synaptic contact in a manner that may be activity-dependent. Some of the brightly labeled gephyrin puncta that are colocalized with mCherry, aren’t colocalized with YFP. This suggests NLGN2-driven changes to the expression of postsynaptic proteins without changes in presynaptic innervation. These non-synaptic gephyrin aggregates indicate a saturation of molecules, whereby clusters of gephyrin or gephyrin/NLGN2 complexes, are trapped intracellularly, meaning they cannot be translocated to the cell surface (Tyagarajan et al., 2011).

Taken together, this data suggests that high levels of NLGN2 overexpression drives synaptic and nonsynaptic gephyrin clustering, as well as increased GABAergic synaptic innervation as seen by higher densities of YFP⁺ terminals and larger axonal swellings. These results are preliminary, but both conflict with and support data from other groups studying in-vivo NLGN2 overexpression. AAV-mediated NLGN2
overexpression studies in adult rats that showed that an increase in GAD65 in neuronal terminals in regions of NLGN2 overexpression, yielded no increase in gephyrin mRNA expression (Kohl et al. 2013). However, clonal overexpression of NLGN2 in the adult rat cerebral cortex following gene transfer by in utero electroporation showed an increase in VGAT and GAD65 in the GABAergic contacts that the overexpressing neurons received, along with juxtaposed gephyrin clusters. Directly comparing my results will require further quantifications and experiments, but the aforementioned experiments were performed in rats, which may account for some differences. To the best of my knowledge, AAV-mediated NLGN2 overexpression experiments have not been done in mice.

It is well documented that in epileptic models GABAergic interneurons exhibit high levels of plasticity throughout the hilus and CA1 regions (de Lanerolle et al. 1989; Long et al 2011; Houser and Esclapez, 1996). Interneurons also exhibit plasticity throughout the adult hippocampus in the dentate gyrus in healthy mice and are thought to play an activity-dependent role in modulating adult neurogenesis in the mammalian hippocampus (Masiulis, Yun, and Eisch, 2011). The extent of this interneuron plasticity in the healthy, adult hippocampus and cortex is unclear, but viral-mediated NLGN2 delivery is a promising approach for altering the structure of GABAergic synapses and increasing the formation of GABAergic synapses in both the developing and adult nervous system.
V. FUTURE DIRECTIONS

The present study focused on the effects of NGLN2 overexpression on GABAergic synapse formation in healthy mice. I have shown that cells infected with the overexpression vector exhibit large gephyrin clusters and robust GABAergic innervation. However, it will be important to quantify NLGN2 overexpression, compare gephyrin clusters on cells infected with the overexpression vector versus the control, and quantify presynaptic GABAergic input using the YFP\(^+\) axon terminals to outline mCherry-expressing cells of interest, and then performing counts of NLGN2 puncta and gephyrin puncta in adjacent sections with Image J, using the Puncta Analyzer (Image J add-on; Bary Wark). As I showed in Fig. 14, Gephyrin puncta quantification in this manner probably wouldn’t be possible in the GCL. This region would be important to quantify, as NLGN2 has been shown to be especially critical in maintenance of synapses at perisomatic sites and in controlling GC excitability (Pouloupolos et al., 2009; Jedlicka et al., 2011). If AAV labeling is consistent throughout the GCL, we may be able to do fluorescence intensity measurements per area of GCL. Another option may be to look at and quantify collybistin in this region, though distribution patterns would likely be similar to those of gephyrin.

The VGAT-ChR2-eYFP mice that I used in this study clearly demarcate sites of putative GABAergic synapses, though do not present as distinct presynaptic puncta, because these cells tend to form en passant synapses characterized by swellings all
along the axon. Thus, accurately quantifying changes in presynaptic GABAergic input would require labeling of VGAT or PV. It is important as well to analyze any changes in excitatory markers to verify possible changes in the composition of excitatory synapses as well. It would be necessary to analyze proteins that would illustrate putative excitatory synapses that cells are both making and receiving, labeled by VGLUT and PSD95, respectively.

My results showed a putative increase in gephyrin puncta clusters and size in regions with high expression of the overexpression vector. The presence of NLGN2, YFP, and gephyrin indicate that the contacts made by the Venus\(^+\) boutons were likely synaptic. However, we do not yet know if they are functional. The study that looked at clonal NLGN2 overexpression following gene transfer by \textit{in utero electroporation} showed an increase in VGAT and GAD65 in the GABAergic contacts that the NLGN2-overexpressing neurons received, along with juxtaposed gephyrin clusters, but \textit{no} increased innervation from PV containing GABAergic terminals (Fekete et al, 2014). This suggested that the increase in VGAT was not due to increased GABAergic innervation, but rather increased numbers of VGAT-containing synaptic vesicles. Thus, it will be necessary in future experiments to test if the apparent increase in presynaptic GABAergic innervation onto cells infected with the overexpression vector is functional. To test the functionality of the GABAergic innervation we can record spontaneous IPSCs from cells infected with the control versus overexpression vector, and/or optogenetically stimulate the ChR2-expressing GABAergic interneurons and record IPSCs from virally labeled principal cells in
developing and adult mice. A functional increase in GABAergic innervation, or an increase in density and stability of GABA\(_\text{A}\)Rs due to an increase in gephyrin, should yield an increase in the size and frequency of IPSCs.

NLGN2 overexpression may increase the formation of inhibitory synaptic circuitry in both the developing and adult nervous system. To investigate the role of NLGN2 in stem-cell mediated GABAergic synapse formation, we could inject adult mice with the overexpression or control vector, followed by GABAergic progenitor transplants into the same region. Using immunohistochemistry we could verify changes in pre and postsynaptic proteins in cells overexpressing NLNG2, and optogenetically stimulate the transplanted cells and record IPSCs from the NLGN2-overexpressing cells. I would expect increased innervation of the NLGN2-overexpressing cells by the transplants, as well as increased IPSCs in the cells innervated by transplanted cells. NLGN2 overexpression could be a viable approach to augment stem-cell mediated circuit repair in TLE.

To test the role of NLGN2 in GABAergic synapse formation from endogenous compensatory sprouting we could induce status epilepticus (SE) followed by injections of the control or experimental virus into the DG. Optogenetic and immunohistochemical experiments could be done to analyze changes in synaptogenic proteins at GABAergic synapses and IPSCs in GCs from sprouted SOM\(^+\) and NPY\(^+\) interneurons. Normally the compensatory sprouting post SE doesn’t create postsynaptic inhibition that effectively prevents seizures. Perhaps an increase in NLGN2 in the hyperexcitable GCs in an epileptic DG could increase innervation
from the cells sprouting in a way that might create effective postsynaptic inhibition and prevent seizure activity. This could be a novel approach to strengthen endogenous repair mechanisms rather than adding new cells.

In the context of repairing GABAergic circuits in models of TLE, it may be important to study NLGN2 overexpression specifically in adult-born GCs. When born into an epileptic environment, adult-born GCs are subject to aberrant changes that make them hyperexcitable. They alter their inhibitory and excitatory synaptic proteins and integration when encountering brain inflammation during initial stages of synapse formation, and show reduced levels of NGLN2 post SE (Chugh et al., 2013; Jackson et al., 2012). Would compensatory sprouting by GABAergic interneurons create more effective postsynaptic inhibition onto newborn GCs if they were overexpressing NLGN2? This could be addressed experimentally by inducing SE and then concurrently injecting AAV and a retrovirus to look at the new synaptic contacts onto adult born GCs infected with a control or overexpression AAV vector. Perhaps AAV-mediated NLGN2 overexpression could rescue the hyperexcitable phenotypes in adult-born GCs.

Research suggests that NLGN2 alters the formation and stabilization of GABAergic synapses, but relatively unaddressed is how the NLGN2 overexpression influences the development of GABAergic synapses over time. Current research in the Naegele lab manipulates NLGN2 expression in an ex-vivo hippocampal slice culture system that may allow for such analysis. It is a good system for studying development, as it facilitates examination of cellular activity in a tissue context that
maintains aspects of in-vivo biology, like functional synaptic circuitry and preserved brain architecture (Cho et al., 2007). Perhaps a time course of live imaging using this system could highlight correlations between molecular changes in NLGN2 expression and GABAergic synapse development. This would also allow better detection of possible morphological, and thus functional, changes to these cells following AAV-mediated gene delivery.

Future experiments looking at the role of NLGN2 in GABAergic synapse formation might consider an AAV vector with a different promoter. The CMV promoter did not consistently drive mCherry expression throughout dendrites of infected cells. The construct was taken up very well in the axons of principal cells (Fig. 13), but expression wasn’t high throughout the dendrites. Research suggests that NLGN2 plays a significant role at perisomatic synapses, without much of an effect on axo-dendritic synapses (Poulopoulos et al., 2009; Gibson et al., 2009). However, many of these experiments were performed in KO animals or in a culture system and show varying results. It would be important to analyze synaptic changes at axo-dendritic synapses induced by AAV-mediated NLGN2 overexpression. A truncated human synapsin-1 promoter could be a viable option for driving higher expression; it has been shown to be effective for neuron-selective gene expression and only occupies 10% of the rAAV genome (Glover et al., 2002; Kugler et al., 2003). Another reason to consider a rAAV vector with a different promoter is due to preferential infection of GABAergic interneurons. Throughout my experiments I observed that NLGN2 expression, as seen by immunohistochemical analysis, seems
to be correlated with mCherry expression (Fig. 15). The AAV DJ appears to drive the highest expression in GABAergic interneurons, perhaps due to the promoter activity in conjunction with higher metabolic rates in these cells. For the initial experiments that examine NLGN2 overexpression-induced synaptic changes on the single cell level, this does not pose a real problem. However, if we move to a system characterized by loss of GABAergic interneurons, like severe TLE, and if NLGN2 overexpression does increase GABAergic synaptic innervation onto infected cells, then one would not want to drive high NLGN2 expression in GABAergic interneurons. This might trigger a disinhibitory circuit, leading to even less inhibition. One possible way to address this issue would be to use a vector with a cell-specific promoter. Advancements in AAV technology are working on the development of rAAV-compatible promoters that are selective to certain neuronal populations. Other ways to overcome this issue would be to use Cre-lines of mice to drive expression via specific viruses.

In summary, the experiments that I performed and propose begin to address important questions about GABAergic synapse formation in healthy brain tissue. Better understanding the role of NLGN2 in GABAergic synapse formation could help inform or augment stem-cell therapies aimed at repairing dysfunctional circuits. AAV-mediated NLGN2 overexpression may also show potential to one day replace stem cell-mediated circuit repair with gene therapy. Manipulating endogenous cells rather than adding new ones could be a powerful approach to stimulate endogenous circuit repair.
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