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ELUCIDATING THE ROLE OF GODZ-MEDIATED PALMITOYLATION IN GABA-ERGIC
INHIBITION

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ABSTRACT

Neurologin 2 (NL2) is a postsynaptic cell adhesion protein that is selectively found at GABAergic but not glutamatergic synapses. Previous unpublished experiments by the Luscher lab have indicated that NL2 interacts with gamma-aminobutyric acid type A receptors (GABA_ARs) and that this interaction might be important for targeting these receptors to synapses. Another line of research has found that NL2 is palmitoylated when co-expressed in heterologous cells with the palmitoyltransferase, Golgi-specific DHHC zinc finger protein (GODZ). Neurons that have the gene for GODZ deleted show a selective reduction in the density of GABAergic synapses on dendrites of neurons, indicating that GODZ-mediated palmitoylation of NL2 might contribute to synapse formation.

The first aim was to assess if palmitoylation of NL2 is required for normal postsynaptic accumulation of GABA_ARs. Two lentiviral plasmids were constructed for either an HA-tagged WT or an HA-tagged mutant version of NL2 that had the codons of the two cytoplasmic Cys residues representing putative palmitoylation sites mutated to Ala codons. The prediction was that some or all of the mutated HA-NL2 viruses would show reduced GABA_ARs accumulation at synapses when compared to WT HA-NL2. However, when these lentiviruses were used to transduce neurons they proved to be toxic, which precluded this approach from being informative. As an alternative strategy, the lentiviral plasmids were then directly electroporated into neurons and the neurons immunostained for HA-NL2 and the inhibitory pre- and postsynaptic markers, VGAT (vesicular GABA transporter) and gephyrin, respectively. This method showed efficient transfection of neurons but preliminary immunostaining was weak and not suitable for quantitative analyses of synapses. The second aim was to determine whether GODZ is more localized to the cis or trans face of the Golgi to allow predictions on how GODZ-mediated palmitoylation might affect the trafficking of substrate proteins. HEK 293T cells were transfected with either the cis or the trans Golgi markers, GFP-N3 and GalT-YFP, respectively, and immunostained for these cis and trans markers and GODZ. It was predicted that GODZ is more likely to be localized in the cis face of the Golgi

than the trans face, as this would support the theory that GODZ facilitates the trafficking of GABA_ARs from the endoplasmic reticulum (ER) to the Golgi. This hypothesis was supported when I found that GODZ was significantly more colocalized with the cis-Golgi marker, GFP-N3, than the trans-Golgi marker, GalT-YFP.

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Chapter 1

Introduction

GABA_A receptors

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. Particularly, the ionotropic GABA_A receptors (GABA_ARs) function by binding GABA and allowing the influx of chloride to hyperpolarize the postsynaptic membrane (Sieghart et al., 1999). Structurally, they are heteropentamers of subunits that belong to the superfamily of Cys loop ligand-gated ion channels (Sigel and Steinmann, 2012).

GABA_ARs are extremely diverse because they can be composed from a variety of subunit combinations. There are 19 distinct genes that code for the subunits of GABA_ARs (α 1- α 6, β 1- β 3, γ 1- γ 3, δ , ϵ , θ , π , ρ 1- ρ 3); most GABA_ARs are comprised of 2 α , 2 β , and a γ 2 subunit (Luscher et al., 2011). In addition, some of the subunits can be alternatively spliced, increasing heterogeneity and thereby presenting an opportunity for even more structural and functional variation (Luscher et al., 2011). The γ 2 subunit is involved in postsynaptic clustering of GABA_ARs; it has been shown that there is a reduction in GABAergic inhibition when there is a knockout of this subunit (Essrich et al., 1998).

Because GABA_ARs operate in critical aspects of neurotransmission, they have been implicated in a number of neurological disorders, including depression (Luscher and Fuchs, 2015), epilepsy (Gonzalez et al., 2015), schizophrenia (Charych et al., 2009), and autism spectrum disorders (Nakamura et al., 2016). These disorders could be a result of differences in the expression of GABA_ARs; therefore, understanding and clarifying the underlying mechanisms

of the trafficking of these receptors is crucial. Changes in the functional efficiency of GABAergic synapses are in large part mediated by dynamic changes in the trafficking of GABA_ARs and their varying accumulation at synapses (Luscher et al., 2011). In particular, the trafficking of GABA_ARs may be modulated by their interaction with cell adhesion protein NL2 (Luscher lab, unpublished results). Moreover, it has been proposed that the trafficking of both GABA_AR and NL2 is regulated by a reversible lipidation reaction catalyzed by the palmitoyltransferase, GODZ.

Neuroligins

Neuroligins are a family of postsynaptic cell-adhesion proteins that interact with neuroligin proteins that are in the presynaptic membrane (Luscher et al., 2011). Structurally, neuroligins consist of a beginning signal peptide that is cleaved off following insertion of the newly translated protein into the endoplasmic reticulum (ER) membrane, a large choline-esterase-like domain, a transmembrane domain, and an intracellular cytoplasmic domain (Lise and El-Husseini, 2006) (Figure 1). Additionally, neuroligins have been shown to homo and heterodimerize within the synapse (Shipman and Nicoll, 2012). Neuroligins modulate the development of excitatory and inhibitory synapses (Varoqueaux et al., 2006), and aid in the trafficking of receptors to the postsynaptic membrane. Some studies have suggested that while they are not necessary for the initial formation of synapses, neuroligins are critical to the maintenance and function of the synapse (Varoqueaux et al., 2006; Chubykin et al., 2007).

Currently, the exact mechanisms by which GABA_ARs are trafficked to the synapse is unclear. NL2 is of particular interest as a possible mediator of GABA_AR trafficking because it is found selectively at GABAergic, but not glutamatergic, synapses (Varoqueaux et al., 2004). The

NL2 forms a trans-synaptic interaction with presynaptic neurexins, and further recruits and binds to gephyrin, a sub-membrane scaffolding protein that is localized underneath the postsynaptic membrane and is believed to contribute to tethering of the synapse (Poulopoulos et al., 2009) (Figure 2). However, in $\alpha 2$ KO neurons, which lack GABA_ARs and gephyrin, NL2 continues to accumulate normally, showing that NL2 can function independently of GABA_ARs and gephyrin (Panzanelli et al., 2011).

The role of NL2 as a possible mediator of GABA_ARs has been supported by several studies. One study showed that NL2-deficient mice had a significant decrease in overall inhibitory synaptic transmission (Gibson et al., 2009). Additionally, overexpression of NL2 in transgenic mice leads to an increased number of GABAergic synapses along with severe behavioral abnormalities, including signs of anxiety and impaired social interactions (Hines et al., 2008). Previous unpublished experiments from the Luscher lab have indicated that NL2 interacts with GABA_ARs and that this interaction might be important for trafficking and accumulation of these receptors at synapses.



Figure 1: Construct of NL2

NL2 consists of an N-terminal signal peptide (SP), a large choline-esterase-like domain, a transmembrane domain (TMD), and an intracellular cytoplasmic domain which contains the binding site for gephyrin (Poulopoulos et al., 2009) as well as the two putative sites of palmitoylation (C710, C766).

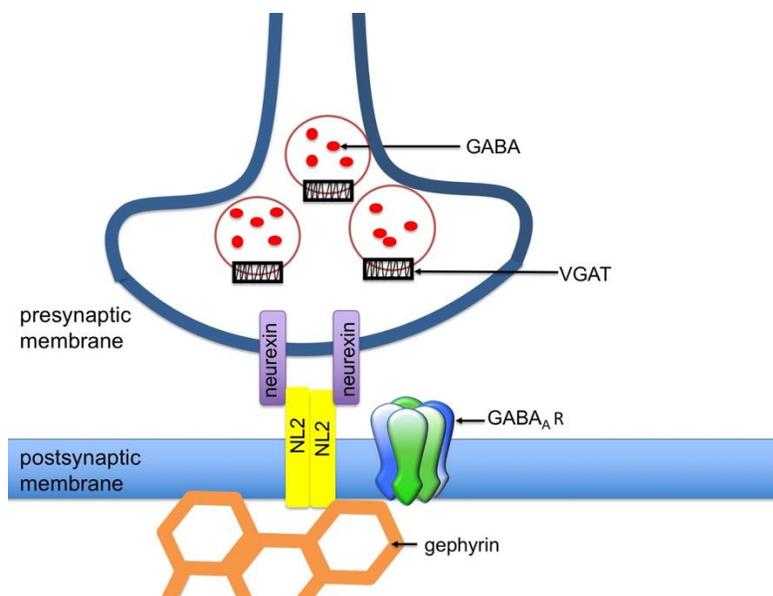


Figure 2: Schematic of interactions at GABAergic synapse

At GABAergic synapses, the vesicular GABA transporter (VGAT) serves as a widely used presynaptic marker of GABAergic synapses. Neurexin and NL2 interact to anchor the presynaptic membrane to the postsynaptic membrane. NL2 recruits and binds to gephyrin, a scaffolding protein, that also interacts with GABA_ARs and is widely used as a postsynaptic marker of GABAergic synapses.

Palmitoylation

Palmitoylation is a reversible post-translational covalent lipid modification that attaches palmitate, a 16-carbon fatty acid, to a Cys residue in a protein through a thioester linkage. Initially palmitoylation was thought to serve solely as a method of anchoring soluble proteins at the membrane. However, palmitoylation is also a common post-translational modification of integral membrane proteins, including ion channels and cell adhesion proteins, which points to functions other than mere association with membranes (Chamberlain et al., 2013). In particular, abundant evidence from the Luscher lab and others suggests that palmitoylation has important

functions in the context of synaptic transmission in neurons (Keller et al., 2004; Fang et al., 2006; Thomas and Huganir, 2013).

In previous studies, palmitoylation has been shown to regulate the accumulation of GABA_ARs at synapses (Rathenberg et al., 2004; Fang et al., 2006). More specifically, GODZ-mediated palmitoylation of GABA_ARs occurs in the cytoplasmic domain of the γ 2 subunit (Keller et al., 2004). In addition, NL2 has been shown to have two Cys residues (C710, C766) in its cytoplasmic intracellular domain, one of which can be palmitoylated by co-expression with GODZ in heterologous cells (C710) (Murakami, PhD thesis, 2008; Kilpatrick, PhD thesis, 2016). Therefore, and given that GABA_ARs and NL2 are part of a larger complex, palmitoylation may contribute to trafficking of GABA_ARs to synapses either by palmitoylation of GABA_ARs directly, or indirectly by palmitoylation of NL2, or both. It has yet to be shown whether palmitoylation affects the function and trafficking of NL2 in neurons independently of GABA_AR palmitoylation.

GODZ

Golgi-specific DHHC zinc finger protein (GODZ) belongs to the DHHC (Asp-His-His-Cys) family of palmitoyl acyltransferases (PATs), a group of proteins shown to function as enzymes. Most DHHCs are thought to have four transmembrane domains, with the N-terminal and C-terminal exposed in the cytoplasm (Lai and Linder, 2013) (Figure 3). The DHHC area of GODZ contains a cysteine-rich domain (CRD), which is necessary for its function as a PAT (Roth et al., 2002) (Figure 3).

GODZ has been found to be highly contained within the Golgi of the cell (Keller et al., 2004). Additionally, GODZ specifically has been shown to palmitoylate the γ 2 subunit of

GABA_ARs; its closest related DHHC paralog, SERZ- β , also palmitoylates the γ 2 subunit to a lesser extent (Fang et al., 2006). Knockdown of GODZ has been shown to induce a reduction of GABA_ARs at the synapse (Fang et al., 2006). Therefore, it was hypothesized that GODZ could play a direct role in the trafficking of GABA_ARs through palmitoylation occurring in the Golgi.

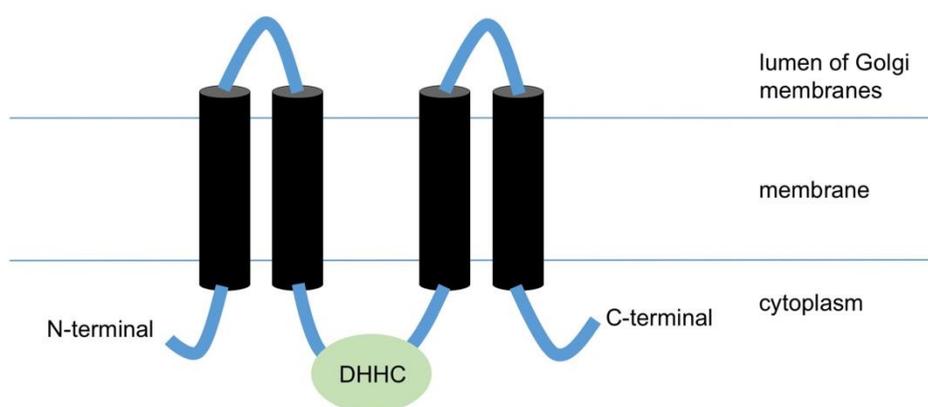


Figure 3: Construct of GODZ

GODZ is a member of the DHHC family, which is thought to have 4 transmembrane domains. The DHHC domain is part of the cysteine-rich domain (CRD), which is essential for GODZ's function as a palmitoyl acyltransferase. The N-terminal and C-terminal are located in the cytoplasm.

Purpose of the study

The first objective of this thesis was to examine the localization of GODZ in the Golgi. The precise localization of GODZ in the secretory pathway provides important clues as to how palmitoylation might affect the trafficking of GODZ substrate proteins. On one hand, the cis face of the Golgi will likely impact the transport of proteins from the ER to the Golgi. In addition, it might affect the formation of protein complexes, and for example, determine whether GABA_ARs and NL2 traffic to the membrane independently or as part of a larger complex. Given that NL2

engages in a trans-synaptic interaction with presynaptic neurexin, its palmitoylation-dependent interaction with GABA_ARs could contribute to palmitoylation-dependent postsynaptic accumulation of GABA_ARs. On the other hand, the trans face of the Golgi is thought to regulate trafficking from the Golgi to the plasma membrane. It is difficult to come up with a trans-Golgi dependent mechanism that would involve both GODZ and NL2 palmitoylation for their postsynaptic localization. Therefore, we hypothesized that GODZ would be more highly localized in the cis face of the Golgi, and help assist in the trafficking of GABA_ARs-NL2 complexes from the ER to the cis-Golgi. In order to examine this, cis and trans face markers of the Golgi were transfected into HEK 293T cells, then immunostained for the Golgi markers as well as GODZ, and quantitatively analyzed to determine the differential colocalization of GODZ with cis vs. trans Golgi markers.

The second aim of this thesis was to elucidate whether postsynaptic accumulation of NL2 is altered in NL2 KO neurons when NL2 is unable to be palmitoylated. We hypothesized that the palmitoylation-deficient NL2 would cause a significant reduction in the accumulation of GABA_ARs at the synapse. In order to examine this, we took advantage of NL2 expression plasmids encoding an HA-tagged NL2 plasmid and a mutated version in which the codons for the two cytoplasmic Cys residues (C710 and C766) were mutated to Ala codons. From these plasmids, we constructed lentiviral derivative plasmids and used them to produce lentiviral particles suitable for infection of neurons. To eliminate possible complications due to dimerization of transfected NL2 with endogenous NL2, we performed these experiments in cultured neurons isolated from NL2 KO embryos. The quantification of the puncta overlap between NL2, VGAT and gephyrin in the neurons would clarify if there is a reduction of GABA_ARs trafficking when comparing the WT plasmid to the mutated NL2 plasmids.

Chapter 2

Methods

Plasmid Construct

An HA-tagged cDNA of NL2A in pNICE (Clontec) was obtained from Dr. Peter Scheiffele (BioCenter, Basel) and is available also from Addgene (plasmid #15259) (Luscher lab p780) (Chih et al., 2006). In addition, I made use of a palmitoylation-deficient derivative of this plasmid in which both cytoplasmic Cys residues of NL2 had been mutated by site directed mutagenesis (HA-pNICE-NL2A^{C710,766A}, Luscher Lab p797, Kilpatrick, PhD thesis, 2016) or a single residue had been mutated (HA-pNICE-NL2^{C710A}, Luscher Lab p788, Kilpatrick, PhD thesis, 2016) (HA-pNICE-NL2^{C766A}, Luscher Lab p789, Kilpatrick, PhD thesis, 2016). The lentiviral NL2-expressing plasmids were created by cutting the HA-NL2 cDNA region from p780, p797, p788, and p789 using the restriction enzymes BglII and Spe1 (New England Biolabs). The backbone lentiviral plasmid pWPI (Addgene, Plasmid #12254) was linearized by digesting with Spe1 and BamH1 (New England Biolabs). The five digests were run through a low electroendosmosis (EEO) agarose (1%) gel electrophoresis in order to confirm fragmentation (11,100 bp pWPI, 2,700 bp and 3,950 bp WT HA-NL2 and HA-NL2^{C710,766A}, HA-NL2^{C710A}, HA-NL2^{C766A}, respectively). The digests were purified from the gel and the linearized pWPI vector was ligated to the BglII and Spe1 fragment isolated from p780, p797, p788, or p789. The following day, the ligation was transformed into electrocompetent *E. coli* cells, and then plated onto LB agar plates with kanamycin (3.33 μ L/mL) (Sigma LB Kanamycin) and grown overnight at 37°C. Two plates were made for each plasmid. The following day, single

colonies were picked and put in LB-Kanamycin broth (3 mL) and incubated overnight at 37°C. In order to screen the colonies to determine which contained the plasmid, isolated colonies were taken from each plate and a standard MiniPrep protocol (Thermo Fischer Scientific) was used to extract and purify the DNA. The growth of colonies ranged from 75 to 120 colonies per plate. Four stock plates were made for each isolated colony chosen from the original plate, creating a total of 8 digests per plasmid. The extracted DNA was digested with Kpn1 (New England Biolabs) and run through a 1% agarose gel to determine which samples were positive for the DNA insertion. Positive samples would have three fragments of 1928 kb, 1566 kb, and 10 kb each if the sequence was correct. Only 1 of 8 digests per plasmid was shown to be positive for the DNA sequence. A colony was taken from the stock plate shown to be positive for the plasmid, and put in LB-Kanamycin broth (200 mL) and incubated overnight at 37°C. The following day the DNA was purified using a standard MaxiPrep protocol (Omega Bio-Tek).

Lentivirus Production

HEK 293T cells were used for transfection when they had reached a confluence of ~80-90%. A first mixture (Mixture 1) was created by combining 40 µL of lipofectamine 2000 and 1.5 mL of Opti-MEM media (Thermo Fischer Scientific, Grand Island, NY), then incubated for 5 min at room temperature. A second mixture (Mixture 2) was created by mixing 20 µg of the WT HA-NL2 or HA-NL2^{C710,766A} lentiviral plasmid, 10 µg of RRE plasmid, 5 µg of REV plasmid, 6 µg of VSVG envelope plasmid, and 1.5 mL of Opti-MEM media which was then incubated at room temperature for 5 min. Next, Mixture 1 and Mixture 2 were combined and incubated together at room temperature for 20 min. The solution was then transfected onto the cells. The media containing the lentivirus was collected at 12 hour intervals for 48 hours. After the final collection, the media was centrifuged at 3000 rpm for 5 min at room temperature, then the

supernatant was collected and filtered through a 0.45 μm filter. HEK 293T cells with serial dilutions of the virus were used to determine the viral titer (10^6 colony-forming-units/mL to 10^8 colony-forming-units/mL). The lentivirus was then transduced into neurons as described below, or frozen at -80°C in 20 μL aliquots in Eppendorf tubes until neurons were available.

Making Cover Slips

Cover slips for neuron culture and cell culture were made by coating cover slips in Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO) overnight at room temperature. The cover slips used for neuron culture had added paraffin wax feet.

Neuron Culture

Cortical neuron cultures were prepared from embryonic day 14 (E14) mouse embryos. They were cultured by Yao Guo as previously described (Alldred et al., 2005). Neuronal suspension of 2.3×10^5 cells/mL was added at 1.0 mL per well of a 24-well plate onto Poly-L-Lysine coated cover slips. Cover slips were positioned with the neurons facing the glia feeder cells grown on the bottom of the dish.

Lentivirus Transduction

The lentivirus was transduced into cultured cortical neurons at DIV 18. The cover slips were inverted to expose the neurons to the top. Half of the culture medium was removed from the cells and 1-5 μL of the virus was added to each well. After 24 hours, the cover slips were flipped so that the neurons were once again facing the bottom of the well as they were originally positioned. The transduced neurons were then incubated for 72 hours.

Cell Culture and Transfection

Human Embryonic Kidney (HEK) 293T (#CRL-11268, ATCC) were used for GODZ localization immunostaining. 10 cm plates were used to culture the cells at a 1:9 dilution, as well as a media solution of 10 ml of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. When the cells reached ~80% confluence (~4 days), they were split into new 6 cm plates at a 1:4 dilution. At 48 hours after plating, the cells were transfected. The plasmid DNA (1 μ g) was added to Opti-MEM media. Then, polyethyleneimine (PEI) (Sigma-Aldrich, St. Louis, MO) was added at a ratio of 4x μ g of DNA. After mixing and letting sit for 5 min, the solution was added to the cells.

Neuronal Transfection

Mouse cortical neurons were transfected at the time of plating by electroporating (BTX ECM 830 Electro Square Porator) 10 μ g of the lentiviral plasmid into a suspension of 250 μ L dissociated neurons (500 V, 800 μ sec pulse 1x) in a 4 mm gap electroporation cuvette and immediately plating onto cover slips. At DIV 21, neurons were immunostained.

Immunofluorescent Staining

48 hours after culture transfection, or DIV 21 for neurons, the cells were washed 3x in PBS (pH 7.4). The cells were then fixed in a 4% paraformaldehyde solution in PBS for 15 min then washed in PBS 3x. The cells were permeabilized in a 0.1% Triton-X solution in PBS for 5 min and washed 3x. The cells were incubated overnight in a primary antibody solution at 4°C. The following day, the cells were washed 3x in PBS for 15 min, and then incubated at room temperature with a secondary antibody solution for 45 min. After 3x 15 min PBS wash, the cells

were mounted on slides using a mounting solution. The following primary antibodies were used for the NL2 KO neuron staining: 1:500 rabbit anti-VGAT (#131002, SYSY), 1:1000 mouse anti-HA (#2367, Cell Signaling), 1:500 mouse anti-gephyrin (#147021, SYSY), 1:1000 chicken anti-HA (#9111, Abcam). The following secondary antibodies were used for the NL2 KO neuron staining: 1:400 goat anti-rabbit 488 Alexa (#84B1-1, Molecular Probes), 1:200 goat anti-mouse Cy3 (#A10521, Molecular Probes), 1:400 goat anti-chicken 488 Alexa (#A11039, Invitrogen). The cells were imaged with a Zeiss Axiophot2 fluorescent microscope and OpenLab imaging software. The following primary antibodies were used for the GODZ localization staining: 1:500 rabbit anti-GODZ (#31837, Abcam), 1:500 mouse anti-GFP (#A11120, Molecular Probes). The following secondary antibodies were used for the GODZ localization staining: 1:200 goat anti-rabbit Cy3 (#51515, Jackson Immuno. Res.), 1:400 goat anti-mouse 488 Alexa (#A11001, Molecular Probes). The cells were imaged with an Olympus FV100 Confocal microscope and ImageJ imaging software.

Quantification

Staining for Golgi localization was quantified according to the Intensity Correlation Analysis (ICA) protocol used previously (Li et al., 2004). The merged image obtained from the confocal microscope was separated into individual channel images for either the cis or trans marker and GODZ. The images were converted into 8-bit images. The background was then subtracted using the BG subtraction from ROI macro. Then, the cell to be analyzed was selected as a region of interest (ROI) in the Golgi area from the first channel image. This ROI was then copied into the second channel image. The ICA plugin was run, which generated the Intensity Correlation Quotient (ICQ) value for the colocalization of the two channels.

Sequencing

All DNA sequencing was done through the Penn State University Nucleic Acid Facility (University Park, PA, 16802). A 5 μ L DNA sample of the plasmid was sent along with 10 μ M primer. The primers used for the sequencing were the E1-F α primer and the cytoplasmic NL2 detection primer.

Chapter 3

Results

Localization of GODZ and cis vs. trans Golgi markers shows a higher GODZ localization in the cis face

Previous experiments have shown that GODZ-mediated palmitoylation plays a role in the trafficking of GABA_ARs and the formation of GABAergic synapses. The location and function of proteins tend to be interdependent, and therefore knowing the localization of GODZ within the Golgi will help clarify the function of GODZ-mediated palmitoylation of specific substrates. Regulation of protein secretion is more likely to occur at the cis face of the Golgi or the interface of the ER and Golgi than in the trans Golgi, where proteins are prepared for export to the plasma membrane. In order to determine whether GODZ is more likely to be found in the cis or trans face of the Golgi, HEK 293T cells were transfected with either a cis-Golgi marker (GFP-N3) or a trans-Golgi marker (GalT-YFP). GFP-N3 (GPP-130) is a glycosylated phosphoprotein of 130 kDa that is localized in the cis face of the Golgi (Linstedt et al., 1997). GalT-YFP is a glycosyltransferase that is localized in the trans face of the Golgi (Roth, 1982). Previously, these two markers have been used together to determine localization of proteins within the Golgi (Linstedt et al., 1997). Immunofluorescent images were collected of the transfected HEK 293T cells (Figure 4, A-F), and quantified using ICA of the colocalization of GODZ and either the cis-

Golgi or the trans-Golgi marker (Figure 4, G). Figure 4 shows that GODZ is more highly localized with the cis face of the Golgi than with the trans face of the Golgi.

Generating lentiviral constructs

Lentiviral constructs were generated in order to examine the accumulation of GABA_ARs in NL2 KO neurons when NL2 is palmitoylation-deficient. In addition to the initial confirmation of the DNA sequence by fragment analysis, the lentiviral plasmid DNA sequence was confirmed after the MaxiPrep purification protocol was completed. The plasmid DNA was digested using the restriction enzyme Kpn1. By cutting with this enzyme, it would create three fragments of 1928 kb, 1566 kb, and 10 kb each if the sequence was correct. After digesting, the sample was run through an agarose gel in order to visualize the fragments. After the fragments were initially confirmed, the plasmids were sent to the Penn State Nucleic Acid facility for the DNA to be sequenced, which served as a secondary confirmation. The facility provided the DNA sequence, which showed that the DNA was successfully inserted and that in the HA-NL2^{C710,766A}, HA-NL2^{C710A}, and HA-NL2^{C766A}, the codons for the Cys residues had been mutated to Ala codons at their respective putative sites of palmitoylation, rendering them incapable of having this interaction (Figure 5).

Synaptic labeling of NL2 KO neurons infected with lentivirus

We hypothesized that neurons with palmitoylation-deficient NL2 will show reduced GABA_AR accumulation at synapses compared to WT NL2. To test this, lentiviral constructs were generated for WT HA-NL2 and palmitoylation-deficient HA-NL2^{C710,766A}, as well as single point mutants HA-NL2^{C710A} and HA-NL2^{C766A}. HA-NL2 and HA-NL2^{C710,766A} were used to

generate lentivirus particles in HEK 293T cells, which were subsequently used to transduce NL2 KO neurons. The cells were transduced at DIV 18 and fixed and immunostained at DIV 21 for HA-NL2 together with either presynaptic VGAT or postsynaptic gephyrin. Inexplicably I found that lentivirus infection killed most neuron cultures before staining could be carried out. Figure 6 shows the results of a rare experiment in which a few neurons survived. The untransduced neurons can be readily identified by the lack of HA-NL2 immunostaining (Figure 6, B, H). The neurons transduced with the lentivirus show staining for HA-NL2 (Figure 6, E, K). Surprisingly, HA-NL2^{C710,766A} lentivirus transduced neurons were entirely devoid of gephyrin staining (Figure 6, D, F), even though immunostaining for gephyrin was successful for the untransfected neurons as indicated by the prototypical punctate immunoreactivity (Figure 6, A, C). The data indicated that the virus is toxic to neurons, which prevented meaningful quantification of synaptic localization of HA-NL2. The staining for VGAT appeared greatly increased in HA-NL2^{C710,766A} lentivirus transduced neurons, suggesting that at least some of the HA-NL2 protein was expressed at the cell surface and, through trans-synaptic interaction with presynaptic neurexin, led to recruitment of GABAergic terminals (Figure 6, J, L).

Synaptic labeling of NL2 KO neurons transfected with lentiviral plasmids

The lentivirus proved to be ineffective for quantifying the synaptic accumulation of GABA_ARs; as an alternative method, the lentiviral plasmids were used to transfect the neurons directly instead. At the time of plating, the plasmids were electroporated with freshly dissociated neurons and the cells then placed in culture for 21 days before immunofluorescent staining. The neurons were then stained for HA-tagged NL2 and either the presynaptic marker (VGAT) or the postsynaptic marker (gephyrin). The immunostained images (Figure 7, A-L) showed an overall

lack of defined puncta, and therefore were unsuitable for quantification (see Discussion, Chapter 4).

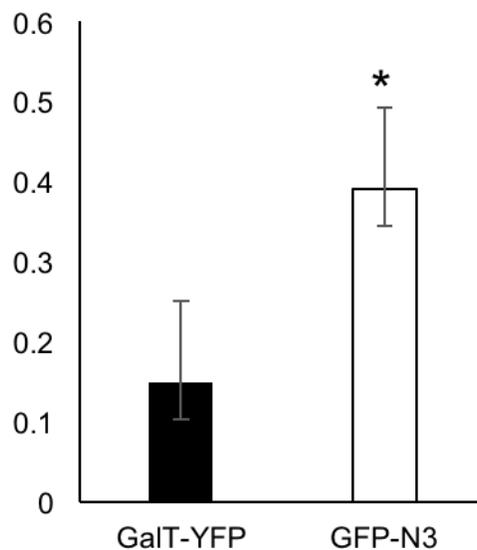
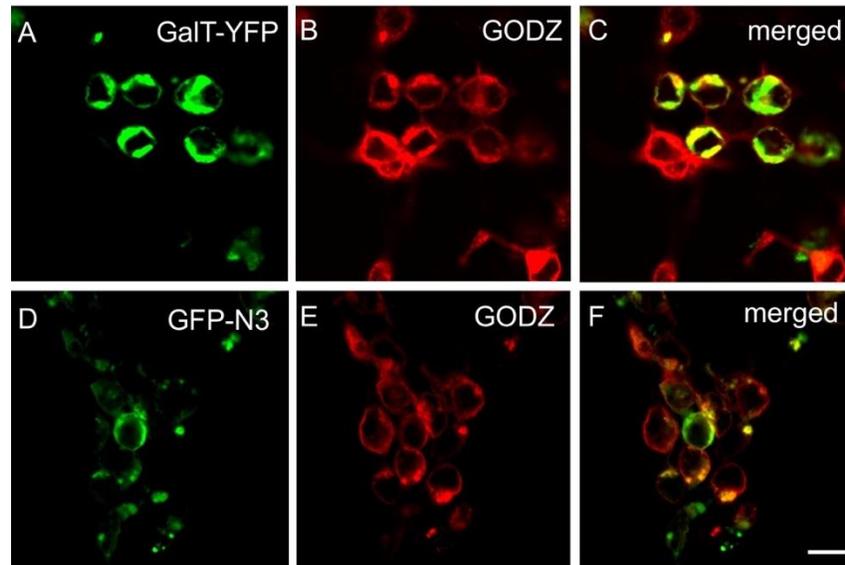


Figure 4: GODZ is more highly localized with the cis-face Golgi marker (GFP-N3) than the trans-face Golgi marker (GalT-YFP)

(A-C) HEK 293T cells were transfected with trans-Golgi marker GalT-YFP and immunostained for GalT-YFP (green, A) and endogenous GODZ (red, B). Merged images are shown in (C). (D-F) HEK 293T cells were transfected with cis-Golgi marker GFP-N3 and immunostained for GFP-N3 (green, D) and endogenous GODZ (red, E). Merged images are shown in (F). Scale bar, 10 μ M. (G) ICA analysis confirmed that GODZ is more positively correlated with the cis-Golgi marker, GFP-N3 (ICQ value: 0.390 \pm 0.046) than with the trans-Golgi marker, GalT-YFP (ICQ value: 0.148 \pm 0.103, n=10, p < 0.05, t-test)

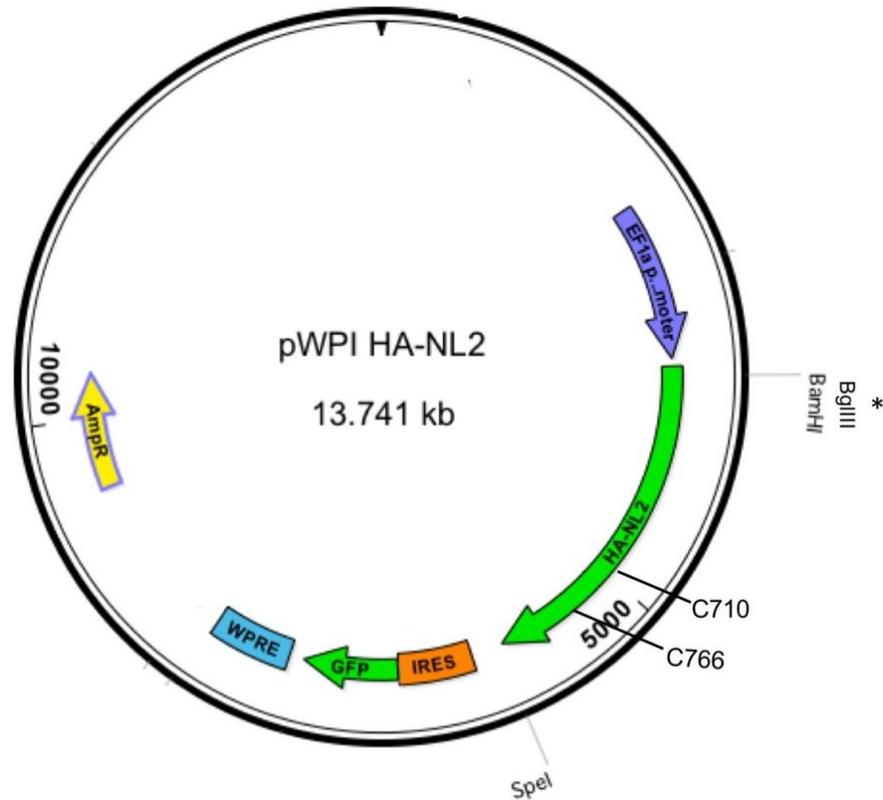


Figure 5: pWPI HA-NL2 Plasmid Construct

HA-tagged NL2 was ligated with pWPI, an empty backbone plasmid used for construction of a lentiviral plasmid. The constructed backbone contains human elongation factor-1 alpha (EF-1 α) which serves as a promoter for the HA-NL2 sequence containing the putative sites of palmitoylation (C710, C766). The HA-NL2 sequence is followed by an internal ribosome entry site (IRES) which allows downstream translation initiation. The sequence for GFP follows the IRES, which allows the plasmid to be visualized in neurons when viewed with a fluorescent microscope. The Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) increases the overall expression of the plasmid (Lee et al., 2005). The AmpR sequence confers bacterial resistance to ampicillin, allowing them to grow on LB plates with ampicillin. The total size of the plasmid is 13, 741 kb. *Ligation of BglII and BamHI sites leads to loss of both enzyme recognition sites.

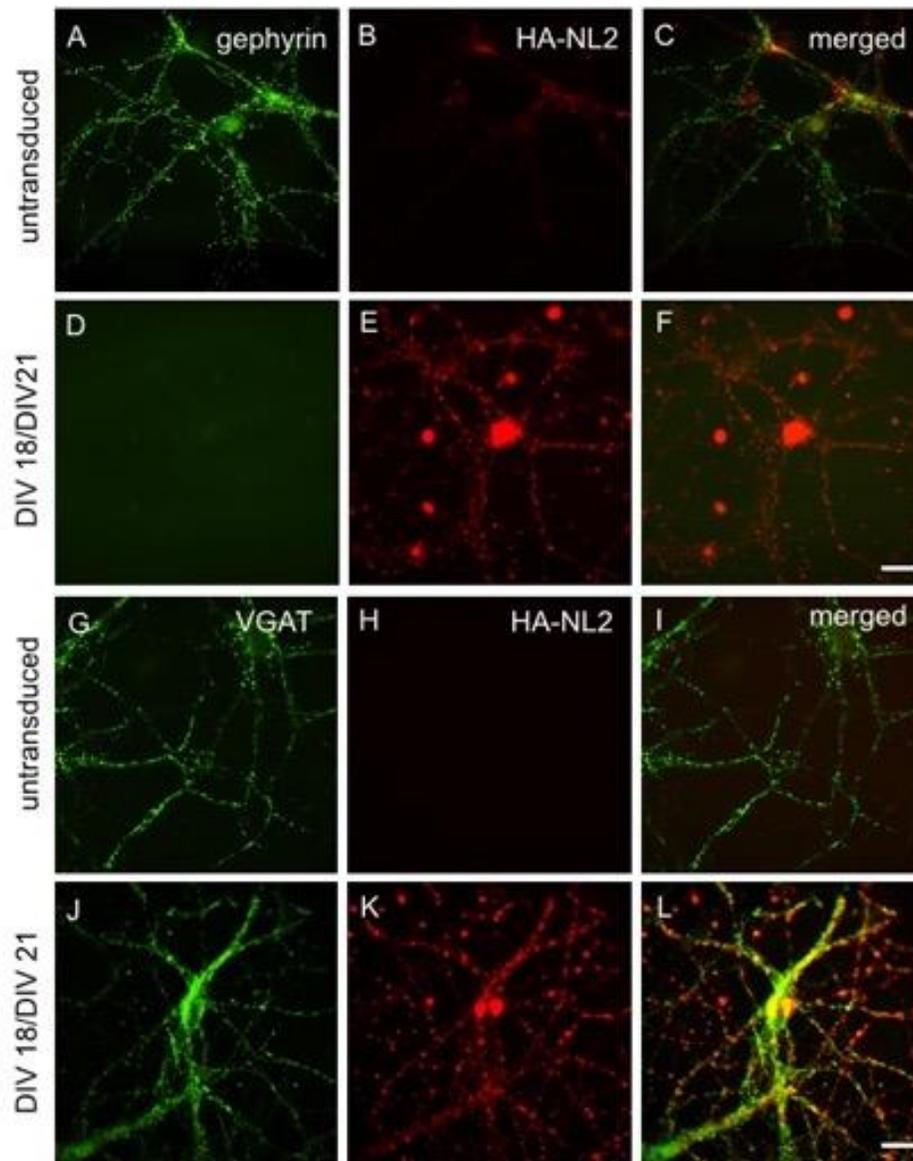


Figure 6: Lentiviral infection of NL2 KO neurons

(**A-F**) NL2 KO neurons were either transduced with the HA-NL2^{C710,766A} lentivirus at DIV 18 (D-F) or left untransduced (A-C). Neurons were immunostained at DIV 21 for gephyrin (green, A, D) and HA-NL2 (red, B, E). Merged images are shown in (C, F). (**G-L**) NL2 KO neurons were either transduced with the HA-NL2^{C710,766A} lentivirus at DIV 18 (J-L) or left untransduced (G-I). Neurons were immunostained at DIV 21 for VGAT (green, G, J) and HA-NL2 (red, H,K). Merged images are shown in (I, L). Scale bar, 10 μ M.

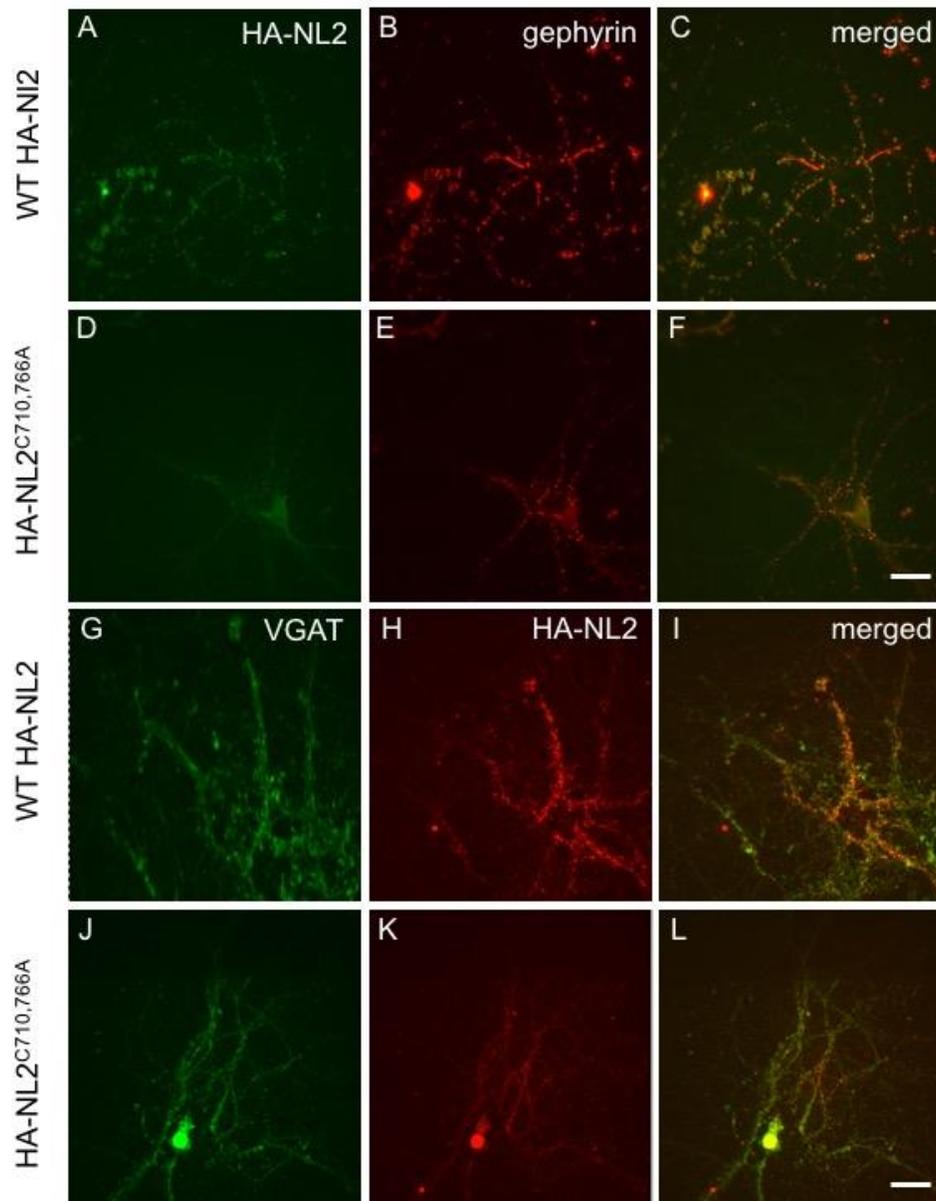


Figure 7: Electroporation of lentiviral plasmid into NL2 KO neurons

(A-F) The lentiviral plasmid for either the WT HA-NL2 (A-C) or the HA-NL2^{C710,766A} (D-F) was electroporated into NL2 KO neurons at the time of plating. At DIV 21 the neurons were immunostained for HA-NL2 (green, A, D) and post-synaptic marker gephyrin (red, B, E). Merged images are shown in (C, F). (G-L) The lentiviral plasmid for either the WT HA-NL2 (G-I) or the HA-NL2^{C710,766A} (J-L) was electroporated into NL2 KO neurons at the time of plating. At DIV 21 the neurons were immunostained for the pre-synaptic marker VGAT (green, G, J) and HA-NL2 (red, H, K). Merged images are shown in (I, L). Scale bar, 10 μ M.

Chapter 4

Discussion

One aim of this thesis was to elucidate whether GODZ was more highly localized in the cis or the trans face of the Golgi. To do this, either a cis face Golgi marker (GFP-N3) or a trans face Golgi marker (GalT-YFP) was transfected into HEK 293T cells and subsequently immunostained and quantified for the Golgi markers and endogenous GODZ. It was hypothesized that GODZ would be more localized in the cis face of the Golgi than the trans face of the Golgi, as this would support the theory that GODZ aids in the trafficking of GABA_ARs from the ER to the Golgi. I found that GODZ was more highly localized in the cis-face of the Golgi than the trans-face of the Golgi. After conducting ICA for the colocalization of fluorescence between GalT-YFP and GODZ versus GFP-N3 and GODZ, there was a significantly higher ICQ value for the colocalization between GFP-N3 and GODZ (Figure 4, G) when compared to GalT-YFP and GODZ. Because GODZ is more localized in the cis face of the Golgi, this could indicate that GODZ serves to modify GABA_ARs, or GABA_ARs-NL2 complexes, between the ER and the cis-Golgi.

There are several possible follow-ups to the GODZ localization experiment. Primarily, SERZ- β could be analyzed using the same methods to determine whether it localizes similarly to GODZ. SERZ- β is the closest related paralog of GODZ, and can palmitoylate the γ 2 subunit of GABA_ARs, at least when co-expressed with its substrate in HEK 293T cells (Fang et al., 2006). If GODZ and SERZ- β function in the same way to palmitoylate the γ 2 subunit of GABA_ARs, then one might expect that SERZ- β is similarly localized in the cis face of the Golgi apparatus. If

it was found to not be localized in the cis face of the Golgi, then this could be a step towards discernment of GODZ and SERZ- β function.

In addition to testing SERZ- β localization in the Golgi apparatus, this experiment could be repeated in COS cells instead of HEK 293T cells. COS cells have a much larger cell body, and therefore it would be easier to visualize the Golgi in immunostaining images. However, COS cells can be more difficult to grow and transfect. If successful, the staining for the cis vs. trans face markers of the Golgi and for GODZ (or SERZ- β) would be more visible than those in HEK 293T cells.

The second aim of this thesis was to examine the effects of palmitoylation-deficient NL2 on the trafficking of GABA_ARs and the formation of GABAergic synapses. Plasmids were created for WT HA-NL2, two single point mutants of C710 (HA-NL2^{C710A}) and C766 (HA-NL2^{C766A}), and one double point mutant of both C710 and C766 (HA-NL2^{C710,766A}). A lentivirus was then generated from the WT HA-NL2 and HA-NL2^{C710,766A} plasmids using HEK 293T cells. The lentiviruses were then used to infect NL2 KO neurons, which were subsequently immunostained for HA-NL2 and either a presynaptic marker (VGAT) or a postsynaptic marker (gephyrin). Due to the technical difficulties with the toxicity of the virus, only WT HA-NL2 and HA-NL2^{C710,766A} were used to infect NL2 KO neurons. It was hypothesized that the neurons infected with HA-NL2^{C710,766A} would show a reduced accumulation of GABA_ARs at the synapse when compared to neurons infected with WT HA-NL2.

After several attempts at infecting and immunostaining NL2 KO neurons, it was shown that the lentivirus was highly toxic to the neurons. When infected with the lentivirus, most neurons were killed as a result. In one attempt, the lentivirus killed some but not all of the neurons, and a few images were obtained using a fluorescent microscope (Figure 6, A-L). In the

untransduced neurons (Figure 6, A-C, G-I) the immunostaining for the presynaptic and postsynaptic markers had well defined puncta (Figure 6, A, G), and as expected there was no visible presence of HA-NL2 (Figure 6, B, H), showing that there was no endogenous NL2 in the NL2 KO neurons. In the infected neurons (Figure 6, D-F, J-L), the immunostaining for HA-NL2 was present, showing that the lentivirus successfully introduces HA-NL2 into neurons (Figure 6, E, K). However, there were severe effects seen in the immunostaining for the pre- and postsynaptic markers as a result of the lentivirus infection. The immunostaining for the postsynaptic marker, gephyrin, was eliminated from the neurons (Figure 6, D, F). The immunostaining for the presynaptic marker, VGAT, was overexpressed in the neurons transduced with the lentivirus (Figure 6, J, L).

There are several possibilities that would explain the heavily altered expression of the pre- and postsynaptic markers as a result of lentiviral infection. The images show increased localization of VGAT along the dendrites, which might indicate that NL2 is expressed at the cell surface. This could in turn increase attraction of GABAergic axons, which would explain the increased labeling of VGAT in dendrites of infected neurons. In the future, this VGAT staining along dendrites could be used to quantify NL2 cell expression. One possibility that would explain the elimination of the gephyrin staining would be that overexpression of NL2 results in intracellular aggregates that interact with gephyrin, enhancing the natural interaction between NL2 and gephyrin. This would result in gephyrin being clustered in the cytoplasm instead of the synapses, and therefore diminish the gephyrin staining at the synapse. Future experiments would have to be conducted in order to elucidate the factors which prevented the successful use of the lentivirus.

One approach to decrease chance of lethal toxicity of the lentivirus would be to infect neurons at a much earlier DIV. Although attempts were made to infect the neurons in a range of days from DIV 14-21, these infection points still proved toxic to the neurons. By infecting neurons as early as DIV 3, there could be a marked improvement in the survival of the neurons. At this point, synapses are still forming, and therefore are much less fragile than they would be at a later DIV. Therefore, the neurons might be more resistant to the toxicity of the lentivirus at this point. In addition, an attempt could be made to introduce the lentivirus into a mouse through stereotaxic injection; the lentivirus would then be infecting more robust cells, and the toxicity could have a lesser impact.

Although the lentivirus proved toxic to the neurons, it is unclear whether it was due to the strength of the lentivirus itself, or because of the specific HA-NL2 sequence of the lentivirus. To clarify this, an empty lentivirus could be generated and used to infect neurons in the same way. If that lentivirus proved to not be toxic to the neurons, then it would be clear that the toxicity is due to the HA-NL2 sequence specifically as opposed to the nature of the lentivirus itself.

After the lentivirus was shown to be toxic to the neurons, an alternate approach was used to examine the effects of the palmitoylation of NL2 on the accumulation of GABA_ARs at the synapse. The lentiviral plasmids were instead electroporated directly into the neurons at the time of plating, then immunostained for HA-NL2 and the pre- and postsynaptic markers at DIV 21. Although this was less likely to kill the neurons, it also allowed for a 21-day period in which the neurons could adjust to the possible deficit in synaptic accumulation of GABA_ARs, and therefore compensate for the deficit that it was the aim to quantify.

The lentiviral plasmid for either WT HA-NL2 (Figure 7, A-C, G-I) or HA-NL2^{C710,766A} (Figure 7, D-F, J-L) was electroporated into NL2 KO neurons at the time of plating, and

immunostained at DIV 21 for HA-NL2 and either the presynaptic marker, VGAT (Figure 7, G-L) or the postsynaptic marker, gephyrin (Figure 7, A-F). The transfection method was successful, but further optimization is needed of both the immunostaining and microscopic imaging. The pre- and postsynaptic markers had a few areas of well-defined puncta, but the HA-NL2 staining was very weak and showed a lack of defined puncta. Because of this, the images were unsuitable for quantification.

Following this experiment, the same procedure could be repeated with an attempt at creating a higher fluorescence of staining for HA-NL2. This method could be executed by altering the antibody concentration and the plasmid concentration at the time of plating. Though the method of plasmid electroporation was successful, the lack of clear immunostaining prevented quantification.

In conclusion, I have demonstrated that the palmitoyltransferase GODZ is more heavily localized in the cis face as opposed to the trans face of the Golgi. This suggests that GODZ acts upon GABA_ARs during their transition from the ER to the Golgi, perhaps to ensure that GABA_ARs and NL2 leave the cis Golgi towards trans-Golgi as a complex rather than as independent proteins. In addition, this aids in elucidating the mechanism by which GODZ mediates GABAergic inhibition. This can be further pursued in future experiments by looking at the GODZ paralog, SERZ- β , in order to understand differences in function. It was found that the lentivirus approach to understanding the palmitoylation of NL2 was unsuccessful, due to the lentivirus toxicity in neurons. Alternatively, the lentiviral plasmids were successfully electroporated into NL2 KO neurons, but the immunostaining was weak and unsuitable for quantification. If the lack of palmitoylation of NL2 showed a significant reduction in the synaptic trafficking of GABA_ARs, this would have vital implications in understanding the

mechanism of these receptors. GABA_ARs have been shown to play a critical role in disorders such as schizophrenia (Charych et al., 2009), depression (Luscher and Fuchs, 2015), epilepsy (Gonzalez et al., 2015) and autism spectrum disorders (Nakamura et al., 2016); any elucidation of the mechanism by which they are controlled furthers our understanding of these diseases.

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