ELUCIDATING THE ROLE OF GODZ-MEDIATED PALMITOYLATION IN CONTROLLING GABAERIC INHIBITION

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by
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ABSTRACT

Palmitoylation involves the addition of a 16-carbon fatty acid chain to cysteine residues, which increases the hydrophobicity of the target protein. This modification functionally influences membrane targeting, protein conformational state, complex formation and protein-protein interactions. Palmitoylation is a rapidly reversible and dynamically regulated post-translational modification that affects the function or localization of many neuronal proteins including GABA\textsubscript{A} receptors (GABA\textsubscript{A}Rs).

GABA\textsubscript{A}Rs are heteropentameric chloride channels responsible for mediating phasic inhibitory neurotransmission in the brain. The γ2 subunit of GABA\textsubscript{A}Rs is critically important for trafficking of these receptors to the synapse and for normal GABAergic synaptic transmission. GABA\textsubscript{A}R γ2 heterozygous knockout mice have moderate functional deficits in GABAergic neurotransmission that result in behavioral and other alterations that are analogous to depression in humans. Diverse post-translational modifications of the γ2 subunit intracellular loop affect the membrane dynamics of GABA\textsubscript{A}Rs and therefore the function of inhibitory synapses. The γ2 subunit is subject to palmitoylation by Golgi-specific DHHC-type zinc finger protein (GODZ, DHHC3) and its paralog, Sertoli cell gene with a zinc finger domain (SERZ-β, DHHC7). Knockdown of GODZ by shRNA or a dominant negative construct indicated that GODZ-mediated palmitoylation regulates the accumulation of GABA\textsubscript{A}Rs at synapses, as well as GABAergic innervation. Neurons derived from GODZ knock-out (KO) and GODZ/SERZ-β double KO mice grown in co-culture with wild-type (WT) neurons, exhibit a loss of punctate immunostaining for presynaptic glutamate decarboxylase (GAD) and postsynaptic γ2 indicating a loss of GABAergic innervation. The loss of synapses could potentially be a result of GODZ palmitoylating other proteins involved in synapse
formation and maintenance such as the postsynaptic adhesion molecule, neuroligin 2 (NL2).

The main objective of this dissertation was to further elucidate the mechanism by which GODZ-mediated palmitoylation controls GABAergic inhibition *in vivo*, by utilizing GODZ KO mice. I screened for candidate novel substrates of GODZ using a combined acyl-biotin exchange assay and quantitative proteomics with isobaric tags for relative and absolute quantitation (iTRAQ) approach. Proteomic analyses of putative palmitoylated proteins revealed that there were a wide variety of proteins that were differentially palmitoylated or expressed in brain tissue of WT compared to GODZ KO mice. This result is consistent with previous evidence that GODZ has broad substrate specificity. Through proteomics, growth associated protein (GAP-43) was identified as a palmitoylated protein that is less abundant or less palmitoylated in brain of KO compared to WT mice. We confirmed that GAP-43 could be palmitoylated by GODZ in transfected HEK 293T cells. Moreover, steady state palmitoylation of the $\gamma_2$ subunit and GAP-43 was significantly reduced in brain extracts of GODZ KO vs. WT mice, thereby identifying both proteins as important substrates of GODZ *in vivo*. In contrast, palmitoylation of PSD-95, which can be palmitoylated by GODZ in transfected HEK 293T cells, was unaffected.

The next objective was to assess the precise localization of GODZ in the Golgi apparatus using *cis*- and *trans*-Golgi markers in HEK 293T cells to gain further insights into the subcellular role of GODZ-mediated palmitoylation. GODZ was more strongly associated with the *cis*- than with the *trans*-Golgi network, suggesting a role in controlling the exit of substrate proteins from the endoplasmic reticulum (ER) to the Golgi. In addition to Golgi membranes in the soma, GODZ was also found localized to
Golgi outposts in dendrites. These results suggest that GODZ-mediated palmitoylation occurs on de novo synthesized proteins and contributes to trafficking of its substrates from the ER to the Golgi complex.

Finally, synaptic deficits seen in GODZ KO neurons could also be explained by the loss of NL2 palmitoylation. NL2 is localized preferentially to inhibitory synapses where it is important for synapse maturation. Moreover, knockdown of NL2 leads to defects in inhibitory synapse formation similar to those of GODZ KO neurons where deficits are only seen selectively when mutant neurons compete with WT neurons. Indeed, we find that GODZ and SERZ-β can palmitoylate NL2 in transfected HEK 293T cells. However, we could not ascertain a measurable difference in NL2 palmitoylation between WT and GODZ KO mice. A screen of the 23-member DHHC family of PATs in transfected HEK 293T cells identified GODZ and SERZ-β as two of several PATs that can act on NL2. A unique Cys residue (C710) present in the cytoplasmic domain of NL2 but is absent in the other neuroligin isoforms has been identified as the primary site of palmitoylation by site directed mutagenesis. Palmitoylated NL2 was observed in cultured neurons and in the cerebellum of transgenic Histidine-Flag-YFP-NL2 tagged mice. Native NL2 is likely to be palmitoylated as the protein was detected in the proteomics screen of palmitoylated proteins. Both putative palmitoylation sites of NL2 are also subject to nitrosylation, a potential competitive post-translational modification. Nitrosylated NL2 was also isolated from whole brain extracts. These results suggest the interplay between palmitoylation and nitrosylation of NL2 likely influences the synaptic localization of NL2 and could contribute to the GABAergic synaptic deficits seen in GODZ KO mice.
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<th>Full Form</th>
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<tbody>
<tr>
<td>15-HYDA</td>
<td>15-hexadecynoic acid</td>
</tr>
<tr>
<td>17-OYDA</td>
<td>17-octadecynoic acid</td>
</tr>
<tr>
<td>ABE</td>
<td>Acyl-biotin exchange</td>
</tr>
<tr>
<td>ACBP</td>
<td>Acyl-CoA binding protein</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>APT</td>
<td>Acyl protein thioesterases</td>
</tr>
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<td>Tetraspanin-9</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DG</td>
<td>Dystroglycan</td>
</tr>
<tr>
<td>DGC</td>
<td>Dystrophin glycoprotein complex</td>
</tr>
<tr>
<td>DHHC-CRD</td>
<td>Aspartate-histidine-histidine-cysteine-cysteine rich domain</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>GABA$_A$Rs</td>
<td>GABA$_A$ receptors</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamate decarboxylase</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth associated protein 43</td>
</tr>
<tr>
<td>GODZ</td>
<td>Golgi-associated DHHC type zinc finger protein</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidyl inositol</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxylamine</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>Hhat</td>
<td>Hedgehog acyltransferase</td>
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<tr>
<td>HIP-14</td>
<td>Huntingtin-interacting protein 14</td>
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<tr>
<td>HRASLS</td>
<td>H-Ras like tumor suppressors</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingtin</td>
</tr>
<tr>
<td>INCL</td>
<td>Infantile neuronal ceroid lipofuscinosis</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantitation</td>
</tr>
<tr>
<td>KCC2</td>
<td>$K^+\cdotCl^-$ cotransporter 2</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LRAT</td>
<td>Lecithin retinol acyl transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LRP6</td>
<td>Low density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>MBOAT</td>
<td>Membrane bound O-acyltransferase</td>
</tr>
<tr>
<td>mEPSC</td>
<td>Miniature excitatory postsynaptic current</td>
</tr>
<tr>
<td>mIPSC</td>
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</tr>
<tr>
<td>MMTS</td>
<td>Methyl-methane thiosulfonate</td>
</tr>
<tr>
<td>Moco</td>
<td>Molybdenum cofactor</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NL</td>
<td>Neuroligin</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NTSR</td>
<td>Neurotensin</td>
</tr>
<tr>
<td>PAT</td>
<td>Palmitoyltransferase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPT</td>
<td>Protein palmitoyl thioesterase</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein of 95 kDa</td>
</tr>
<tr>
<td>RAC</td>
<td>Resin-assisted capture</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
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<tr>
<td>RPE65</td>
<td>Retinal pigment epithelium-specific protein 65kDa</td>
</tr>
<tr>
<td>SCAM</td>
<td>Synaptic scaffolding molecule</td>
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<td>Sertoli cell gene with a zinc finger domain</td>
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<td>Stable isotope labeling with amino acids in cell culture</td>
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<td>Synaptosomal associated protein 25 kDa</td>
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<td>Sorting nexin 1</td>
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<td>Tumor endothelial marker 8</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>VGCC</td>
<td>Voltage gated calcium channels</td>
</tr>
<tr>
<td>Wg</td>
<td>Wingless</td>
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<td>WT</td>
<td>Wild type</td>
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Chapter 1

Introduction

1.1 Lipid modifications

Regulation of subcellular protein localization and function in the central nervous system can be modulated by post-translational modifications and underlie one of the core mechanisms by which synapse function is regulated. S-palmitoylation involves the post-translational addition of a 16-carbon fatty acid chain called palmitate to a cysteine (Cys) residue through a labile thioester bond (Figure 1.1a) (Schmidt et al., 1979, Magee et al., 1984). Global analyses of rat synaptosomal fractions led to the discovery of nearly 300 palmitoylated neuronal protein candidates underlying the importance of palmitoylation in synaptic regulation (Kang et al., 2008). Other major classes of S-acylation include the additions of both saturated and unsaturated fatty acids such as myristic, stearic, oleic and arachidonic acids (Young et al., 2012). Four types of acylation have been discovered: isoprenylation, myristoylation, palmitoylation and the addition of glycophosphatidyl inositol (GPI) anchors. N-myristoylation involves the addition of a 14-carbon saturated fatty acid chain via an amide bond to N-terminal glycine residues after aminopeptidase cleavage of a nearby methionine. However, this modification is only present in around 0.5% of proteins (Farazi et al., 2001, Maurer-Stroh and Eisenhaber, 2004). Prenylation is the post-translational addition of a 15-20 carbon isoprenoid farnesylpyrophosphate or geranylgeranylnpyrophosphate to a cysteine residue (Zhang and Casey, 1996). GPI anchoring facilitates membrane association and involves the addition of a glycolipid that is attached to the C-terminus by a GPI
transamidase (Mayor and Riezman, 2004). Specific combinations of these lipid modifications can be synergistic. Myristoylated proteins are often subject to palmitoylation of cysteines adjacent to the initial glycine residue or are similarly isoprenylated (Mumby et al., 1994, Dudler and Gelb, 1996).

1.1.1 Palmitoylation

S-palmitoylation (referred to from here on simply as palmitoylation) is different from N-palmitoylation and other S-acylation modifications in that it is rapidly reversible, allowing the protein to undergo multiple cycles of palmitoylation and depalmitoylation (Fukata et al., 2015). Palmitoylation occurs on a wide variety of proteins that are involved in nearly all biological processes. Around 10% of the human proteome has been identified as being subject to palmitoylation by mass spectrometry (Martin et al., 2012). In the brain, palmitoylation of proteins responds dynamically to extracellular signaling cues and can contribute to synaptic plasticity. In addition, palmitoylation has been shown to be involved in regulation of signaling, ion transport, protein degradation, endocytosis, and localization (el-Husseini Ael and Bredt, 2002, Hayashi et al., 2005, Fukata and Fukata, 2010, Salaun et al., 2010, Han et al., 2015).
Figure 1.1 Functional consequences of palmitoylation on target proteins

(a) Palmitoylation is the reversible addition of a 16-carbon fatty acid chain to a cysteine residue on a protein via a thioester bond. (b) Palmitoylation can have various effects on target proteins. (b_i) Palmitoylation of a soluble protein can alter the protein’s membrane association or association with lipid rafts as shown for H-Ras and N-Ras (Swarthout et al., 2005). (b_ii) Conformational state changes in protein secondary structure as a result of palmitoylation which can protect from ER degradation in LRP6 (Abrami et al., 2006). (b_iii) Palmitoylation can protect proteins from ubiquitination and degradation as shown in LRP6 and TEM8 (Abrami et al., 2008). (b_iv) Protein palmitoylation can facilitate interactions with other proteins as has been observed in calnexin and the ribosome translation complex (Lakkaraju et al., 2012). Figure adapted from (Blaskovic et al., 2013).

The addition of a large chain-fatty acid such as palmitate can have a variety of consequences for the protein (Figure 1.1b). Palmitoylation can alter the hydrophobicity of a soluble protein or protein domain and thereby can alter its membrane association or, in case of a bona fide membrane protein, its structural configuration at the membrane. Some of the most studied examples of palmitoylated soluble proteins include the small GTPases H-Ras, N-Ras and K-Ras which regulate cell proliferation and migration (Cuiffo and Ren, 2010). H-Ras and N-Ras must be associated with the plasma membrane in order for signal transduction to occur. Palmitoylation of Ras occurs early in the secretory
pathway and dictates the distribution of Ras to the plasma membrane or to the Golgi (Figure 1.1b) (Swarthout et al., 2005).

In addition to targeting of proteins to the plasma membrane, palmitoylation can translocate transmembrane proteins to micro- and nano-domains such as cholesterol-rich lipid rafts or synapses. For example, neurotensin receptor-1 (NTSR-1) is a G-protein-coupled receptor involved in cancer progression. Palmitoylation sequesters NTSR-1 to structured membrane micro-domains and that localization is necessary for mitogenic signaling of NTSR-1 (Heakal et al., 2011).

Palmitoylation of transmembrane proteins can also have functional consequences on the protein’s conformational state. For example, palmitoylation of the Wnt co-receptor low-density lipoprotein receptor-related protein 6 (LRP6) is predicted to result in tilting and extension of the cytosolic face of the transmembrane domain and is necessary for exit of LRP6 from the ER (Figure 1.1bii) (Abrami et al., 2008). In addition, palmitoylation can affect a protein’s susceptibility to other post-translational modifications such as ubiquitination or nitrosylation, which subsequently can affect its degradation. For example, palmitoylation of the anthrax receptor tumor endothelial marker 8 (TEM8) prevents its association with lipid rafts and leads to its ubiquitination (Figure 1.1biii) (Abrami et al., 2006). Lastly, palmitoylation can directly influence interactions between proteins and facilitate complex formation. For example, palmitoylation of the major endoplasmic reticulum (ER) chaperone calnexin leads to its translocation from the peripheral ER to the perinuclear rough ER face. Once there, calnexin forms a supercomplex with the ribosome-translocon complex and recruits actin to further stabilize the complex (Figure 1.1biv) (Lakkaraju et al., 2012).
The thioester bond that links palmitate with cysteine residues contains an electropositive carbonyl group that renders the carbon atom uniquely susceptible to nucleophilic attack. In contrast, myristoyl- and isoprenyl-lipids are stably attached to proteins via amide or thioether bonds (Castro, 1999, Ferri et al., 2005, Salaun et al., 2010). The labile nature of the thioester bond allows for rapid changes in the palmitoylation state. Dynamic changes result in spatial and temporal fine-tuning of the palmitoylation state of target proteins (Conibear and Davis, 2010).

1.1.2 Interplay with nitrosylation

Nitric oxide (NO) is a free radical and an important biological second messenger. As a post-translational modification, it signals by S-nitrosylation of cysteine residues in a manner similar to palmitoylation (Hess et al., 1993, Hess et al., 2005). Nitrosylation can directly or indirectly compete with other post-translational modifications including phosphorylation, ubiquitination, acetylation, sumoylation and palmitoylation (Lopez-Otin and Hunter, 2010, Hess and Stamler, 2012). For example, incubation of cells with a nitric oxide donor results in reduced palmitoylation of growth associated protein 43 (GAP-43) and synaptosomal associated protein 25kDa (SNAP-25), implicating the cross-talk between palmitoylation and nitrosylation being important in neurons (Hess et al., 1993).

The postsynaptic density protein of 95 KD (PSD-95) serves as the primary postsynaptic scaffolding protein of excitatory synapses. It is dynamically regulated by synaptic activity and, in turn, modulates synapse strength (Migaud et al., 1998, Han et al., 2015). The lattice-like structure of PSD-95 allows its multiple binding domains to spatially organize proteins at the synapse. PSD-95 localization at the synapse is
dependent on palmitoylation at Cys-3 and Cys-5 where it functions to cluster AMPA receptors and binds to neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996, Christopherson et al., 1999). However, more recent studies have shown the C-terminus of PSD-95 to be responsible for activity dependent AMPAR trafficking and the N-terminus for AMPA function (Xu et al., 2008). Palmitoylation of PSD-95 is influenced by glutamate receptor synaptic activity and results in depalmitoylation of PSD-95 which, in turn, reduces the number of AMPA receptors at synapses (El-Husseini Ael et al., 2002). Activation of NMDA receptors causes an influx of calcium that increases the production of NO through binding to calmodulin associated with nNOS. Neuronal activity also depalmitoylates PSD-95 further facilitating nitrosylation. Nitrosylation of PSD-95 prevents its re-palmitoylation and reduces the amount of PSD-95 at the synapse. The interplay between nitrosylation and palmitoylation is emerging as an important factor in modeling synaptic efficacy (Ho et al., 2011).

1.1.3 Enzymes involved in palmitoylation

Palmitoyl-CoA is the unbound bio-accessible form of palmitate that is generated by acyl-CoA synthetase in the cell and is present at below 5nM concentrations (Faergeman and Knudsen, 1997). Acyl-CoA binding protein (ACBP) binds acyl-CoA at low concentrations and inhibits non-enzymatic palmitoylation (el-Husseini Ael and Bredt, 2002). Due to inhibition by ACBP and the low concentration of palmitate in the cell, palmitoylation does not occur spontaneously. Palmitoylation requires specific sets of palmitoyltransferase (PAT) enzymes. There are three major types of proteins that exhibit PAT activity: lecithin retinol acyl transferase (LRAT), membrane bound O-
acyltransferase (MBOAT) and DHHC (Asp-His-His-Cys) motif family proteins (Bartels et al., 1999, Lobo et al., 2002, Xue et al., 2004, Fukata et al., 2006a).

LRAT was thought to play a role in vision by palmitoylating retinal pigment epithelium-specific protein 65kDa (RPE65) that is involved in converting all-trans retinol (vitamin A) to 11-cis retinal. Subsequently, 11-cis retinal is used in synthesis of rhodopsin and cone photopigments in photoreceptor cells in the retina (Xue et al., 2004). Unpalmitoylated RPE65 acts as a chaperone for the synthesis of vitamin A in non-neural tissues such as the intestines, pancreas, lung and liver (Batten et al., 2004). Palmitoylation of RPE65 is believed to be involved in its membrane association and contributes to maximal isomerase activity. However, palmitoylation of RPE65 has not been observed in vivo (Jin et al., 2007, Yuan et al., 2010). LRAT also binds to and is necessary for the activation of STRA6, the membrane receptor for binding plasma retinol binding protein; this underlies its importance in vitamin A processing (Kawaguchi et al., 2012). The LRAT family shares significant sequence homology with H-Ras-like tumor suppressors (HRASLS). HRASLS are thiol proteases that function in vitamin A metabolism by swapping domains with LRAT. This domain swapping results in a chimeric thioester catalytic intermediate state. The conformational state change promotes efficient acyl transfer and thereby contributes to the function of LRAT (Golczak et al., 2015).

The MBOAT-family proteins are multi-pass (8-12) transmembrane-domain proteins. They have a short conserved sequence that utilizes a histidine residue to catalyze the transfer of fatty acids to a hydroxyl groups in proteins, alcohols, diacylglycerol or to cholesterol (Hofmann, 2000). Several members of the MBOAT-family also exhibit lysophospholipid acyltransferase activity that contributes to membrane
organization (Shindou and Shimizu, 2009). Two members of the MBOAT family are involved in palmitoylation of target proteins. They catalyze the acylation of numerous extracellular secreted signaling proteins such as ghrelin, Hedgehog (Hh) and Wg/Wnt (Chang and Magee, 2009). For example, Hh acyltransferase catalyzes N-palmitoylation at the N-terminus of Hh, while Porcupine catalyzes the acylation of Wnt. Both MBOAT proteins play an important role in the secretion of their effector molecules as depalmitoylation redistributes Ras to endomembranes for diffusion back to the Golgi (Rocks et al., 2005, Rocks et al., 2010). N-palmitoylation of Hh differs from S-palmitoylation in that it occurs via a thioester intermediate to form a stable amide bond at a lysine residue near the N-terminus. N-palmitoylation is irreversible, but it contributes to regulation of secreted signaling molecules (Aicart-Ramos et al., 2011).

While S-palmitoylation was discovered almost 40 years ago, identification of the enzymes involved was hindered by difficulties in purifying integral membrane proteins and the labile nature of the modification (Schmidt et al., 1979, Magee and Courtneidge, 1985). A breakthrough was made in 2002 when Erf2 was identified as the enzyme responsible for palmitoylating Ras2p in yeast (Lobo et al., 2002). Erf2 is localized in the peripheral ER membrane and contains a highly conserved DHHC domain within a ~50 amino acid cysteine rich domain (CRD) that is essential for PAT activity (Lobo et al., 2002). A second PAT was identified in yeast named Akr1P; it palmitoylates the casein kinase Yck2p, which only shares homology to Erf2 in the DHHC domain (Roth et al., 2002). Due to the specific homology in the DHHC domain between Ark1P and Erf2 it was suggested that proteins that contain the DNA sequence for DHHC domains are PATs. Subsequent studies have conclusively identified seven DHHC-family PATs in Saccharomyces cerevisiae. Based on homology, 22 DHHC-family PAT genes have
been predicted in Drosophila melanogaster and 23 in mammals (Fukata et al., 2004, Ohno et al., 2006, Roth et al., 2006, Bannan et al., 2008).

The first mammalian DHHC protein shown to exhibit PAT activity was Golgi-specific DHHC domain zinc finger protein (GODZ, DHHC3) (Uemura et al., 2002, Keller et al., 2004). GODZ shares about 60.9% amino acid identity with its closest related paralog, Sertoli cell gene with a zinc finger domain -β (SERZ-β, DHHC7). The DHHC PATs that are expressed in the brain and most closely related to GODZ are shown in Figure 1.2 a.

![Figure 1.2 Homology and structural model of DHHC enzymes found in the brain that are closely related to GODZ](image)

(a) Phylogenetic tree representing the PATs expressed in the brain that are most closely related to GODZ (DHHC3) and SERZ-β (DHHC7) and their domain structure based on their human genes. (b) Structural model of the palmitoyltransferase, GODZ, containing four transmembrane domains with the catalytic DHHC-CRD on the cytoplasmic face between the second and third transmembrane domain and a C-terminal PDZ binding domain motif.

Based on heterologous co-expression studies, GODZ and SERZ-β may palmitoylate a wide variety of proteins including the GABA_{A}R \gamma 2 subunit (Fang et al.,
SNAP25, PSD-95 (Fukata et al., 2004), the GluA1-4 subunits of AMPARs (Hayashi et al., 2005), NCAM (Ponimaskin et al., 2008), endothelial NOS (eNOS) (Fernandez-Hernando et al., 2006), integrin α6β4 (Sharma et al., 2012) and GAP-43 (Fukata et al., 2004). However, it is unclear which of these proteins are primary substrates of GODZ in vivo. In the brain, GODZ and SERZ-β are specifically expressed in neurons, which suggest a role in regulation of synaptic transmission by palmitoylating neuronal proteins (Keller et al., 2004, Fang et al., 2006).

### 1.1.3.1 DHHC enzymes

The prototypical membrane topology of most DHHC proteins is depicted in Figure 1.2 b. The enzymes consist of four transmembrane (TM) domains, with the hydrophobic DHHC-CRD localized in the second and extending into the third TM domain in the cytosol and both the N- and C-terminus localized in the cytoplasm (Linder and Jennings, 2013). However, a few DHHC proteins are predicted to have six TM domains plus N-terminal ankyrin repeats, with the DHHC domain localized between the fourth and fifth TM domains (Figure 1.2a (DHHC 23)) (Politis et al., 2005). DHHC proteins have considerable diversity in their N and C-terminal regions; this diversity is likely responsible for enzyme-substrate specificity (Huang et al., 2009).

The cysteine residue in the DHHC domain catalyzes the transfer of palmitate to target substrates and is necessary for autoacylation of the enzyme (Lobo et al., 2002, Roth et al., 2002). Autoacylated DHHC proteins transfer their acyl chain to the substrate via a two-step ping-pong mechanism with the autoacylated enzyme as the intermediate. For GODZ it was shown that the rate of autoacylation and palmitate transfer is significantly reduced with fatty acid chains longer than 16-carbons (Jennings and Linder,
DHHC enzymes vary widely in their degree of specificity for different protein substrates, with some members only palmitoylating one known substrate (DHHC9, 17, 18), and others palmitoylating numerous substrates (GODZ, SERZ-β) (Fukata et al., 2004, Fang et al., 2006, Fernandez-Hernando et al., 2006, Greaves et al., 2008). To date, there is no known consensus sequence for palmitoylation of protein targets. However, some DHHC enzymes appear to favor certain protein topologies. For example, GODZ and SERZ-β appear to favor cysteine residues near transmembrane domains such as the putative palmitoylation sites on the cytoplasmic loop of the γ2 subunit of GABAARs and NCAM, but they can palmitoylate other sites as well. In contrast, DHHC17 preferentially palmitoylates cysteine residues adjacent to prolines (Fang et al., 2006, Greaves et al., 2008, Greaves et al., 2009, Huang et al., 2009).

The conserved DHHC-CRD of DHHC-type PATs was originally classified as a zinc-finger-domain based on homology to other zinc finger domain proteins. However, the function of binding metals among DHHC proteins has not been well understood (Putilina et al., 1999). In support of a zinc finger structure, modeling of the yeast PAT Swf1 suggested that the CRD is similar to the CTCHY zinc finger domain of mouse ubiquitin ligase Prih2. The zinc finger domain coordinates two zinc atoms per protein and zinc binding is essential for Swf1 PAT function (Gonzalez Montoro et al., 2013). Additionally, the treatment of GODZ with metal chelators results in loss of PAT activity indicating metal binding might be important for mammalian PAT function. Consistent with the above findings, using a zinc fluorescent indicator GODZ was found to bind two zinc atoms per GODZ protein (Gottlieb et al., 2015). The zinc-binding CRD is required for the secondary structure and functional integrity of DHHC proteins analyzed by limited proteolysis assays (Gottlieb et al., 2015).
The transmembrane topology of DHHC proteins (Figure 1.2 b) mimics that of certain ion channels. Two of the DHHC PATs have been shown to be able to transport metal ions across membranes when overexpressed in heterologous cells. For example DHHC17, also known as huntingtin-interacting protein 14 (HIP-14), plays not only an important role as a PAT of huntingtin but has also been shown to contribute to palmitoylation-dependent membrane transport of Mg\(^{2+}\) when expressed in frog oocytes (Goytain et al., 2008). Moreover, upon similar overexpression of GODZ in frog oocytes the protein reaches the plasma membrane and mediates membrane transport of Ca\(^{2+}\) in a palmitoylation-dependent manner (Hines et al., 2010). It is not known whether the native versions of these proteins exhibit ion transporter properties when expressed at physiological levels at their normal membrane locations.

DHHC enzymes are segregated into different subcellular compartments within the cell. The targeting of enzymes to different compartments generates spatially restricted patterns of PAT distribution and influences their function. In this manner, the localization of PATs influences the substrate specificity and enzyme function. Eighteen of the 23 DHHC enzymes localize to the early secretory pathway where they palmitoylate proteins in the ER or the Golgi apparatus. Other DHHC family members accumulate at synapses (DHHC2, 5) or at the plasma membrane (DHHC5, 8, 20, 21) (Ohno et al., 2006). The differential localization plays an important role in the modulation of palmitoylation in response to physiological stimuli. Blocking activity of neurons by inactivating voltage-gated sodium channels using tetrodotoxin or a glutamate receptor antagonist causes an increase in palmitoylation and clustering of PSD-95 at the synapse. Activity-dependent palmitoylation of PSD-95 is controlled in part by DHHC2 translocation to the synapse (Noritake et al., 2009). Additionally, DHHC5 forms a
trimeric complex with PSD-95 and the Src family tyrosine kinase Fyn. Neuronal activation disrupts this complex and then leads to endocytosis, translocation of DHHC5 to dendritic shafts and DHHC5 association with δ-catenin. Palmitoylation of δ-catenin leads to its trafficking to dendritic spines. Once at synapses, δ-catenin recruits AMPA receptors and strengthens cadherin stabilization in a palmitoylation dependent manner (Brigidi et al., 2015).

DHHC enzymes interact with each other to form dimers in the cell. Co-immunoprecipitation experiments suggest that GODZ and SERZ-β can form homo- and hetero-multimers, and this dimerization is partially responsible for the mechanism of inactivation in the catalytically inactive mutant, GODZ\textsuperscript{C157S}, where the cysteine in the catalytic domain is mutated to serine (DHHS) (Fang et al., 2006). Similarly, bioluminescence resonance energy transfer revealed that both DHHC2 and GODZ self-associate \textit{in vitro}. Catalytically inactive DHHC proteins exhibit greater affinity to form dimers than their active versions. This association likely inactivates their wild type counterparts, potentially implicating dimerization in regulating PAT activity (Lai and Linder, 2013).

1.1.3.2 Depalmitoylation enzymes

S-palmitoylation can be reversed in the cytoplasm or lysosome by acyl protein thioesterases (APTs). However, in contrast to the large number of PATs identified, only a few depalmitoylating enzymes have been identified to date. APTs belong to a superfamily of serine hydrolases that utilize serine for hydrolysis of amides, esters and thioesters and for protein degradation (Long and Cravatt, 2011). Protein palmitoyl thioesterase 1 (PPT1) was identified as the enzyme responsible for removing palmitate
from H-Ras and Gα subunits (Camp and Hofmann, 1993). PPT1 contains an S-H-D catalytic domain and a GXSXG (where x is any residue) motif that is typical of other lipases (Camp and Hofmann, 1993, Camp et al., 1994). PPT2 is a paralog of PPT1 that shares 26% sequence identity but has different substrate preferences (Calero et al., 2003). PPT1 operates at both neutral and acidic pH suggesting that it has a role in removing fatty acids in the lysosomal lumen (Hellsten et al., 1996, Verkruyse and Hofmann, 1996). In neurons, PPT1 is also present at synapses and axons and in synaptic vesicles, implicating it in depalmitoylation outside of the lysosome (Salonen et al., 2001, Ahtiainen et al., 2003, Virmani et al., 2005). Mutations in the PPT1 gene leads to infantile neuronal ceroid lipofuscinosisis (INCL), a lysosomal storage disorder from the accumulation of lipid-modified proteins (Vesa et al., 1995). While PPT1 is an important depalmitoylation enzyme it is regulated in part by palmitoylation. PPT1 is palmitoylated on Cys6 by GODZ and SERZ-β and this palmitoylation acts as a noncompetitive inhibitor reducing the function of PPT1. The disease progression seen in INCL may be due in part to a positive feedback loop generated by palmitoylation of PPT1; the feedback loop leads to a reduction in overall depalmitoylation in the cell and increased protein degradation (Segal-Salto et al., 2016).

APT1 is a cytoplasmic acylprotein thioesterase that contains the same catalytic domain as PPT1 (Devedjiev et al., 2000). APT1 shares 64% homology with APT2; however, direct in vivo evidence of depalmitoylation activity of APT2 has yet to be discovered. Knockdown of APT2 leads to changes in localization of GAP-43 and H-Ras and depalmitoylation of N-Ras has been observed in vitro (Tomatis et al., 2010, Rusch et al., 2011). APT1 has a number of candidate neuronal substrates such as, eNOS, Gα, synaptosomal associated protein 23 (SNAP-23) and H-Ras (Duncan and Gilman, 1998,
Depalmitoylation by APTs in the cytosol is believed to play a role in removing mislocalized proteins by redirecting them to the Golgi that is inhabited by numerous PATs (Rocks et al., 2010). The N-terminal Cys2 of both APT1 and APT2 are palmitoylated. Palmitoylation of APTs has been proposed to enhance their localization to the plasma membrane and facilitate depalmitoylation. Palmitoylated APT1 and APT2 are found partially in the Golgi complex where they depalmitoylate each other in trans causing their translocation to the cytosol to develop a balance between palmitoylation-depalmitoylation cycles in the cell (Vartak et al., 2014). The interplay between palmitoylation and depalmitoylation contributes to the spatial organization of the enzymes in the cell as well as plays an important role modulating their substrates. While APT1 is the most studied depalmitoylating enzyme, it has narrow substrate specificity and there are likely other proteins with APT activity. Abhydrolase (α-β-hydrolase) 17 (ABHD17) was recently identified as a potential depalmitoylation enzyme that accelerates the palmitoylation turnover for PSD-95 and N-Ras (Lin and Conibear, 2015). It is clear that APTs are critical for dynamic palmitoylation cycles and further work will be paramount for understanding the role it plays in the cell.

1.1.4 Measuring palmitoylation

Historically the only method to measure palmitoylation involved metabolic radio-labeling with [³H]-palmitate and fluorography, which involved lengthy exposure times of days to weeks (Figure 1.3 a). This method allows for measure of rate of incorporation as well as pulse-chase turnover experiments to determine half-life. However, it is insufficient for high throughput studies and is hampered by the high signal-to-noise ratio because the probe is incorporated into every palmitoylated protein. Using radio-label
incorporation further immunopurification is necessary to resolve a protein of interest (Waterborg and Matthews, 1994).

**Figure 1.3 Direct methods to assay for palmitoylation**

(a) **Metabolic Radiolabeling** - Tritium labeled palmitate is given to living cells and is incorporated into palmitoylated proteins by PATs. At a given time point, proteins can be harvested and further analyzed by SDS-PAGE and fluorography. (b) **Acyl-biotin exchange assay** provides a steady state look at palmitoylation at the time of harvest. It involves blocking free thiols, selective cleavage of palmitate, biotin labeling, and pull-down to enrich for palmitoylated proteins. (c) **Metabolic Click chemistry assay** – A terminal alkyne probe is fed to cells for a given time point and is incorporated into palmitoylated proteins. After harvest, through copper catalyzed cyclo-addition proteins are be conjugated to a reporter molecule to measure the incorporation of the palmitate analog.

Two complementary non-radioactive methods have been developed in recent years to enrich palmitoylated proteins and both allow for a mass spectrometry based approach to studying palmitoylation. The first method, acyl-biotin exchange (ABE) is useful for measuring the steady state level of palmitoylation at the time the cells are harvested (Figure 1.3 b) (Drisdel and Green, 2004, Roth et al., 2006, Kang et al., 2008).
This multistep procedure involves first blocking free thiols by alkylation using N-ethylmaleimide (NEM) or methyl-methane thiosulfonate (MMTS) to prevent non-specific background. Next, the samples are split in two: one half is used as a control and one half is subject to hydroxylamine (HA) that reacts with the electrophilic carbonyl group of palmitoylated cysteines and cleaves the thioester bonds. The newly exposed thiols are subject to disulfide capture of a thiol-reactive biotin or another reporter molecule this allowing for purification or identification (Wan et al., 2007). Some groups have simplified this method using a thiol-reactive resin, thiopropyl sepharose, to eliminate the biotin step. While these methods have a great advantage over metabolic radiolabeling by enriching for palmitoylated proteins via pull-down, it also enriches other thioester containing proteins such as lipoamide dehydrogenases (Forrester et al., 2011).

The second nonradioactive method utilizes bio-orthogonal fatty acids and click chemistry conjugation (Figure 1.3 c) (Hang et al., 2007, Kostiuk et al., 2008). The fatty acid probes contain a terminal alkyne group that does not interfere with the hydrophobic nature of the fatty acid and allows for diffusion into the cell and incorporation into proteins. The most common probe for palmitoylation is 17-octadecynoic acid (17-OYDA) that has a fatty acid chain that is 18 carbons in length instead of 16 normally present in endogenous palmitate. Proteins incorporate 17-OYDA and subsequently can be conjugated to biotin-azide or rhodamine-azide by reacting with the terminal alkyne group through Cu (I)-catalyzed (3+2) Huisgen cycloaddition (click reaction) (Rostovtsev et al., 2002, Tornoe et al., 2002). This procedure combines the advantages of metabolic time-sensitive labeling and the specificity of the ABE-assay. Biotin conjugation is useful for streptavidin enrichment and rhodamine conjugation for localization and rapid detection. A dual functional fatty acid x-alk-16 contains an alkyne and a diazirine that can be used
for photo-crosslinking palmitoylated protein complexes and will be useful for elucidating the role of palmitoylation in the cell (Peng and Hang, 2015).

These enhanced isolation methods have been used to globally analyze the palmitoylated proteome by combining enrichment techniques with mass spectrometry in numerous different animal models (Roth et al., 2006, Wan et al., 2007, Kang et al., 2008, Forrester et al., 2011). Direct methods of detecting palmitoylation by mass spectrometry have been achieved but have proven difficult due to the labile nature of the thioester bond and complexity in separating hydrophobic peptides by C18 reversed-phase liquid chromatography (Ji et al., 2013). Palmitoylation purification techniques can be combined with numerous quantitative proteomic methods such as isobaric tagging (iTRAQ) or stable-isotope labeling with amino acids in cell culture (SILAC) to quantitate relative palmitoylation levels simultaneously for multiple proteins. Proteomics approaches have identified roughly 300-600 palmitoylated proteins in various cell types; this reinforces the importance of palmitoylation for a vast number of proteins (Martin et al., 2012, Hemsley et al., 2013).

1.2 Importance of studying GABA\(\alpha\)Rs

The work presented in this thesis focuses on the palmitoylation of proteins that affect the trafficking of gamma-aminobutyric acid receptors (GABA\(\alpha\)Rs) to the synapse. GABA\(\alpha\)Rs are GABA-gated chloride channels that bind synaptically released GABA and are the primary phasic inhibitory receptors in the brain. The functional strength of GABAergic synapses depends largely on the number of receptors located in the postsynaptic membrane because neurotransmitter release at synapses tends to saturate its receptors (Otis et al., 1994, Clements, 1996, Nusser et al., 1997). The importance of
modulating GABA<sub>A</sub>R concentration at the synapse can be seen in the variety of neurological diseases associated with aberrant GABAergic transmission. GABAergic alterations have been implicated as a causal factor in anxiety disorders (Crestani et al., 1999, Malizia, 2002, Lydiard, 2003), autism spectrum disorders (Schmitz et al., 2005), Down syndrome (Deidda et al., 2015), schizophrenia (Lewis et al., 2005, Petryshen et al., 2005) and epilepsy (Baulac et al., 2001, Wallace et al., 2001, Reinthaler et al., 2015). Therefore, the study of GABA<sub>A</sub>Rs is critically important for understanding the mechanisms that underlie the etiology of these diseases.

### 1.2.1 Structure and function of GABA<sub>A</sub>Rs

GABA<sub>A</sub>Rs are heteropentameric GABA-gated ion channels that are part of the superfamily of Cys−loop receptors (Barnard et al., 1998). Each subunit exhibits a shared homologous structure consisting of both the N and C−terminus located extracellularly, four TM domains, and a large cytoplasmic loop sequence between TM3 and TM4, which is subject to a variety of post-translational modifications and interacts with numerous proteins (Chen and Olsen, 2007, Luscher et al., 2011). In immature neurons early in development, the intracellular chloride concentration is higher than it is in mature neurons; therefore, activation of GABA<sub>A</sub>Rs leads to membrane depolarization because the response to activation is dependent on the equilibrium potential for Cl<sup>-</sup>. This depolarization activates voltage gated calcium channels (VGCC) leading to calcium influx that induces transcription of the K<sup>+</sup>- Cl<sup>-</sup> cotransporter 2 (KCC2) (Ben-Ari, 2002). The increased expression of KCC2 controls the gradual developmental reduction of the equilibrium potential for Cl<sup>-</sup> to levels below the resting membrane potential such that
activation of GABA_ARs leads to an outward hyperpolarizing inhibitory current found throughout adulthood (Rivera et al., 1999).

The subunits of GABA_ARs are encoded by 19 genes that are grouped into eight subclasses based on their homology (α, β, γ, δ, ρ, ε, π and θ) (Sieghart et al., 1999, Sieghart and Sperk, 2002, Olsen and Sieghart, 2009). The intracellular loop region of these subunits is the most diverse region and involved in interaction with trafficking and other proteins, whereas overall there is a 70-80% sequence identity present within each subunit class. The heterogeneity of the subunit combinations leads to at least 11 structurally and functionally different types of GABA_ARs, and probably many more (Olsen and Sieghart, 2009). Additional structural heterogeneity exists due to alternative splicing of subunit mRNAs, as shown for the γ2 subunit that exists as a short (γ2S) and long (γ2L) isoform; the long isoform is distinguished by an additional eight amino acids of γ2L in the major cytoplasmic loop region. The γ2S variant is found at higher concentrations in the cortex, hippocampus, olfactory bulb and in early development (Gutierrez et al., 1994, Miralles et al., 1994). Receptors containing the γ2S subunit show less sensitivity to zinc blockade than γ2L containing receptors (Boileau et al., 2010). The multitude of different subunit compositions leads to functional heterogeneity demonstrated by variance in the pharmacology, agonist affinity, cell type expression, channel conductance and gating properties of different GABA_AR subunit combinations (for review see (Luscher et al., 2011)). Despite the large number of possible subunit combinations, specific GABA_AR subtype combinations are present in distinct neuronal synapse populations (Fritschy and Mohler, 1995).
GABA_\text{A} receptors at inhibitory synapses are composed of 2\(\alpha\) (\(\alpha_{1-3}\)), and 2\(\beta\) (\(\beta_{2-3}\)) subunits with a single \(\gamma_{2}\) subunit and mediate phasic inhibition (Baumann et al., 2001). These receptors are ideal for responding to comparatively high concentrations (0.3-1 mM) of GABA reached following synaptic GABA release because they have low affinity for GABA and rapidly desensitize (Perrais and Ropert, 1999). In contrast, tonic inhibitory currents are mediated by extrasynaptic receptors containing \(\alpha_{4}, \alpha_{5}, \alpha_{6}, \beta_{2}, \beta_{3}\) and \(\delta\) subunits and are ideal for responding to low concentrations of GABA present outside the synaptic cleft (Farrant and Nusser, 2005, Brickley and Mody, 2012). However, \(\alpha_{5}\) subunit-containing receptors have also been observed at GABAergic synapses in neuronal development (Brady and Jacob, 2015) Most receptors are trafficked to the plasma membrane at extrasynaptic sites. A variety of mechanisms facilitate their clustering at synaptic sites through lateral diffusion and protein interactions (Bogdanov et al., 2006). GABA_\text{A}R\_s located in extrasynaptic membranes tend to be more laterally mobile than synaptically located receptors, with the exception of \(\alpha_{5}\) containing receptors that are stabilized in the extrasynaptic membrane by radixin-mediated interaction with the cytoskeleton (Jacob et al., 2005, Bogdanov et al., 2006, Hausrat et al., 2015). The \(\gamma_{2}\) subunit is essential for the clustering of GABA_\text{A}Rs and the clustering of the postsynaptic scaffolding protein gephyrin at the synapse (Essrich et al., 1998, Schweizer et al., 2003, Alldred et al., 2005). Therefore, studying the mechanism by which the \(\gamma_{2}\) subunit contributes to clustering GABA_\text{A}Rs at the synapse is a key component in understanding mechanisms that regulate neuronal inhibition in the brain.
1.2.2 Palmitoylation of the γ2 subunit of GABA_A Rs

Trafficking of GABA_A Rs is influenced by a number of post-translational modifications such as phosphorylation, ubiquitination and palmitoylation (Luscher et al., 2011). Palmitoylation of the γ2 subunit occurs at multiple cysteine residues in the intracellular loop region and is critical for the clustering of GABA_A Rs at synapses (Rathenberg et al., 2004, Fang et al., 2006). A rare point mutant variant of the human γ2 subunit gene (GABRG2) leads to impaired palmitoylation of the γ2 subunit has been identified in patients with rolandic epilepsy, suggesting that palmitoylation of GABA_A Rs is critically important for normal inhibitory synapse function in vivo (Reinthaler et al., 2015).

GODZ and SERZ-β were first identified as γ2 subunit interacting proteins in a yeast two-hybrid screen. Indeed, GODZ interacts with γ1-3 subunits and upon co-transfection in human embryonic 293T (HEK 293T) cells. GODZ palmitoylates the γ2 subunit and palmitoylated γ2 is found in neurons (Keller et al., 2004). A screen of all known 23 DHHC PATs by metabolic radiolabeling revealed that GODZ and SERZ-β were capable of palmitoylating the γ2 subunit in transfected HEK 293T cells (Fang et al., 2006). Palmitoylation likely occurs on multiple cysteine residues in the cytoplasmic loop of the γ2 subunit and is required for clustering of GABA_A Rs at the synapse (Rathenberg et al., 2004, Fang et al., 2006). Blocking palmitoylation using α-brominated 2-bromopalmitate or by mutating the cytoplasmic cysteines (Cys 359, 368, 375 or 376) to alanine reduces the synaptic clustering of GABA_A Rs in neuron cultures (Rathenberg et al., 2004). Neurons transfected with GODZ shRNA or dominant negative GODZ resulted in reduced GABA-evoked whole cell currents and miniature inhibitory postsynaptic current (mIPSCs) amplitude and reduced frequency but did not affect
AMP A-evoked whole cell currents, nor miniature excitatory postsynaptic currents (mEPSCs) (Fang et al., 2006). However, it has not been firmly established whether these functional defects are due to reduced palmitoylation of GABA\_Rs or of other substrates involved in inhibitory synapse function.

1.2.2.1 Generation of GODZ and SERZ-\(\beta\) KO mice

A previous student from our laboratory generated GODZ and SERZ-\(\beta\) KO mice to further investigate the role of palmitoylation \textit{in vivo}. Global GODZ and SERZ-\(\beta\) KO mice were found to be viable. However, GODZ and SERZ-\(\beta\) double knockout (DKO) mice showed a partially penetrant perinatally lethal phenotype, with rare survivors being fertile and showing normal life expectancy despite a severely runted phenotype (Murakami, 2008). Cultured cortical neurons derived from GODZ KO and GODZ/SERZ-\(\beta\) DKO mice exhibit normal inhibitory synapse development as evidenced by unaltered density of postsynaptic clusters for the \(\gamma_2\) subunit. They also showed normal colocalization with punctate immunoreactivity for the presynaptic markers glutamate decarboxylase (GAD), in apparent conflict with data obtained with GODZ shRNA- or dominant negative GODZ-transfected neurons (Fang et al., 2006, Murakami, 2008). However, when the mutant neurons were analyzed in co-culture with WT neurons (i.e. competitive conditions that mimic sparse transfections of WT cultures with shRNA) they largely reproduced data from shRNA transfected neurons. Co-cultured mutant neurons showed prominent defects in GABA\_ergic synapses, including a reduction in the amplitude and frequency of mIPSCs. This phenotype of GODZ KO neurons mimics a very similar deficit in GABA\_ergic innervation seen when \(\gamma_2^{+/−}\) neurons are grown in co-culture with WT
neurons, suggesting that the GODZ KO phenotype in co-cultures is at least in part caused by reduced accumulation of GABA\(_{\alpha}\)Rs in the postsynaptic membrane of mutant neurons (Ren et al., 2015). GABA and glutamate induced whole cell currents are normal in GODZ KO and DKO neurons suggesting that defects in GABA\(_{\alpha}\)Rs are limited to synapses and do not extend to non-synaptic GABA\(_{\alpha}\)Rs (Murakami, 2008). The severity of deficits in GABAergic transmission observed in GODZ KO neurons was comparable to that seen in DKO mice, indicating that SERZ-\(\beta\) did not contribute materially to the function of GABAergic synapses, even in the absence of GODZ. Therefore, for the purpose of this dissertation we chose to focus on GODZ-mediated palmitoylation as the PAT important for development and function of inhibitory synapses.

1.2.3 Proteins implicated in clustering of GABA\(_{\alpha}\)Rs

1.2.3.1 Gephyrin

Gephyrin plays a critical role in clustering of GABA\(_{\alpha}\)Rs at the synapse by forming oligomerized lattice like clusters at synapses as shown in Figure 1.4 a (Kneussel and Betz, 2000). Gephyrin binds to the cytoplasmic loop region of GABA\(_{\alpha}\)R \(\alpha1, \alpha2, \alpha3, \alpha5, \beta2\) and \(\beta3\) subunits and contributes to receptor subtype specificity and clustering at the synapse (Tretter et al., 2008, Saiepour et al., 2010, Mukherjee et al., 2011, Tretter et al., 2011, Kowalczyk et al., 2013). The oligomerization and clustering of gephyrin is dependent on its domain organization (Figure 1.4 b). Gephyrin G and E-domains share homologous sequences with molybdenum cofactor (Moco) biosynthesis enzymes (MoeA) in \textit{Escherichia coli} (Kamdar et al., 1994). Replacing the gephyrin E-domain with a homologous bacterial MoeA domain resulted in reduced gephyrin clustering, implying that Moco biosynthesis is not necessary for clustering (Lardi-Studler et al., 2007).
However, a patient with a missense mutation in the gephyrin gene (G375D) that, based on in vitro experiments, affects both Moco synthesis and gephyrin clustering, showed both epileptic encephalopathy and metabolic functional deficits (Dejanovic et al., 2015). Therefore, the precise role of Moco biosynthesis plays in clustering of gephyrin is not well understood.

Crystal structure analysis revealed that the G-domain forms a trimerized structure, while the E-domain forms a dimer; together this leads to the hypothesis that clustered gephyrin forms a hexagonal subsynaptic lattice structure (Figure 1.4 a, b) (Sola et al., 2001, Xiang et al., 2001, Lardi-Studler and Fritschy, 2007, Saiyed et al., 2007). Gephyrin targeting and clustering at inhibitory synapses requires neuron-specific mechanisms that are not present in other cell-types. In contrast, when gephyrin is overexpressed in vitro it tends to form large intracellular aggregates instead of discrete puncta (Kirsch et al., 1995, Saiyed et al., 2007). Gephyrin is subject to numerous post-translational modifications that may facilitate targeting and clustering instead of non-targeted aggregate formation.

Extracellular signal-regulated kinase (Erk) phosphorylates gephyrin at Ser-268 and negatively modulates clustering at the synapse. Erk phosphorylation is synergistically coupled to phosphorylation at Ser-270 by glycogen synthase kinase 3-β (GSK3-β) (Tyagarajan et al., 2013). Inhibition of Ser-270 phosphorylation by GSK3-β antagonist and mood stabilizing drug lithium enhances gephyrin clustering and GABAergic inhibitory synapse function, thereby suggesting that lithium might exert its mood stabilizing effects at least partially through potentiation of GABAergic inhibition (Luscher et al., 2011, Tyagarajan et al., 2011b).
Figure 1.4 Structure and interaction of gephyrin, NL2 and GABA$_A$Rs at inhibitory synapses.

(a) Clustering of GABA$_A$Rs involves a complex interplay of interactions between numerous postsynaptic proteins. Those of particular interest in the context of this thesis are shown above. Gephyrin forms a postsynaptic scaffolding that binds to GABA$_A$Rs through their $\alpha_2$ subunit and forms a complex with NL2 and collybistin to facilitate clustering at the membrane (Luscher et al., 2011). (b) Gephyrin consists of an N-terminal G domain, C domain and E domain. The G domain forms trimers while the C-terminal E domain forms dimers leading to a hexagonal matrix structure (Sola et al., 2001, Xiang et al., 2001). Gephyrin is palmitoylated at two Cys residues 212 and 284 in the C-domain. Gephyrin binds to collybistin and nNOS (Tyagarajan et al., 2011a, Dejanovic and Schwarz, 2014) (c) NL2 consists of an N-terminal signal peptide (SP) shown in yellow, followed by a large choline-esterase-like domain with the alternatively spliced exon A as is most common form in the brain. Proceeded by a short TM domain and an intracellular cytosolic domain containing the tyrosine containing binding site for gephyrin and a C-terminal domain (Ichtchenko et al., 1996). NL2 contains two cytoplasmic cysteine residues that are potential sites of palmitoylation: Cys 710 and Cys 766.
The postsynaptic clustering of gephyrin is also regulated by palmitoylation (Dejanovic et al., 2014). Gephyrin is palmitoylated at two cysteines, Cys 212 and Cys 284 in the C-domain by DHHC12, a Golgi-localized PAT. This post-translational modification is required for normal plasma membrane association and postsynaptic clustering of gephyrin (Figure 1.2 b). Overexpression of DHHC12 in cultured neurons increases the amplitude and frequency of mIPSCs, consistent with the established role of gephyrin in regulating GABAergic inhibitory synapse function. Interestingly, unlike DHHC12, GODZ was ineffective in the clustering of gephyrin despite its otherwise broad substrate specificity. Palmitoylation of gephyrin is upregulated in response to GABA\(\alpha\)R activation and reduced by the GABA\(\alpha\)R antagonist bicuculline, suggesting that palmitoylation by DHHC12 plays an important role in modulation of the strength of GABAergic synapses (Dejanovic et al., 2014).

At synapses, gephyrin forms a complex with collybistin, a neuron specific dbl-like GDP/GTP exchange factor and postsynaptic adhesion molecule neuroligin 2 (NL2) that together facilitate gephyrin clustering (Figure 1.4 a, c). Collybistin contains a DBL homology domain that binds the small GTPase Cdc42, a pleckstrin homology domain involved in lipid binding and plasma membrane association, and a Src homology 3 (SH3) domain that is absent in rare splice variants but otherwise auto-inhibits the collybistin-gephyrin clustering function (Kins et al., 2000, Harvey et al., 2004, Tyagarajan et al., 2011a, Korber et al., 2012). However, when gephyrin and collybistin form a complex with a GABA\(\alpha\)R \(\alpha2\) subunit and NL2, they synergistically induce clustering of gephyrin at the plasma membrane (Figure 1.4 a) (Poulopoulos et al., 2009, Saiepour et al., 2010). Gephyrin also binds to the Cdc42 paralog, TC10 in the CA1 region of the hippocampus.
and the binding facilitates gephyrin clustering (Mayer et al., 2013). The release of inhibition by complex formation may be due in part due to a switch in the conformational state of collybistin from a closed to an open/active state upon binding of collybistin to NL2. Binding of NL2 to collybistin stabilizes the active open conformation of collybistin and thereby facilitates gephyrin binding (Soykan et al., 2014). Additionally, gephyrin binds to NL2 through a conserved tyrosine (Tyr770) residue that is required for colocalization of gephyrin and NL2 in cultured neurons (Poulopoulos et al., 2009, Kang et al., 2014). Phosphorylation of similar tyrosine residue (Tyr 2) in neuroligin-1 (the NL2 paralog typically present at excitatory synapses) prevents binding of NL1 to gephyrin controlling the balance between excitatory and inhibitory clustering of gephyrin (Giannone et al., 2013).

1.2.3.2 GAP-43

GAP-43 was recently identified as an important modulator of inhibitory synapses and is potentially a substrate of GODZ. GAP-43 stimulates GTP binding of G-protein G_{11} and facilitates actin polymerization by enabling the association of phosphatidylinositol-4,5-bisphosphate (PIP2) with the actin cytoskeleton (Pfenninger et al., 1991). GAP-43 is highly expressed in neuronal growth cones and during development (Benowitz and Routtenberg, 1997). The actin stabilizing effect of GAP-43 depends on phosphorylation at serine 41 by protein kinase C (Chan et al., 1986, Skene, 1989).

GAP-43 modulation has been implicated in synaptic plasticity, memory storage and long-term potentiation (Lovinger et al., 1986, Holahan and Routtenberg, 2008). Palmitoylation of GAP-43 is specific to Cys3 and 4 with Cys4 being the predominant palmitoylation site. Palmitoylation affects membrane association of GAP-43 and
interaction with PKC (Liu et al., 1993). Steady-state proteomics analyses in COS-1 cells showed that only 35% of GAP-43 is found in a lipidated state suggesting that palmitoylation is not required for its retention at the plasma membrane. Retention at the membrane requires an additional separate polybasic amino acid sequence (Figure 1.5) (Zuber et al., 1989, Liu et al., 1993, Liang et al., 2002).

Figure 1.5 GAP-43 palmitoylation affects membrane trafficking and synapse development
Palmitoylation targets GAP-43 to the plasma membrane where it is phosphorylated by protein kinase C and enables the association of PIP2 with actin to polymerize and influence neuron structure, filipodia, regeneration and synaptic plasticity. Gephyrin
clustering is negatively influenced by dephosphorylated GAP-43 and therefore GAP-43 through gephyrin can impact clustering of GABA\textsubscript{A}Rs at synapses in immature neurons.

Dephosphorylated GAP-43 can interact with the inhibitory postsynaptic scaffolding protein gephyrin causing it to misfold and form aggresomes thereby influencing clustering of GABA\textsubscript{A}Rs at the synapse and modulating synaptic function (Wang et al., 2015a). Transient oxygen-glucose deprivation (tOGD) mimics neonatal hypoxia and leads to calcineurin mediated GAP-43 dephosphorylation and gephyrin misfolding indicating a role in activity dependent neuroprotective mechanisms (Wang et al., 2015a).

1.2.4 Adhesion molecules involved in GABAergic innervation

The spatial and temporal development of synapses involves recruitment of the presynaptic terminal to the postsynaptic cell and stabilization of synapses by trans-synaptic cell adhesion complexes. Adhesion molecules bridge the gap in the synaptic cleft (~20 nm) to mediate interactions between the presynaptic and postsynaptic specializations that are essential for proper synapse function (Akert, 1972). Adhesion molecules implicated in synapse formation and maturation include neurexins and neuroligins, Eph receptors and ephrins, leucine-rich repeat transmembrane neuronal proteins (LRRTMs), SynCAM, integrins and N-cadhedrin/\(\beta\)-catenins (Yang et al., 2014).

1.2.4.1 Neurexins and neuroligins

This dissertation focuses on the palmitoylation of NL2 as an important mechanism that potentially regulates the formation and maturation of GABAergic synapses. Neuroligin-Neurexin \textit{trans}-synaptic complexes are critical for early synapse formation and contribute to learning and memory in adulthood (Bang and Owczarek,
The interaction between NL and neurexin facilitates numerous facets of synapse development and maintenance. Synapse formation and receptor specificity was induced in axons of neurons grown in co-culture with HEK 293T cells transfected with NL1 or NL2 (Scheiffele et al., 2000). Similarly, HEK 293T cells transfected with β-neurexin clustered both excitatory and inhibitory scaffolding proteins and receptors on neurons grown in the same culture (Graf et al., 2004). These landmark studies demonstrated for the first time that adhesion molecules can influence synapse development in neurons.

Neurexins (Nrxs) are localized to the presynaptic and postsynaptic plasma membranes in neurons by a stop-transfer anchor sequence (Berninghausen et al., 2007, Zhang et al., 2010). In mammals there are three genes encoding neurexins that each are driven by two independent promoters thereby generating both long α-neurexin and short β-neurexin isoforms (Baudouin and Scheiffele, 2010). Intracellular binding of proteins is driven in part by the PDZ domain binding site on the C-terminus of neurexins (Missler and Sudhof, 1998). Short β-neurexins contain only one laminin neurexin sex-hormone binding domain (LNS) in their extracellular region whereas long α-neurexins contain six LNS domains separated by three epidermal growth factor (EGF) domains that mediate their extracellular synaptic interactions (Missler and Sudhof, 1998). There are five alternative splice sites in α-neurexins and two in β-neurexins leading to potentially thousands of neurexin variations (Tabuchi and Sudhof, 2002).

Neuroligins share similar structural diversity, with four genes found in mice and rats and five in humans (Ichtchenko et al., 1996, Lise and El-Husseini, 2006). Structurally, neuroligins contain an extracellular domain with homology to acetylcholinesterase (AChE) but lacking cholinesterase activity, a transmembrane domain and a cytoplasmic C-terminal PDZ domain (Figure 1.5 c) (Missler and Sudhof,
Further structural diversity of neuroligins is generated by alternative splicing of the extracellular domain. This splicing affects the localization and function of the protein in neurons. NL1 includes two sites for alternative splicing that lead to the presence or absence of exons A and B and a total of four isoforms [NL1(-), NL1A, NL1B and NL1AB] whereas NL2 shares only the first splice site to generate [NL2(-) and NL2A]. In transfected neurons NL1(-) and NL1A increased the density of GABAergic and glutamatergic synapses whereas NL1B and NL1AB selectively affected glutamatergic synapses (Song et al., 1999, Chih et al., 2006, Koehnke et al., 2010). The most common alternative splicing of NL1 at the B site leads to the majority of NL1 being restricted to glutamatergic terminals whereas the predominant form of NL2 (NL2A) is found at GABAergic synapses (Graf et al., 2004, Varoqueaux et al., 2004, Chih et al., 2005). Neuroligin 3 and 4 are less specific and have been observed at both excitatory and inhibitory synapses (Budreck and Scheiffele, 2007, Bolliger et al., 2008).

The binding of neuroligins and β-neurexins involves a high affinity Ca$^{2+}$ dependent mechanism in which the ectodomain of neuroligin binds Ca$^{2+}$ directly. This binding is necessary for synaptogenesis (Nguyen and Sudhof, 1997, Dean et al., 2003). Binding of NL1 to α-neurexin has been observed only in the NL1B splice variant whereas NL2 can bind with α-neurexin implicating differential organization of the various splice isoforms (Boucard et al., 2005). Neuroligin 1 is extensively glycosylated and mutagenesis analysis has revealed a glycosylation at asparagine 303 located in splice site B that negatively impacts binding of β-neurexin. This implicates complementary methods for modulating neurexin-neuroligin interactions (Comoletti et al., 2003). Knockout of α-neurexin results in reduced neurotransmitter release from action potentials as a consequence of reduced presynaptic Ca$^{2+}$ channel function. However, it
is not yet known whether this is due to a direct interaction between neurexin and the neurotransmitter release machinery (Missler et al., 2003). Neurexin has several other interaction partners such as dystroglycans, neurexophilins, cerebellin1-GluRδ2, and leucine-rich repeat transmembrane proteins (LRRTMs) indicating diverse differential roles at synapses (Sugita et al., 2001, Linhoff et al., 2009, Siddiqui and Craig, 2011).

### 1.2.4.2 The role of NL1 and NL2 in neurotransmission

Overexpression of neuroligins in cultured neurons results in an isoform specific increase in the number of synapses (i.e. NL1 increases excitatory and NL2 increases inhibitory) indicating that neuroligins have synaptogenic properties (Chih et al., 2004). Conversely, down regulation of neuroligins using RNAi leads to a reduction of the number of synapses supporting the role of neuroligin-neurexin interactions in synaptic stability (Varoqueaux et al., 2004, Chih et al., 2005, Levinson and El-Husseini, 2005). Global triple-NL (1/2/3) KO mice show a functional reduction of both GABAergic and glutamatergic synaptic transmission but does not affect the number of synapses. Based on these results, neuroligins have been proposed to mediate synaptic maturation rather than assembly of synapses (Varoqueaux et al., 2006). This at first seems like a direct conflict between numerous in vitro studies, however Kwon et al. (2012) discovered that absolute synapse number is dependent on trans-cellular interactions of NL1 with neurexins rather than the absolute level of NL1. Deficits in synaptogenesis were observed in NL1 KO neurons under competitive conditions where the neighboring cells express NL1 (Kwon et al., 2012).

The synaptogenic activity of the neuroligin-neurexin complex is dependent on intracellular interactions of neuroligins with scaffolding proteins and receptors. At newly
forming glutamatergic synapses, axodendritic contact preferentially recruits NL1. NL1 and \(N\)-methyl-\(d\)-aspartate (NMDA) receptors form a stable complex with PSD-95 and are trafficked to the synapse (Gerrow et al., 2006, Barrow et al., 2009, Budreck et al., 2013). The subsequent recruitment of \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors requires recruited NMDAR synaptic activity (Nam and Chen, 2005). Similarly, NL2 binds directly to and recruits postsynaptic gephyrin that is critical for the maintenance of GABAergic synapses (Varoqueaux et al., 2006). The gephyrin/NL2 interaction is negatively regulated by proline-directed phosphorylation at S714-P on NL2. Phosphorylation of NL2 recruits peptidyl-prolyl cis-trans isomerase (Pin1) and prevents the NL2/gephyrin interaction thereby negatively regulating GABAergic transmission (Antonelli et al., 2014). NL2 knockout mice exhibit a 50% reduction in unitary IPSC amplitude from fast-spiking interneurons recorded from cortical slices (Gibson et al., 2009). Neurons cultured from NL2 KO mice show reduced postsynaptic gephyrin clustering and an increase in the cytoplasmic aggregates of gephyrin. In hippocampal neurons, these mice have reduced GABAergic transmission at perisomatic synapses (Poulopoulos et al., 2009). This regional specificity likely accounts for the abnormal behavior observed in NL2 KO mice. NL2 KO mice exhibit increased anxiety in the open field and light/dark box test as well as decreased pain sensitivity, suggesting the importance of NL2 in proper synapse function (Gibson et al., 2009).

Neuroligins undergo endogenous oligomerization similar to other AChE domain containing proteins. Oligomerization is necessary for their surface expression because monomers are retained in the early secretory pathway. This oligomerization controls the neuroligin-neurexin binding because mutations in the AChE domain leads to loss of NL1
binding with β-neurexin. Therefore, both oligomerization and association with β-neurexin are necessary for synaptogenesis (Comoletti et al., 2003). Interestingly, coimmunoprecipitation studies indicated that neuroligins can form homodimers and heterodimers with NL1/3 and to a lesser degree NL1/2 in hippocampal neurons. This leads to an interesting question about potential regulation of the excitatory: inhibitory balance in the brain (Poulopoulos et al., 2012).

Synaptic activity can modulate synapse function and organization by cleavage of neuroligins. NMDA receptor activity causes an influx of Ca$^{2+}$ that activates calmodulin-dependent kinase and facilitates the proteolytic ectodomain cleavage of NL1 by matrix metalloprotease 9 (MMP9) (Peixoto et al., 2012). Activity-dependent cleavage of NL1 also occurs via disintegrin and metalloproteinase 10 (ADAM10) extracellularly and γ-secretase intracellularly. ADAM10 is localized at excitatory synapses and does not act upon NL2, however, cleavage of NL2 might occur through other enzymes that have yet to be identified (Suzuki et al., 2012). Muscle arm development defective phenotype 4 (MADD-4) was identified as a novel neuroligin-neurexin interacting partner that promotes clustering of receptors at GABAergic synapses of the C. elegans neuromuscular junction (NMJ) (Maro et al., 2015). MADD-4 is a secreted metalloprotease with a thrombospondin-like repeat, but it lacks proteolytic activity (Pinan-Lucarre et al., 2014). C. elegans only have one neuroligin homolog (NL1) and it binds to neurexin-1 to promote GABA$_{A}$R clustering. MADD-4 binds directly to NL1 and neurexin-1, and its binding of neurexin-1 promotes the association with NL1, indicating they likely form a complex (Maro et al., 2015, Tu et al., 2015). This tripartite model is similar to the complex formed when cerebellin bridges the gap between neurexin and GluRδ but the
exact mechanism of how MADD-4 influences synaptogenesis in the brain is unknown (Zhang and Craig, 2015).

### 1.2.4.3 NL2 interactions with other adhesion molecules

NL2-knockout mice exhibit deficits in inhibitory synapses in a circuit-dependent manner. They also display relatively subtle behavioral deficits indicating there are likely other inhibitory adhesion molecules involved in regulating GABAergic transmission (Gibson et al., 2009, Poulopoulos et al., 2009). Supporting this hypothesis, several recent adhesion molecules have been identified as NL2 interacting partners (Figure 1.6).

Dystrophin is a cytoskeleton protein of the α-actinin/β-spectrin family that provides a transmembrane linkage between the extracellular matrix and the cytoskeleton. The dystrophin glycoprotein complex (DGC) contains dystroglycan (DG) bound to utrophin in the cytosol and agrin, laminin and perclean outside of the cell (Figure 1.6). Both α- and β-dystroglycan together with dystrophin are clustered at a subset of GABAergic synapses that contain α1 or α2 and γ2 subunits (Levi et al., 2002).

β-dystroglycan binds to S-SCAM at inhibitory synapses. S-SCAM contains multiple PDZ binding domains, a guanylate kinase domain and two WW domains (also known as rsp5-domains) (Irie et al., 1997). The WW domains of S-SCAM bind to β-dystroglycan and the PDZ domain is responsible for binding to NL2. S-SCAM is localized at both inhibitory and excitatory synapses. However, SCAM alone is insufficient to recruit NL2 to synapses (Sumita et al., 2007). Neuronal activity up-regulates α-DG glycosylation and localization at inhibitory synapses. Knockdown of DG blocks homeostatic up-scaling of inhibitory synaptic strength that normally occurs under
chronic elevation of neuronal firing rate. The homeostatic increase in GABAergic inhibition requires both protein synthesis and glycosylation of DG. As a result, the response may be in part due to DG binding to agrin which recruits additional GABA\(_A\)Rs to the synapse (Pribiag et al., 2014). This mechanism may explain why mutations in DG result in cognitive deficits and epilepsy (Mehler, 2000, Pane et al., 2013).

![Figure 1.6 Neuroligin 2 interacts with numerous adhesion molecules](image)

**Figure 1.6 Neuroligin 2 interacts with numerous adhesion molecules**

1) NL2 forms trans synaptic partnerships with presynaptic neurexins. 2) NL2 binds to S-SCAM intracellularly and links NL2 to the DGC and facilitates GABA\(_A\)R clustering (Pribiag et al., 2014). 3) IGSF9b is preferentially localized at inhibitory synapses where it binds to NL2 through S-SCAM (Woo et al., 2013). 4) NL2 forms a complex with collybistin and gephyrin to facilitate GABAergic synapse strength (Poulopoulos et al., 2009). 5) MGDA1 interferes with the binding of NL2 and neurexin thereby inhibiting NL2 function (Pettem et al., 2013).
IgSF9b is another adhesion molecule implicated in regulating NL2 function. IgSF9b is an Ig superfamily protein strongly expressed in GABAergic interneurons. Mutations in IgSF9b have been associated with depression through a genome-wide association study (Shyn et al., 2011). Through shRNA studies, IgSF9 was originally believed to be involved in regulating dendrite arborization and excitatory synapse maturation. However, IgSF9 KO mice do not exhibit the same phenotype (Shi et al., 2004a, Shi et al., 2004b, Mishra et al., 2008). IgSF9b is preferentially localized at inhibitory synapses where it binds to NL2 through the synaptic scaffolding molecule S-SCAM (Figure 1.6). NL2 contains a PDZ-binding domain that interacts with S-SCAM to bridge the interaction with IgSF9b (Irie et al., 1997, Sumita et al., 2007). Unlike most adhesion molecules, IgSF9 exhibits distinct homophilic trans-synaptic interactions however it is believed that the postsynaptic side is more important for inhibitory synapse modulation (Woo et al., 2013). NL2 serves as a synaptic organizer binding to gephyrin, S-CAM, IgSF9b, and neurexins to facilitate inhibitory synaptic clustering.

Interestingly, negative regulators of NL2 function have also been identified. MAM-domain-containing GPI anchor proteins (MDGAs) are members of the Ig superfamily of adhesion molecules that mediate heterophilic cell adhesion. Small nucleotide polymorphism sequencing has linked mutations in MDGA1 to patients with schizophrenia. Additionally, protein-truncating variants of MDGA2 were found in patients with autism (Kahler et al., 2008, Bucan et al., 2009, Li et al., 2011). MDGA1 interferes with the binding of the NL2 ectodomain to neurexin (Figure 1.6). Additionally, MDGA1 reduces the cell surface level and total levels of NL2 but does not affect trafficking to the synapse (Pettem et al., 2013). MDGA1 binds with nanomolar affinity (Kd ~150 nm) to the ectodomain of NL2 near the A splice site but does not bind to NL1 or NL3 (Lee et al.,
The discovery that MDGAs interact directly with neuroligins further supports the broad hypothesis of deregulation of the E: I balance in the etiology of many neuropsychiatric disorders.

1.3 Rationale for the study

GODZ KO neurons grown in a competitive environment exhibit a loss of both GABA\(_A\)R \(\gamma_2\) puncta and presynaptic GAD puncta (Murakami, 2008). These observations suggest that GODZ KO neurons show reduced palmitoylation of the \(\gamma_2\) subunit, however, this link has not been established. Transfecting postsynaptic neurons with GODZ shRNA reduces GABAergic innervation by untransfected presynaptic cells indicating that GODZ is required for proper innervation. Loss of innervation might be due to loss of GABA\(_A\)Rs at synapses because GABA\(_A\)R deficient neurons show similar defects in GABAergic innervation (Ren et al., 2015). Alternatively, the phenotype could be due to reduced postsynaptic accumulation of other unknown substrates of GODZ. Of particular interest as a candidate substrate of GODZ is NL2. NL2 is similar to GABA\(_A\)Rs in that it is largely dispensable for synapse formation in KO mice but required for inhibitory synapse formation under competitive conditions involving sparse deletion by shRNA (Scheiffele et al., 2000, Varoqueaux et al., 2004). Thus, the defect in GABAergic innervation seen in mixed cultures of GODZ KO and WT neurons but not in pure KO cultures might be due partially to reduced palmitoylation of NL2. Consistent with this idea, preliminary results obtained in collaboration with M. Fukata (National Institute for Physiological Sciences, Japan) indicate that NL2 but not NL1 can be palmitoylated selectively by GODZ and SERZ-\(\beta\) in transfected HEK 293T cells. GODZ shRNA transfected neurons showed drastically reduced NL2 punctate staining that was larger
than the deficit seen in gephyrin staining suggesting an important role for GODZ in trafficking of NL2 and GABA\(_{\alpha}\)Rs. However, the precise role NL2 palmitoylation plays in contributing to deficits observed upon knockdown of GODZ is not known. We hypothesize that NL2 and other unidentified substrates are palmitoylated by GODZ and that serves to facilitate clustering of GABA\(_{\alpha}\)Rs at the synapse and thereby control GABAergic inhibition.

1.3.1 Aims of the study

**Specific aim 1:**

Preliminary experiments indicate that GODZ is localized in the Golgi network and contributes to palmitoylation of numerous substrates *in vitro*. We hypothesize that GODZ will contribute to the steady-state level of palmitoylation in wide variety of proteins *in vivo*. In this dissertation, we will further analyze the global and cellular phenotype of GODZ KO in brain tissue by using immunohistochemical stainings, palmitoylation detection assays and quantitation of global levels of protein palmitoylation by proteomics. Given our preliminary results, we hypothesize that GODZ KO mice will exhibit significant alterations in palmitoylation levels of other proteins associated with inhibitory synapses. Novel substrates identified in GODZ KO mice will be confirmed in heterologous cell lines.

**Specific aim 2:**

Analysis of neurons from GODZ KO mice indicate selective deficits in GABAergic innervation. We hypothesize that the deficits observed in GODZ KO neurons may be in part due to loss of palmitoylation of NL2. In this dissertation, we further address the
effect of post-translational modifications on NL2 by identifying the enzymes responsible for palmitoylation and the sites of palmitoylation and other post-translational modifications such as nitrosylation in vitro.
Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 HEK 293T cell culture and transfection

Human Embryonic Kidney (HEK) 293T cells were used for all \textit{in vitro} palmitoylation experiments and for localization experiments. Cells were cultured in 10 cm plates with 10 ml High-Glucose Dulbecco’s Modified Eagle Medium (DMEM) (11965, Gibco, Life Technologies Co, Grand Island, NY), 10% fetal bovine serum (FBS) and penicillin/streptomycin (081M0845, Sigma, St. Louis, MO). Upon reaching 80-90% confluency the cells were split 1/20 into new 6 cm plates for palmitoylation assays or onto coverslips for colocalization experiments. Cells were transfected approx. 24-48 hours after plating using lipid transfection in all experiments. First, plasmid DNA (1-4 µg) was thoroughly mixed in Opti-MEM (31985062, LifeTechnologies, Carlsbad, CA) (200µl/6-cm plate). Secondly, polyethyleneimine (PEI) (408727, Sigma-Aldrich) was added to the mix at a ratio of four times the amount of DNA. The solution was thoroughly mixed, left alone for five min, and subsequently distributed evenly drop-wise across the plate. The medium was changed between four and five hours after transfection and the cells were harvested at different time points depending on the experiment.
2.1.2 Neuron culture

Coverslips were coated with 0.5 mg/ml Poly-L-Lysine (P1524, Sigma, Saint Louis, MO) in borate buffer (150 mM boric acid, adjusted pH to 8.4) overnight at room temperature (RT) or three h at 37°C. Cortical neuron cultures were prepared from E14 mouse embryos as previously described (Alldred et al., 2005). Embryos were removed from the mother, tails were removed for genotyping and the mice were beheaded and the heads kept in Hibernate E media (BrainBits™, Springfield, IL) supplemented with 2% B27 (17504, Life Technologies, Grand Island, NY) at 4 °C while the genotyping was performed. The next day or when genotyping was complete, the cortices of selected genotypes were removed using a dissecting microscope and placed in cold PBS with 10 mM glucose. Tissue was digested in a papain solution (PBS containing 10 mM glucose, 0.1% BSA, 0.5 mg/ml papain (P4762, Sigma-Aldrich, MO), and 10 µg/ml DNase I) for 15 min at room temperature and dissociated by trituration with a fire-polished Pasteur pipette. The papain reaction was stopped by adding one volume of DMEM with 2% FBS/10 mM HEPES (15630, Invitrogen) and the cell suspension was spun down at 160 x g for five min at RT. For immunocytochemistry, the neurons were seeded Minimal Essential Medium (MEM) (51200, Life Technologies) supplemented with 2 mM Glutamax™ I (35050, Life Technologies), 2% fetal bovine serum (FBS) (100, Gemini BioProducts, West Sacramento, CA), 1 mM pyruvic acid, 5.2 mg/ml glucose and 100 u/ml penicillin/streptomycin. For immunocytochemistry staining, neurons were seeded onto poly-L-lysine-coated coverslips at 3.6 x 10^4 cells/cm^2 supplemented with paraffin wax feet. The next day, neurons were washed with PBS three times and then changed into Neurobasal-A (NBA) medium (10888, Life Technologies) containing 2% B27, 2 mM Glutamax® I, 100 u/ml penicillin/streptomycin at 37 °C and 5% CO₂/95% air. Neurons on coverslips were flipped over glial feeder cells and left undisturbed until analysis. For
immunohistochemistry, neurons were kept inverted on glia feeder until 21 days in vitro (DIV). For palmitoylation assays, neurons were seeded on 6cm dishes and harvested at DIV 14.

2.1.3 Preparation of Glia cells

Glial feeder cells were prepared from newborn day old mice as described (Banker, 1998). Newborn pups were decapitated on postnatal day 1-2 (P1-2), their cortices were dissected into cold Hanks’ Buffered Salt Solution [1 x HBSS (14025076, Invitrogen), 1 mM HEPES, 100 u/ml penicillin/streptomycin]. Diced cortices tissues were digested at 37 °C with 2.5% trypsin and 1% DNase in 1 x HBSS for 15 min at room temperature, and then triturated with a fire polished Pasteur pipette and filtered through a 72 µm nylon filter to remove undigested tissue. Cells were centrifuged, re-suspended in glial media (MEM, 0.6% glucose, 100 u/ml penicillin/streptomycin, 10% FBS, 2 mM Glutamax I) and seeded on a T-25 flask and maintained at 37 °C in a 5% CO₂/95% air incubator. Once they reached confluency, the cells were passaged at a 1:3 ratios and kept in culture until confluent. The glial cells were then seeded onto 24-well plates for immunohistochemistry. 24 h before use, the cell division was stopped with 1:100 volume of poly-uridine (U+FdU) [3.5 mg/ml uridine (U6381, Sigma) and 1.5 mg/ml fluoro-deoxyuridine (F0503, Sigma) in water].

2.2 Plasmid preparation and use

2.2.1 Plasmid transformation

Plasmids used in experiments were transformed into electrocompetent E. coli XL-1 Blue cells that were made in house. 1 µg of DNA was used per 50 µl of bacteria
and electroporated in an ice cold cuvette using a BioRad Pulser (0.1 µM cuvette, 200 Ohms, 25 µF, 1.8 kV). After electroporation, 950 µl of SOC medium was immediately added (15544-034, ThermoFisher) and incubated at 37 °C for 1 h at gentle rocking speed (225 rpm). Cells were plated on agar plates with appropriate antibiotic selection and grown overnight at 37 °C.

2.2.3 Plasmid generation

Transformed *E. coli* were grown in 200 ml Lysogeny broth (LB) (L3022, Sigma) for 12 h at 37 °C. The following day plasmids were isolated using QIAGEN Plasmid Maxi Kit (12165, QIAGEN) and plasmid concentrations and purity were determined using a Nanodrop 2000 (Thermo-scientific).

2.2.4 Plasmid constructs

All 23 DHHC PATs were obtained from the Fukata lab (Myodaiji, Okazaki, Japan). Each contains the cDNA for the corresponding PAT in a pEF-BOS plasmid backbone (Fukata et al., 2004). pNICE-HA tagged NL2 construct containing mouse NL2 and a CMV promoter was obtained from Dr. Scheiffele’s lab (University of Basel, Basel). Point mutant versions of NL2, C710A and C766A were generated in our lab by site directed mutatgenesis. Palmitoylation deficient mutant Flag-GODZ<sup>C157S</sup> was generated by Cheng Fang (Fang, 2007) in a pEGFP-C2 backbone. For GODZ localization, pGPP130-GFP and pGalT-YFP were described in (Mukhopadhyay et al., 2010, Jarvela and Linstedt, 2012) and obtained from A.D. Linstedt (Carnegie Mellon, Pittsburgh, PA). pEGFP-GAP-43 wild type and palmitoylation deficient mutants were described in (Trenchi et al., 2009) and were a gift from J.L. Daniotti (Universidad Nacional de Cordoba, Argentina).
2.3 Immunohistochemistry

2.3.1 Transcardial perfusion

Mice were anesthetized with a lethal dose of Avertin (30 μl/g), 2, 2, 2-tribromoethanol (T48402, Sigma Aldrich) in 2-methyl-2-butanol (152463, Sigma Aldrich). After confirmation of loss of consciousness by toe pinch, mice were pinned to a dissection board and sprayed with 70% ethanol. A mini-pump with variable flow rates (VWR, Randor, PA) was used at a rate of 10 ml/min with a two-way valve that allows for switching between PBS and PFA. The chest wall was opened using sharp scissors and the ribs and diaphragm that cover the heart were carefully removed as to not puncture the lungs or the heart. A cannula pre calibrated with ice cold PBS and at the lowest flow rate was inserted into the aorta through the left ventricle with the heart held in place with forceps. After the heart began to swell the right ventricle was severed and the PBS flow rate adjusted to 10 ml/min for two min and 25 s followed by a five min flow of cold 4% paraformaldehyde. After perfusion the brain was removed and placed into 5 ml of 4% PFA for post fixation for 12 h at 4ºC. Brains were washed three times and stored in PBS until slicing.

2.3.1.1 Vibratome preparation of brain slices

The cerebellum was removed from the fully fixed brain and superglued onto the vibratome stage with the anterior portion facing up. The brain was stabilized using 4% UltraPure low melting agarose (16520, Invitrogen) and sliced into 50 μM coronal sections for staining.
2.3.1.2 Immunofluorescent staining of brain slices

Sections were permeabilized with 1% Triton-X100 for one hour followed by one hour of blocking in 5% normal goat serum or donkey serum and three PBS washes. Primary antibodies were incubated overnight at 4 °C. Sections were washed three times for five min in PBS and incubated with secondary antibody for two hours at room temperature. Sections were washed three times for 15 min and mounted using ThermoScientific Immu-Mount (9990402, Thermo) and sealed with nail polish.

2.3.2 Immunofluorescent staining of neurons and HEK 293T cells

Neurons at DIV 21 or HEK 293T cells were washed three times with PBS (136 mM NaCl, 1.75 mM NaH₂PO₄, 8.25 mM Na₂HPO₄), fixed with 4% (w/v) paraformaldehyde in 0.15 M phosphate buffer (pH 7.4) at room temperature for 12 minutes and washed again three times with PBS. Neurons were permeabilized for five min at room temperature with PBS containing 0.2% Triton X-100 and 10% normal goat serum (Jackson ImmunoResearch). The neurons were washed three times with PBS and incubated with either mouse anti Map2 (1:1000, Ab11267, Abcam, MA), rabbit anti GODZ (1:500, Ab31837, Abcam) mouse anti gephyrin mAb7a (1:2000, gift of H. Betz, Max Plank Institute, Frankfurt, Germany), mouse anti HA (2367, Cell Signalling), mouse anti GFP (A11120, Molecular Probes), mouse anti FLAG (F1804, Sigma) and washed and incubated with secondary AlexaFluor 488-conjugated donkey anti-mouse, AlexaFluor 647-conjugated goat anti-rabbit (1:500, Molecular Probes, OR) and Cy3 donkey anti-guinea pig (1:500, Jackson ImmunoResearch).

2.3.3 Fluorescence microscopy
Fluorescent images were captured using a Zeiss Axiophot2 microscope equipped with a 40 x 1.3 NA objective and an ORCA-100 video camera linked to an OpenLab imaging system (PerkinElmer, Waltham, MA). Digital gray scale images were pseudo-colored using green, red and blue for the sequentially recorded fluorescences. Images were adjusted for contrast using OpenLab and assembled into figure palettes using ImageJ.

2.3.2 Structured Illumination Microscopy

All images were obtained using an AAA-Nikon N-SIM microscope with a CFI Apochromat TIRF 100×oil objective (NA1.49) with Nikon Elements Software utilizing the Center for Biologic Imagining (Pittsburgh, PA) under the grant number 1S10OD10624-01A1.

2.3.2.1 Intensity correlation analysis

Images that were free of image saturation were used for analysis (saturation was measured before taking images). Comparisons were made from single image planes taken from an N-SIM microscope. Images were converted into 8-bit format and mean background intensity was subtracted from each file using the BG-subtraction ImageJ macro. Fluorescence intensity was quantified in the Golgi region for each channel. ICQ analysis was performed using the ICQ analysis ImageJ macro described in (Li et al., 2004). Briefly, this macro assumes that for any set of values the sum of the differences from the mean are equal to zero $\sum_N (A_i - a) = 0$ where a is the mean distribution with N values of A. N is the number of pixels and A is the pixel intensity. When comparing two sets of random staining (A and B) the sum of the product of their differences tends toward zero thus, $\sum_N (A_i - a)(B_i - b) = 0$ unless the two sets of staining are dependent or
anti-correlated. This macro generates paired pixel staining intensities and normalize them to 0-1.0 range, generates an arithmetic mean and generates a scatterplot of the normalized A vs. B staining intensities and calculates the \((A_i - a) (B_i - b)\) relationship to generate the ICQ value.

2.4 Palmitoylation assays

2.4.1 ABE assay

Proteins extracted from 4-month-old female mouse brains were analyzed by the acyl-biotin exchange assay with minor modifications (Wan et al., 2007). Briefly, the whole brain was lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 4% SDS, 10 mM NEM, supplemented with protease inhibitors Complete Protease Inhibitor cocktail (11697498001, Roche, Switzerland). 1.7% Triton x-100 was added and the tissue was homogenized using a tip sonicator (three 10-s pulses) followed by incubation on ice for one min. Proteins were diluted in LB buffer (0.15 M NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 50 mM NEM, protein inhibitors and incubated at 4 °C overnight with gentle rocking. Proteins were precipitated with chloroform/methanol, resolubilized with 4SB buffer [50 mM Tris (pH 7.4), 5 mM EDTA, and 4% SDS] and precipitated and solubilized repeatedly again. Total protein was quantified using a Bradford (B6916, Sigma-Aldrich) assay using BSA as the standard. Equal amounts of protein (0.5–2.0 mg for immunoblot experiments and 10–20 mg for mass spectrometry experiments). After the third precipitation the proteins were labeled with biotin-HPDP 0.8 mM (21341, ThermoFisher) in buffer containing either 0.56 M hydroxylamine, pH 7.4, or 0.56 M Tris, pH 7.4, for 1 h at room temperature. Proteins were precipitated with acetone (4 volumes -20°C 2 h). Biotinylated proteins were purified with high capacity-streptavidin
agarose (20359, ThermoFisher), eluted off the beads using 200mM DTT (D9779, Sigma) and separated by SDS-PAGE.

2.4.2 Acyl-Resin Assisted Capture (Acyl-RAC)

Proteins from cultured cells and mouse brains were collected and washed in cold PBS. Cells were lysed in lysis buffer (25 mM HEPES, 25 mM NaCl, 1 mM EDTA, pH 7.5) containing protease inhibitor cocktail (Roche). Samples were homogenized using a tip sonciator (three 10-s pulses) followed by incubation on ice for one min. For enrichment of membranes, nuclei were removed via centrifugation at 800 g for five min. The supernatant was centrifuged at 20,000 g for 30 min to enrich for membrane components, and the pellet was resuspended in lysis buffer containing 0.5% Triton X-100. Total protein was quantified using a Bradford assay using BSA as the standard. Equal amounts of protein (2.0 mg for immunoblot experiments and 20 mg for mass spectrometry) were diluted to a concentration of 2 mg/ml in blocking buffer (100 mM HEPES, 1.0 mM EDTA, 2.5% SDS, 0.1% MMTS, pH 7.5) and incubated at 40°C for 10 min with frequent vortexing. Four volumes of cold acetone were added, and proteins were allowed to precipitate at −20°C for 2 h or overnight until precipitates were visible. Following centrifugation of the solution at 23,000 rpm for 20 min, the pellet was extensively washed with 70% acetone, resuspended in 300 μl of binding buffer (100 mM HEPES, 1.0 mM EDTA, 1% SDS, pH 7.5) and added to 50 μl of prewashed thiopropyl Sepharose (17-0420-01, GE-Amersham). To this mixture was added 40 μl of either 2 M NH₂OH (freshly prepared in H₂O from HCl salt and brought to pH 7.5 with concentrated NaOH) or 2 M NaCl. Binding reactions were incubated on a rotator at room temperature for 2–4 h. Approximately 20 μl of each supernatant was saved as the total input for comparison by western blot. Resins were washed at least four times with binding buffer.
For immunoblot analysis, elution was performed using 60 μl of binding buffer containing 50 mM DTT at 37°C for 20 min.

### 2.4.2 S-nitrosoylated-Resin Assisted Capture (SNO-RAC)

The protocol for SNO-RAC involved the same protocol as Acyl-RAC except the selective cleaving reagent is ascorbate instead of hydroxylamine. In cultured cells, a nitric oxide donor CysNO was added to living cells 5 min before harvest. CysNO was prepared by mixing ice-cold solution 1 (100 mM NaNO₂, 1 mM EDTA) and solution 2 (100 mM cysteine, 0.5 M HCl) at a 1:1 ratio then diluted with HEN buffer at a 1:5 ratio and protected from light. Cells were harvested in ice-cold 400 μl lysis buffer (50 mM NaCl, 250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA, 1% (v/v) NP-40, 0.1 mM Neocuproine, and freshly added protease inhibitors). Lysates were incubated on ice for 10 min followed by centrifugation at 16,000 g for 10 min. Protein concentration was analyzed by Bradford and diluted to a final concentration of 0.2-0.8 mg/ml in HEN buffer (250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA, 0.1 mM Neocuproine). Methyl methanethiosulfonate (MMTS) was dissolved in dimethylformamide to a final concentration of 2 M in 1ml cell lysate. To the pellet, 100 μl of 25% (v/v) SDS and 10 μl of 2M MMTS was added to the 1 ml cell lysate and incubated at 50 ºC for 20 min and vortexed every four minutes. Samples were then resuspended in HEN with 1% SDS. Thiopropyl sepharose (6B100, Sigma-Aldrich) was rehydrated for 25 min in HEN. Following rehydration, 40 μL of the resin slurry was added to blocked samples and rotated for four hours in the dark at room temperature. Resin-bound proteins were then washed with 5 × 0.5 mL HENS buffer. Elution was performed using 60 μl of binding buffer containing 50 mM DTT at 37°C for 20 min.
2.4.3 Metabolic click chemistry assay

One hour prior to labeling deprive cells of fatty acids by incubation with 1% fatty acid free BSA in DMEM. 15-HYDA or 17-OYDA (15968, 90270, Cayman Chemical) was dissolved in DMSO to make 25 mM (1000X) stock. The probe was diluted to 100 μM in fatty-acid free Opti-MEM labeling media and bath sonicated at room temperature for 15 min. Cells were labeled for desired time period (optimum 6-8 h). Cells were harvested in ice cold PBS in lysis buffer with from ABE-assay with protease inhibitors and 1% Triton X-100. Lysates were incubated at 4 °C 1 h with end over end rotation. Excess probe was removed by acetone precipitation. Protein concentration was measured by Bradford assay, 1.5 mg of the lysate was reacted with biotin-azide (B10184, ThermoFisher) in 1 ml of PBS for 1 h with 100 μM biotin azide dissolved in DMSO, 1 mM Tris (2-carboxyethyl)phosphine hydrochloride TCEP (PG82080, ThermoFisher) in water, 100 μM Tris (1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) (678937, Sigma) TBTA in DMSO/t-butanol (20%/80%) and 1 mM CuSO₄ in PBS. The click chemistry reaction was carried out in the dark at 37 °C for 1 h. The reaction was quenched by adding 10 volumes of ice cold acetone and proteins were precipitated at -20 °C for 2 h or overnight. Proteins were resuspended in PBS with 1.2% SDS. Samples were heated at 95 °C for five min and diluted six fold with PBS and frozen at -80 °C overnight. Pre-equilibrated streptavidin-agarose beads were added to the lysates and rotated 90 min at room temperature. Beads were washed once with 0.2% SDS in PBS and three times with PBS and three times with Tris-Cl pH 6.8. Proteins were eluded as described above.

2.4.4 Metabolic radiolabeling with ³H-palmitate
All pipette tips, centrifuge tubes and conical tubes were coated with SIGMACOAT® (Sigma-Aldrich), dried and rinsed with ddH$_2$O before use. Palmitic acid, [9,10-³H(N)] (NEN-Perkin-Elmer, Wellesley, MA) was concentrated in a Speed Vac vacuum concentrator at low heat to approximately 300 mCi/ml. HEK 293T cells were transfected the following day switched to low serum media. The cells were incubated for 30 min with 4.5 ml DMEM with 2% dialyzed, heat inactivated FCS (10082139, ThermoFisher). Concentrated [³H]-palmitic acid was diluted into 500 µl in media and mixed into the plates, incubated and followed by cell extraction as desired.

2.5 Quantitative proteomics

Lysates for proteomics were processed as listed above with the ABE-assay, after elution the samples were completely dried using a vacuum concentrator. The samples were resuspended in 20 µl dissolution buffer (0.5 M triethylammoniumbicarbonate (TAEB) at pH 8.5) (17902, Sigma) diluted in water. 1 µl of 2% SDS and 1 µl of 100 mM tris-(2-carboxylethyl) phosphine (TCEP) was added to each sample to make a 5 mM solution. Samples were vortexed and spun down and the samples were incubated for 60 ºC for 1 h. Samples were spun down and 1µl of iodoacetamide was added and incubated for 30 min in the dark at room temperature. Promega sequencing grade trypsin was resuspended in 21 µl of the included resuspension buffer and diluted to 1 mg/ml and 10 µl was added to each sample. Trypsin incubation was carried out over night at 48 ºC. iTRAQ reagents were brought to room temperature, iTRAQ Multiplex (8-plex) kit was used (4390881, Applied Biosystems). Each iTRAQ sample was added to a tube and incubated at room temperature for 2 h. 100 µl of water was added to quench the reaction and incubated for 30 min, after 30 min the samples were combined and dried using a vacuum concentrator.
2.5.1 Mass spectroscopy

All mass spectroscopy was performed by the Mass Spectroscopy Core at Penn State College of Medicine, Hershey, PA. The combined eight iTRAQ-labeled samples were separated offline into 15 strong cation exchange fractions, using a 4.6 X 250 mm PolySULFOETHYL Aspartamide Strong Cation exchange column (PolyLC, Columbia, MD) with an ammonium formate gradient in 20% acetonitrile. Samples were dried by SpeedVac, resuspended in H$_2$O and repeated for all fractions (3X) to remove all acetonitrile and ammonium formate. Each of eight SCX fractions were then isolated, using a 120-min gradient from an Eksigent NanoLC-Ultra-2D Plus and Eksigent cHiPLC Nanoflex through a 200µm x 0.5 mm Chrom XP C18-CL 3 µm 120 Å Trap Column and elution through a 75 µm x 15 cm Chrom XP C18-CL 3 µm 120 Å Nano cHIPLC Column and direct injection into an ABSciex 5600 TripleTOF mass spectrometer. The instrument was calibrated with an injection of beta-gal digest standard before each sample injection.

A 250 ms parent scan was acquired, then 50 MS/MS spectra over 2.5 s, then repeated with the next LC eluent fraction. Combination of the MS/MS data from all eight SCX fractions were used for a Paragon algorithm search (ProteinPilot 4.5 Software) and against a concatenated normal and reversed database (NCBI). The false discovery rate was determined by comparing the data to a concatenated normal and reversed database (NCBI) and identifications were only considered valid if they were less than 5% similar to what was expected using a p < 0.05, 95% cutoff threshold. The total protein score is a measurement of all the peptide evidence for identification for a protein and the unused score is a measurement of all the evidence for a protein that is not better explained by a higher ranking protein. The unused protein score is based on the number of unique peptides attributed to the protein. The cutoff used for this study was at
least two peptides with a 95% confidence. Protein abundance was determined using ProteinPilot software and statistical significance was determined using non-parametric permutation analysis and R-Studio.

2.6 Western blotting

Protein samples were heated at 95 °C for 10 mins and separated by 4-12% NuPAGE® Bis-Tris (NW04125BOX, ThermoFisher) precast polyacrylamide gels for 45 min at 165 mV. Proteins were transferred onto 0.2 µm Westran S Polyvinylidine Fluoride (PVDF) membrane (GE Healthcare) (activated by immersion in 100% methanol for 20 s and equilibrated with transfer buffer for 5 min) using a semidry blotter at 15 volts OWL Hep-1 (Thermo Scientific). The membrane was then blocked with 5% nonfat dry milk (Carnation) in Tris-buffered saline with 1% tween-20 (TBST) buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Tween-20) for 2 h at room temperature and incubated with primary antibody in 5% nonfat dry milk in TBST buffer overnight at 4 °C. Primary antibodies used were, mouse anti PSD-95 (1:1000, MABN68, Millipore, MA), rabbit anti γ2 subunit (1:1000, 224003, Synaptic Systems, Germany), and rabbit anti GAP-43 (1:1000, AB5220, Millipore, MA), rabbit anti HA (H6908, Sigma). The membrane was washed with TBST buffer for 15 min and repeated twice. The membrane was incubated with appropriate secondary antibody conjugated to the appropriate LiCor IRDye at a 1:15,000 dilution for two h at room temperature. The membrane was washed with TBST buffer for 3 x 15 min and one final wash with TBS without tween. Membranes were developed using Odyssey® Clx Imaging system (LI-COR Biosciences, Lincoln, NE). For chemiluminescence detection, HRP conjugated secondary antibodies were used and protein signals were detected using ECL Plus Kit (GE Healthcare, Pittsburgh, PA).
2.6.1 Quantification of Western blots

The intensity of the protein bands in immunoblotting were scanned by Odyssey® Clx (LI-COR Biosciences, Lincoln, NE). The value of each palmitoylated protein band was first normalized to the value of its individual input control lane. The palmitoylated protein levels were subtracted from the negative control (HA-) lane of experiment group was then normalized to the input lane. Statistical comparisons were performed using two-tailed student’s \( t \)-test or one-way ANOVA with Tukey’s post-hoc tests.

2.7 Animal Husbandry

2.7.1 Animal housing

A standard 12 h light-dark cycle was used for all mouse lines with food and water supplied \textit{ad libitum}. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University, and strictly adhered to guidelines and regulations of the National Institutes of Health (NIH).

2.7.2 PCR and genotyping

GODZ KO mice were described previously (Murakami, 2008). The PCR reaction consists of two separate reactions to identify the WT and KO locus. The first containing the primers for exon 3: GZ1 primer 5’-GAGGC TTCAG AATAG TCTCT TAC-3’ and GZ4 primer 5’-GCTCCC CAACTC TTACTT GAATG-3’ results in a 2.4 kb band for the WT locus and a 0.5kb band for GODZ KO. The second primer set is for the downstream region contains GZ3 primer 5’-TGCCA GCCCA GCTCT TTTTT ATT-3’ combined with GZ1 results in a 1.3kb band for WT mice and no band for KO mice. For depiction of the deleted locus and primer locations see Figure 3.1.
SERZ-β similarly requires two separate PCR reactions using the following primer sets: SZ1 primer 5’-TGAGC CAGGA TGGATT TCAGA CA-3’ and SZ6 primer 5’-TGCCC TCGGA CGCAG GAGAT GAA-3’ results in a 1.49 kb band for WT and no band for KO. The second primer set is for exon 3: SZ4 primer 5’-TCCCC TGATG TATGC GAATG TCC-3’ SZ5 primer 5’-AACA GGTGC CTTTT GAATG TCAG-3’ results in a 2.6 kb band for WT and a 0.45 kb band for KO.

NL2 KO mice were generated by Thomas Sudhof and obtained from Jackson laboratory by our collaborator at Penn State University, Dr. Gong Chen’s lab (008139, The Jackson Laboratory)(Varoqueaux et al., 2006). A targeting vector containing a neomycin resistance cassette was used to disrupt the first coding exon. The reaction consists of two separate PCR reactions with the following primers: NL2 KO: 5’ CGCCT AGACT ACCCT CCCCT CATA 3’ NL2 WT locus: 5’ CCCAT CAGTG TACCA TTCCC TAAA 3’ NL2 Neo 5’ GAGCG CGCGC GGCGG AGTTG TTGA C 3’. WT PCR reactions result in a 1.3 kb band and no band in the KO. KO PCR reactions result in a 2 kb WT band and a 1.5 kb KO band.

Histidine-Flag-YFP-NL2 (HFY-NL2) transgenic mice were obtained from A.M. Craig (University of British Columbia) [described in (Kang et al., 2014)] and genotyped using XFP primers 3’ primer 5’-GCAAG CTGAC CCTGA AGTTC ATCTG CACCA 3’ and 5’ primer 5’-AAGTCG ATGCC TTCAG CTCGA TGCGG TTC 3’. Producing a PCR product of 700-bp selectively in Histidine-Flag-YFP-NL2 transgenic mice.

Genomic DNA was isolated from tail biopsies taken from juvenile mice (P21-28) at the time of weaning. Tails were digested and PCR reaction mixes were generated as per manufacturer’s instructions using AccuStart II GelTrack PCR SuperMix kit (95135, Quanta BioSciences).
Chapter 3

Results

Part I: Analysis of the functional deficits in GODZ KO mice

3.1.1 Introduction to the study

Knockdown of GODZ by transfection of GODZ shRNA or dominant negative GODZ\textsuperscript{C157S} leads to a loss of both GABA\textsubscript{A}R \( \gamma \)2 puncta and presynaptic GAD puncta, indicative of a deficit in GABAergic synapse formation (Fang et al., 2006). Similar deficits in inhibitory synapse formation were observed in GODZ KO neurons grown in co-culture with WT neurons (Murakami, 2008). The loss of GABAergic innervation in all these experiments is consistent with similar loss of innervation seen when GABA\textsubscript{A}R \( \gamma \)2 KO neurons are grown in competition with WT neurons (Ren et al., 2015). By contrast, pure cultures prepared from \( \gamma \)2 KO embryos or GODZ KO embryos are normally innervated (Essrich et al., 1998, Murakami, 2008). However, despite these similarities between the synaptic phenotypes of GODZ- and \( \gamma \)2-deficient neurons, it cannot be excluded that other, so far unknown substrates of GODZ also contribute to defects of inhibitory synapses in GODZ-KO neurons. Consistent with the latter idea, studies involving overexpression of GODZ in heterologous cells suggest that GODZ exhibits broad substrate specificity (Fukata et al., 2004, Fang et al., 2006, Fernandez-Hernando et al., 2006, Greaves et al., 2008). However, whether GODZ has a broad substrate specificity \textit{in vivo} or if other proteins contribute to the deficit seen upon knock down of GODZ has not been established and is one of the primary goals of this dissertation. We hypothesize that GODZ mediated palmitoylation occurs on other proteins involved in
GABAergic synapse formation, maturation or stability. Additionally, we hypothesize that NL2 palmitoylation contributes to the clustering of NL2 and GABA<sub>R</sub>Rs at the synapse.

### 3.1.2 Confirmation of knockdown of GODZ in vivo

Deletion of protein coding exons three to four of the GODZ gene is predicted to result in a shift in the reading frame and introduce a stop codon after the second exon that prevents the expression of exons five and six downstream of the deletion. A representation of the targeted genomic locus, targeting vector and mutated allele are shown in Figure 3.1a. Preliminary characterization of GODZ KO mice by (Murakami, 2008) using RT-PCR of RNA derived from brain extracts confirmed that exons three and four had been successfully deleted (Figure 3.1 b). The frame shift mutation is predicted to create a truncated protein lacks the DHHC-CRD required for enzymatic activity and is likely degraded, however, this had not been previously demonstrated in the KO mice.

![Figure 3.1 Strategy of targeted inactivation of the GODZ locus (by Shoko Murakami).](image)

(a) Representation of the targeted genomic region of GODZ showing from top to bottom the (i) genomic locus including the protein coding exons of GODZ (exons are shown as black boxes, the DHHC domain containing exon is shown in red), (ii) targeting vector, (iii) mutated allele resulting from homologous recombination, (iv) the floxed allele after Cre-mediated deletion of the PGK-neo cassette (grey shaded box), and the mutated allele lacking the fourth and fifth protein coding exon. The yellow triangles indicate the position of the l<i>oxP</i> sites. The locations of the primers used for RT-PCR (GP1-3, 5’-3’ direction) are shown as black arrowheads. Restriction sites are indicated as follows: B, Bam HI, Bg, Bgl II; ERI, EcoRI; N, Nco I. (b) RT-PCR analyses of brain RNA from GODZ and WT, heterozygous and homozygous KO mice with the size of amplified cDNA fragments representing WT and deleted alleles indicated on the right (Murakami, 2008).
Figure 3.2 Verification of targeted inactivation of GODZ in vivo

(a) Immunofluorescent staining of DIV 14 cultured cortical neurons derived from WT (top row) and GODZ KO (bottom row) embryos immunostained for GODZ (green, left column) and MAP2 (red, middle column), with merged images shown in the right column. (b) Confocal immunostaining of a brain section of a WT (top row) and a GODZ KO mouse brain (bottom row) for GODZ (green, right) and gephyrin (red, middle) in the cerebral cortex. Note the complete absence of GODZ signal in both GODZ KO cultured neurons and brain sections (bottom rows of part (a) and (b)). Scale bars, 10 μm.

The loss of GODZ at the protein level was confirmed using immunofluorescent staining of WT and GODZ KO neuron cultures. In WT neurons, GODZ was found
localized throughout the soma, concentrated near the Golgi and was absent in KO neurons (Figure 3.2 a). The loss of GODZ was also observed in 50 µM coronal brain sections from adult GODZ KO mice, confirming the loss of GODZ in vivo (Figure 3.2 b).

Golgi outposts in primary dendrites are important for dendrite morphology and arborization (Ori-McKenney et al., 2012). To determine if GODZ-mediated palmitoylation occurs in Golgi outposts in primary dendrites, we imaged cultured neurons with a dendrite marker and looked at localization of endogenous GODZ. Interestingly, GODZ was observed in dendrite Golgi outposts (Figure 3.3a). To confirm that this represents GODZ localized in the Golgi we utilized a cis-Golgi marker and stained for endogenous GODZ (Figure 3.3b). Golgi outposts in the dendrites appear during neuron development and are involved in the trafficking of integral membrane and secreted proteins to further neuron growth (Horton and Ehlers, 2003). These results implicate GODZ-mediated palmitoylation in contributing to protein trafficking to distal dendrites.

Figure 3.3 GODZ is localized in primary dendrites
(a) Immunofluorescent staining of representative WT cultured cortical neurons (DIV 14) with GODZ (green, left panels) and dendrite marker MAP2 (red, middle) demonstrating GODZ localized in the Golgi region and extending into dendrites. Arrows show localization in dendrites near the soma. (b) SIM imaging of GODZ and GFP-N3, Scale bar, 10 µm.
3.1.3 Determining the precise localization of GODZ in the Golgi

Previous Immuno-electron microscopy (EM) experiments had indicated that GODZ was distributed asymmetrically to one side of the Golgi network (Keller et al., 2004). However, Immuno-EM cannot distinguish the cis from the trans face of the Golgi. Similarly, the limited resolution of confocal microscopy (200-250 nm) is insufficient to conclusively resolve cis vs trans-Golgi structures. By contrast, super resolution structure illuminated microscopy (SIM) allows for a two-fold enhanced resolution of 100-125 nm in the horizontal plane. Therefore, SIM was used to garner a better understanding of where GODZ was localized in the Golgi to give insight to its function in the cell.

The localization of GODZ in the Golgi was determined by comparing to known cis and trans- Golgi markers. Golgi phosphoprotein of 130 kDa (GPP-130 also known as N3) is a 130-kDa glycosylated phosphoprotein that is localized to the cis/medial Golgi complex (Linstedt et al., 1997). Galactosyltransferase (GalT) is a glycosyltransferase that localizes to the farthest two or three trans cisternae of the Golgi (Roth and Berger, 1982). Together, these two proteins were used to determine the localization of novel proteins within the Golgi complex (Strous, 1986, Linstedt et al., 1997).

Optical sections of the Golgi complex were analyzed by intensity correlation analysis (ICA) (Figure 3.4). ICA makes the assumption that if two proteins are colocalized in a complex, then the pixel intensity of both channels should vary in synchrony over a given distance or area and this synchrony is considered dependent or colocalized staining. Random staining would result in an Intensity Correlation Quotient
Figure 3.4 GODZ localization in the Golgi complex is more correlated with the cis Golgi network marker.

(a) HEK 293T cells were transfected with GFP-GPP130 (N3) and immuno-stained for endogenous GODZ (a1) and GFP (a2). Merged images are shown in (a3) and an enlargement of the Golgi complex (marked area in a3) is shown in (a4). (b) HEK 293T cells were transfected with GalT-YFP and immunostained for endogenous GODZ (b1) and GFP (b2). Merged images are shown in (b3) and exhibit minimal colocalization. Enlargement of the Golgi complex is shown in (b4). Scale bars, 2.5 μM. (c) Unbiased ICQ analysis confirmed that GODZ staining is more positively correlated with staining for GFP-N3 (ICQ value: 0.218 ± 0.057) than with staining for the trans Golgi marker YFP-GalT (ICQ value: 0.078 ± 0.049, \( p < 0.001, n = 7, t\)-test).

HEK 293T cells were transfected with either the cis- or trans- Golgi markers and immunostained for endogenous GODZ and the Golgi markers to determine co-localization (Figure 3.4 a, b). ICA analysis revealed that endogenous GODZ was more positively correlated with the cis-Golgi marker compared to the trans-Golgi marker (Figure 3.4 c). Similar results were observed using confocal microscopy (data not shown). However, the average ICQ values were higher for confocal than for SIM imaging, consistent with the increased resolving power seen in SIM imaging (N3: 0.39 ± 0.04, GalT: 0.22 ± 0.12, \( p < 0.001, n = 10 \)). GODZ localization in the cis-Golgi network
suggests that the primary function of GODZ is to palmitoylate proteins at the Golgi-ER interface and facilitate the exit of proteins from the ER. Alternatively, palmitoylation could influence retrograde transport of proteins back to the ER. Supporting this hypothesis, the overexpression of GODZ and NL2 alters the ratio of mature NL2 to the immature version that has not been fully glycosylated (data not shown). Neurexin 2 resolves as two distinct bands in western blots of transfected cells with the lower immature band representing an N-linked glycosylated ER localized protein (Poulopoulos et al., 2012). Both bands are palmitoylated indicating that some of the protein may be sequestered or transported back to the ER by GODZ.

3.1.4 Analysis of global palmitoylation in GODZ KO mice

GODZ contains a Post Synaptic Density 95, Drosophila disc large tumor suppressor, and Zonula occludens-1 (PDZ) domain binding motif that facilitates interaction with other PDZ domain-containing proteins (Tao and Johns, 2004). The PDZ domain is common in numerous neuronal proteins, such as glutamate receptor interacting protein (GRIP), S-SCAM, nNOS, β-catenin and many others (Tao and Johns, 2004). The PDZ binding motif on GODZ potentially contributes to the large diversity of proteins palmitoylated by GODZ through interaction with PDZ domain-containing proteins. Additionally, the frequency of mEPSCs were significantly reduced in DKO co-culture experiments suggesting that GODZ and SERZ-β are essential for glutamatergic excitatory synaptic transmission (Murakami, 2008). Therefore, we hypothesized that GODZ-mediated palmitoylation contributes to the function of numerous other neuronal proteins.

Potential substrates of GODZ were identified using a quantitative proteomics approach with isobaric tags for relative and absolute quantitation (iTRAQ). After ABE-
enrichment for palmitoylated proteins, the samples were digested and labeled using an iTRAQ kit that utilized eight different labeling molecules that have the same mass but fractionate differently during tandem mass spectrometry (MS/MS) to yield reporter ions of different size. The isobaric tags allow for direct comparison of palmitoylated protein WT: KO ratios in the same mass spectrometry run. Protein abundance was determined using the ProteinPilot™ software and only proteins with at least two unique peptides were considered. Additionally, around 400 proteins were eliminated from the list if the ratio of hydroxylamine (HA+) (proteins enriched by palmitoylated pull-down) compared to hydroxylamine (HA-) (negative control for nonspecific binding of proteins to the beads) was less than 1 indicating that the protein was not enriched in the pull-down.

Globally, there were 1099 proteins identified as potential palmitoylated proteins (i.e. these proteins were more enriched in the pull-down). Among these, 42 proteins were significantly down regulated in GODZ KO mice. In contrast, 12 proteins were upregulated in KO mice compared to WT (Table 1 and 2, uncorrected p-values). It is important to note that this experiment did not differentiate between an increase in palmitoylation or an increase in the total level of substrate proteins. Therefore, it is likely that the upregulated proteins included proteins whose palmitoylation was unchanged but whose abundance was increased, for example due to changes in neural activity. Similarly, proteins that appeared less palmitoylated in KO compared to WT brain include some proteins with an unaltered palmitoylation state but that were simply less abundant for other reasons.
| disco-interacting protein 2 homolog B isoform 2 | 4.897 | 0.525 | 0.004 |
| neurofilament heavy polypeptide | 4.218 | 0.606 | 0.002 |
| nuclear pore complex protein Nup155 | 3.826 | 0.051 | 0.006 |
| acyl-CoA:lysophosphatidylglycerol acyltransferase 1 isoform 2 | 2.898 | 0.169 | 0.002 |
| APC membrane recruitment protein 2 isoform 1 | 2.753 | 0.723 | 0.001 |
| glutamyl-CoA dehydrogenase, mitochondrial | 2.609 | 0.433 | 0.004 |
| NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4 | 2.335 | 0.761 | 0.001 |
| pancreas/duodenum homeobox protein 1 | 2.262 | 0.514 | 0.008 |
| calnexin precursor | 2.252 | 0.845 | 0.026 |
| potassium voltage-gated channel subfamily KQT member 2 iso 5 | 2.118 | 0.051 | 0.004 |
| synaptic vesicle glycoprotein 2A | 2.032 | 0.507 | 0.036 |
| myelin-oligodendrocyte glycoprotein precursor | 1.997 | 0.370 | 0.009 |
| CD44 antigen isoform a precursor | 1.909 | 0.120 | 0.002 |
| BMP/retinoic acid-inducible neural-specific protein 1 precursor | 1.890 | 0.318 | 0.005 |
| isobutyryl-CoA dehydrogenase, mitochondrial precursor | 1.889 | 0.547 | 0.043 |
| 60S ribosomal protein L14 | 1.877 | 0.533 | 0.024 |
| KH domain-containing, RNA-binding, signal transduction-associated sorting nexin-1 | 1.849 | 0.809 | 0.003 |
| multidrug resistance protein 1B | 1.807 | 0.359 | 0.040 |
| ELAV-like protein 3 | 1.726 | 0.213 | 0.035 |
| uncharacterized protein KIAA1671 | 1.707 | 0.198 | 0.008 |
| sodium/potassium-transporting ATPase subunit alpha-2 precursor | 1.704 | 0.715 | 0.011 |
| adenylate kinase isoenzyme 5 | 1.687 | 0.691 | 0.026 |
| junctional adhesion molecule C precursor | 1.653 | 0.173 | 0.010 |
| cyclin-dependent kinase 16 | 1.650 | 0.547 | 0.024 |
| oral-facial-digital syndrome 1 protein homolog | 1.583 | 0.520 | 0.009 |
| proline-rich transmembrane protein 3 isoform b precursor | 1.517 | 0.142 | 0.020 |
| probable ATP-dependent RNA helicase DDX17 isoform 4 | 1.453 | 0.483 | 0.014 |
| arylsulfatase B precursor | 1.451 | 0.804 | 0.002 |
| plexin-B2 precursor | 1.449 | 0.772 | 0.003 |
| FAD-dependent oxidoreductase domain-containing protein1 iso1 | 1.443 | 0.895 | 0.042 |
| opioid-binding protein/cell adhesion molecule precursor | 1.428 | 0.886 | 0.042 |
| rho GTPase-activating protein 23 | 1.367 | 0.676 | 0.014 |
| phosphoprotein associated with glycosphingolipid-enrich. | 1.354 | 0.723 | 0.014 |
| alpha-1,3/1,6-mannosyltransferase ALG2 | 1.346 | 0.364 | 0.041 |
| amyloid beta A4 precursor protein-binding family A member 1 | 1.341 | 0.881 | 0.007 |
| TBC1 domain family member 10B | 1.318 | 0.785 | 0.050 |
| prostaglandin reductase 2 isoform 1 | 1.293 | 0.722 | 0.046 |
| cat eye syndrome critical region protein 6 homolog | 1.285 | 0.287 | 0.008 |
| inactive phospholipase C-like protein 2 | 1.279 | 0.852 | 0.020 |
| leucine-rich repeat-containing protein 7 isoform 1 | 1.217 | 0.739 | 0.003 |
Table 2 Palmitoylated proteins upregulated in KO mice

<table>
<thead>
<tr>
<th>Protein</th>
<th>Avg WT/KO</th>
<th>HA-/HA+</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>signal-induced proliferation-associated 1-like protein 1 isoform 2</td>
<td>0.877</td>
<td>0.388</td>
<td>0.023</td>
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<tr>
<td>EH domain-containing protein 3</td>
<td>0.846</td>
<td>0.875</td>
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<td>vimentin</td>
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<td>0.040</td>
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<td>ubiquitin-conjugating enzyme E2 D1</td>
<td>0.820</td>
<td>0.624</td>
<td>0.022</td>
</tr>
<tr>
<td>immunoglobulin superfamily member 21 precursor</td>
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<td>0.676</td>
<td>0.047</td>
</tr>
<tr>
<td>nitrilase homolog 1 isoform 1</td>
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<td>0.692</td>
<td>0.038</td>
</tr>
<tr>
<td>plexin-A1 precursor</td>
<td>0.759</td>
<td>0.748</td>
<td>0.026</td>
</tr>
<tr>
<td>ras-related protein Rab-1A</td>
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<td>0.847</td>
<td>0.041</td>
</tr>
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<td>myelin proteolipid protein isoform 1</td>
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<td>0.032</td>
</tr>
<tr>
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<td>0.050</td>
</tr>
<tr>
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<td>0.914</td>
<td>0.050</td>
</tr>
<tr>
<td>ras-related C3 botulinum toxin substrate 3 precursor</td>
<td>0.616</td>
<td>0.207</td>
<td>0.039</td>
</tr>
<tr>
<td>protein disulfide-isomerase A3 precursor</td>
<td>0.569</td>
<td>0.363</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Table 1 and 3 Proteins that are differentially regulated in GODZ KO mice (uncorrected P-values)

Peptide identifications are shown on the left hand column. The combined average of WT/KO values are shown in the next column, followed by the ratio of negative control (HA-) to pulldown (HA+) to eliminate false positives, the final column represents the uncorrected p-values obtained through permutation analysis.

The high number of proteins that were identified as potential substrates of GODZ was consistent with GODZ having a large substrate specificity. However, while many synaptic proteins were successfully identified by mass spectrometry, those that were identified were not as abundant and not as well represented in the sample compared to non-synaptic proteins. It is likely that enriching for synaptosomal fractions before performing the ABE-assay would have revealed more changes in synaptic proteins compared to proteins elsewhere in the cell. There were several proteins that were more palmitoylated or abundant in the KO mice. This most likely reflects a compensatory increase in the total amount of the protein or the upregulation of other DHHC PATs. Supporting this hypothesis, DHHC15 mRNA is upregulated in GODZ KO mice indicating that there is likely compensation by other enzymes (Murakami, 2008).
The protein with the largest deficit in palmitoylation or abundance in the KO mice was disco-interacting protein 2 homolog B (DIP2B) which was reduced four-fold. DIP2B contains a binding site for methyltransferase 1 associated proteins potentially implicating the protein in DNA methylation (Winnepenninckx et al., 2007). Calnexin was also identified as a palmitoylated protein that was significantly reduced in KO mice. Calnexin is a chaperone protein normally localized to the ER. Upon palmitoylation, calnexin becomes enriched in the mitochondria-associated membranes where it interacts with sarcoendoplasmic reticulum (SR) Ca\textsuperscript{2+} transport enzymes to modulate mitochondrial Ca\textsuperscript{2+} signaling. Upon ER stress, calnexin becomes depalmitoylated and transported back to the ER to serve as a chaperone (Lynes et al., 2013). Calnexin palmitoylation was downregulated 2.25 fold in GODZ KO mice compared to WT mice, supporting the hypothesis that GODZ is important at the Golgi to ER interface and may regulate the trafficking to different routes of the secretory pathway. Further detailed examination of candidate GODZ substrates is addressed in Chapter 4: Discussion.

3.1.4.1 Testing of candidate proteins

3.1.4.2 GAP-43 is a substrate of GODZ

Neurofilament heavy chain was also downregulated four-fold in GODZ KO mice. Neurofilaments are intermediate filaments of the cytoskeleton that are involved in determining axon diameter of neurons [for review see (Robert et al., 2016)]. Neurofilaments interact with GAP-43, another palmitoylated protein also identified as a potential GODZ substrate (Uchida et al., 2002). Although GAP-43 did not meet the strict cutoff criteria for unique peptide identification (described in Materials and Methods), its
palmitoylation was reduced was three-fold in GODZ KO compared to WT mice. GAP-43 is of particular interest as it was recently discovered to play a role in trafficking of gephyrin and GABA<sub>A</sub>Rs of developing neurons (Wang et al., 2015a).

GAP-43 is palmitoylated two N-terminal Cys residues (Cys 3 and Cys 4) (Benowitz and Routtenberg, 1997). Palmitoylation and a polybasic sequence on GAP-43 facilitate the trafficking of GAP-43 to the membrane (Trenchi et al., 2009). While GAP-43 was identified as a putative substrate through proteomics, further confirmation was required to demonstrate direct palmitoylation by GODZ. HEK 293T cells were transfected with GFP-GAP-43 alone, GFP-GAP-43 plus HA-GODZ or palmitoylation deficient GFP-GAP-43<sup>C3.4S</sup> plus HA-GODZ, to determine the amount of palmitoylation by GODZ (Figure 3.5).

**Figure 3.5 Palmitoylation of GAP-43 is mediated by GODZ in HEK 293T cells.**

(a) Representative images of western blots of HEK 293T cells that were transfected for 12 h with GAP-43 plasmids WT GFP-GAP-43, GFP-GAP-43 plus GODZ or GFP-GAP-43<sup>C3.4S</sup> as indicated. Palmitoylation was assayed using the ABE-assay and HA-reactions as negative controls. Palmitoylation levels were determined with ImageStudio by subtracting (HA-) from (HA+) and normalizing to the input level for each sample. (b) Quantification of the percent GAP-43 palmitoylated compared to the input levels. Normalized to GAP-43 alone (100%), GAP-43 with GODZ increased palmitoylation to 620% ± 106. GAP43<sup>C3.4S</sup> with GODZ reduced palmitoylation to 10.85% ± 2.85 [F<sub>(2,6) = 21.08, p < 0.01, n = 3, Tukey's test>](F<sub>(2,6) = 21.08, p < 0.01, n = 3, Tukey's test)
Co-transfection of GODZ with GAP-43 led to a significant increase in the amount of palmitoylation as shown in HA+ lanes. To confirm this represented palmitoylation a double point mutant GAP-43$^{C3,4S}$ that is incapable of being palmitoylated was used (Figure 3.5 a). Palmitoylation of GAP-43$^{C3,4S}$ was significantly reduced, thereby confirming that GAP-43 is a direct substrate of GODZ in heterologous cells (Figure 3.5 b).

3.1.4.3 GODZ is one of many PATs that can pamitoylate gephyrin in HEK 293T cells

Gephyrin is a putative GODZ substrate that plays an important role in clustering of GABA$_A$Rs at the synapse through interaction with numerous postsynaptic proteins. We hypothesized that gephyrin palmitoylation could contribute to the synaptic deficit seen upon knockdown of GODZ in neurons. As part of a collaboration with the Schwarz lab (University of Cologne, Germany) the palmitoylation of gephyrin was analyzed using transfected HEK 293T cells first with GODZ alone, then subsequently with all 23 DHHC PATs (Figure 3.6 a, b).
Figure 3.6 Gephyrin is palmitoylated by GODZ and numerous other PATs
(a) HEK 293T cells were transfected for six hours with GODZ or GODZ_{C157S} with full length gephyrin P2 isoform (Ramming et al., 2000). The degree of gephyrin palmitoylation was analyzed by ABE-assays. Note the GODZ-dependent increase in palmitoylation in the HA+ lanes for gephyrin (top panel) but not with the dominant negative GODZ_{C157S} (lower panel). (b) Palmitoylation of gephyrin was analyzed by co-transfection with all 23 DHHC PATs utilizing metabolic click chemistry assay with 15-HYDA as a substrate, for six hours. Note that GODZ was one of many PATs that were able to palmitoylate gephyrin, while some of these enzymes appeared to inhibit the activity of endogenous PATs.

Gephyrin palmitoylation (HA+ lane) was increased upon co-transfection of full-length gephyrin and GODZ. Moreover, co-transfection of gephyrin with GODZ_{C157S} completely abolishes gephyrin palmitoylation by acting as a dominant negative enzyme. This indicates that GODZ_{C157S} inactivates the endogenous PATs that are palmitoylating transfected gephyrin in this heterologous expression situation (Figure 3.6 a). A screen of all 23 DHHC PATs using metabolic click chemistry revealed that gephyrin can be palmitoylated by almost all of the PATs and there is a high level of endogenous palmitoylation in HEK 293T cells. Therefore, it is unlikely that GODZ significantly contributes to the overall level of gephyrin palmitoylation (Figure 3.6 b). This result was
consistent with what the Schwarz lab reported that numerous PATs acted on gephyrin in 293T cells, possibly indicating that gephyrin’s consensus sequence for palmitoylation is ubiquitously accepted by numerous PATs. The Schwarz lab determined the enzyme responsible for palmitoylating gephyrin using a different assay that involved transfecting the 23 DHHC PATs into cultured neurons and quantifying the acute clustering of gephyrin. Cluster analysis of gephyrin revealed that DHHC-12 was responsible for palmitoylation mediated gephyrin clustering (Dejanovic et al., 2014). Therefore, the role GODZ plays in gephyrin palmitoylation was not explored further.

3.1.4.4 GODZ KO mice exhibit deficits in previously established substrates

GODZ KO mice exhibited deficits in GABAergic neurotransmission under competitive conditions (Murakami, 2008). However, a deficit in palmitoylation of known substrates that may contribute to the GABAergic deficit has thus far not been observed. Previously, our lab identified GODZ and SERZ-β as the sole PATs responsible for palmitoylating the γ2 subunit in cultured HEK 293T cells (Fang et al., 2006). Therefore, we hypothesized that GODZ KO mice should exhibit similar deficits in γ2 palmitoylation as well as newly identified GAP-43. We analyzed levels of PSD-95 as a control because it is palmitoylated by DHHC 2/15 in addition to GODZ/SERZ-β (Fukata et al., 2004). Whole brain extracts from WT and GODZ KO mice were analyzed for the steady state level of palmitoylation using the ABE-assay (Figure 3.7a, b). Consistent with our hypothesis, a 38% reduction in γ2 palmitoylation was observed in GODZ KO mice. A moderate reduction in palmitoylation likely contributes to the selective phenotype in competitive KO cultures. Under normal conditions having reduced palmitoylation may
not be as detrimental compared to conditions where neurons have to compete for innervation with WT neurons (Buffelli et al., 2003).

Figure 3.7 Palmitoylation deficits in GABA\(_2\) subunit and GAP-43 in GODZ KO mice

(a, b) Brain extracts from WT and GODZ KO mice were analyzed using the ABE-assay. Palmitoylation levels were determined by subtracting band intensities of (HA-) from (HA+) lanes and normalized to the input bands for each sample. Hydroxylamine minus (HA -) lanes represent the negative control reaction for each sample. Palmitoylation of PSD-95 showed no difference between WT and GODZ KO mice (108% ± 11% of WT, \(p = 0.589, n = 4\)). A significant genotype difference was seen in both \(\gamma_2\) (67.19% ± 5% of WT, \(p < 0.001, n = 7-8\)) and GAP-43 (67.4% ± 10.3% of WT, \(p < 0.05, n = 10-11, t\)-tests) palmitoylation in GODZ KO vs. WT brain tissue. Results represent means ± S.E.M. *: \(p < 0.05\); **: \(p < 0.01\) \(t\)-test.

A similar reduction was observed in GAP-43 palmitoylation confirming that GAP-43 is a substrate of GODZ \textit{in vivo}. GAP-43 heterozygous mice exhibit impaired spatial learning, stress induced anxiety, axon path-finding deficits and hippocampal abnormalities (Latchney et al., 2014). Given the reduction of GAP-43 palmitoylation in GODZ KO mice and the necessity of palmitoylation for GAP-43 membrane localization, it is likely that GODZ KO mice exhibit similar deficits to GAP-43 heterozygous mice.
Further work will need to be done to establish the functional effect of reduced palmitoylation of GAP-43 in GODZ KO mice.

No deficit was detected in palmitoylation of PSD-95 consistent with PSD-95 having numerous other PATs that can compensate for the loss of GODZ. This detection method is more sensitive than a proteomics approach as it allows for direct identification of proteins using antibodies. In contrast, mass spectrometry favors the identification of the most abundant proteins. While proteomics was more useful in identifying new substrates, a western blot approach is more advantageous for quantifying individual palmitoylation levels.

Part II: Elucidating the role of palmitoylation of neuroligin 2

3.2.1 GODZ contributes to the palmitoylation of NL2

The loss of presynaptic GABAergic innervation observed in GODZ and DKO co-cultures indicate there are likely other substrates of GODZ that contribute to the deficit besides the post-synaptic $\gamma_2$ subunit. GODZ shRNA transfected neurons have lower accumulation of NL2 at synapses that could be due to the loss of $\gamma_2$ or an autonomous function of NL2 trafficking (Fang, 2007). NL2 forms trans-synaptic complexes with neurexins in the presynaptic cell; therefore, we hypothesized the deficits in GODZ KO cultures may be due to palmitoylation of NL2. HEK 293T cells were transfected with HA-NL2 alone or co-transfected with HA-GODZ, harvested at different time points and subjected to the ABE-assay to test if NL2 is a substrate of GODZ (Figure 3.8 a).
Figure 3.8 GODZ palmitoylates NL2 in cultured HEK 293T cells

(a) Cells were transfected with HA-NL2 alone or together with HA-GODZ for six (top) or twelve hours (bottom), respectively. Note that after 12h of transfection NL2 was overtly palmitoylated by endogenous PATs in HEK 293T cells, independent of transfected GODZ (HA+ lanes, lower panel). (b) After six hours there was a significant increase in level of palmitoylation of NL2 with GODZ compared to NL2 alone (53% ± 8.36 NL2 alone vs. 100% NL2 plus GODZ ± 16.31, p = 0.05, n = 4-5) (HA+ lanes, top panel of (a)). (c) Transfected cells with HA-GODZ and HA-NL2 were metabolically labeled with the either 15-HDYA or 17-ODYA for an additional one to six hours followed by click chemistry conjugation. There was an overt GODZ-dependent increase in NL2 palmitoylation seen around 4-5 hours with 15-HYDA but not 17-OYDA. Results represent means ± S.E.M. *: p < 0.05 t-test.

Six hours after transfection there was a significant increase in the amount of NL2 palmitoylation by GODZ compared to NL2 alone (Figure 3.8 a, b). However, transfection for twelve hours led to an increase in the level of palmitoylation of NL2 by endogenous PATs. Previous experiments (Figure 3.4) demonstrated that there was endogenous GODZ in HEK 293T cells making them less than ideal for this type of experiment. The time dependent increase in GODZ mediated palmitoylation was also demonstrated using the metabolic click chemistry assay (Figure 3.8 c). There was a transient GODZ mediated increase seen in the 15-hexadecyonic acid (15-HYDA) treated cells around four to five hours after transfection that may reflect dynamic turnover of palmitoylation of NL2. There was no apparent increase in palmitoylation of NL2 in the 17-OYDA treated
cells. As GODZ cannot effectively utilize 17-OYDA, palmitoylation of NL2 is likely from another enzyme present in HEK 293T cells (Jennings and Linder, 2012).

3.2.2 NL2 is palmitoylated by numerous enzymes in HEK 293T cells

While GODZ palmitoylates NL2 as demonstrated in Figure 3.8, it is possible that other PATs contribute to palmitoylation. Our collaborators, Masaki and Yuko Fukata from the Fukata lab (National Institute for Physiological Sciences, Japan) analyzed NL2 palmitoylation using [3H] - palmitate radiolabeling of transfected HEK 293T cells (Figure 3.9 a). Each of the known 23 DHHC-PATs was transfected into HEK 293T cells together with HA-NL2. GODZ and NL2 were transfected alone as a negative control. The protein extracts were solubilized and divided in half: one-half was used to detect transfected NL2 by western blot and the second half was used for fluorography. Out of the 23 DHHC proteins, both GODZ and SERZ-β palmitoylated NL2 in HEK 293T cells. However, in the negative control lane containing only transfected HA-GODZ (Figure 3.9 a, first lane) there were distinct bands that ran in the same pattern as NL2 therefore further experiments were required to confirm that GODZ and SERZ-β are responsible for NL2 palmitoylation.
Figure 3.9 NL2 is palmitoylated by NL2 and variety of other DHHC enzymes. HEK 293T cells were transfected with individual DHHC plasmids with HA-NL2. (a) The cells were metabolically labeled with [³H]-palmitate and the proteins separated by SDS-PAGE and analyzed by fluorography. Immunoblot (IB) to confirm HA-NL2 expression is shown below. The bands marked with an asterisk represent palmitoylated NL2 and are increased in both DHHC3 (GODZ) and DHHC7 (SERZ-β). The remaining background bands likely represent other palmitoylated proteins not identified. Note that transfection of GODZ alone (first lane) results in a background band that runs at the same size as HA-NL2 (b) Metabolic labeling with an alkyne palmitate analog (15-HYDA) for six hours (top) or 12 hours (bottom). There are bands that correspond to palmitoylated NL2 in DHHC3 among other PATs. NL2 typically resolves as two distinct bands with the upper band corresponding to the mature, fully glycosylated form.

Due to the high background levels of palmitoylation using [³H]-palmitate detection, we repeated the experiment using metabolic 15-HYDA incorporation to enrich for palmitoylated proteins and directly detect proteins using an antibody for the HA-tag of
transfected HA-NL2. Cells were transfected with both HA-NL2 and individual DHHC PATs for six or twelve hours and analyzed for palmitoylation levels by click chemistry. After six hours an increase in palmitoylation of NL2 was observed in the lanes for GODZ and DHHC21 (Figure 3.9 b, top panel). DHHC 21 is expressed in the brain and is known to palmitoylate eNOS, LCK, FYN and Gαi2 but does not typically share common substrates with GODZ (Fukata and Fukata, 2010). After twelve hours palmitoylation was observed in DHHC 1, 2, GODZ, SERZ-β, 10, 11, 14, 17 and 21 (Figure 3.9 b, lower panel). However, only DHHC 2, GODZ, SERZ-β, 17 and 21 are known to be expressed in neurons (Ohno et al., 2006). Strong palmitoylation by SERZ-β was not observed potentially indicating that NL2 may not be an ideal substrate for SERZ-β. Interestingly, DHHC2 shares numerous other substrates with GODZ such as PSD-95 and GAP-43 therefore it may also contribute to NL2 palmitoylation in the brain (Fukata and Fukata, 2010). DHHC2 is localized in the dendrites and mediates activity-sensitive palmitoylation of PSD-95 (Fukata et al., 2013). Potentially GODZ mediates steady-state levels of NL2 and DHHC2 translocates to the postsynaptic membrane and palmitoylates NL2 in an activity dependent manner. Further work is needed to determine the contribution of GODZ to NL2 palmitoylation in vivo.

3.2.3 NL2 is palmitoylated primarily on Cys 710

Neuroligin 2 has a large extracellular AChE domain, a transmembrane domain and a small cytosolic C-terminus. Neuroligin 2 contains two Cys residues C710 and C766 in the cytoplasmic domain that potentially serve as palmitoylation sites (Figure 3.10 a). Palmitoylation can occur on single or multiple Cys residues depending on the
nature of the protein. For example, palmitoylation of GAP-43 is specific to Cys 3 and Cys4 however Cys 4 is the predominant palmitoylation site implicating potential sequence specificity in determining palmitoylation sites (Liu et al., 1993). Therefore to elucidate the palmitoylation sites on NL2, single point mutant HA-NL2\textsuperscript{C710A}, HA-NL2\textsuperscript{C766A} and double mutant HA-NL2\textsuperscript{C710A/C766A} constructs were made using site directed mutagenesis where cysteine residues were mutated to alanine thereby eliminating palmitoylation. Palmitoylation of NL2 point mutants was assessed using the ABE-assay in transfected HEK 293T cells.

**Figure 3.10 Palmitoylation by GODZ occurs at C710 in neuroligin 2**

(a) The domain structure of NL2 containing potential palmitoylation sites: NL2\textsuperscript{C710}, NL2\textsuperscript{C766} (see Figure 1.4 for more information). (b) HEK 293T cells were transfected with HA-NL2 alone or HA-NL2, HA-NL2\textsuperscript{C710A}, HA-NL2\textsuperscript{C766A} together with HA-GODZ for six hours followed by quantitation of palmitoylation by the ABE-assay. (c) Quantification of HA-NL2 palmitoylation normalized to HA-NL2 + GODZ shows a GODZ dependent difference (40% of NL2 with GODZ ± 4, p < 0.05, n = 3) in palmitoylation. A loss of palmitoylation was observed in NL2\textsuperscript{C710A} + GODZ compared to HA-NL2 + GODZ (24.7% ± 10 of NL2 + GODZ, p < 0.001, n = 3) and in the double mutant NL2\textsuperscript{C710A/C766A} + GODZ (45.6% ± 11.5, p < 0.05, n = 3) no difference was observed between HA-NL2 + GODZ and NL2\textsuperscript{C766A} + GODZ. Results represent means ± S.E.M. *: p < 0.05; ***: p < 0.001 t-test.
A similar GODZ dependent increase was observed for WT NL2 (Figure 3.10 b). Co-transfection of NL2<sup>C710A</sup> and GODZ led to a reduction in the amount of palmitoylation compared to WT NL2 with GODZ. A similar reduction was observed in NL2<sup>C710/766A</sup> transfected cells with GODZ. However, no significant reduction was observed in NL2<sup>C766A</sup> with GODZ compared to WT (Figure 3.10 c). Taken together these data indicate that palmitoylation by GODZ likely occurs on Cys 710, a residue that is unique to NL2. Palmitoylation of NL2 potentially influences targeting NL2 to the membrane and to GABAergic synapses.

### 3.2.4 The effect of palmitoylation on trafficking of NL2

The interaction between the γ2 subunit and GODZ depends on both palmitoylation of the intracellular loop portion of γ2 and the Cys residue in the DHHC domain of GODZ (Fang et al., 2006). Overexpression of the γ2 subunit and GODZ results in trapping of the γ2 subunit in the Golgi (Fang et al., 2006). We therefore hypothesized that the association of NL2 and GODZ might be facilitated by palmitoylation and lead to their association in the cell upon overexpression. Transfection of HEK 293T cells with GODZ and NL2 led to an intracellular localization of GODZ and NL2 in the region of the Golgi, consistent with the localization of GODZ (Figure 3.11 a). Co-transfection of palmitoylation deficient NL2<sup>C710/766A</sup> and GODZ led to a loss of localization in the Golgi and NL2<sup>C710/766A</sup> localization at the cell surface (Figure 3.11 b). A similar phenotype is seen in dominant negative GODZ<sup>C157S</sup> with NL2 (Figure 3.11 c) indicating that both proteins require palmitoylation for association in the Golgi.
Figure 3.11 Wild type NL2 is localized with GODZ in the Golgi

(a) Transfected HEK 293T cells with WT HA-NL2 (a’) and Flag-GODZ(a) show perinuclear colocalization of both proteins (a’’) indicating GODZ and NL2 interact in a palmitoylation manner with GODZ sequestering NL2 in the Golgi. (b) WT Flag-GODZ (b) co-expressed with palmitoylation deficient HA-NL2\textsuperscript{C710/766A} (b’) demonstrates a loss of colocalization of HA-NL2\textsuperscript{C710/766A} and Flag-GODZ (b’’). (c) Similarly, co-transfection of the palmitoylation deficient Flag-GODZ\textsuperscript{C157S} (c) and HA-NL2 (c’) led to a loss of colocalization in the Golgi (c’’). Arrows represent localization in the Golgi region. Scale bar, 10 μM.

3.2.5 Neuroligin 2 is palmitoylated and nitrosylated in the brain

We have demonstrated GODZ dependent NL2 palmitoylation in cultured HEK 293T cells; however, it is not known if NL2 is palmitoylated in the brain or what functional impact it has on NL2. Palmitoylation of NL2 was observed using the ABE-assay in the
cerebellum of histidine-flag-YFP (HFY) tagged NL2 transgenic mice (Figure 3.12 a). In addition, palmitoylation of NL2 was observed in cultured neurons using the metabolic click chemistry assay (Figure 3.12 b). Neuroligin 2 palmitoylation was also identified in the quantitative proteomics screen of GODZ KO mice; however, the levels of palmitoylation were not significantly changed and thus not reported. Attempts at quantifying NL2 palmitoylation in GODZ KO mice by western blot were not successful to date due to the apparent variability in the amount of palmitoylation and ability to pull-down NL2 (data not shown). We established palmitoylation of NL2 in vivo; however, the contribution of GODZ to palmitoylation of NL2 in the brain is not yet known.

Figure 3.12 Palmitoylation of NL2 in the brain and cultured neurons
(a) Cerebellum brain extracts from 8-week-old HFY-NL2 transgenic mice demonstrate a distinct band corresponding to palmitoylated HFY-NL2. (b) Cultured neurons treated with 17-HYDA for 12 hours and analyzed using biotin-azide conjugated using click chemistry and purified validates NL2 palmitoylation in neurons.

3.5.2.1 Nitrosylation of NL2

The interplay between different post-translational modifications is rapidly emerging as a mechanism to modulate their function. Nitrosylation influences multiple post-translational modifications such as phosphorylation, ubiquitination, acetylation, sumoylation and palmitoylation (Lopez-Otin and Hunter, 2010, Hess and Stamler, 2012). Nitrosylation directly competes for the binding site of palmitoylation on PSD-95 and
impacts its clustering at excitatory synapses (Ho et al., 2011). Gephyrin was discovered to be associated with neuronal nitric oxide synthase (nNOS) which is the major source of NO in the brain (Dejanovic and Schwarz, 2014).

Therefore, NO production occurs at GABAergic synapses and could potentially play an important role in nitrosylating and modulating other synaptic proteins. Due to the localization of NL2 with gephyrin and nNOS, we hypothesized that NL2 might also be subjected to nitrosylation in addition to palmitoylation. A modified biotin switch assay utilizing a thiol-reactive resin (Acyl-RAC) is used to assay for palmitoylation. A similar technique is used for nitrosylation except using ascorbate to cleave the S-nitro bond on the proteins (SNO-RAC) (Chen et al., 2013). It is unclear if HEK 293T or other heterologous cell lines contain endogenous nitric oxide synthase therefore an exogenous NO donor was added to the cells (CysNO) shortly before harvest to provide NO to the cells. Transfected HEK 293T cells with HA-NL2 alone or with HA-GODZ resulted in both palmitoylated and nitrosylated NL2 (Figure 3.13 a). NL2 nitrosylation was not affected by overexpression of GODZ in heterologous cells.

**Figure 3.13** The interplay between palmitoylation and nitrosylation.

(a) Transfected HEK 293T cells with HA-NL2 alone or together with HA-NL2 and HA-GODZ, five minutes before harvest cells were incubated with a NO donor (CysNO) and subjected to Acyl-RAC and SNO-RAC. Negative control lanes represent HA- or ascorbate (-) lanes (b) Hippocampal protein extracts were analyzed for endogenous levels of nitrosylation and palmitoylation using selective cleavage palmitate by HA or nitrosylation by ascorbate using the biotin-switch ABE-assay. Both palmitoylation and
Nitrosylation were present in the hippocampus of WT mice.

NO is a highly reactive second messenger molecule having off target effects aside from S-nitrosylation. Therefore, providing additional NO may not reflect an accurate depiction of what occurs in the cell. Therefore, we analyzed mouse brain extracts for palmitoylation and nitrosylation of NL2. Endogenous levels of palmitoylation and nitrosylation were analyzed from hippocampal brain extracts of WT mice by dividing protein extracts into two and processing using biotin-switch assays for S-NO and palmitoylation simultaneously. Both palmitoylation and nitrosylation of NL2 were observed in vivo indicating that at the steady state level NL2 is subject to both post-translational modifications (Figure 3.13b).

Nitrosylation could potentially compete with palmitoylation at the same sites on NL2 and prevent palmitoylation or re-palmitoylation cycles. Both single and double point mutant versions of NL2 were used to analyze which sites are nitrosylated on NL2. Both point mutants, NL2<sup>C710A</sup> and NL2<sup>C766A</sup> were subject to nitrosylation and a complete reduction was observed in the double point mutant NL2<sup>C710A/C766A</sup>. These results indicated that nitrosylation of NL2 is specific to the same two cysteines that are subject to palmitoylation (Figure 3.14).
Figure 3.14 Nitrosylation occurs at both C710 and C766 in NL2

a) Transfected HEK 293T cells with HA-NL2 alone, HA-NL2\textsuperscript{C710A}, HA-NL2\textsuperscript{C766A} with HA-GODZ and five minutes before harvest CysNO was added to the cells as a NO donor. There was no overt difference in nitrosylation between the presence or absence of overexpressed GODZ. HEK 293T cells transfected with HA-NL2\textsuperscript{C710A/C766A} showed no detectable nitrosylation.

There is no difference between the nitrosylation levels in the presence or the absence of over expression of GODZ potentially implicating that nitrosylation might be independent of GODZ mediated palmitoylation. Further experiments are needed to determine if nitrosylation competes with palmitoylation of NL2 \textit{in vivo} and whether or not it influences the function or trafficking of NL2.
Chapter 4

Discussion

4.1 Overview of findings

The work presented here was designed to elucidate the function of GODZ-mediated palmitoylation in the brain. GODZ was localized to the cis-Golgi and also detected in Golgi outcroppings of primary dendrites. The loss of GODZ was confirmed in brain slices of GODZ KO mice and in neuron culture. The localization of GODZ to the cis-Golgi face suggests a role for palmitoylation of GODZ substrates in the early secretory pathway. New substrates of GODZ were identified using a combination of palmitoylated pull-down ABE-assays and a quantitative proteomics approach (iTRAQ). Several proteins were found to show reduced palmitoylation in GODZ KO mice. Of particular interest, GAP-43 was revealed as a potential substrate of GODZ by mass spectrometry. Palmitoylation of GAP-43 by GODZ was confirmed using an in vitro ABE-assay to measure steady state levels of palmitoylation. Gephyrin, another potential GODZ substrate, was found to be palmitoylated by GODZ but it was not the functionally relevant PAT in neurons (Dejanovic et al., 2015). Palmitoylation of known substrates, GABA\(_A\)R \(\gamma2\) subunit and GAP-43 palmitoylation was reduced in GODZ KO mice but not palmitoylation of PSD-95.

The loss of presynaptic GAD observed in GODZ KO co-cultures could potentially be explained by alterations in palmitoylation of the trans-synaptic adhesion molecule NL2. Palmitoylation of NL2 by GODZ was confirmed in HEK 293T cells, and a screen of
all 23 DHHC PATs revealed that GODZ is not the only PAT that can catalyze palmitoylation of NL2. Neurologin 2 palmitoylation occurs on Cys710, a residue unique to NL2 and not on Cys766 in heterologous cells. Palmitoylation of NL2 was also discovered in the brain, however, the pull-down levels were too variable to determine if there was a reduction of NL2 palmitoylation in GODZ KO mice. Neurologin 2 was subject to nitrosylation independent of palmitoylation by GODZ. Nitrosylation was observed on both cytoplasmic cysteine residues on NL2. NL2 nitrosylation was also found in vivo implicating a potential physiological role of competing modifications.

4.2 The functional consequences resulting from the loss of GODZ-mediated palmitoylation in the brain

4.2.1 GODZ localization in the cis-Golgi potentially influences the stability of substrate proteins

DHHC PATs exhibit differential localization in the cell and in different tissues and that localization likely impacts the functional effect palmitoylation has on their substrates. Most of the 23 DHHC proteins are found in brain tissue. Eighteen of the 23 mammalian DHHC enzymes exhibit colocalization with the Golgi apparatus, ER or both compartments. In contrast, DHHC 2, 5, 20 and 21 were localized to the plasma membrane in neurons and are involved in activity-dependent regulation of palmitoylation because they can respond rapidly to extracellular cues (Ohno et al., 2006). The localization of DHHC2 on recycling endosomes in dendrites helps mediate palmitoylation response to activity due to its localization to the cell surface (Noritake et al., 2009).

ER and Golgi localized DHHC PATs likely act on de novo synthesized proteins and facilitate trafficking of the substrate protein to the cell surface, mitochondria or
lysosomes. GODZ localization in the cis-Golgi may facilitate the substrates exit from the ER by promoting ER transport vesicles or by preventing lysosomal degradation. A similar mechanism was observed in the closest GODZ paralog, SERZ-β. SERZ-β palmitoylates the death receptor Fas in the Golgi and protects Fas from lysosomal degradation (Rossin et al., 2015). Palmitoylation can prevent degradation of substrate proteins through disruption of ubiquitin ligase activity or prevention of ubiquitination by steric hindrance (Abrami et al., 2008). Palmitoylation also confers additional hydrophobic properties to its substrate. In this manner, palmitoylation can rescue a protein from degradation by correcting hydrophobic mismatching of transmembrane domain length to plasma membrane thickness in the Golgi. A study using transmembrane adaptor proteins (TRAPs) discovered that in addition to TM domain length, palmitoylation and the flanking sequence are necessary determinants for membrane localization (Chum et al., 2016). Lipid modifications are thought to provide the free energy needed for membrane association of proteins such as N-Ras that are initially localized in the cytoplasm. Surface plasmon resonance (SPR) spectroscopy revealed that the binding energy arising from N-Ras membrane association increases with lipid modifications. With one palmitate moiety the binding energy was increased 21% and dual lipidation of N-Ras with farnesylation and palmitoylation increased binding energy to up to 70%. These experiments support the hypothesis that lipid modifications are crucial for overcoming hydrophobic mismatch for membrane stability in the early secretory pathway and provide a mechanism for the role GODZ plays in the cis-Golgi (Weise et al., 2013).

Supporting the role GODZ plays in the cis-Golgi, calnexin was identified as a potential GODZ substrate in vivo through quantitative proteomics. Palmitoylation
controls the switch in localization of calnexin from the ER to the sarcoendoplasmic reticulum. The localization of GODZ substrates in early secretory pathway strengthens the finding that GODZ is localized in the cis-Golgi complex (Lynes et al., 2013). One of the proteins identified as more abundant or palmitoylated in GODZ KO mice, Ras-related protein Rab-1A (RAB1A) plays an important role in regulating vesicular protein trafficking from the ER to the Golgi (WT:KO ratio 0.73) (Mukhopadhyay et al., 2011). This upregulation of RAB1A potentially compensates for the loss of GODZ mediated palmitoylation in sorting of proteins the early secretory pathway in KO mice.

4.2.2 The interplay between palmitoylation and other post translational modifications

4.2.2.1 Palmitoylation and ubiquitination

Ubiquitination serves as an important mechanism to regulate proteolysis, localization and activity of proteins. GODZ mediated palmitoylation may directly compete with ubiquitination on substrates in addition to modulating ubiquitin ligases directly (Hochstrasser, 1996, Schnell and Hicke, 2003). Tripartate motif protein 32, (TRIM32) is a ubiquitin E3 ligase that contains a conserved RING finger domain (Frosk et al., 2002). TRIM32 was also identified as a putative GODZ interacting protein through a yeast two-hybrid screen (Fang, 2007). The γ2 subunit, another GODZ substrate also interacts with an ubiquitin ligase, RNF34, and is subject to ubiquitin-mediated degradation (Jin et al., 2014). Interestingly, one of the proteins that was more abundant in GODZ KO compared to WT mice based on our proteomic screen was ubiquitin-conjugating enzyme E2 (E2). This could mean that KO of GODZ leads to an increase in either the amount of or the palmitoylation of E2 in brain tissue. This increase may be
due to the increased need for ubiquitin mediated degradation as a consequence of reduced palmitoylation in GODZ KO mice. The ubiquitin proteasome has been shown to be crucial for the activity-dependent regulation of AMPA and NMDA receptors (Yi and Ehlers, 2007). GODZ KO mice were shown to have increased hind leg clenching that is indicative of increased excitability in the spinal cord (Murakami, 2008). Therefore, it is possible that E2 is upregulated in response to increased activity in GODZ KO mice.

Palmitoylation can directly regulate ubiquitin ligase function by modulating the localization of the ligase in the cell. Ring finger protein 11 (RNF11) is an E3-ligase implicated in signal transduction and apoptosis. RNF11 palmitoylation drives its association with early and recycling endosomes. However, de-palmitoylated RNF11 is inactive and retained in the early secretory pathway. Altering the localization of RNF11 through palmitoylation impacts its ubiquitin ligase function (Santonico et al., 2010). Alternatively, palmitoylation prevents subsequent ubiquitination of substrate proteins. We hypothesize GODZ KO mice would exhibit more ubiquitination of known substrates and potentially impact the function of numerous ubiquitin ligases. Interestingly, the intracellular loop region of γ2 subunit interacts with an E3 ubiquitin ligase, RNF34, and negatively impacts clustering of GABA\(_A\)Rs by γ2 ubiquitination (Arancibia-Carcamo et al., 2009, Jin et al., 2014). Therefore, it would be interesting to assess the amount of γ2 ubiquitination and degradation in GODZ KO mice. The amount of global ubiquitination in KO mice compared to WT can be assessed using a specific antibody that recognizes trypsin digested diglycine tag (K-ε-GG) on lysine residues left behind by ubiquitin cleavage combined with stable isotope labeling of amino acids (SILAC) proteomics approach to identify proteins whose ubiquitination state is modulated by GODZ and potential sites of ubiquitination (Na et al., 2012, Udeshi et al., 2012).
Nitrosylation likely modulates other inhibitory synaptic proteins

Nitric oxide (NO) is an important second messenger molecule that is implicated in modulating a variety of processes in the brain. After synthesis through NOS, NO can bind to soluble guanylyl cyclase (sGC) that converts guanosine-5’-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) that regulates the activity of many downstream targets (Alderton et al., 2001). Alternatively, NO acts as a post-translational modification through S-nitrosylation of target proteins. Nitrosylation involves the addition of NO directly to cysteine residues and modulates the function of numerous pre-synaptic proteins.

Neurotransmitter (NT) release is dependent on vesicular transporters that load presynaptic vesicles with their respective neurotransmitters. NT transport in the brain is dependent on vesicular monoamine transporter 2 (VMAT2) for the uptake of monoamines (dopamine, noradrenaline and serotonin), vesicular glutamate transporters 1 and 2 (VGLUT1/2) that uptake glutamate and vesicular acetylcholine transporter (VACht) for acetylcholine. Purification of all S-nitrosylated proteins in the brain revealed that VMAT2, VGLUT1/2 along with VACht were all nitrosylated. An increase of NO by adding a nitric oxide donor (nitroglutathione (GSNO)) led to a decrease in the amount of uptake of dopamine, acetylcholine and glutamate indicating NO inhibits vesicular uptake of neurotransmitters (Wang et al., 2015b). Neurotransmitter release is further modified by nitrosylation of syntaxin a protein involved in vesicle fusion at the membrane (Prior and Clague, 2000).

Postsynaptically, S-nitrosylation has been shown to affect clustering of receptors and scaffolding proteins. NMDA receptor subunit NR2A is nitrosylated at Cys 399 and results in inhibition of NMDA-evoked currents (Choi and Lipton, 2000). Additionally, two
cysteine residues on NMDA NR1 subunits are nitrosylated under hypoxia-induced stress further inhibiting excitatory neurotransmission (Takahashi et al., 2007). AMPA receptor activation leads to an increase in intracellular calcium through NMDA receptor activation. Increased calcium also stimulates activity of neuronal nitric oxide synthase by calmodulin leading to further production of NO (Garthwaite, 2008). NMDAR activity leads to the increase in nitrosylation of stargazin that subsequently increases the surface expression of AMPA receptor subunit GluA1 (Selvakumar et al., 2009). The increase of calcium also results in AMPA receptor subunit GluA1 phosphorylation at S831 by calcium-dependent kinases. Nitrosylation of GluA1 at C875 enhances S831 phosphorylation and reduces AMPA receptor endocytosis (Selvakumar et al., 2013). Nitrosylation of PSD-95 negatively impacts NMDA receptor clustering at the synapse further implicating a complex relationship between nitrosylation and modulation of synaptic strength (Ho et al., 2011).

Nitrosylation at inhibitory synapses is not as well studied. nNOS is present at inhibitory synapses through interactions with gephyrin and the dystroglycan complex (Yu et al., 2008, Giudice et al., 2013, Dejanovic and Schwarz, 2014). nNOS over-expression results in decreased size of gephyrin clusters in hippocampal neurons. In contrast, gephyrin palmitoylation increases clustering at the synapse (Dejanovic and Schwarz, 2014, Dejanovic et al., 2014). Although the authors did not look into the levels of both modifications simultaneously, it is likely that palmitoylation and nitrosylation are reciprocally regulated similar to what was observed in PSD-95 (Ho et al., 2011). The synaptic deficits observed in GODZ KO co-cultures may be due to an increase in nitrosylation of gephyrin, NL2 or other putative substrates such as the γ2 subunit. Future work is needed to establish the role nitrosylation plays at inhibitory synapses.
4.3 Neuroligin 2 palmitoylation in regulation of synaptogenesis

Palmitoylation is required for the function of several other cell adhesion molecules. Therefore, palmitoylation may play a critical role in the interaction between neuroligins and neurexins. Cadherins are a class of synaptic adhesion molecules essential for activity-dependent structural remodeling and enhancement of synapse density (Bozdagi et al., 2000, Mendez et al., 2010). Cadherin clustering at the synapse is increased by activation of NMDA receptors and essential for the establishment of long-term potentiation (Tang et al., 1998).

Cadherin interacts with β-catenin trans-synaptically bridging the gap between the pre- and post-synaptic cell. However, clustering of cadherin is regulated by cis-interactions with δ-catenin, p120-catenin, p0071 and armadillo repeat protein deleted in velo-cardio-facial syndrome (ARVCDF) proteins (Brigidi and Bamji, 2011). δ-catenin functionally links cadherins to the actin cytoskeleton, PSD-95 and glutamate receptor interacting protein (GRIP) (Silverman et al., 2007). δ-catenin is transiently palmitoylated by DHHC5 in response to neuronal activity and this palmitoylation increases the interaction between δ-catenin and cadherins at the synapse. Context-dependent fear conditioning in mice increased palmitoylation and colocalization of δ-catenin and cadherin. This study implicated activity-dependent palmitoylation changes of adhesion molecules in memory formation (Brigidi et al., 2015).

The neural cell adhesion molecule (NCAM) is an immunoglobulin (Ig) superfamily adhesion protein that acts via non-receptor tyrosine kinase or fibroblast growth factor (FGF) receptors. Out of the three isoforms of NCAM, only NCAM140 has been shown to influence synaptic plasticity, neuron regeneration and learning in the brain (Ditlevsen

Similarly, cis-dimerization of neuroligins is required for synapse formation and for trans-synaptic interactions with presynaptic neurexins (Shipman and Nicoll, 2012). Neuroligins 1-3 form homodimers. Heterodimers between NL1/3 and NL1/2 but not between NL2/3 were found by in situ crosslinking experiments in neurons indicating there is specificity in which NL isoforms dimerize (Poulopoulos et al., 2012). NL2 palmitoylation may contribute to homo- or heterodimerization interaction specificity as has been demonstrated for other adhesion molecules such as NCAM.

Alternatively, palmitoylation of adhesion proteins may impact their endocytosis and degradation. For example, sortilin is a lysosomal sorting receptor that cycles between the Golgi and endosomes based on its palmitoylation state. Non-palmitoylated sortilin is ubiquitinated and subject to lysosomal degradation (Dumaresq-Doiron et al., 2013). A combined affinity purified and proteomics approach identified that NL2 interacts with numerous proteins involved in the endocytic pathway such as, clathrin-associated adaptor protein complex 3 (AP-3), dynamin-3, Rab21, neurochondrin, myosin VI, and vacuolar protein sorting-associated protein 35 (VSP35). Subsequent experiments using dynasore, a selective inhibitor of dynamin-dependent endocytosis, demonstrated NL2 undergoes dynamin dependent endocytosis in neurons (Kang et al., 2014). Therefore, palmitoylation of NL2 potentially influences NL2 stability at the membrane through endocytosis.
Neuroligins have been implicated in trafficking of Slitrk; another cell adhesion molecule at synapses. The Slitrk family consists of six structurally related transmembrane proteins that contain a conserved leucine rich repeat domain that shares homology with other cell adhesion molecules such as neurotrophin (Aruga and Mikoshiba, 2003). Deficits in Slitrk family proteins have been implicated in obsessive-compulsive disorder, schizophrenia and bipolar disorder (Smith et al., 2009, Piton et al., 2011). Slitrk3 selectively induces inhibitory presynaptic differentiation in HEK 293T and neuron co-culture. Slitrk3 binds trans-synaptically with protein tyrosine phosphatase PTPδ and is required for induction of inhibitory synapses (Takahashi et al., 2012). In a similar manner, PTPσ binds to TrkC to induce excitatory presynaptic differentiation. PTPσ binds to NL3 to regulate formation of excitatory synapses implying potentially that PTPδ similarly binds to NL2 (Woo et al., 2009). Emerging roles involving inhibitory synapse organizers provide novel insights into understanding the maintenance of proper E: I balance in the brain.

4.3.1 Potential cooperative function of NL2 and GABA\textsubscript{A}R palmitoylation

Knockout of \( \gamma 2 \) also led to a reduction of NL2 at the cell surface but not a change in the total level (Murakami, 2008). These results suggest trafficking of NL2 to the cell surface and clustering at the synapse depends on the presence of GABA\textsubscript{A}Rs with the \( \gamma 2 \) subunit. The presence of the \( \gamma 2 \) subunit itself is required for proper GABAergic synapse formation (Li et al., 2005, Ren et al., 2015). However, coexpression of \( \alpha 2\beta 3 \) GABA\textsubscript{A}R subunits and NL2 in HEK 293T cells alone is sufficient to induce functional GABAergic synapses in co-cultured neurons, indicating the interaction between NL2 and GABA\textsubscript{A}Rs may not act directly through the \( \gamma 2 \) subunit (Dong et al., 2007). However, co-transfection
of NL2 together with α1β2γ2 GABA<sub>A</sub>Rs significantly increased the amount of synapses formed compared to GABA<sub>A</sub>Rs alone (Fuchs et al., 2013). It is likely that NL2 forms a complex with GABA<sub>A</sub>Rs and this interaction facilitates their translocation to the cell surface. Palmitoylation of the γ2 subunit is required for the clustering of receptors at the synapse (Rathenberg et al., 2004, Fang et al., 2006). The loss of GABA<sub>A</sub>Rs from the loss of palmitoylation potentially induces the loss of NL2 at the synapse in GODZ and DKO co-cultures. The interdependence of GABA<sub>A</sub>Rs and NL2 trafficking and palmitoylation makes trying to separate the effect of palmitoylation on either substrate challenging.

4.3.2 Deficits in GABAergic inhibition under competitive conditions

Deficits in GABAergic inhibition were observed through shRNA-mediated knockdown of the γ2 subunit in cultured neurons where the presynaptic cell exhibits no deficit in GABA<sub>A</sub>Rs (Fang et al., 2006). However, cultured neurons derived from γ2 subunit knockout embryos exhibited no deficit in GABAergic innervation (Essrich et al., 1998). However, when γ2<sup>+</sup> neurons are grown in co-culture with GFP-WT neurons, they exhibit a distinct loss of GABAergic synapses (Ren et al., 2015). A similar phenotype was observed following shRNA-mediated knockdown of NL2 but not in NL1-3 KO mice or analysis of cultures derived from these mice (Varoqueaux et al., 2004, Chih et al., 2005, Varoqueaux et al., 2006). Moreover, a deficit was observed under competitive conditions where NL1-KO neurons were grown under competitive conditions or following sparse knockdown of NL1 in mice (Kwon et al., 2012).

Subtle mutations that result in receptor deficits in neurons may not exhibit significant reduction in the number of synapses when grown in a homogenous culture of
only mutant neurons (Fang et al., 2006, Varoqueaux et al., 2006). Subtle deficits in receptor function or number may only exhibit an effect on synapse number under competitive conditions due to the inability of the neurons with a deficit to compete with trans-synaptic interaction partners for innervation with the surrounding WT neurons. Synapse formation and elimination depends more on the relative function of the neuron rather than the absolute activity level (Buffelli et al., 2003). The neuron that exhibits strongest ability to recruit presynaptic proteins trans-synaptically will win the competition for innervation (Buffelli et al., 2003, McClelland et al., 2010, Kwon et al., 2012). As shown in this dissertation, the loss of GODZ in KO mice results in significant deficits in palmitoylation of the γ2 subunit. Palmitoylation of the γ2 subunit and the γ2 subunit itself are required for normal GABAergic inhibition (Fang et al., 2006, Ren et al., 2015). Therefore, the loss of palmitoylation in GODZ KO mice likely results in the inability of KO neurons to compete with WT neurons for trans-synaptic partners.

4.3.2 Neuroligin 2 palmitoylation may indirectly affect GABAergic inhibition through interactions with other transporters

Neuroligin 2 palmitoylation may impact interactions with other receptors. The response to GABAAR activation depends on the equilibrium potential of Cl− (ECl−) across the membrane. The equilibrium potential is determined mainly by the expression of two different chloride transporters, KCC2 and NKCC1 that control the concentration of Cl− in the cell. Early in development of neurons the expression of NKCC1 predominates, leading to comparatively high intracellular Cl− concentration. Under these conditions, activation of GABAARs has a depolarizing effect (Ben-Ari, 2002, Aguado et al., 2003). Gradual upregulation of KCC2 during development leads to net export of Cl− from the cell
and a shift in the reversal potential to more hyperpolarized levels. This gradual shift in $E_{Cl}$ results in a switch of GABA from depolarizing to shunting and eventually hyperpolarizing effects of GABAA receptors (GABA<sub>A</sub>R) activation. While this shift is seen universally in all species, there is considerable variation in the time course of upregulation of KCC2 in different brain regions and species (Ben-Ari, 2002, Rivera et al., 2004).

Neuroligin 2 expression occurs prior to the developmental increase in KCC2 expression, potentially implicating NL2 in regulation of the developmental shift of the $E_{Cl}$. Knockdown of NL2 by shRNA in cultured cortical neurons resulted in a down regulation of KCC2 expression. Neuroligin 2 knockdown resulted in a shift in the equilibrium potential of GABA (Sun et al., 2013). The down regulation of NL2 delays the developmental switch between inhibitory and excitatory GABA effects in neuron culture. These results suggest a key role for NL2 in governing the chloride equilibrium potential and thereby regulating GABAergic inhibition in early development by modulating the equilibrium potential of chloride (Sun et al., 2013). It is possible that NL2 palmitoylation could affect the interaction between NL2 and KCC2.

Neuroligin 2 is also implicated in controlling serotonergic neurotransmission in the midbrain and could be affected by palmitoylation of NL2. The neurotransmitter serotonin (5-hydroxytryptophan (5-HT)) modulates overall homeostasis in cognition, emotion and sensory perception in the central nervous system (Azmitia, 2007). Availability of serotonin is limited by the expression of antidepressant sensitive 5-HT transporter (SERT) that is responsible for the reuptake of serotonin at the synapse (Bunin and Wightman, 1998). A proteomic analysis identified NL2 as a SERT interacting partner. NL2: SERT complexes were co-immunoprecipitated in a Ca<sup>2+</sup> independent manner from the midbrain of mice but not from the hippocampus that supports a cis-
interaction of NL2 and SERT. Additionally, NL2 KO mice exhibit reduced SERT expression in the midbrain and decreased 5-HT synaptosomal uptake. Behaviorally, NL2 KO mice exhibit a similar phenotype to SERT or 5-HT receptor manipulated mice (Ye et al., 2015). While the precise mechanism of NL2 regulation of SERT has not been elucidated, the interaction suggests interplay between GABAergic and serotonergic signaling in the midbrain.

4.4 Proteomics revealed other substrates of GODZ that may explain the GABAergic deficits seen upon GODZ knockdown

4.4.1 GODZ KO mice may exhibit similar neuronal deficits seen in GAP-43+/− mice

A robust decrease in palmitoylation of GAP-43 was observed by western blot and in the proteomics screen of GODZ KO mice. GAP-43 is a central nervous system protein that exists in the cytoplasm and in the plasma membrane based on a dual palmitoylation sequence and a poly-basic region in the N-terminal domain that targets GAP-43 to lipid rafts (Zuber et al., 1989, Liu et al., 1993, Liang et al., 2002). The association of GAP-43 at the membrane facilitates the interaction with PIP2 and actin. This interaction promotes actin polymerization and the subsequent restructuring of neurons. The activity-dependent remodeling of synaptic terminals is dependent on PKC phosphorylation of GAP-43 (Benowitz and Routtenberg, 1997). Homozygous knockdown of GAP-43 in mice is lethal shortly after birth due to the failure of telencephalic commissures to form (Shen et al., 2002). A mental retardation phenotype is also observed in humans with deletion in one allele of GAP-43 (Mackie Ogilvie et al., 1998). By contrast, overexpression of GAP-43 in a transgenic mouse model leads to
enhanced learning and excitatory long-term potentiation solidifying its importance in the brain (Routtenberg et al., 2000).

GAP-43 heterozygous mice exhibit stress-induced anxiety and a dislike for social novelty consistent with an autistic like behavioral model (Zaccaria et al., 2010). Heterozygous mice exhibit poorly arborized thalamocortical axons and aberrant pathfinding (McIlvain et al., 2003). GODZ KO mice exhibit a significant loss in palmitoylation of GAP-43 and this deficit potentially mimics the cellular phenotype observed in GAP-43 heterozygous knockout mice. Loss of palmitoylation results in a reduction in the localization of GAP-43 at the growth cone of developing axons. Palmitoylation facilitates GAP-43 piggybacking on exocytic vesicles, which is thought to facilitate GAP-43 transport to neurite tips (Gauthier-Kemper et al., 2014). Thus one might predict that GODZ KO neurons exhibit reduced accumulation of GAP-43 in axon growth cones and subsequently show deficits in axon pathfinding. A simple experiment to quantify the amount of GAP-43 in growth cones of WT mice compared to GODZ KO early in development would confirm this hypothesis.

4.4.2 Reduction in GABAergic inhibition from the loss of GODZ may be influenced by semaphorin adhesion molecules

PlexinB2 interacts with semaphorin adhesion molecules and were significantly down regulated in GODZ KO mice. An RNAi based genetic screen approach identified a potential role of class four semaphorins in the development of GABAergic synapses (Paradis et al., 2007). Semaphorins are secreted, transmembrane or GPI-anchored proteins that contain an extracellular Sema domain and a conserved cysteine-rich region near the N-terminus (Yazdani and Terman, 2006). They play an important role in
glutamatergic synapse formation, maintenance and axon guidance cues (Koncina et al., 2007). Sema3A regulates dendritic branching and spine maturation, whereas Sema5B mediates synapse elimination in neurons (Morita et al., 2006, O'Connor et al., 2009). Sema4D promotes rapid GABAergic synapse formation likely by recruiting other effector molecules to the presynaptic and postsynaptic sites. Sema4D mediated upregulation of GABAergic synapses occurs through the receptor PlexinB1 (Kuzirian et al., 2013).

The semaphore receptor, PlexinB1 forms heterodimers with PlexinB2 that together are necessary for Sema4A binding. While it has not been studied to date, it is possible that the action of Sema4D on GABAergic synapses similarly involves the dimerization of PlexinB1 and PlexinB2 (Nkyimbeng-Takwi and Chapoval, 2011). Our proteomics screen did not identify any PlexinB1 peptides, however it did show a 1.45-fold down regulation of PlexinB2 peptides in GODZ KO mice. Plexin proteins are not known to be palmitoylated but it represents a potential mechanism for explaining the GODZ mediated deficits in GABAergic inhibition.

4.4.3 Multiple putative substrates of GODZ allow speculation that GODZ mediated palmitoylation might alter tumor progression

Quantitative proteomics identified multiple new putative substrates of GODZ that are implicated in metastatic tumor progression. The adhesion glycoprotein CD44 receptor binds to hyaluronic acid and regulates cell differentiation, growth, survival and mobility in most cells (Ponta et al., 2003). Brain extracts of GODZ KO mice revealed a two-fold reduction in the amount of CD44 palmitoylation or abundance. CD44 also acts as a co-receptor for growth factors; and its cytoplasmic tail helps organize the actin cytoskeleton through linker proteins such as ezrin/radixin/moesin (ERM) (Terawaki et al.,
Increased expression of CD44 was correlated with the epithelial-mesenchymal transition (EMT) contributing to metastasis and tumor invasion (Xu et al., 2015). Palmitoylation of CD44 enhances its localization in cholesterol and sphingolipid enriched lipid raft domains in the plasma membrane. Mutating the palmitoylation sites on CD44 resulted in enhanced migration of breast cancer cells potentially facilitated by increased binding of ezrin and subsequent actin reorganization (Babina et al., 2014). GODZ KO mice potentially exhibit increased breast cancer tumor metastasis progression based on the significant reduction in palmitoylation of CD44.

Sorting nexin 1 (SNX1) was another protein implicated in cancer that was almost two-fold less abundant or palmitoylated in GODZ KO mice. SNX1 acts as a component of the retromer membrane-deforming complex which is critical for formation and stabilization of tubules that mediate cargo recycling (van Weering et al., 2012). In addition, SNX1 mediates epidermal growth factor receptor (EGFR) signaling by suppressing retrograde trafficking of EGFRs from endosomes to the trans-Golgi network. The suppression of trafficking facilitates EGFR/PI3K/AKT signaling (Cozier et al., 2002, Nishimura et al., 2015). Expression of SNX1 was significantly reduced in colorectal cancer tissues and is associated with poor overall survival of patients with the disease (Bian et al., 2015). However, it is not yet known if SNX1 is a palmitoylated protein. Reduced levels of SNX1 in GODZ KO mice may result in reduced endosomal recycling and increased susceptibility to colorectal cancer.

Another adhesion molecule, tetraspanin-9 (CD9), was significantly (63%) more abundant or more palmitoylated in GODZ KO mice. CD9 is involved in migration, adhesion, proliferation and interaction with other growth factors and tetraspanins. Reduced CD9 expression is associated with a poor outcome in various cancers such as,
breast cancer, lung cancer and colon cancer (Higashiyama et al., 1997, Huang et al., 1998, Mori et al., 1998). CD9 negatively modulates EGFR signaling by enhancing receptor internalization in carcinoma cells (Murayama et al., 2008). Palmitoylation of CD9 increases the association with CD151 and protects both from lysosomal degradation (Sharma et al., 2008). CD9 is predominately palmitoylated by DHHC2 and is explicitly not a substrate of GODZ (Sharma et al., 2008). As palmitoylation of CD9 was increased in GODZ KO mice this likely represents a compensatory increase in DHHC2 protein levels in GODZ KO mice. Consistent with this finding, an increase of mRNA of DHHC15 (GODZ KO; 129.3 ± 10.9% \( p < 0.05, n = 4 \)) was observed in GODZ KO mice indicating that there is likely upregulation of other PATs (Murakami, 2008). Upregulation of DHHC2 and DHHC15 could potentially explain why GODZ KO mice do not exhibit a more pronounced cellular phenotype.

The loss of DHHC gene expression has been linked to an increase in metastasis in some cancers. Reduced expression associated with metastasis protein (REAM) also known as DHHC2 mediates palmitoylation of various substrates including, eNOS, PSD-95, SNAP25, CD9 and CD151 (Sharma et al., 2008, Fukata and Fukata, 2010). The gene is localized in a region of the chromosome where frequent loss of heterozygosity and mutations have been observed in colorectal, hepatocellular carcinoma and nonsmall lung cell metastatic cancers (Oyama et al., 2000). In human gastric adenocarcinoma the amount of DHHC2 expression analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was reduced in the gastric tumor tissues in almost half of the patients analyzed (Yan et al., 2013). Reduced expression of mRNA of DHHC2 was also significantly reduced in the metastatic center of advanced colorectal cancer and knockdown of DHHC2 in A431 epithelial cells mimics tumor metastasis.
(Oyama et al., 2000, Sharma et al., 2008). Taken together, these studies support the idea that DHHC2 exhibits tumor suppressor properties.

Integrin α6/β4 is known substrate of GODZ that is implicated in metastasis and tumor growth (Sharma et al., 2012). Integrin α6/β4 mediates cell adhesion with laminin and tetraspanins in epithelial cells (Rabinovitz et al., 2001) and has become a potential drug target for pharmacological suppression of tumor invasion, endothelial mesenchymal transition, and angiogenesis (Nikolopoulos et al., 2004, Lipscomb and Mercurio, 2005, Stipp, 2010). Knockdown of GODZ resulted in reduced surface expression and accelerated degradation of α6/β4 due to endocytosis (Sharma et al., 2012). Thus, reduced GODZ mediated palmitoylation of α6/β4 integrins can readily be predicted to facilitate metastasis of tumor cells.

Gene expression of ZDHHC3 is elevated in patients with breast carcinoma and prostate cancer. Additionally, ZDHHC3 gene expression is upregulated in numerous cancer cell lines such as lung carcinoma, leukemia, and colorectal cancers (Lukk et al., 2010, Petryszał et al., 2014). Therefore, it is not surprising we found changes in numerous proteins implicated in cancer in GODZ KO mice.

4.5 Technical limitations in measuring palmitoylation

In order to understand how palmitoylation regulates pathways and proteins it is critical to link DHHC enzymes to their substrates. Traditionally, enzyme substrate pairs are identified by co-transfecting plasmids with the cDNA for each of the 23 DHHC palmitoyltransferases together with the substrate and assayed for increased palmitoylation (Fukata et al., 2006b). However, the expression of DHHC plasmids in HEK 293T cells is highly variable and not all of the transfected PATs are capable of
autoacylation and therefore likely do not function (Ohno et al., 2012). Additionally, some DHHC enzymes require the binding of other proteins to function. For example, DHHC9 requires binding of golgin A7 (GCP16) in order to palmitoylate H-Ras and N-Ras (Swarthout et al., 2005). Alternatively, a knockdown strategy using RNAi against each of the DHHC proteins was used to establish palmitoylation of calcium activated potassium channels. However, this method is further complicated by uneven knockdown efficiency and compensation by other DHHC enzymes (Tian et al., 2012). We experienced similar technical issues while trying to determine the enzymes responsible for palmitoylation of NL2. An alternative approach was used to determine DHHC specificity of gephyrin by utilizing a functional palmitoylation assay (Dejanovic et al., 2014). Dejanovic and colleagues analyzed gephyrin clustering following overexpression of each individual DHHC enzymes in neurons. Potentially this approach may be suitable also for other synaptic proteins, including NL2, as it provides an alternative way to assay for palmitoylation if the function of the protein is directly impacted by its palmitoylation state.

Another common method for analyzing post translational modifications of proteins involves site directed mutagenesis against putative target residues. In the case of palmitoylation, replacing cysteine with alanine potentially affects the overall secondary or tertiary structure of the protein, in addition to altering protein interactions, stability or surface trafficking. Indeed, replacement of cysteines with alanine disturbed membrane binding of SNAP-25 in a palmitoylation-independent manner and was restored when the same residues were mutated to leucine. Using these two types of mutagenesis the authors concluded that SNAP-25 membrane association occurs through a hydrophobic cysteine rich domain and palmitoylation is only necessary for stable membrane binding (Greaves et al., 2009). Arguably, substitution of cysteines with serine or threonine would
confer similar amino acid properties while not being able to be palmitoylated and may be better candidates for site-directed mutagenesis studies.

Enzyme-substrate identification through quantitative isobaric tag proteomics provides a large scale method to identify new substrates. However, proteomics does have an intrinsic bias towards more abundant peptides. An alternative proteomics approach utilizes heavy and light chain amino acids to reduce the variability due to isobaric tagging. For example, DHHC5 neurons labeled with heavy and light chain amino acids and enriched for palmitoylated proteins identified about 50 proteins that were potential substrates of DHHC5 (Li et al., 2012). Interestingly, the authors found a reduction in the abundance level of the total protein extract indicating that DHHC5 palmitoylation impacts the stability or expression of its substrates. Therefore, further validation of candidate proteins is necessary to confirm enzyme substrate specificity.

While we have identified multiple potential substrates of GODZ further in vitro and in vivo testing is required to confirm these proteins as substrates. In this dissertation I have shown a loss of palmitoylation of both γ2 and GAP-43 in GODZ KO mice but the amount of NL2 palmitoylation remains elusive. Potentially this could be due to inherent variability in the amount of palmitoylation of NL2 that cannot be assayed using the steady state ABE-assay. Alternatively, the kinetics of palmitoylation of NL2 might have a rapid turnover rate or it does not remain palmitoylated at the membrane. The generation of a NL2<sup>C710/766A</sup> knock in mouse might be a better tool to study the role of NL2 palmitoylation in vivo.
4.6 Overall conclusions and outlook

The current study has strengthened our understanding of the role GODZ mediated palmitoylation plays in the brain. We have identified several novel putative substrates of GODZ and confirmed the reduction of palmitoylation in GODZ KO mice for two of these substrates. Additionally, understanding the interplay between GODZ substrates is critical for understanding the function of GODZ in controlling GABAergic inhibition. Consistent with GODZ having broad substrate specificity in vitro, our proteomics screen revealed potential substrates involved in a wide variety of pathways and may significantly contribute to the excitatory: inhibitory balance in the brain.

Several adhesion molecules were identified as candidate GODZ substrates that are involved in metastatic tumor progression, possibly pointing to a key role for GODZ-mediated palmitoylation in tumor growth. Additionally, GODZ may alter the function of several ubiquitin ligases, either directly by palmitoylating them or indirectly by preventing ubiquitination of their substrates.

We found that GODZ is predominantly localized in the cis-Golgi network and in dendrites where it likely contributes to the steady-state level of palmitoylation of de novo synthesized proteins and facilitates ER to Golgi transfer. We have identified GAP-43 as a novel substrate of GODZ through proteomics and confirmed using palmitoylation assays in heterologous cells. Given that GAP-43 is important for axon grown and remodeling, we hypothesize that GODZ KO neurons exhibit deficits in axon growth and pathfinding similar to what is seen in GAP-43 +/- mice.

We have identified NL2 is subject to dual post-translational modifications, palmitoylation and nitrosylation. It is likely that palmitoylation of NL2 contributes to the
clustering of NL2 and GABA\(_A\)Rs at the synapse and could serve as an important mediator of GABAergic inhibition. The precise role of palmitoylation on the \(\gamma\)2 subunit and NL2 remains to be elucidated. Total internal reflection fluorescent microscopy can be used to assess if these proteins remain palmitoylated at the cell surface. This technique would also allow for imaging of live neurons to assay for changes in palmitoylation due to receptor activity. GODZ KO neurons did not exhibit a reduction of the surface expression of NL2 or \(\gamma\)2 (Murakami, 2008). Therefore, it is likely that palmitoylation affects the clustering of NL2 and \(\gamma\)2 at the synapse. Numerous inhibitory synaptic proteins are palmitoylated and could potentially impact the complex formation between NL2, gephyrin and GABA\(_A\)Rs. A dual functional fatty acid \(x\)-alk-16 contains an alkyne and a diazirine that can be used for photo-crosslinking palmitoylated protein complexes and will be useful for elucidating the role of palmitoylation at the synapse (Peng and Hang, 2015). This technique could also be used to identify substrates of GODZ \textit{in vivo} to further the work presented in this dissertation and to further our understanding of the role GODZ-mediated palmitoylation plays in the brain.
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