PANDION: The Osprey Journal of Research & Ideas

Volume 3 | Number 1 Article 3

2022

NGLY1 Deficiency Affects Glycosaminoglycan Biosynthesis and Wnt Signaling Pathway in Mice

Amy Batten
University of North Florida, N01109370@unf.edu

Faculty Mentor: Dr. Marie Mooney, Assistant Professor
Biology

NGLY1 Deficiency Affects Glycosaminoglycan Biosynthesis and Wnt Signaling Pathway in Mice

Amy Batten

Faculty Mentor: Marie Mooney, Ph.D.
Department of Biology
University of North Florida

Abstract

Individuals affected by NGLY1 Deficiency cannot properly deglycosylate and recycle certain proteins. Even though less than 100 people worldwide have been diagnosed with this rare autosomal recessive condition, thousands are affected by similar glycosylation disorders. Common phenotypic manifestations of NGLY1 Deficiency include severe neural and intellectual delay, impaired muscle and liver function, and seizures that may become intractable. Very little is currently known about the various mechanisms through which NGLY1 deficiency affects the body and this has led to a lack of viable treatment options for those afflicted. This experiment uses a loss-of-function (LOF) mouse model of NGLY1 Deficiency homologous to a mutation observed in affected humans that prematurely terminates the protein. Mouse pups with two LOF alleles are embryonic-lethal, and we observe gross morphological effects in the liver and brain of these pups starting as early as embryonic day 15.5 (E15.5). Given the potential for pharmaceutically improving cognitive function post-diagnosis in humans, we focused on understanding the neurological gene expression. Therefore, we harvested cortex and full brain tissue from littermates of a carrier cross at E14.5 to acquire transcriptomes for gene expression analysis. NGLY1 gene counts displayed significant dose dependency across the normal, carrier, and affected littermates with correlated effects in genes dealing with glycosaminoglycan biosynthesis, as expected, and Wnt signaling pathways. Previous knowledge of glycosaminoglycan biosynthesis and recent breakthroughs in treatments utilizing the Wnt signaling pathway provide a foundation for future treatment investigations concerning NGLY1 Deficiency and potentially other glycosylation disorders during development.

Introduction

NGLY1 Deficiency is a rare genetic disorder in which individuals lack the ability to successfully deglycosylate and recycle certain proteins (Suzuki et al., 2016). The phenotypes of this disease manifest in early childhood through delayed neural and muscular development (Rare Diseases Clinical Research Network). Less than 100 cases have been identified and therapeutic approaches are ineffective in improving quality of life. Very little is known about NGLY1 pathway interactions, leading to a lack of supported drug treatments.

Known pathway interactions include components of the Endoplasmic Reticulum-Associated Degradation (ERAD) pathway (Enns et al., 2014). The ERAD pathway identifies misfolded proteins and degrades them. NGLY1 encodes *N*-glycanase 1, which aids in the cytosolic degradation of these misfolded proteins. *N*-glycanase acts at the asparagine residues of N-liked

proteins by cutting off the initial GlcNAc (Suzuki et al., 2016). Mutations in this gene lead to an inability to recycle the proteins and block protein recycling. Specific details of the role of NGLY1 in the ERAD pathway are still unclear. Transcriptomic identification of drug targetable pathways in NGLY1 Deficiency have not been reported, though transcriptomic data have recently been published for a *Drosophila* model of the disorder and our group has generated both zebrafish and mouse transcriptomics. It is important to gain insight into gene expression of a mammalian model of NGLY1 due to the lack of phenotype in zebrafish. Mouse models of NGLY1 are embryonic-lethal which provide a druggable phenotype for future research. The purpose of this experiment was to find drug targetable pathways in an NGLY1 mouse model using 'omics' technology. Zebrafish transcriptomes point to a downregulation of cholesterol biosynthesis which was the acting hypothesis for this experiment. Transcriptomes were obtained through RNA sequencing of wildtype (WT), carrier (heterozygous), and mutant brain tissue, then analyzed using BioJupies to find the upregulated and downregulated pathways across the unaffected (WT and heterozygous) and the mutants (Torre et al., 2018).

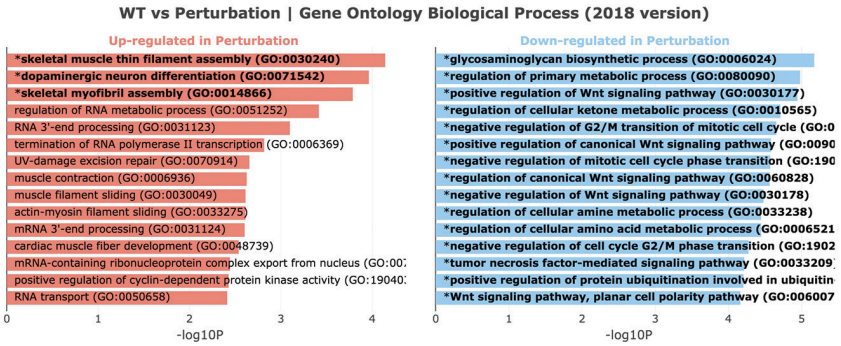
Materials and Methods

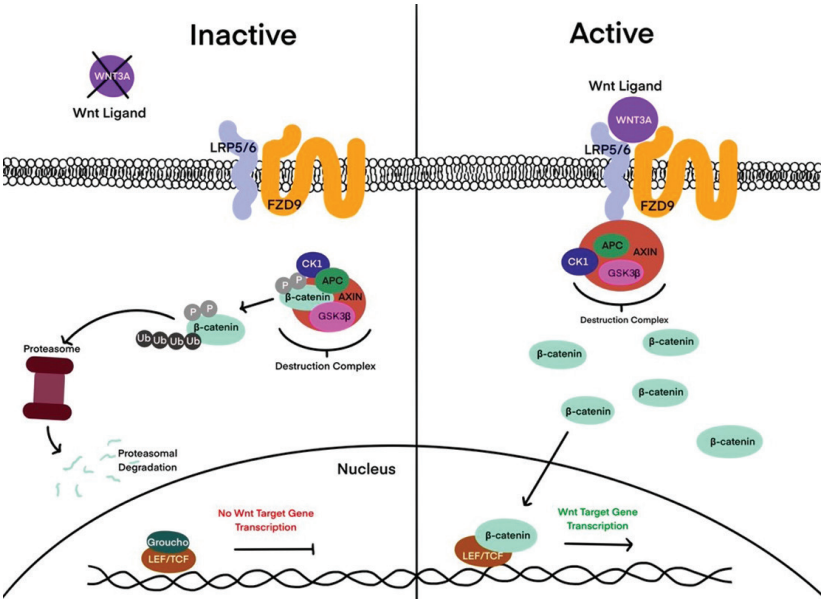
Carrier crosses were conducted, and the pups were harvested at day E14.5. Whole brain and cortex tissue were collected separately. The tissues were genotyped and subjected to 50bp single-end sequencing on Illumina HiSeq 4000 platform at the NUSeq Core at Northwestern University. RNA sequencing provided .fastq files that were analyzed using the cloud-based program, BioJupies. The BioJupies analyses combined with further analysis of raw and normalized gene counts in Excel led to the identification of pathways and individual genes that were downregulated in the mutants. Gene counts were averaged for each phenotype and the differential expression was calculated. Reactome software facilitated the creation of schematics that outlined the gene interactions between the most significantly downregulated genes. Further literature research provided insight into possible explanations and hypotheses for unknown interactions.

Results

The BioJupies analysis of .fastq files containing NGLY1 mouse RNA sequencing data showed glycosaminoglycan biosynthesis to be significantly downregulated among the mutants. Various instances of cell cycle and metabolic downregulation were also noted. Wnt signaling was downregulated as well, specifically in the canonical Wnt signaling pathway using β-catenin (Figure 1).

Figure 1. Specific metabolic and growth pathways are significantly downregulated in mutant mice. Analysis of the transcriptomic data allowed the affected genes in the mutant animals to be classified as up-regulated (highlighted in red) or down-regulated (highlighted in blue). The function of each transcript was determined using the Gene Ontology Biological Process (2018 version).





Expression of NGLY1 displayed dose dependency across the phenotypes (Figure 2). A similar dose dependency to that of NGLY1 was seen for transcripts RSPO2 and WNT2B, which are both implicated in the Wnt signaling pathway (Figure 2). Although expression levels were decreased among mutants for most of the main genes involved in Wnt signaling, there was a lack of dose dependency for the majority. The expression level for WNT3A, a Wnt ligand, was decreased in mutants with greater disparity between mutant and WT than seen in NGLY1 itself (Figure 2). FZD9, the WNT3A receptor, was decreased in mutants as well (Figure 2 and Figure 3).

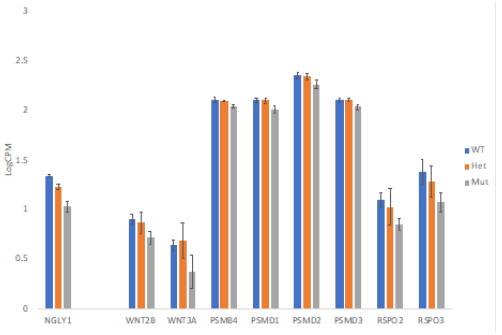


Figure 2. NGLY1 and Wnt signaling components were dose dependent across the phenotypes examined. LogCPM gene counts for wild-type (WT, blue), heterozygous (Het, orange), and mutant (Mut, gray) mice for genes associated with the Wnt signaling pathway were plotted. The error bars represent the standard error of the mean.

Figure 3 (see above). Canonical Wnt-signaling pathway in the inactive and active states. In the inactive state, β-catenin is bound by the destruction complex and eventually degraded by the proteasome. Ligand-bound receptors in the activate state sequester the destruction complex, which allows β-catenin to accumulate and translocate into the nucleus, where it binds TCF/LEF transcription factors to stimulate Wnt-signaling target genes. These genes encourage cell growth and differentiation. (Figure generated with Procreate software).

Dose dependency was less evident among genes implicated in glucosaminoglycan (GAG) biosynthesis (Figure 4). It is interesting to note that B4GALT7 and B3GLAT6 showed increased expression in mutants even though the pathway was identified as downregulated. Other genes involved in GAG biosynthesis did have lower expression levels in mutants, but overall looked to be insignificant. BCAN and XYLT1, which carry out the initiation

of the tetrasaccharide linkage formation, displayed lower levels of expression (Figure 5). Heparan sulfate (HS) was identified by Gene Ontology and Reactome as a major GAG affected by the downregulation of necessary biosynthetic genes (Figure 5).

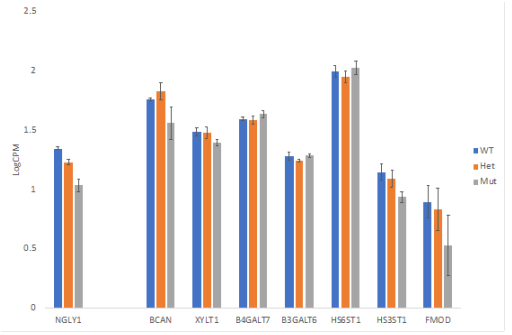


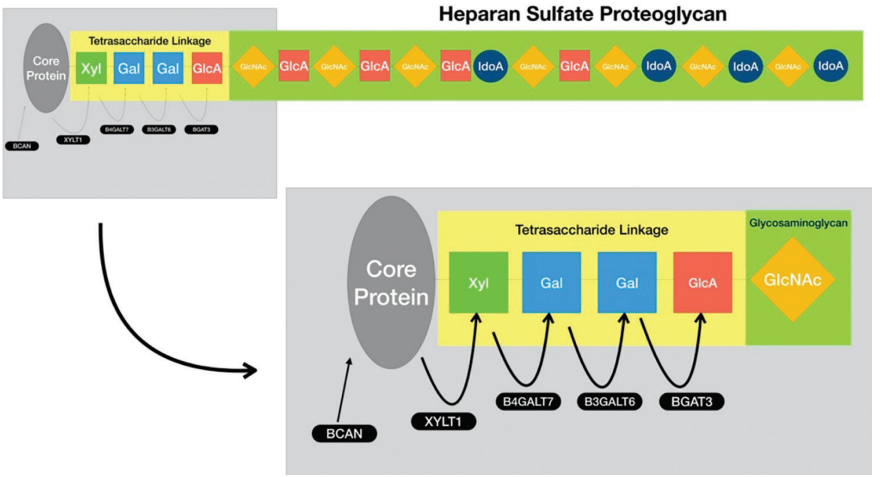
Figure 4. Glucosaminoglycan (GAG) biosynthesis components did not have dose dependent expression across the phenotypes examined. LogCPM gene counts for wild-type (WT, blue), heterozygous (Het, orange), and mutant (Mut, gray) mice for NGLY1 and genes associated with the GAG biosynthesis pathway were plotted. The error bars represent the standard error of the mean.

Figure 5. Interaction of genes identified by the Gene Ontology Enrichment Analysis in production of the GAG heparan sulfate. Carbohydrates are added to the core protein using enzymes indicated by the black ovals. (Figure generated using PowerPoint software).

Discussion

GAG biosynthesis and canonical Wnt signaling were found to be downregulated among NGLY1 deficient mice. The original hypothesis of mutant mice experiencing cholesterol biosynthesis downregulation was not supported as a main affected pathway. However, cholesterol biosynthesis may be hidden under the larger group of metabolic processes indicated to be downregulated. Downregulation of both Wnt signaling and GAG biosynthesis pathways is known to display phenotypes like NGLY1 mutants. Individually and synergistically, GAGs such as HS and Wnt signaling are crucial to embryonic development. Significant downregulation of both processes could account for the embryonic lethality of the NGLY1 mutation when homozygous.

Through analysis of the biochemical structure of the Wnt signaling components, their possible interactions with NGLY1 become clearer. The Wnt-signaling pathway relies on Wnt ligands to cause downstream sequestration of the β-catenin destruction complex by other membrane bound proteins. This is the mechanism by which β-catenin accumulates within the cell and moves down its concentration gradient into the nucleus (Komiya & Hapas, 2008). The application of this mechanism has not been studied in models of NGLY1 Deficiency. Therefore, β-catenin nuclear localization may not be occurring



as in normal models. The Wnt ligand, WNT3A, is *N*-glycosylated at two asparagine (Asn) residues along the molecule. A study by Komekado and colleagues demonstrated that glycosylation at Asn87 was critical for the secretion of WNT3A (2007). The second N-glycosylation at Asn298 has little effect on secretion when compared to the WT. Interestingly, the lack of deglycosylation seen in NGLY1 deficiency should theoretically help Wnt ligand secretion, yet an overall downregulation of the canonical Wnt signaling pathway was seen. This may indicate other areas of malfunction in the pathway, or disruption of the cycle of glycosylation all together. When dealing with N-linked fucosylation of Wnt ligands in zebrafish, Feng and colleagues discovered that increased levels of Wnt N-fucosylation decreases Wnt signaling through a negative feedback mechanism, as well as diminishes the range of Wnt signaling all together (2014). Although this study deals with fucosylation, it could possibly explain the downregulation of Wnt signaling without impaired secretion.

When looking at downstream Wnt target genes, DPAGT1 is another possible mode of negative feedback. DPAGT1 is a gene involved in the regulation of the *N*-glycosylation pathway (Vargas et al., 2016). After the initial decrease in Wnt signaling due to excessive *N*-glycosylation of the Wnt ligands, decreased DPAGT1 could propagate lower glycosylation states in the cell. This may be a cause for subsequent issues in Wnt ligand secretion. RSPO2 shows dose dependency like that of NGLY1. A study by Kim and colleagues demonstrated that RSPOs act to amplify the Wnt ligand signal by inhibiting DKK1, a negative regulator of the Wnt pathway (2008).

The end goal of the canonical Wnt signaling pathway is the facilitation of transcription of downstream target genes through increased nuclear b-catenin. Therefore, downregulation of PIN1 could lead to an inability to retain nuclear β-catenin. PIN1 is a prolyl isomerase that keeps β-catenin in the confirmation necessary to remain in the nucleus (Shin et al., 2016). An inability to keep β-catenin in the nucleus makes it less likely for the Wnt ligand stimulation to have any effect on Wnt target gene transcription. A PIN1 knockout mouse model has significantly decreased nuclear β-catenin and neuronal differentiation (Nakamura et al., 2012). The same study was able to rescue wild-type neuronal differentiation in neuronal progenitor cells through overexpression of β-catenin (Nakamura et al., 2012). With the downregulation of PIN1, decreased expression of Wnt signaling components downstream of the Fzd receptor are seen.

In terms of signaling and the binding of ligands to target receptors, Wnt ligands and GAGs like HS are connected. Binari and colleagues studied a *Drosophila* model of *kiwi* mutants (1997). The *kiwi* gene codes for UDP-glucose dehydrogenase, which is a key player in GAG biosynthesis. They found that the kiwi mutants have identical phenotypes to *wingless* (Wnt) mutants which alludes to the importance of GAGs in Wnt-mediated development. More specifically, they were able to rescue wild-type cuticular development through supplementation of HS. Wild-type individuals were given heparinase, a heparan degrader, and displayed *wingless* phenotypes as well. HS modulates Wnt signaling by binding and releasing Wnt ligands in variation (Gao et al., 2017).

Specific *O*-sulfations are needed for maximum binding affinity of Wnt ligands (Gao et al., 2017). Wnt molecules were found to have high affinities for HS chains with six to eight saccharide residues and 2-*O*, 3-*O*, and 6-*O* sulfations. In a study by Mii and colleauges, clustering of HS along the cell surface by *N*-sulfo-rich and *N*-acetyl-rich chains facilitates the colocalization of Wnt ligands and associated receptors (2020). Wnt ligands readily bind to *N*-sulfo-rich HS proteoglycans and form signalosomes that are internalized and lead to the phosphorylation of LRP5/6 (Bilic et al., 2007). Wnt ligand association with *N*-acetyl-rich HS proteoglycans is implicated in long range Wnt signaling (Mii et al., 2020).

Downregulation of GAG biosynthesis decreases the amount of HS available to assist Wnt ligands in binding target receptors. HS proteoglycans also play a role in axon guidance and synapse function (Condomitti and de Wit, 2018).

Heparan Sulfate Proteoglycans (HSPGs) are some of the largest glycosylated proteins. They consist of a core protein, a tetrasaccharide linkage, and a GAG region with many possible biochemical modifications. The gene expression data for BCAN, the gene encoding the brevican core protein of HSPGs, was downregulated in mutants (Figure 4). The mutants may be downregulating biosynthesis of core proteins due to the low levels of available GAGs for use in building larger proteoglycans. Furthermore, we propose a new biochemical function for NGLY1 cleavage at the tetrasaccharide linkage. The use of lectins to identify the presence of key HSPG structures post-exposure to NGLY1 could be used to test the proposed interaction.

Limitations of this study include the small sample size and stress responses after the harvest that influence transcription levels. Moving forward, the treatment of an NGLY1 model with a Wnt agonist could provide insight into the sensitivity of the downstream signaling components experiencing downregulation. A study by Chai and colleagues found that structural birth defects in a mouse model of deficient Wnt secretion could be overcome using a Wnt agonist (2021). They were able to rescue the development of vertebrate within the mutant tails. If developmental delay of NGLY1 models can be overcome with Wnt agonist supplementation, future use in humans may provide a simple drug treatment for the afflicted children and their families.

In conclusion, although the original hypothesis stating that cholesterol biosynthesis would be downregulated in mutants was not supported, GAG biosynthesis and Wnt signaling were downregulated. Heparin sulfate is a GAG implicated in Wnt signaling. With continued investigation, targeting Wnt signaling using a Wnt agonist could provide an inexpensive and direct way to treat NGLY1 Deficiency and a possible future treatment option for the children affected by this disorder.

References

Bilic J, Huang Y, Davidson G, Zimmermann T, Cruciat C, Bienz M, Niehrs C. 2007. Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science 316(5831):1619-22.

Binari RC, Staveley BE, Johnson WA, Godavarti R, Sasisekharan R, Manoukian AS. 1997. Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. Development 124(13):2623-32.

Chai G, Szenker-Ravi E, Chung C, Li Z, Wang L, Khatoo M, Marshall T, Jiang N, Yang X, McEvoy-Venneri J, et al. 2021. A human pleiotropic multiorgan condition caused by deficient wnt secretion. N Engl J Med 385(14):1292-301.

Condomitti G and de Wit J. 2018. Heparan sulfate proteoglycans as emerging players in synaptic specificity. Front Mol Neurosci 0.

Enns GM, Shashi V, Bainbridge M, Gambello MJ, Zahir FR, Bast T, Crimian R, Schoch K, Platt J, Cox R, et al. 2014. Mutations in NGLY1 cause an inherited disorder of the endoplasmic reticulum-associated degradation (ERAD) pathway. Genet Med 16(10):751-8.

Feng L, Jiang H, Wu P, Marlow FL. 2014. Negative feedback regulation of wnt signaling via N-linked fucosylation in zebrafish. Dev Biol 395(2):268-86.

Gao W, Xu Y, Liu J, Ho M. 2016. Epitope mapping by a wnt-blocking antibody: Evidence of the wnt binding domain in heparan sulfate. Scientific Reports 6(1):26245.

Kim K, Wagle M, Tran K, Zhan X, Dixon MA, Liu S, Gros D, Korver W, Yonkovich S, Tomasevic N, et al. 2008. R-spondin family members regulate the wnt pathway by a common mechanism. Mol Biol Cell 19(6):2588-96.

Komekado H, Yamamoto H, Chiba T, Kikuchi A. 2007a. Glycosylation and palmitoylation of wnt-3a are coupled to produce an active form of wnt-3a. Genes to Cells 12(4):521-34.

Komiya Y and Habas R. 2008. Wnt signal transduction pathways. Organogenesis 4(2):68-75.

Mii Y and Takada S. 2020. Heparan sulfate proteoglycan clustering in wnt signaling and dispersal. Front Cell Dev Biol 0.

Nakamura K, Kosugi I, Lee DY, Hafner A, Sinclair DA, Ryo A, Lu KP. 2012. Prolyl isomerase Pin1 regulates neuronal differentiation via β-catenin. Mol Cell Biol 32(15):2966-78.

Shin H, Islam R, Yoon W, Lee T, Cho Y, Bae H, Kim B, Woo K, Baek J, Ryoo H. 2016. Pin1-mediated modification prolongs the nuclear retention of β-catenin in Wnt3a-induced osteoblast differentiation. J Biol Chem 291(11):5555-65.

Suzuki T, Huang C, Fujihira H. 2016. The cytoplasmic peptide: N-glycanase (NGLY1); structure, expression and cellular functions. Gene 577(1):1-7.

Torre D, Lachmann A, Ma'ayan A. 2018. BioJupies: Automated generation of interactive notebooks for RNA-seq data analysis in the cloud. Cell Systems 7(5):556-561.e3.

Vargas DA, Sun M, Sadykov K, Kukuruzinska MA, Zaman MH. 2016. The integrated role of wnt/β-catenin, N-glycosylation, and E-cadherin-mediated adhesion in network dynamics. PLoS Comput Biol 12(7).