

Impaired N-Glycosylation Processing Impacts Neuronal Function

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Modifications of membrane proteins are carried out primarily using N-linked glycosylation. N-glycosylation has the potential to co-translationally and post-translationally modify protein function by the addition and processing of oligosaccharides. These oligosaccharides are known as glycans and are broken up into three types: oligomannose, hybrid and complex. Modification of oligosaccharides is performed by enzymes known as N-acetylglucosaminyltransferases (GnTs). Encoding of GnT proteins is performed by genes known as *Mgats*. Kv3.1b is an alpha subunit of a voltage-gated potassium channel known as Kv3 channels. Kv3 channels have the ability to open and close much more rapidly than other K channels, thus allowing neurons to create trains of action potentials. Kv3.1b is N-glycosylated at two sites, the N220 site and N229. The first aim of this study was to examine caudal primary (CaP) motor neuron development and the motor activity of WT AB larval zebrafish when the N220 site of a Kv3.1b alpha subunit is abolished. The zebrafish expressing N220Q Kv3.1b protein in CaP motor neuron displayed maldeveloped CaP neurons and a significant

reduction in motor activity throughout larval development compared to those expressing Wt Kv3.1b protein or EGFP in CaP neurons. The second aim was to determine the effect of atypical N-glycosylation processing in developing zebrafish. Herein, CRISPR/Cas9 was implemented to create the *Mgat1b* *-/-* and *Mgat1a* *-/-* fish strains for current and future experiments. *Mgat1* genes are responsible for the encoding of GnT enzymes. The *Mgat1b* *-/-* strain has increased oligomannose type glycans, as demonstrated by the Galanthus Nivalis Lectin (GNL) lectin binding assay. Up until now, the *Mgat1b* strain has been established, while the *Mgat1a* strain is still in progress. The locomotor studies indicated that the *Mgat1b* *-/-* strain is less active than the WT AB strain. The confocal images suggest cell morphology of CaP neurons are lacking branches. The third and final aim of this study examined the fully glycosylated (Wt) or the un-glycosylated (N220/229Q) Kv3.1b proteins in two different neuroblastoma (NB) cell lines. The NB_1 cell line (control), along with the N-glycosylation mutant (NB_1(-*Mgat1*) cell line were utilized for confocal microscopy experiments. The earlier and later cell lines express primarily complex and solely oligomannose types of N-glycans, respectively. Both cell lines were designed to stably express either Wt or N220/229Q Kv3.1b proteins. To examine whether occupancy influenced the distribution of Kv3.1b to the neurites (outgrowth) and soma (cell body), cell lines expressing Wt or N220/229Q Kv3.1b proteins was compared. To determine the effect due to the type of N-glycan, then Wt Kv3.1b protein expressed in each of the cell lines were compared. It was found that the percent of Kv3.1b protein present in outgrowths was higher when the Kv3.1b was N-glycosylated. Further attachment of complex type N-glycan to the Kv3.1b protein had more Kv3 channels, containing Kv3.1b protein, in outgrowth than when

oligomannose was associated with the Kv3.1b glycoprotein. My studies along with laboratory's research, conclude that N-glycosylation processing of Kv3.1b in zebrafish is critical in CaP neuron development and function, and thereby swimming locomotor activity. Further N-glycosylation processing of Kv3.31b has a vital role in distribution of Kv3.1b protein to the subdomains of NB cells.

Impaired N-Glycosylation Processing Impacts Neuronal Function

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Master of Science in Biomedical Sciences

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Abbreviations

AP – Action Potential

Asn – Asparagine

BP – Base Pair

CaP – Caudal Primary Motor Neuron

Cas 9 – Caspase 9 Nuclease

cDNA – Complementary Deoxyribonucleic Acid

CGD – Congenital Glycosylation Disorders

CRISPR – Clustered Regularly Interspaced Palindromic Repeats

DMEM – Dulbecco's Modified Eagle Media

DNA – Deoxyribonucleic Acid

dpf – days post-fertilization

EDTA – Ethylenediaminetetraacetic Acid

hpf – hours post-fertilization

EGFP – Enhanced Green Fluorescent Protein

EPHA – Erythroagglutinin Phaseolus vulgaris agglutinin

GlcNAc– N-Acetylglucosamine

GNL – Galanthus Nivalis Lectin

GnT – N-acetylglucosaminyltransferase

gRNA – Guide RNA

IREDL– Improved Restriction Enzyme Digest Ligation

Kv – Voltage-gated Potassium Channel

Abbreviations cont.

Man – Mannose

Mgat – Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase gene

MiP – Middle Primary Motor Neuron

MS222 – Tricaine Methanesulfonate

NB – B35 Rat Neuroblastoma

PAM – Protospacer Adjacent Motif

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PVDF – Polyvinylidene Difluoride

RIPA – Radioimmunoprecipitation Assay Buffer

RED – Restriction enzyme Digest

RNA – Ribonucleic Acid

RO – Reverse Osmosis

RoP – Rostral Primary Motor Neuron

SARL– Sinnhuber Aquatic Research Laboratory

SDS– Sodium Dodecyl Sulfate

SEM – Standard Error of the Mean

Sf9 – *Spodoptera frugiperda*

tracrRNA – trans-activating CRISPR RNA

wpf - weeks post-fertilization

Introduction

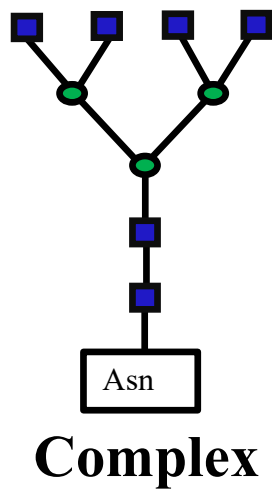
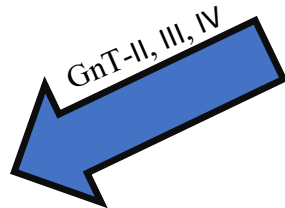
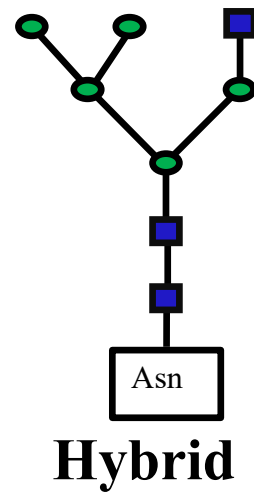
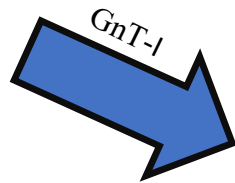
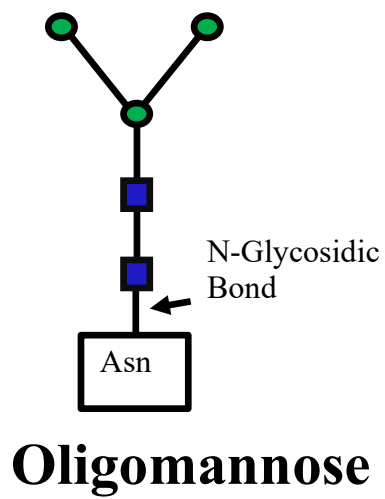
The ability to regulate protein function is critical in the development and processes of vertebrate life. N-glycosylation is one of the primary ways protein modification occurs in many living things. For example, Kv3, voltage-gated potassium channels undergo N-glycosylation processing (Hall et al., 2013). N-glycosylation involves the addition of a glycan to a N-glycosylation site of a protein (Stanley et al., 2015).

Abnormal N-glycosylation patterns can result from several congenital disorders known as congenital glycosylation disorders (CGD). CGD symptoms have been shown to affect neurological development by hindering motor skill as well as other neurological symptoms such as seizures and epilepsy. (Freeze, 2007; Jaeken, 2010). Improper N-glycosylation processing has been shown to be a factor in several neurodegenerative disorders such as Alzheimer's disease (Kizuka et al., 2017). As such, it is vital to address unmet needs of patients suffering from neurodegenerative disease.

N-glycans contain a conserved pentasaccharide with two GlcNAc residues connected to three mannose residues (Stanley et al., 2015). This pentasaccharide structure connects to an amide side chain of asparagine via an N-glycosidic bond at the N-glycan sequence Asn-x-Ser/Thr, where x is any amino acid besides proline (Schwarz & Aeby, 2011). N-glycans occur in three types, classified primarily by type and number of monosaccharides, called oligomannose, hybrid, and complex, each depicted in Figure 1 (Stanley et al., 2015). Oligomannose N-glycans have only mannose residue; hybrid N-glycans have one branch with oligomannose, and the other segment having GlcNAc with further elongation of various monosaccharides. Finally, complex N-glycans

have both branches of the pentasaccharide containing a GlcNAc residue and its extension with various monosaccharides (Stanley et al., 2015). Processing of N-glycans from oligomannose type to hybrid and complex types occurs via enzymes called mannosidases which will remove the mannose residues at the tip, followed by replacement with a GlcNAc residue via GnTs to allow further elongation of the branches of the protein (Stanley et al., 2015). *Mgat* genes encode GnT proteins. Knocking out a *Mgat* gene will alter the modification of glycan structure. *Mgat1* is responsible for the addition of a GlcNAc residue to the pentasaccharide creating hybrid N-glycans (Stanley et al., 2015). *Mgat2* converts hybrid N-glycans to complex type N-glycans (Stanley et al., 2015). N-glycosylation and N-glycans play a key role in controlling protein folding and dispersion to the membrane during neuronal development. (Ioffe & Stanley, 1994; Metzler et al., 1994).

Glycan Branching Structures



Key

-  GlcNAc
-  Man.
-  Asparagine

Figure 1 – Glycan Branching Structures – The three types of glycan branching structures. Each structure is made up of a conserved pentasaccharide made of two GlcNaC residues connected to three mannose (Man) residues, which is connected to a protein via an N-glycosidic bond. Oligomannose is the simplest N-glycan and contains the conserved pentasaccharide with only mannose residues. The hybrid type N-glycans consist of the pentasaccharide with oligomannose on one side and a GlcNaC residue on the other. Complex N-glycans have the same pentasaccharide with GlcNaC residues on the ends.

In vivo knockout studies of *Mgat1* have been performed using drosophila and mice. In drosophila, *Mgat1* null flies were viable, although it resulted in male sterility and decreased locomotor activity, and a shorter life span (Sarkar et al., 2010). A total knockout of *Mgat1* in mice is embryonically lethal at ≈9.5 days (Ioffe et al., 1996), coinciding with the neural tube development in mice that runs from embryonic days 7.0-10.5 (Chen et al., 2017). Zebrafish are distinct since they possess two copies of *Mgat1*, known as *Mgat1a* and *Mgat1b*. The two copies of *Mgat1* should most likely allow for a global knockout of either *Mgat1b* or *Mgat1a* that will survive to maturity. Since there are dual copies of the *Mgat1* gene, we expect that both *Mgat1a* and *Mgat1b* knockout fish will be viable and fertile.

Zebrafish make an ideal model organism, with thoroughly documented studies tracking development at all stages of life (Kimmel et al., 1995) (Singleman & Holtzman, 2014). Zebrafish can be housed in sufficient numbers in small spaces using modern fish husbandry rack systems, reducing maintenance. Crossing of zebrafish requires minimal

equipment, with a single zebrafish cross capable of generating around 30-300 eggs (Hoo et al., 2016) allowing for experiments requiring large sample sizes. Zebrafish develop rapidly, with neural tube formation occurring around 17-24 hours post-fertilization (hpf) (Araya et al., 2016). At 48-72 hpf, zebrafish have hatched from their chorion, ceasing the period of embryonic growth; they are now known as larvae (Kimmel et al., 1995). The larvae will begin freely swimming with the inflation of the swim bladder occurring at 5 dpf (Kimmel et al., 1995). Once free-swimming, the zebrafish larvae start to use an anguilliform swim style to move (Muller et al., 2008). This locomotion style, characterized by all forward momentum generated via an S-shaped motion of the tail and caudal fin, will last for approximately two weeks post-fertilization (Kern & Koumoutsakos, 2006). By ≈ 15 dpf, the larvae begins to take on characteristics more akin to those of adults using either a sub-carangiform or carangiform swim style (Yao et al., 2015). A sub-carangiform or carangiform swim style where the anterior region of the body is also used for more efficient movement (Borazjani & Sotiropoulos, 2010). The zebrafish genome is well studied and cataloged with CRISPR/Cas9 and transposon expression vectors capable of mutating the genome.

CRISPR/Cas9

CRISPR/Cas9 is a genome modification tool that comes from a bacteria's immune system responsible for fighting a bacteriophage by recognizing and destroying viral DNA (Jinek et al., 2012). CRISPR stands for clustered regularly interspaced palindromic repeats (Jiang & Doudna, 2017). The CRISPR sequence contains segments of previously encountered viral DNA in regularly interspaced elements in the bacteria's genome. Once viral DNA is detected, the bacteria will transcribe the viral DNA in its CRISPR sequences into what is known as CRISPR RNA (crRNA). Crisper RNA is combined with Trans-activating CRISPR RNA (tracrRNA) responsible for coding endonuclease activity to form the CRISPR/Cas9 assembly (Jinek et al., 2012). The CRISPR/Cas9 assembly searches the viral DNA for the guide RNA that is approximately three nucleotides upstream of a promotor adjacent motif (PAM) sequence. Cas9 is an endonuclease that recognizes the PAM sequence NGG (Hirano et al., 2016). This sequence signals the Cas9 Nuclease to cut the genome resulting in a double-stranded break preventing the replication of the virus (Jinek et al., 2012). In genome editing, a guide RNA requires construction that targets the desired gene to be modified. The Cas9 nuclease will cut at the selected location, leaving the cell to repair the damage, which in some cases will restore gene function but in other cases, the repair will result in insertions or deletions called indels. If this insertion or deletion is not divisible by three, this will result in a frameshift mutation and most likely silence the expression of a given gene.

Primary Motor Neurons

Primary motor neurons are neurons that begin to develop on the first day after fertilization and produce neural impulses to execute body movement in zebrafish (Kimmel et al., 1995). Zebrafish possess three primary motor neurons that exist permanently to adulthood: caudal primary (CaP), middle primary (MiP), rostral primary (RoP), (Myers et al., 1986) shown below in Figure 2. These motor neurons are present at each somite down the length of the fish. CaP motor neuron structure consists of a cell body located in the spinal cord, with a ventral axonal protrusion extending ventrally past the horizontal myotome to the edge of the axial muscles, with a hook extending dorsally (Ruzicka et al., 2019). The CaP motor neuron is the first primary motor neuron with an axon structure that leaves the spinal cord (Myers et al., 1986). The MiP neuron axon travels toward the posterior end and dorsally around the spinal cord until it reaches the dorsal surface of the muscle, where it branches along the surface of the ventral and dorsal muscle (Myers et al., 1986). The axon of the RoP motor neuron travels on a near-identical path as the CaP axon until it reaches the horizontal myotome, where it shifts direction and extends rostrally into the lateral muscle surface cord (Myers et al., 1986). All of the above-listed primary motor neurons are high firing neurons and are highly active as they are responsible for innervating the axial muscles of the fish, which require rapid signaling for movement (Westerfield et al., 1986).

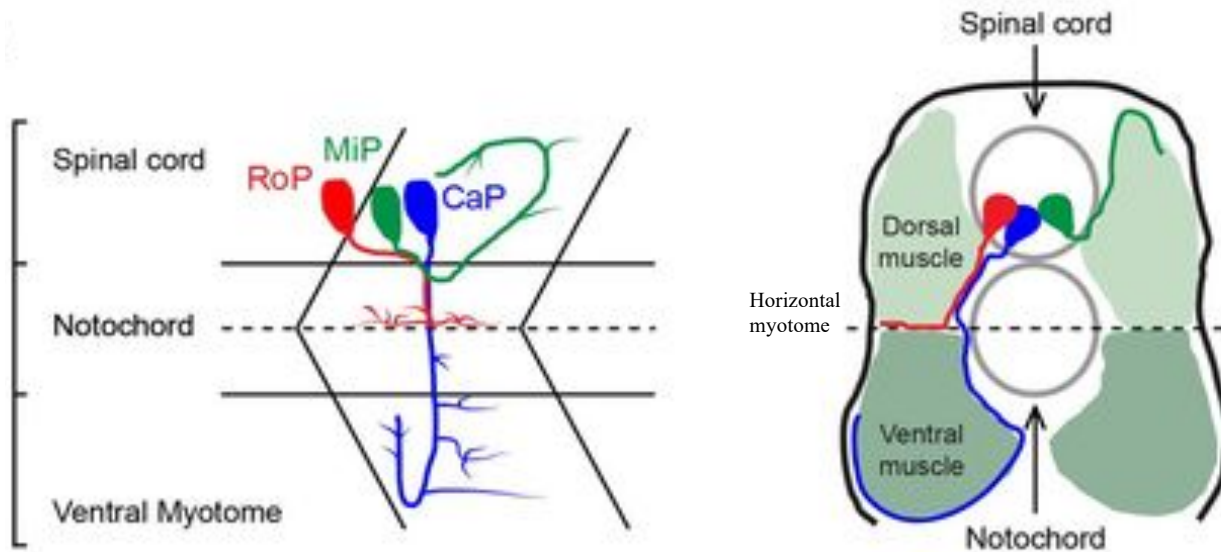


Image modified from Issa, F. A., Mock, A. F., Sagasti, A., & Papazian, D. M. (2012).

Spinocerebellar ataxia type 13 mutation that is associated with disease onset in infancy disrupts axonal pathfinding during neuronal development. *Dis Model Mech*, 5(6), 921-929. <https://doi.org/10.1242/dmm.010157>

Figure 2 – Primary Motor Neurons – Primary motor neurons located in the spinal cord of zebrafish. They can be identified by their axon orientation and the sections of muscle they innervate. Rostral Primary (RoP) has an axon that protrudes down to the horizontal myotome in the center of the notochord and then extends caudally and rostrally to innervate the muscle. Middle Primary (MiP) axon travels slightly downward and then hooks back up towards the neuron's soma. Finally, Caudal Primary (CaP) extends down beyond the horizontal myotome and forms a hook around the ventral muscle with branching innervations beginning once past the horizontal myotome.

Kv3

Voltage-gated potassium channels are broken up into four families: Kv1, Kv2, Kv3, Kv4 (Trimmer, 2015). This research focuses on the Kv3 family, the alpha subunits of Kv3 channels are encoded by: (*KCNC1* (Kv3.1), *KCNC2* (Kv3.2), *KCNC3* (Kv3.3), and *KCNC4* (Kv3.4). The *KCNC1* gene encodes for the Kv3.1b protein, which is used in our studies. Diversity of the Kv3 channel is enriched since each gene undergoes gene-splicing *KCNC1*, for example, codes for both Kv3.1a and Kv3.1b via alternative splicing (Liu & Kaczmarek, 1998). Kv3.1b is a membrane protein with six transmembrane segments (Yellen, 2002). It has two N-glycosylation sites occupied by complex N-glycans in the rat (Schwalbe et al., 2008; Vicente et al., 2018). Function of Kv3 channels has been thought to cause neurological diseases such as epilepsy and Alzheimer's (Kohling & Wolfart, 2016; Noh et al., 2019). The Kv3.1b proteins have two N-glycosylation sites known as N220, and N229 illustrated in Figure 3 below (Shi & Trimmer, 1999). Conservation of these N-glycosylation sites is evident in all Kv3 channels, and they are present along the S1-S2 extracellular loop (Shen et al., 1993), with complex N-glycans usually occupying these sites in the rat (Schwalbe et al., 2008). Previous cell cultures with Sf9 insect cells showed that partially or entirely vacant glycosylation sites did lead to a reduced activation rate and a more positive activation potential (Brooks et al., 2006). Sf9 cells are derived from insects and only express oligomannose N-glycans (Brooks et al., 2006). Alteration in the glycosylation of these sites in the NB_1 cell line led to modification of channel activity, particularly the activation time and deactivation rate (Hall, Cartwright, et al., 2011; Hall et al., 2017;

Issa, 2021). The activation and deactivation rate of the fully glycosylated form occurs approximately twice as fast as the un-glycosylated form (Hall et al., 2011).

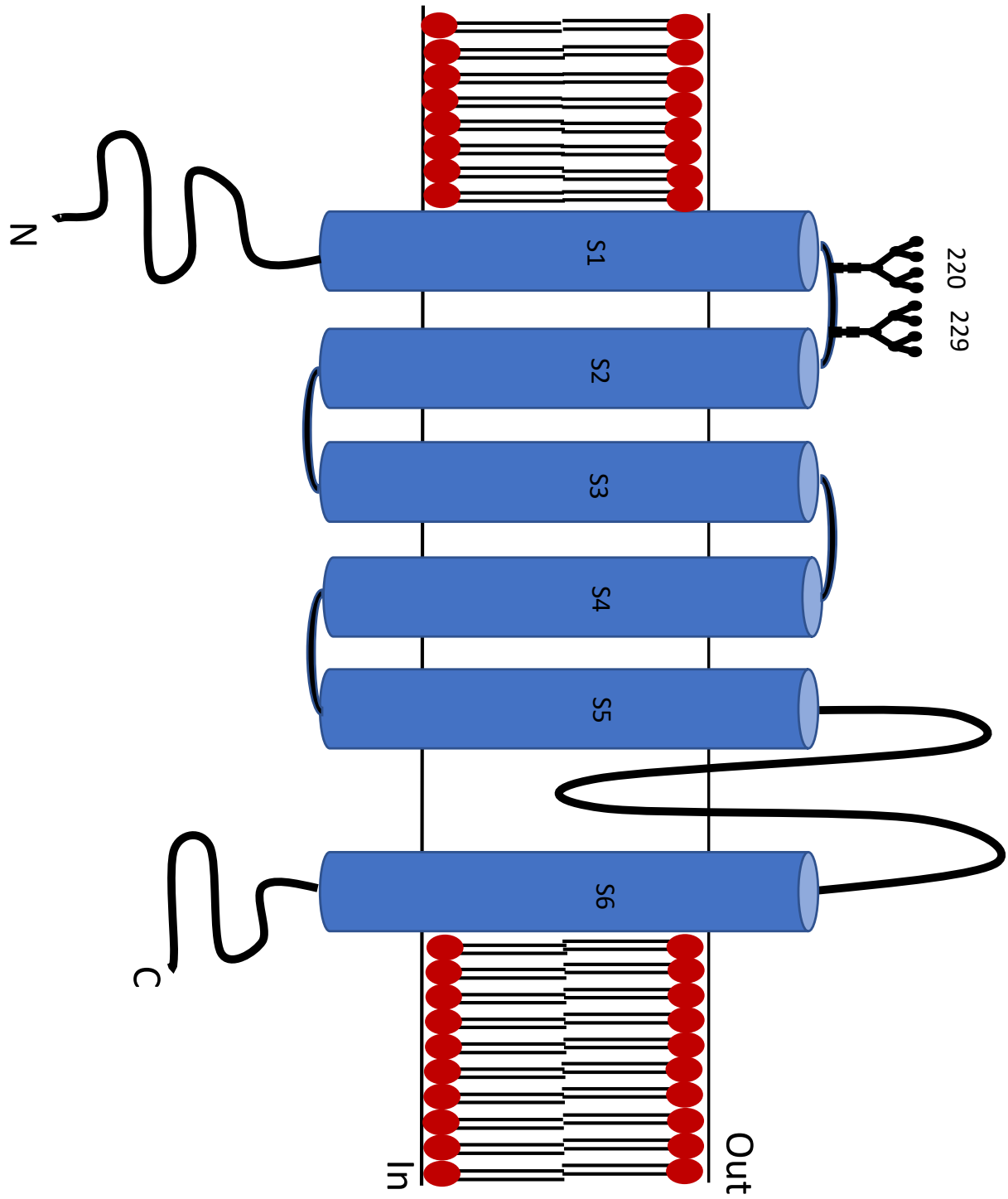


Figure 3 – Topological Model of Kv3.1b Subunit—A topological view of the Kv3.1b subunit illustrating the six transmembrane segments and the N220 and N229 glycosylation sites on the extracellular loop between transmembrane units 1 and 2.

Action potential

Neurons transmit signals throughout the body using what is called an action potential or nerve impulses. An action potential begins by changing a cell's membrane potential, which at rest is usually -65 mV (Barnett & Larkman, 2007). After incurring a stimulus strong enough to overcome the threshold, the change in membrane potential will initiate the three main phases of an action potential. These phases are known as depolarization, repolarization, and hyperpolarization. Depolarization is characterized by the opening of sodium channels. Depolarization continues until the membrane potential gets closer to the positive equilibrium for sodium, causing the sodium channels to begin to close. Repolarization begins after the closing of the sodium channels. Repolarization is characterized by the opening of voltage-gated potassium channels, allowing potassium to flow out of the cell to bring the membrane potential back toward a resting state. The repolarization of the membrane continues till the potential comes closer to the potassium electrochemical resting equilibrium of ≈ -95 mV (Ramahi & Ruff, 2014), where the voltage-gated potassium channels begin to close, leading to hyperpolarization, which gradually subsides to the resting potential. These potentials are generated in the soma and travel down the axon and into the neuromuscular junctions, which innervate the skeletal muscle causing it to contract.

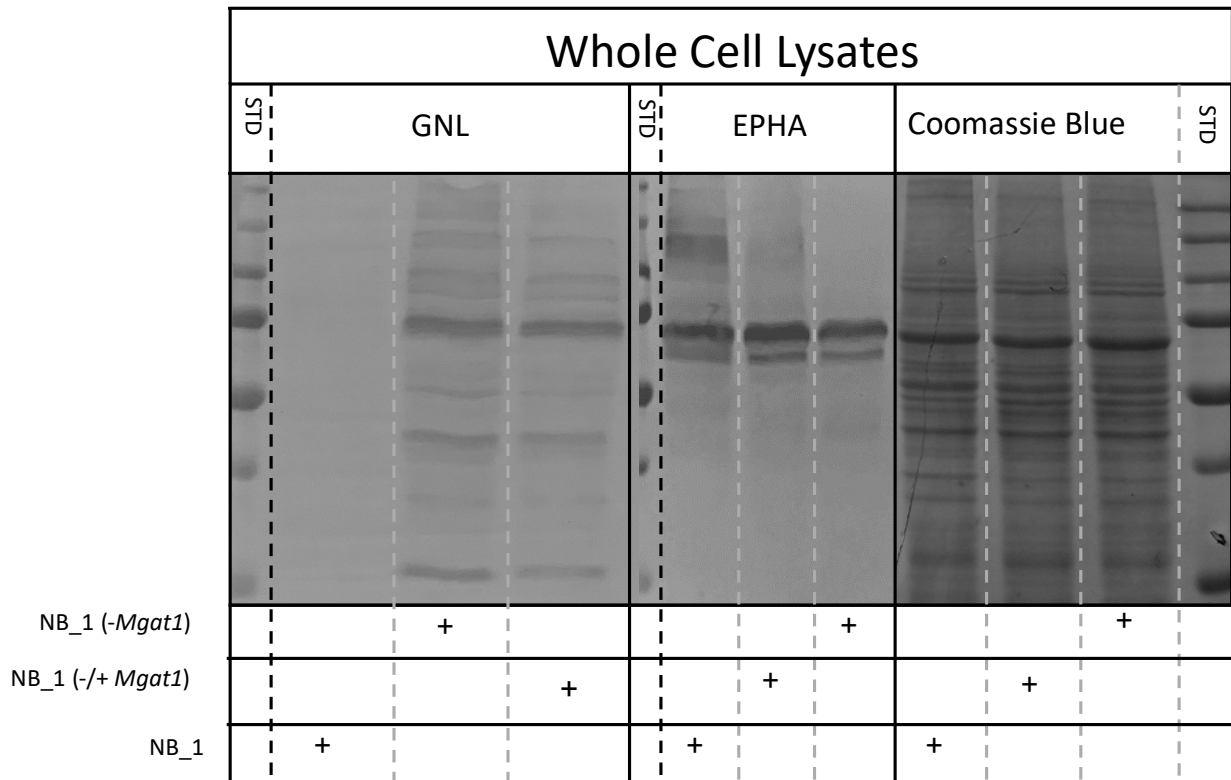
Role of Kv3.1 in an Action Potential

Kv3 channels are usually closed but open up just before the peak of an action potential in excitable tissues. The function of Kv3 in an action potential is to aid in repolarization by opening once the membrane potential depolarizes to allow potassium

to flow out and the membrane to repolarize (Kaczmarek & Zhang, 2017; Rudy & McBain, 2001). *In vitro* patch clamp studies showed a reduction in Kv3 glycan structure delayed both opening and closing rates of the Kv3 channel (Brooks et al., 2006; Hall, Reutter, et al., 2011; Hall et al., 2017). Kv3 channels are found in fast-spiking neurons, so the ability to rapidly open and close is paramount to their function (Kaczmarek & Zhang, 2017). Kv3 channels are found in fast-firing neurons, like primary motor neurons, where they significantly shorten the duration of the action potential (AP) to generate a train of nerve impulses to generate body movement and potentially motor development (Issa et al., 2021).

Cell Lines

The cell lines covered in this paper are Rat B35 neuroblastoma cell lines (Otey et al., 2003). For clarity, we will refer to the B35 cells as NB_1(Hall et al., 2017). The parental cell line (NB_1) was used to create the NB_1(-*Mgat1*) cell line, in which *Mgat1* has been knocked down. A knockout of *Mgat1* resulted in the absence of hybrid and complex N-glycans and sole expression of oligomannose N-glycans. This is supported by the lectin binding assays of the NB_1 and NB_1(-*Mgat1*) in Figure 4 and the partial sequences in Figure 5 showing the indels along at the 54th nucleotide.



Modified from (Issa, F.A.; Hall, M.K.; Hatchett, C.J.; Weidner, D.A.; Fiorenza, A.C.; Schwalbe, R.A. Compromised N-Glycosylation Processing of Kv3.1b Correlates with Perturbed Motor Neuron Structure and Locomotor Activity. *Biology* **2021**, *10*, 486. <https://doi.org/10.3390/biology10060486>)

- Figure 4** – Lectin Binding for NB_1 (-*Mgat1*) Cell Line Characterization. A knockout of *Mgat1* results in an increase in oligomannose N-glycans and a reduction in complex N-glycans. The lectin GNL binds to oligomannose glycans, and EPHA binds to bisecting complex glycans. The darker the lane, the higher the concentration present. The standard (std) adjacent to blots and Coomassie depict molecular weight standards in kDa from top to bottom: 250, 150, 100, 75, 50, and 37. Coomassie blue gel shows equal protein concentration in each lane. NB_1(-*Mgat1*) shows a higher concentration of oligomannose monosaccharides and no complex N-glycans supporting a knockout of *Mgat1*.

```

RatNB_1: ctt gtg ctt tgg ggt gct atc atc ttt gtg ggc tgg aat gcc
RatNB_1(-Mgat1): ctt gtg ctt tgg ggt gct atc atc ttt gtg ggg ctg gaa tgc
RatNB_1(-Mgat1): ctt gtg ctt tgg ggt gct atc atc ttt gtg _gc tgg aat gcc

```

Modified from (Issa, F. A., Hall, M. K., Hatchett, C. J., Weidner, D. A., Fiorenza, A. C., & Schwalbe, R. A. (2021).

Compromised N-Glycosylation Processing of Kv3.1b Correlates with Perturbed Motor Neuron Structure and Locomotor Activity. *Biology (Basel)*, 10(6). <https://doi.org/10.3390/biology10060486>)

Figure 5 – Sequences of NB_1 and NB_1(-*Mgat1*) –Upper line denotes partial genome sequence of *Mgat1* for NB_1 cell. The lower two lines are partial sequences from clones of the NB_1(-*Mgat1*) cell lines. The lower two sequences convey the knockout of *Mgat1* by either an insert of G at the 54th nucleotide location or deletion of the 54th nucleotide altogether.

Previous Studies in Our Lab

The first NB *Mgat* mutant cell line created in our lab was a knockout of *Mgat2*. *Mgat2* is responsible for the synthesis of complex N-glycans from hybrid type N-glycans (Hall et al., 2018). The results from which showed that a knockout of *Mgat2* would minimize cancerous growth. The lesser glycosylation structures impacted the cell morphology by resulting in significantly more outgrowths, but the overall length was truncated (Hall et al., 2018).

The assessment of Kv3.1 distribution in rat neuroblastoma cell lines showed that N-glycosylation plays an essential role in the spatial arrangement of Kv3.1b in the

plasma membrane of the cell. The study compared the fully glycosylated Wt Kv3.1b with the un-glycosylated N220/229QKv3.1b, partially glycosylated N220Q Kv3.1b, and partially glycosylated N229QKv3.1b glycoforms in the NB_1 cell line (Hall et al., 2015). Wt_Kv3.1 distribution was shown to be evenly distributed throughout the cell plasma membrane through a higher percentage was found in the outgrowths than the cell body (Hall et al., 2014). N220/229Q_Kv3.1b resulted in a significant reduction in outgrowth expression with a higher percentage found in the cell body. The single mutant glycoforms showed opposing patterns from the Wt Kv3.1b, with the N220Q and N229Q Kv3.1b protein expression being skewed to the cell body (Hall et al., 2014). By these results, localization is impacted differently based on the glycosylation state of the Kv3.1b protein.

Aims

This thesis aims to discern the effect of N-glycosylation processing on developing primary motor neurons and motor activity in living zebrafish and in the localization of Kv3.1b protein in neuroblastoma cell lines.

Aim 1 and 2 are to determine the impact of aberrant N-glycosylation processing Kv3.1 on motor activity, and motor neuron structure in WT AB zebrafish (aim 1) and *Mgat1b* knockout (aim 2) zebrafish expressing EGFP or the different Kv3.1 glycoforms: fully glycosylated Wt Kv3.1 or partially glycosylated N220Q Kv3.1 proteins in neural tissue. Confocal microscopy was used to examine CaP neuron development. Locomotor activity was monitored to see the *in vivo* effect of altered glycosylation in a model organism.

The *Mgat1b* zebrafish knockout strain was created using CRISPR/Cas9 technology. The *Mgat1b* *-/-* fish were subjected to locomotor assays throughout larval development to determine whether the effect on motor activity was retained throughout development. Confocal microscopy imaging was used to evaluate the cell morphology of the primary motor neurons. Since a knockout of *Mgat2* in cell lines showed a decrease in cell length, it is possible that the knockout of *Mgat1* will reduce neuron branching structure and motor activity (Hall et al., 2018).

The third and final aim was to study the effects of N-glycosylation processing of the protein on sub-domain distribution in rat neuroblastoma (NB) cells, which express either Wt and N220/229Q Kv3.1 proteins in the NB_1 parental cell line and NB_1(-*Mgat1*) knockout cell line. In order to compare, NB_1 and NB_1(-*Mgat1*) were transfected to express either Wt Kv3.1 (fully glycosylated) or N220/9Q (unglycosylated) Kv3.1 proteins. The Kv3.1 proteins were fluorescently tagged with EGFP, so the distribution can be analyzed to determine the percent of Kv3.1 relegated to the outgrowths versus the cell body. The spatial orientation was mapped with confocal microscopy and analyzed on image J software. In order to quantify the spatial arrangement of Kv3.1 proteins in the cell, the percent area of expression in the outgrowths was calculated.

Methods

Creating Recombinant Vectors for Kv3.1 and EGFP

In order to study how inadequate glycosylation affects neuronal development and motor activity, recombinant pTol vectors were created which targeted expressing to neuronal tissue shown below in Figure 6 (Issa et al., 2021). In addition, these vectors had to be fluorescently labeled to make the neuron structure visible. (Issa et al., 2011; Nakano et al., 2005). In short, BamH1 sites were added to both ends of the Kv3.1 cDNA using Polymerase Chain Reaction (PCR) (Issa et al., 2021). These sites left sticky ends when digested, which allowed the Kv3.1 segment to be ligated into the pTol vector. Next, the addition of the variously glycosylated Kv3.1 (Wt, N220Q, N220/9Q) was done upstream of the EGFP segment (Issa et al., 2021) The method used for making recombinant vectors was an Improved Restriction Enzyme Digest Ligation (IREDL) (Wang et al., 2014).

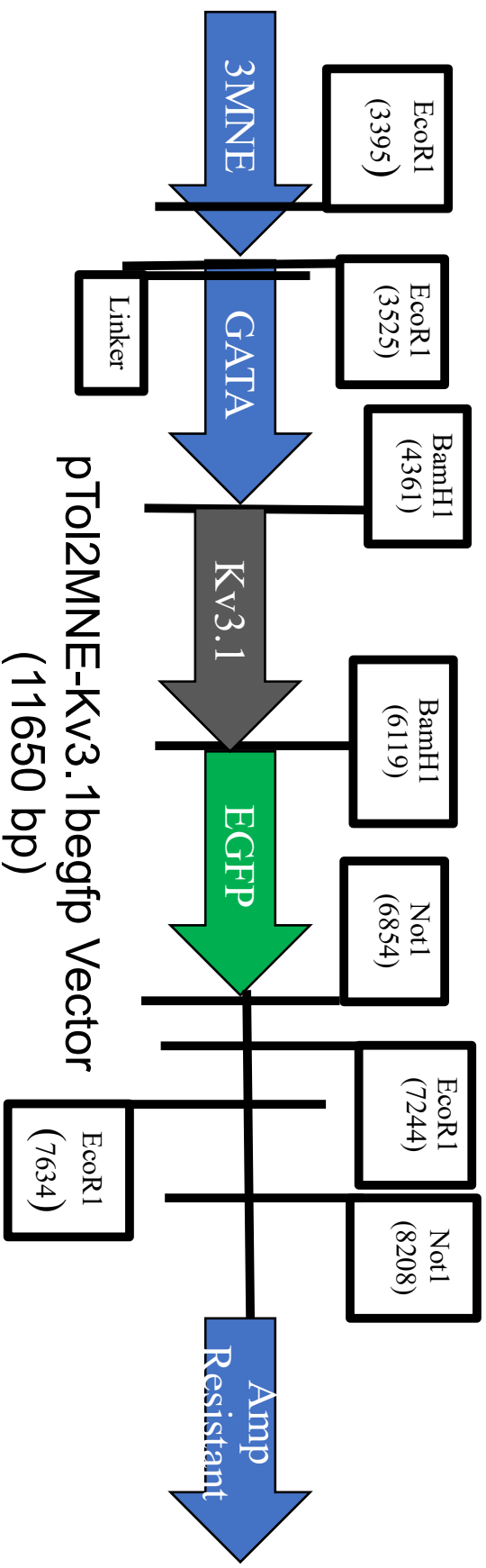
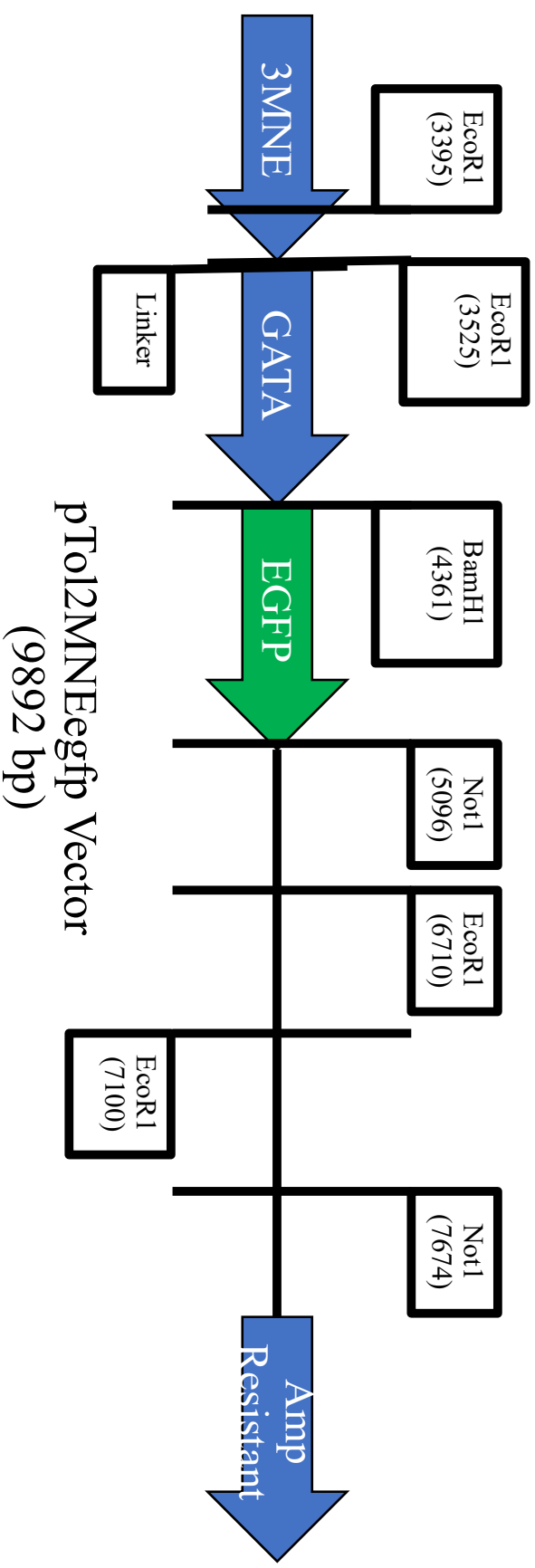


Figure 6 – Partial Vectors for Microinjections of Embryos with EGFP or Kv3.1- Vectors were created using the p_{tol}-MNE vector, which contains the MNE promotor which targets expression to neurological tissue. EGFP expression was used for screening of the injected embryos and confocal microscopy of the motor neurons. The restriction enzyme sites visible were used to ligate the Kv3.1 protein and adjacent sequence into the plasmid.

Zebrafish Husbandry

Adult *Pseudoloma neurophilia*-free WT AB Zebrafish were obtained from Sinnhuber Aquatic Research Laboratory (SARL). All zebrafish were cared for under standard laboratory conditions at 28 °C on a 14-hour light and 10-hour dark cycle and fed 2-4 times daily as previously described in (Issa et al., 2021). Housing was provided for adults using an Aquaneering zebrafish husbandry rack with a pH 7.2 and a conductivity of $\approx 775 \mu\text{S}$ (Avdesh et al., 2012). Embryos from 0 to 120 hpf are housed in 100 mm Petri dishes in a 1x embryo medium. At 5 dpf, the larvae are kept in 250 ml beakers with 50 ml 1x egg water; this concentration is adjusted each day between 5-9 dpf to gradually acclimate the larvae to the 200 ml of system water on day 9. Larvae are kept in the 250 ml beakers with 200 ml of system water until 30 dpf when they are transferred to the rack system. The larvae beakers are water changed and fed twice daily with Gemma Micro size 75 pelleted food (Skretting, Tooele, UT, USA). As the zebrafish larva are grown out, the food size is changed based on body size to Gemma Micro 150 and finally once at the adult stage to Gemma Micro 300.

The Aquaneering rack system is automated to conduct daily water changes which is required to maintain a healthy fish population, and continuously monitored the water parameters such as conductivity and pH. shown in Figure 7 below. In order to sustain the homeostatic levels necessary to support zebrafish life, the system is equipped with an auto dosing tank system that needs to be refilled every ≈ 4 days with solutions of either NaCl (conductivity) and NaHCO_3 (pH) in reverse osmosis (RO) water. Changing of all pump prefilter material was done every two months and failure to do so may result in a loss of flow to the tanks. The automatic water change system is supplied via an RO water filter fed from the tap water faucet. Several safety measures are in place to prevent chloramine from entering the aquatic housing system, such as the carbon block filter in the RO filter. Replacement of these RO filters were performed every three months



Figure 7 – Aquaneering Zebrafish Husbandry Rack – used for large scale zebrafish housing.

Breeding Zebrafish

Breeding of zebrafish was carried out in 2 L poly-carbonate static breeding tanks shown in Figure 8 below, which consist of a grated breeding insert and a divider to separate the fish until the desired crossing time as first described in (Mullins et al., 1994). Zebrafish have few sexually dimorphic traits that begin to appear around six weeks post-fertilization (wpf) that become more visually apparent at approximately 12 wpf (Darrow & Harris, 2004). The most common trait used to distinguish the genders is a protrusion of the belly from eggs as shown in the female, as shown in Figure 9 (Spence & Smith, 2007). Females are also discernable by their overall rounder appearance (Paull et al., 2008). The male to female ratio in a breeding tank is on average 1:2 as previously described in (Christian, 2011). The crosses are set up the

evening before and the following morning were initiated by the removal of the divider; embryos delivered were collected during this time were collected using a strainer and rinsed with tap water and RO water. After rinsing, the embryos were transferred into a 100 mm plate with 1x egg water and cared for as listed above in zebrafish husbandry.



Figure 8 – Static Zebrafish Breeding Tank - Embryos generated fall through the perforated insert and are collected at the bottom. The insert is removable so that the adult fish can be moved to a fresh tank and the embryos harvested.



Figure 9 – Adult Zebrafish Sexual Identification - Female Zebrafish are discernable from males by features such as a protruding belly and overall rounded shape.

Microinjections of Kv3.1

Expression of EGFP, and EGFP tagged Wt and N220Q Kv3.1b proteins in CaP neurons of zebrafish were conducted as previously described (Issa et al., 2021).

Micropipettes were pulled using a Sutter Instrument Company SU-97 Micropipette puller (heat 28, pull 30). Adult *Pseudoloma neurophilia*-free WT AB zebrafish were crossed to generate embryos for microinjection. Embryos were harvested and loaded onto a 4% agar plate with pre-molded egg holding grooves molded in place created using WPI Zebrafish MI Z-Molds. The pulled tip was then cut to the proper size using a razor blade and loaded with a solution of pto12MNE-EGFP plasmid, transposase, and phenol red. Embryos were injected using nitrogen at around 12-15 psi and a WPI PV820

Microinjection pump. After injection, embryos were removed from the agar plate and placed into a clean 100 mm dish, containing egg water for 36 hours. followed by screening for expression of EGFP in motor neurons.

Construction of *Mgat1a/b* Knockout Fish Strains

For the generation of *Mgat1b* and *Mgat1a* knockout fish strains, *Pseudoloma*-free WT AB zebrafish were crossed to generate embryos for microinjections. Embryos were microinjected with the following reagents: *Mgat1b* or *Mgat1a* gRNA, Caspase 9, and phenol red to create the F0 generation, as designed and conducted by Dr. Schwalbe (PI) and Ms. M. Kristen Hall (Research Lab Specialist). After injection, my role was to contribute to maintenance and growth of fish to adulthood for F3 generation of the *Mgat1b* knockout fish strain and F0 generation of the *Mgat1a* knockout fish strain.

Genotyping Zebrafish

Microinjected zebrafish were grown to 3 months of age before conducting the genotype procedure. Genotyping was conducted by Mrs. M. Kristen Hall, Mr. Theodore Blume, Dr. Schwalbe, and I. The fish were transferred from their tank using a net and placed in a 0.025% MS222 solution until operculum movement ceases, rendering the fish anesthetized. A portion of the ventral lobe of the caudal fin was clipped from the distal cleft to the proximal edge of the fin using clean surgical micro-scissors. The clipped fin portion was placed in a labeled clean tube, followed by the addition of 25 μ l of 50 mM NaOH. Dissolution of the fin is carried out using an MJ Research MiniCycler at 99°C for 15 minutes. Primer-directed DNA amplification of the dissolved genomic material was done using a Bio-Rad iCycler. Once amplified, BclI (*Mgat1b*) restriction

enzyme digests were used at either 37°C with 2 hour and 15-minute incubation time, or 65°C for 1 hour, respectively, to cleave the DNA fragment. Digested DNA samples were run using either 1.5% or 1.2% agarose gels with ethidium bromide and a 1 Kb ladder. The presence of a single band at 572 bp indicated a homozygous mutant, two bands at 372 bp and 204 bp indicated a homozygous wild type, and all three band sizes present indicated the fish was heterozygous. The *Mgat1b* homozygous Wt have normal N-glycosylation and were created via the crossing of the *Mgat1b* heterozygous mutant with Wt AB or other heterozygous mutants.

Microinjection of *Mgat1b* Knockout Strain with pToIMNEegfp Plasmid

To obtain confocal images of CaP motor neurons in the knockout *Mgat1b* ^{-/-} zebrafish, microinjections of embryos with pToIMNEegfp recombinant vector, transposase, and phenol red were conducted, as described above under sub-heading: Microinjections of WT AB with Kv3.1. the WT AB fish.

Screening Embryos for EGFP Expression in Primary Motor Neurons

Microinjected embryos were grown to 36 hpf at 28 °C in a 100 mm dish in 1x egg water at 28 °C. Embryos were dechorionated using needles and/or forceps. The screening was carried out using an Olympus fluorescent microscope at 10X and 20X magnification. Careful observations were taken to select embryos expressing EGFP in the cell body and axon of primary motor neurons. These positive embryos were transferred to a 100 mm dish for confocal microscopy. An example of a positive embryo observed during looks like during the screening process using a 20X objective can be

seen below in Figure 10. WT AB expressing EGFP and EGFP tagged N220Q Kv3.1 or Wt Kv3.1 positive embryos were also used for motor activity studies.

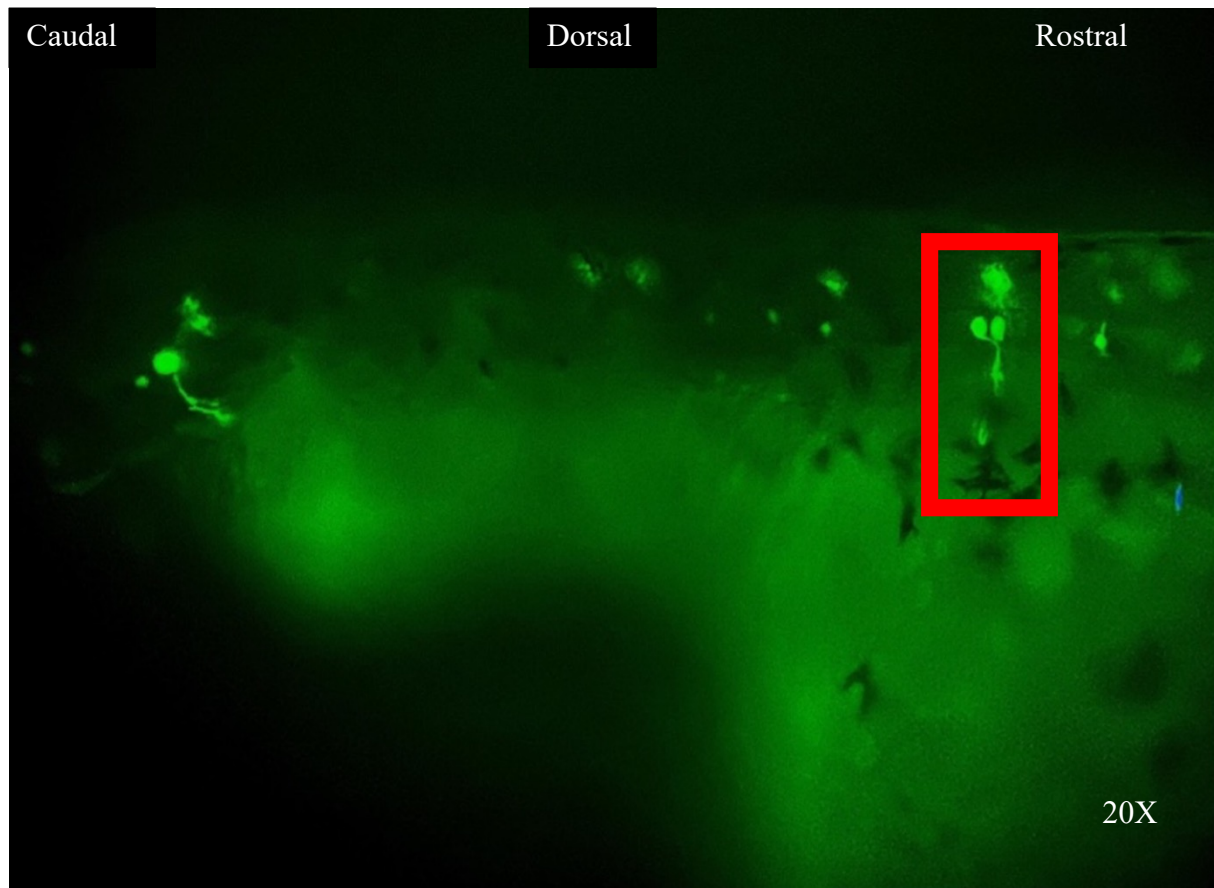


Figure 10 – Image of *Mgat1b* Dechorionated Embryo Expressing EGFP During Screening – This is an example of a positive embryo with motor neurons brightly displaying EGFP. The red box inset shows a CaP neuron with EGFP expression.

Larval Zebrafish Motor Activity

Wt AB adult zebrafish were crossed to generate embryos. These embryos were harvested and then injected with one of the following recombinant vectors: EGFP, EGFP- N220Q Kv3.1 or EGFP- Wt Kv3.1. At 36 hpf, the embryos were screened, and EGFP positive embryos were transferred into 100 mm dishes in embryo medium till 5 dpf at 28 °C when the first time point is recorded. Following the 5 dpf time point, the larvae would be transferred into a 250 mL beaker containing 50 mL 1x egg water with 12 larvae per beaker. The larvae were transferred at each time point using a transfer pipet into a 6-well plate with one larva per well with embryo medium. An acclimation period of 6 minutes was carried out on the benchtop before loading the plate into the Zantiks MW machine. The protocol would then be selected and once started, would track and record in pixels the distance the fish traveled for a total of 15 minutes. Fish with no movement recorded for the 15 minutes were removed and the data discarded. The recorded pixel values were then converted into mm values and the average distance travel (mm)/min and graphed for comparison. An image of the Zantiks MWP unit used to record the motor activity is shown in Figure 11. The protocol as described above was also carried out with *Mgat1b* strain larva beginning at 5dpf.



Figure 11 – Zantiks MWP Locomotor Tracking Unit – Used for tracking larval zebrafish movement from 5 days post fertilization onward.

Zebrafish Motor Neuron Morphology using Confocal Microscopy

Embryos actively expressing: EGFP- N220Q Kv3.1 or EGFP- Wt Kv3.1 were anesthetized with 0.02% Tricaine MS-222 at 48 hours and embedded in 1% low melting temperature agar on a depression microscope slide. The agar was cooled over ice before adding it to the fish to prevent harming the fish. Confocal imaging was performed with a Zeiss LSM 800 upright confocal microscope with 20X and 40X water immersion lenses. Image stacks were taken in 1um segments for analysis—3D models of the captured neurons were created using Imaris neuronal image processing software. Given that zebrafish neurons vary significantly in size as the spinal cord travels down the length of the body, so to remove size as a variable, all neurons used for analysis were located between the 10th and 18th somite range.

To conduct confocal microscopy studies of the *Mgat1b* strain, a procedure was established to use an inverted confocal microscope. *Mgat1b* strain embryos expressing EGFP were embedded in 0.5% agar using distilled water was made in 1.5 ml aliquots and kept in a heat block to keep it from solidifying. A single EGFP expressing dechorionated embryo at 48 hpf was transferred onto a poly-lysine coated 35 mm glass-bottom dish for mounting. A pipet set to 4 μ l was used to remove excess water from around the embryo. Hot agar is drawn up into a transfer pipet and cooled till it was warm to the touch over an ice pack. Testing the temperature can be done by placing a single drop on the back of the hand to ensure a safe temperature. Once cooled to a safe temperature, 1-2 drops of agar were used to secure the embryo to dish on its side, and using a pipet tip, the agar was smoothed to ensure the fish is touching the glass. Once the agar was added, allow it to cool on the benchtop for 2-3 minutes before placing the plate on the ice pack to accelerate the cooling process. Attempting to move the plate too early after adding the agar can lead to the embryo moving into an unfavorable position or away from the glass. It was imperative during the mounting phase that the fish be mounted directly to the glass and not be floating in the agar. Embryos not correctly mounted to the glass will lead to focusing issues at a magnification greater than 10X. 1-2 drops of MS-222 at a concentration of 0.025% to anesthetize the embryo preventing the embryo from twitching inside the agar during imaging. *Mgat1b* Confocal images were taken using a Zeiss LSM 700 inverted confocal using a 488 nm laser setting; all confocal images were taken using either a 20X objective or a 40X oil immersion objective. Image slices were taken at 1 μ m intervals to create a 3D structure of the neuron to study branching patterns of the neuron. Once the

imaging was completed, embryos can be removed from the plate by adding warmed egg water to soften the agar before pulling it apart with forceps to free the embryo.

Lectin Blotting of *Mgat1b* Zebrafish

The larvae were harvested flash frozen and placed in a RIPA buffer+EDTA with ten larvae per tube. The RIPA buffer consists of the following: PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS. The larvae are then homogenized via sonication to release the protein from the fish. The homogenized protein was treated with reducing buffer plus DTT and incubated for 2-3 min at 99 °C. Samples were loaded onto an SDS-PAGE acrylamide gel and ran for 90 minutes at 20mA. Once the protein has run down the gel, the stacking gel portion was removed and thrown away. The separating gel is placed in Coomassie blue stain for 30 minutes at room temp to ensure equal protein concentration, although the same number of fish was used per strain, the size of the fish was variable. After staining, the stained gel was placed in a de-staining solution for 45 minutes, so only the protein on the gel is stained. The gel was then visually analyzed to determine if the proteins from each strain were of similar concentration. If they were not, then the loads were adjusted and reran to ensure the modified load was indeed equal across the genotypes.

A new gel was run with the adjusted protein concentration levels from the Coomassie for the lectin binding assay. The gel removed from the frame, and the stacking gel was removed and discarded. The separating gel was placed in an immunoblotting transfer insert against a washed 0.45 PVDF membrane; the tank is then filled with CAPs buffer and methanol solution and ran at 0.25mA for 2 hours. After the

transfer, the gel was discarded, and the transferred membrane was placed in a blocking buffer. The blocked membrane was incubated with a lectin solution for 24hrs. The lectin incubation was followed up by incubation with streptavidin for an hour. The membrane was then washed four times at five minutes each. Proteins were visualized by incubation of the membrane in developing solution. Next, the membrane is placed in water to stop the development and then placed on a paper towel to dry before imaging.

NB Cell Lines

Neuroblastoma (NB) cell lines were acquired, engineered, and maintained as previously described (Hall et al., 2018) (Issa et al., 2021). Parental NB cell lines expressing EGFP tagged Wt and N220/N229Q Kv3.1b proteins are called NB_1_Wt Kv3.1b and NB_1_N220/229Q Kv3.1b cell lines. The N-glycosylation mutants are referred to as NB_1(-*Mgat1*)_Wt Kv3.1b, and NB_1(-*Mgat1*)_N220/229Q Kv3.1b cell lines.

Confocal Microscopy of NB Cells

Cells were provided on 35 mm glass bottom poly-lysine coated dishes for confocal microscopy recordings. One-micron image slices using a 488 nm laser were acquired for each cell line. These stacks were compressed, giving an extended depth of focus so that all focal planes were visible in 2D images. To determine the percentage of Kv3.1b expression in the outgrowths, ImageJ software was used. The area of fluorescence with a threshold of (125-255) and particle size (5-10,000) was calculated for the whole cell and then just the outgrowth.

Statistical Data Analysis

Statistical analysis of the presented data was carried out using GraphPad Prism software v9.1.2. Adobe Photoshop was used for the preparation of the lectin blots and agarose gel images. Image J and Zen software was used to prepare the confocal images. The data is shown with mean and standard error of the mean. Statistical comparison was achieved using One-way Anova for the locomotor activity and confocal images of the cells. Statistical significance was considered at $P < 0.05$ unless otherwise illustrated.

Results

Zebrafish Kv3 Glycosylation Site

To ascertain the effect of Kv3.1b protein glycosylation has on zebrafish primary motor neuron development and locomotor activity, zebrafish were designed to heterologously express Wt or N220Q Kv3.1b proteins. Zebrafish were microinjected with recombinant vectors expressing either partially glycosylated N220Q Kv3.1 or fully glycosylated Wt Kv3.1b proteins. The expression of these proteins was in addition to endogenous Kv3.1 but was targeted to motor neurons via the motor neuron enhancer (MNE) from mice. Since Kv3 channels are found in faster firing neuronal tissue, including CaP neurons (Issa et al., 2011; Issa et al., 2012), CaP neurons were the primary target as they are responsible for innervating the muscles for movement in developing zebrafish. AB strain zebrafish embryos were microinjected with the recombinant vectors Wt Kv3.1b or N220Q Kv3.1 tagged with EGFP, or with EGFP vector only, and then embryos were screened for EGFP expression in CaP motor neurons at 36 hpf. At 48 hpf, the zebrafish larvae were mounted for confocal microscopy by our collaborator to image CaP neuron structure. These images are shown in Figure 12 see below.

Neuron length, neuron volume, and number of nodes were determined to evaluate the neuron structure. A decrease in these measurements would reduce the neuron's ability to innervate the muscles needed for locomotion. The results of CaP neurons from Wt Kv3.1 or EGFP expressing zebrafish were not significantly different. The N220Q strain on the other hand, resulted in a significant reduction in branching

patterns and fewer synapses. The injection of the completely un-glycosylated form of Kv3.1, the N22022/9Q was embryonically lethal.

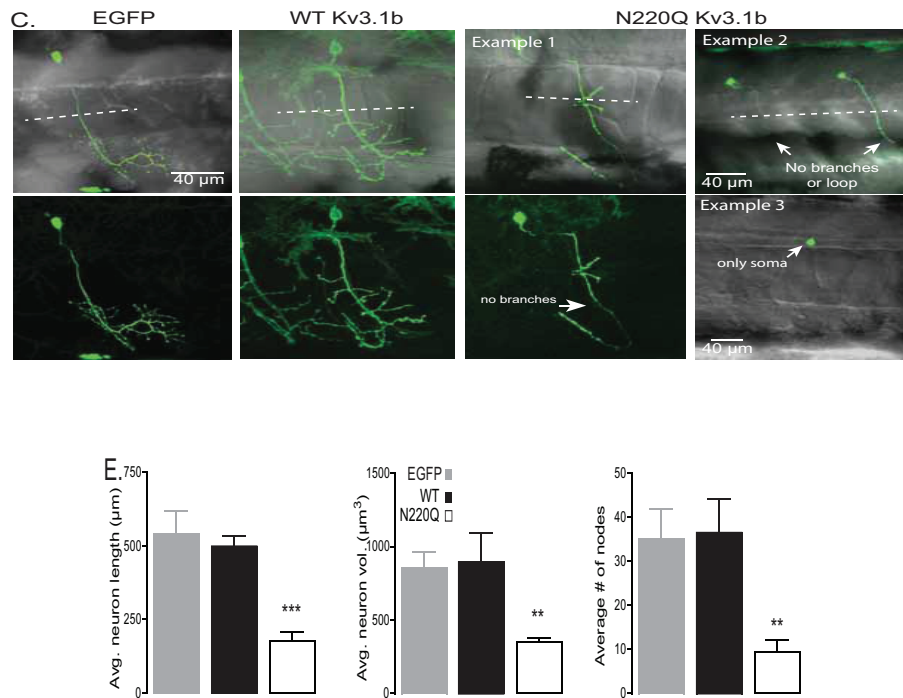
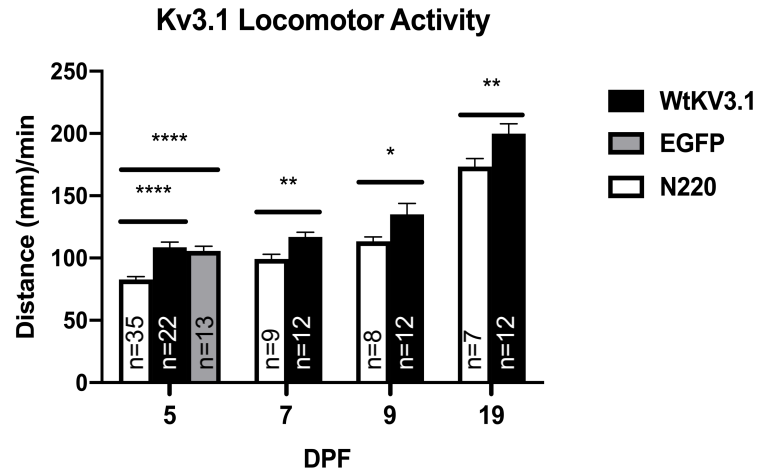


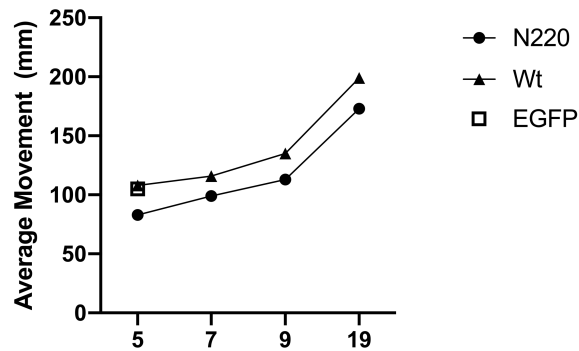
Figure 12 – Confocal Projections of CaP Neurons Expressing EGFP, WT and N220Q Kv3.1b Proteins. The bottom row shows confocal images, and top row images are overlaid with transmitted images to show the horizontal myoseptum (dashed line). Average axonal length, neuron axonal volume and number of nodes of CaP neurons expressing EGFP ($n = 12$), WT ($n = 10$) and N220Q ($n = 11$) Kv3.1b proteins. Graphs denote mean $\pm$ SEM and were compared by one-way ANOVA followed by Kruskal-Wallis adjustment (** $p < 0.0005$, *** $p < 0.0001$).

Modified from (Issa, F. A., Hall, M. K., Hatchett, C. J., Weidner, D. A., Fiorenza, A. C., & Schwalbe, R. A. (2021). Compromised N-Glycosylation Processing of Kv3.1b Correlates with Perturbed Motor Neuron Structure and Locomotor Activity. *Biology (Basel)*, 10(6). <https://doi.org/10.3390/biology10060486>)

To see if the reduction in axon length also coincides with reduced motor activity, the larvae motor activity of larvae detected to express EGFP, Wt or N220QKv3.1b proteins in embryonic CaP neurons was assayed. Locomotor activity was recorded beginning on day five of post-fertilization. Figure 13 below shows the mean distance traveled in mm per min. The results showed no significant difference between Wt Kv3.1 and EGFP, only expressing larvae. However, N220Q expressing zebrafish showed a 30% reduction in locomotor activity compared to both the Wt Kv3.1 and EGFP expressing larvae. This trend continues through at least 19 dpf, with the N220Q showing retarded motor activity throughout development. This supports the idea that N-glycosylation occupancy of Kv3.1b is essential for Kv3 channel function, and on a larger scale, organismal development.



Kv3.1 Developmental Locomotor Activity



Modified from (Issa, F.A.; Hall, M.K.; Hatchett, C.J.; Weidner, D.A.; Fiorenza, A.C.; Schwalbe, R.A. Compromised N-Glycosylation Processing of Kv3.1b Correlates with Perturbed Motor Neuron Structure and Locomotor Activity. *Biology* **2021**, *10*, 486. <https://doi.org/10.3390/biology10060486>)

Figure 13 - Motor Activity of Kv3.1b Zebrafish- This shows the average motor activity as the fish develop over time comparing the larvae injected with EGFP, EGFP tagged Wt Kv3.1, or EGFP tagged N220Q. The embryos with the N220Q glycosylation site knocked out saw a significant reduction in motor activity. The Wt Kv3.1 larva showed no significant difference from the EGFP only larva. Compared via one-way Anova (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$)

Creation of *Mgat1b* and *Mgat1a* KO Fish Strains

To determine the effects of increased oligomannose N-glycans in zebrafish neuron morphology and motor development, *Mgat1a* and *Mgat1b* was knocked down. CRISPR/Cas9 and gRNAs was used to knockout the aforementioned genes resulting in a frameshift mutation. The location of the indel was at a specific restriction enzyme site relative to the PAM sequence. Cas9 cuts at the restriction enzyme site located three nucleotides upstream of the PAM sequence. Identifying the different mutant genotypes was carried out using PCR for DNA amplification followed by a restriction enzyme digest.

After amplification with PCR, samples were digested using BclI and ran on a 1.5% agarose gel shown in Figure 14 below. If a frameshift mutation occurred, resulting in a *Mgat1b* homozygous mutant, only one band would be present at 576 base pairs (bp), which is the size of the whole amplified fragment. If two bands were present, the restriction site was not knocked out indicating a WT. These bands are found at 376 and 204 bp. If three bands were present at: 576, 376 and 204 bp, then a heterozygous mutant was present. F0 and F1 generations were also sent for DNA sequencing to identify the specific indels. The F0 generation was crossed with Wt AB zebrafish to generate the F1 generation. Heterozygous genotypes, which were confirmed via genotyping and DNA sequencing showing a 5 nt indel present. A male and female with identical mutations from the F1 generation zebrafish were crossed with itself to generate the F2 generation and resulted in the following genotypes: +/+, +/-, -/-. The F2 generation is what was used to produce embryos for injection and locomotor studies.

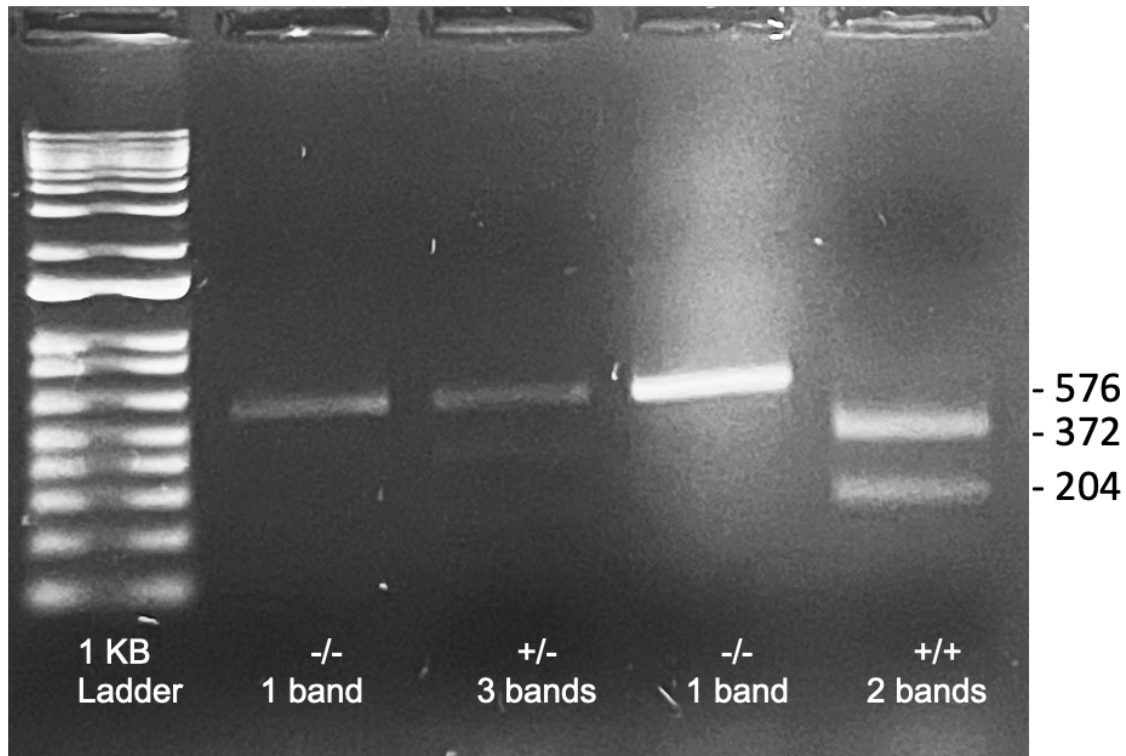


Figure 14 – Generation of *Mgat1b* Fish Strain – BclI restriction enzyme digest used to identify *Mgat1b* strain genotypes.

Next, the construction of the *Mgat1a* knockout fish strain was carried out. *Mgat1a* mutated zebrafish were identified using the Tsp45I (NmuCI) restriction enzyme after amplification with PCR. The *Mgat1a* strain at the moment of writing this thesis is in the process of generating the F1 generation. So the only genotyped generation is the F0 generation. The F0 genotyping results can be seen below in Figure 15. A heterozygous mutant will have three bands at: 486, 282, 136 bp. A WT will have two bands at 286 and 136 bp. Although none have been generated yet, a *Mgat1a* homozygous mutant will have only one band at 486 bp as an indel will most likely knock out the restriction site. The amplified *Mgat1a* fragment from the F1 generation of *Mgat1a* zebrafish will also be sequenced to identify the indels once generated.

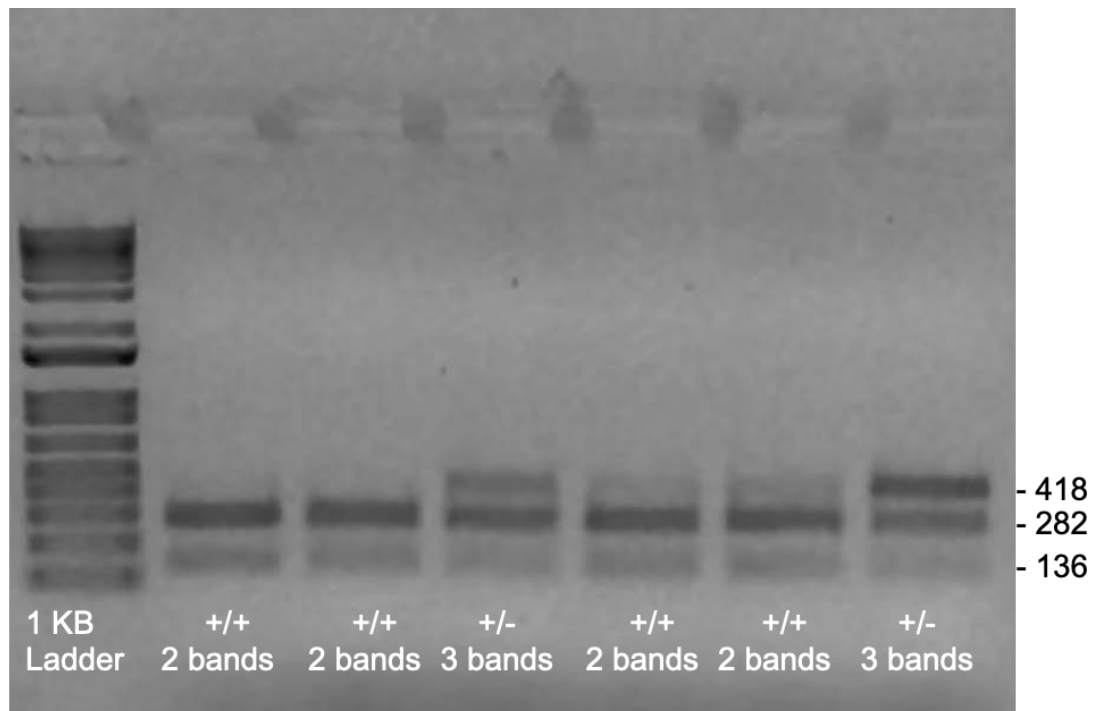


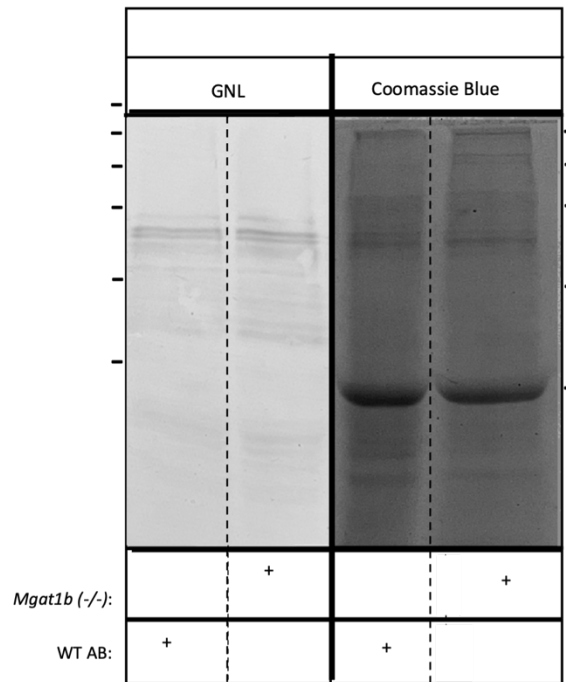
Figure 15 – *Mgat1a* Zebrafish Strain Generation – Restriction enzyme digest of F0 *Mgat1a* zebrafish with Tsp45I.

***Mgat1b* KO Results**

The research so far supports the idea that glycosylation site occupancy is essential in motor neuron development and function. However, what if we change the glycan type globally? Will we see similar results in retarded motor activity? WT AB strain zebrafish were used to create the *Mgat1a* and *Mgat1b* knockout line using Crispr/Cas9. Zebrafish have two copies of the *Mgat1* gene, which are known as *Mgat1a* and *Mgat1b*. Knockout of one or both genes will have a global effect within the fish, unlike the Kv3 channel studies which targeted neural tissue, as described above. As mentioned, genotyping of the F0 generation was performed to identify fish with a mutated *Mgat1b* or *Mgat1a* gene. DNA sequencing was performed to verify a loss of function in *Mgat1b*. An indel of 5bp was identified in the fish which can be found in Figure 13 see below. A

Mgat1 knockout in an organism with only one copy was found lethal in vertebrate (Stanley, 2016). However, a loss of one copy of the *Mgat1* gene in zebrafish would likely be viable and generate higher levels of oligomannose type N-glycans or it may result in no change in the N-glycan population from the WT AB as the other *Mgat1* gene could completely compensate. A lectin blot was run to examine the levels of various types of N-glycans. An increase in oligomannose type N-glycans would show darker color intensity per lane. If the other *Mgat1* gene would compensate, then there should be no discernable difference in the band intensity for Wt AB and *Mgat1b* knockout fish. A Coomassie blue stained gel was used to determine protein loads per sample, prior to conducting a GNL lectin blot. GNL is a lectin that binds to oligomannose type N-glycans, so more intense bands correlate with a higher concentration of oligomannose N-glycans. The image below in Figure 16 shows one of the GNL lectins run to see if the oligomannose type N-glycan concentration increased.

As shown by the lectin blot, the *Mgat1b* knockout larvae were shown to have an increase in oligomannose type N-glycans when compared to WT AB.



AB strain: gga gct aac gga ttg gcc ggt gaa gtg atc aga ttg gct gaa
Mgat1b strain: gga gct aac gga ttg gcc ggt gaa gt- --- -ga ttg gct gaa

Figure 16 - *Mgat1b* GNL lectin and Coomassie Blue Stained Gel of *Mgat1b* Knockout Fish – A knockout of *Mgat1b* should result in a higher concentration of oligomannose type N-glycans. GNL is a lectin that binds oligomannose oligosaccharides, so darker shades and more bands support the knockout of *Mgat1b*.

CaP Motor Neuron Morphology in the *Mgat1b* Knockout Fish

Does changing the type of N-glycan present result in reduced neuron branching patterns in primary motor neurons? These neurons are responsible for innervating the trunk musculature needed for tail movement. The typical branching pattern is very dense as these branches end in neuromuscular junctions. Figure 17 below shows confocal images taken of 48 hpf zebrafish larvae injected with the EGFP expression vectors. The images show a clear visual difference in the branching patterns when comparing CaP neurons from WT AB to the *Mgat1b* ^{-/-}. The results suggest that *Mgat1b* strain has significantly reduced branching when compared to Wt AB. Future studies will be established this difference in axon branching. Does this difference in axon branching patterns result in decreased motor activity as these neurons innervate the muscles necessary for swimming?

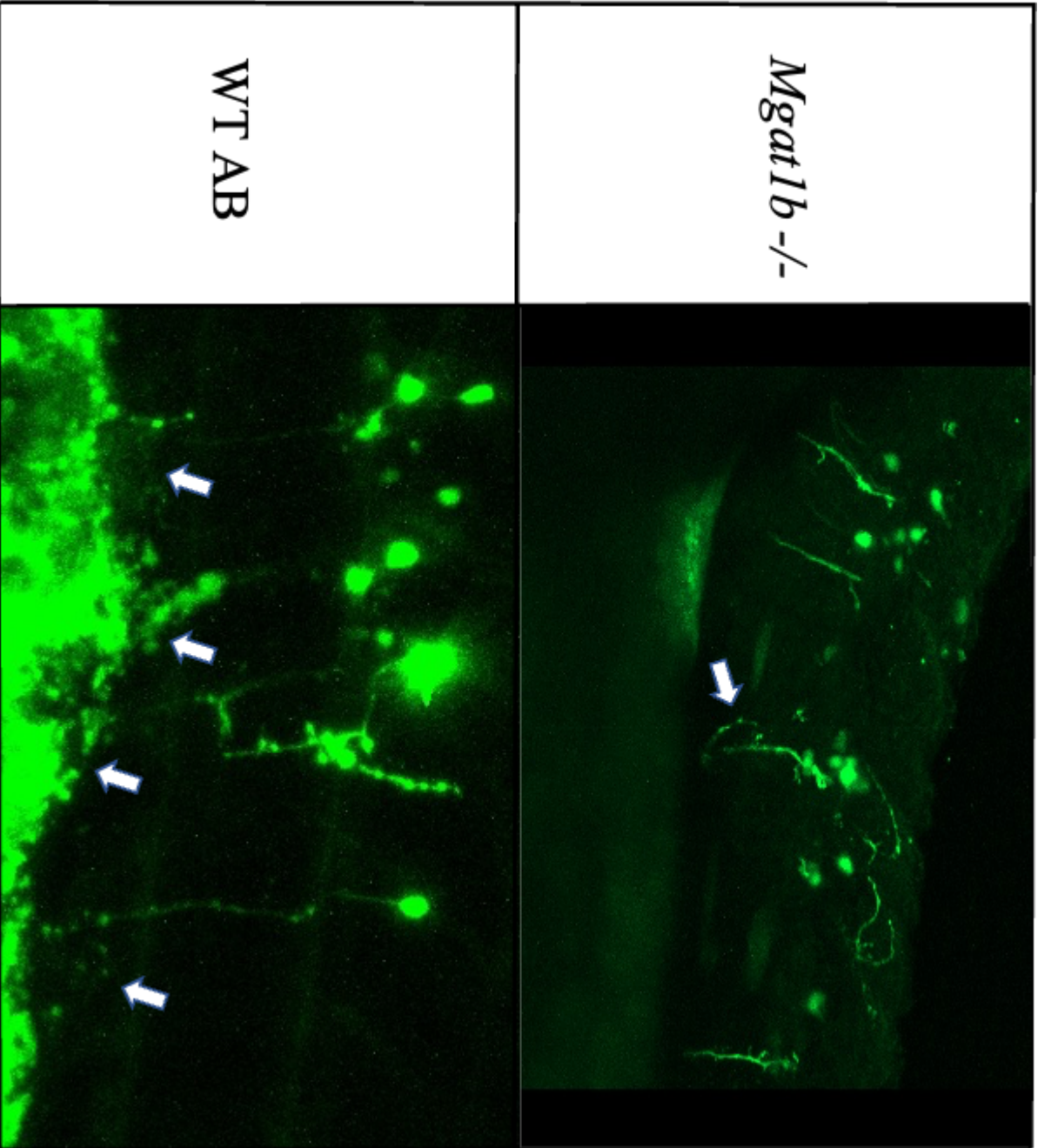


Figure 17 – *Mgat1b* ^{-/-} Motor Neurons Versus WT AB - The *Mgat1b* zebrafish show a considerably reduced branching pattern when compared to WT AB zebrafish. Location of branching indicated by white arrows.

Mgat1b Zebrafish Motor Activity

The locomotor activity shown in Figure 18 was recorded at five dpf using the F3 generation of *Mgat1b* strain zebrafish with the genotypes $-/-$, $+/-$, $+/+$ in addition to WT AB. To show there are no off-target mutations present that would be affecting the assay, the $+/+$ and $+/-$ genotypes of the *Mgat1b* line were ran in parallel to the $-/-$ genotype and WT AB. They were found to be not significantly different from that of WT AB. However, the *Mgat1b* knockout larvae showed a significant decrease in motor activity compared to WT AB and the $+/+$, $+/-$ genotypes of the *Mgat1b* larvae. Furthermore, this trend of decreased activity continued till at least 24 dpf. This decrease in motor activity supports the hypothesis stating proper N-glycosylation is essential in neuronal development and that a knockout of the *Mgat1b* will cause decreased motor activity which will likely be due to hampered CaP motor neuron development.

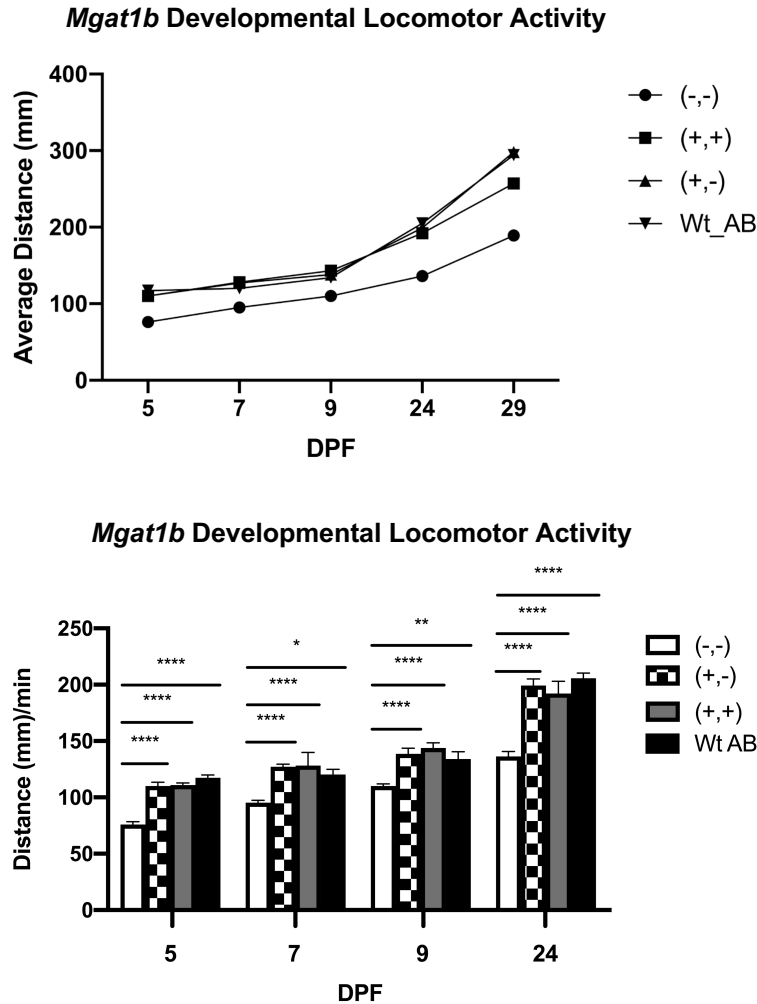


Figure 18 – *Mgat1b* Motor Activity – This shows the average motor activity as the fish develop over time, comparing the larvae with more complex N-glycans +/+ and WT AB, +/-, with those of -/- which possess a higher concentration of oligomannose N- glycans. The *Mgat1b* -/- larvae showed a decrease in motor activity throughout development. The +/+ and +/- genotypes showed no significant difference throughout larval development. The asterisk denotes the level of significance using a one-way ANOVA and 95% confidence interval. Sample sizes used were no less than 35 larvae per genotype. Compared via one-way Anova (*p<0.05, **p<0.01, ****p<0.0001)

Kv3.1 Distribution in Neuroblastoma Cells

N-glycosylation of Kv3.1b has been shown in previous studies to affect the distribution of various glycosylated forms of the Kv3.1b protein at or near the plasma membrane in the NB_1 cell line using total internal reflection fluorescence (TIRF) microscopy (Hall et al., 2014) These cells had a Kv3.1b subunit, completely unglycosylated with the N-glycosylation sites being knocked out with the asparagine residue being changed to glutamine, referred to as N220/229Q Kv3.1b protein. However, will the distribution of the Kv3 channels to the outgrowths and cell body differ if total Kv3.1b protein in the sub-domain is evaluated via confocal microscopy? The cell lines used in the study were the following: NB_1_Wt Kv3.1b, NB_1_N220/9Q and NB_1 (-*Mgat1*) _ Wt Kv3.1b and NB_1 (-*Mgat1*) _ N220/9Q. Examples of images taken using confocal microscopy can be found in Figure 19.

As depicted in Figure 20 the NB_1_Wt Kv3.1b showed significantly more expression in the outgrowth than the NB_1_N220/229Q cell line. It was supporting the hypothesis that unoccupied N-glycosylation sites result in lower expression of Kv3.1b in the outgrowths. The NB_1(-*Mgat1*) _Wt Kv3.1 had a greater percentage of Kv3.1b expression than its un-glycosylated counterpart the NB_1(-*Mgat1*) _N220/9Q in the outgrowth. When comparing the NB_1_Wt Kv3.1b cell line to the NB_1(-*Mgat1*) _Wt Kv3.1b cell line, the NB_1_Wt Kv3.1b cell show a drastic increase in outgrowth expression of Kv3.1b. This shows that the distribution of Kv3.1b expression is dependent on the type of N-glycan occupying the N-glycosylation sites.

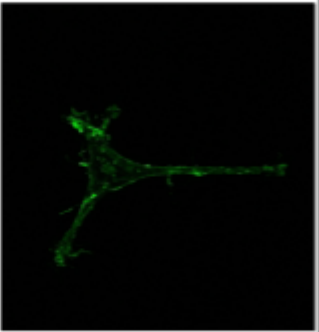
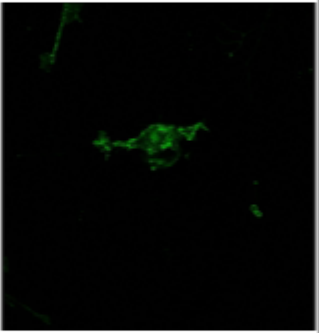
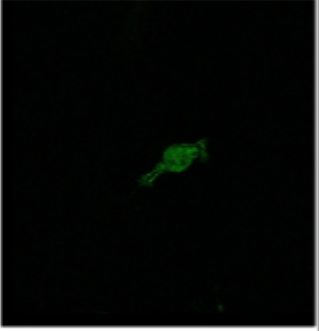
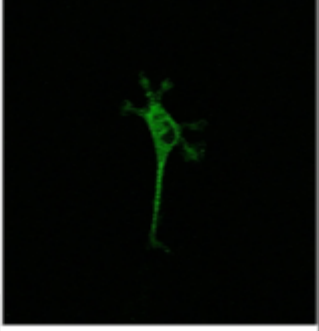
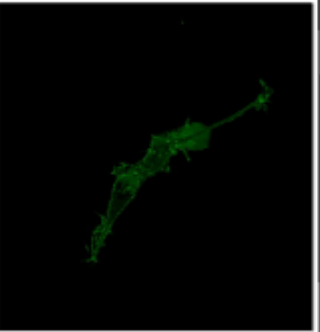
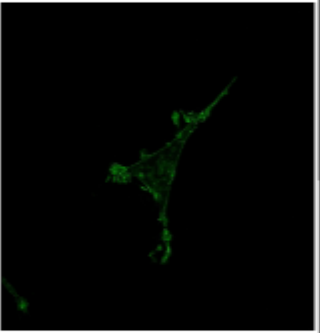
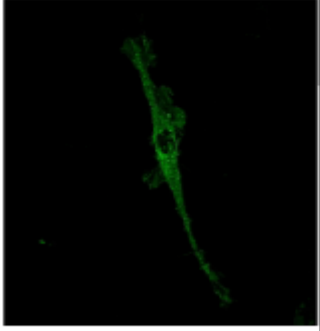
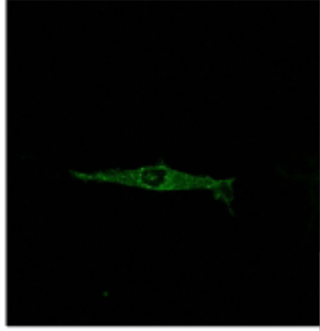
	Wt Kv3.1		N220/9QQ	
NB_1 (- <i>Mgat1</i>)				
NB_1				

Figure 19 – Confocal Images of NB_1 and NB_1(-*Mgat1*) Cell Lines with EGFP Tagged Kv3.1 – Shows examples of the images taken for the Kv3.1 outgrowth distribution analysis.

Kv3.1 Distribution to the Outgrowth

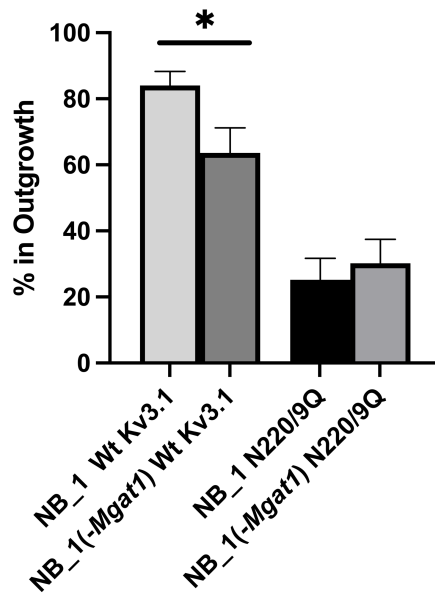


Figure 20 – Distribution of Kv3.1 in NB_1 and NB_1(-*Mgat1*) - The fluorescently tagged Kv3.1b showed that the percent of Kv3.1b in the outgrowths of the cell was limited when the cell only has oligomannose N-glycans. Knocking out the glycosylation sites on the Kv3.1b subunit reduced the percentage of expression in the outgrowth though it was not significantly different between the NB_1 and NB_1(-*Mgat1*) cell lines. Compared via one-way Anova (* $p < 0.05$). Smallest sample size used was 14.

Discussion

Kv3.1 Expressed in Primary Motor Neurons

Atypical N-glycosylation patterns are linked to several disorders known as congenital glycosylation disorders (CGD). The N-glycosylation-related disorders can be broken down into two types (Type 1 and Type 2) (Freeze, 2007). Type I disorders are linked to improper makeup and/or absence of glycan attachment to the protein being glycosylated (Freeze, 2007). Type II disorders are caused by issues with the enzymes such as GnTs, which are responsible for modifying the oligosaccharide once linked to the proteins via an asparagine glycosylation site (Freeze, 2007). Aim 1 of the thesis illustrates an example of Type 1 N-glycosylation disorder where one of the N-glycosylation sites of the Kv3.1b subunit was knocked out, preventing an oligosaccharide from attaching to a protein. The results from the in-vivo studies of transiently expressed Kv3.1b subunits tagged with EGFP expressed in CaP neurons illustrated that one unoccupied N-glycosylation site, resulted in maldeveloped neurons (Issa et al., 2021). For example, the terminal neuron branches that innervate the muscle fibers at the neuromuscular junction are significantly reduced. It was this lack of innervation at the neuromuscular junction that is a possible cause of the reduction seen in motor activity with the N220Q expressing larva. The removal of one N-glycosylation site was not entirely mitigated by the other site, so it is possible these sites are responsible for different modifications of the Kv3.1 subunit. However, future studies are needed with just the N229 site knocked out to see if a similar reduction in motor activity and neuronal structure was present. The N229 site must provide some form of

compensation, as an attempted transient expression of the completely un-glycosylated N220/9Q via microinjection was shown to be fatal in the first 48 hpf. The neurons that Kv3.1b are known to innervate are fast-spiking neuron (Kaczmarek & Zhang, 2017). As fast-spiking neurons, Kv3.1b protein or a different Kv3 protein must be present since it is their ability to rapidly open and close that allows these neurons to fire at an extended rate. Thus, the N-glycosylation sites of Kv3.1b must be occupied to support neuron development and efficient motor activity, and furthermore vacancy of the sites is likely to contribute to neurological complications in patients suffering from CDG.

Previous studies with the glycosylation sites on the Kv3.1b protein channel show that partial glycosylation of the Kv3.1b alpha subunit resulted in a delay in Kv3 channel activation and deactivation (Brooks et al., 2006; Hall, Cartwright, et al., 2011; Hall et al., 2015). The rapid activation and deactivation of the channel is a primary characteristic of the Kv3 channel and what allows neurons to fire rapidly (Kaczmarek & Zhang, 2017). The reduced glycosylation can result in a slower action potential. An action potential contains 3 main phases depolarization, repolarization, and hyperpolarization (Barnett & Larkman, 2007). The depolarization phase is carried out by sodium channels (Andavan & Lemmens-Gruber, 2011). After depolarization the voltage gated potassium channels such as Kv3 open to repolarize the cell (Kaczmarek & Zhang, 2017). The partial glycosylation results in a slower activation and deactivation rate which will affect the action potential from neurons expressing the reduced glycosylation variant.

Motor Activity of *Mgat1b* Knock Out Fish

The *Mgat1b* ^{-/-} zebrafish have a global knockout of *Mgat1b*, which results in an overall increase of oligomannose N-glycans and a decrease in complex and hybrid N-glycans. The locomotor activity for the *Mgat1b* ^{-/-} showed a drastic reduction when compared to other fish strains with typical glycan processing. One of the possible causes of reduced locomotion is a change in the axon branching structure thereby disabling the axonal innervation of the trunk musculature which is necessary for movement. Although further studies and analysis are necessary, the images attained for this thesis appear to show that the *Mgat1b* ^{-/-} strain zebrafish transiently expressing EGFP appear to have minimalistic branching patterns when compared to that of the Wt AB zebrafish strain. Another possible reason for the decrease in motor development may be caused by altered membrane proteins, which require complex N-glycans for proper function. Kv3.1 would be a prime example of this, as previous studies have shown that *in vitro* *Mgat1* knockouts in NB_1 cell lines resulted in a change in the duration of opening and closing of the Kv3 channels (Issa et al., 2021). A change in the spatial arrangement of membrane proteins like Kv3.1 could also be a possible cause for the decreased motor activity as the repolarization needs to be able to occur throughout the axon for a proper response time (Issa et al., 2021). Further it was shown that N-glycosylation of a Kv3 channel by oligomannose glycans instead of the complex caused a different spatial arrangement of Kv3.1b in the cell membrane.

Spatial Arrangement of Kv3.1 in Neuroblastoma Cells

Previous studies have shown that glycan site occupancy of the Kv3.1b channel affects the overall distribution of Kv3.1b in the plasma membrane (Hall et al., 2014). Does changing the type of glycan present in these sites affect distribution? The results in this study show the distribution of Kv3.1b to the outgrowths does change with a significant difference between glycosylated Kv3.1b expressed in NB_1 _Wt Kv3.1 cells versus NB_1(-*Mgat1*) _Wt Kv3.1 cells. When the Kv3.1 glycosylation sites were removed in the N220/229Q lines, the differences between NB_1 _ N220/229Q Kv3.1 and NB_1 (-*Mgat1*) _ N220/229Q Kv3.1 were insignificant. Since the Kv3.1 glycoprotein with oligomannose had reduced levels to the outgrowths, it could alter the action potential, and thus a delay in motor activity.

Axon Growth and Formation

The first phase of axon growth is for neurites to appear around the cell body by what are called filopodia which form from the actin filament network inside the cell (Chia et al., 2014). These neurites are formed using the push-pull forces generated by dynein and myosin motor proteins acting on microtubules (de Rooij et al., 2018). Actin is used to adjust the shape of the filopodia and control the direction of the growth cone (Arnold & Gallo, 2014). Actin is added at the distal end of the filopodia and removed at the proximal end allowing the axon to grow (Arnold & Gallo, 2014). Once the axon has grown to its desired length a more localized filopodia is sent out to develop the branches by using actin to pull along the microtubules of the filopodia. It is possible that N-glycosylation of Kv3.1 influences the cytoskeleton network, and therefore it would be

of interest to evaluate how axon growth and formation result in maldeveloped CaP motor neurons.

Conclusion

The conclusions are that N-glycosylation is essential for proper development in zebrafish and Kv3.1 spatial arrangement in NB cell lines. Abnormal N-glycosylation patterns impact primary motor neuron structure by limiting proper branching patterns in larval zebrafish. This branching is necessary for muscle innervation, which is the likely cause of the decrease in motor activity. Furthermore, in NB cell line studies, atypical glycosylation results in improper distribution to the outgrowths of the cell. This could indicate an issue with neurons being able to initiate an action potential.

Future Studies

A new strain of zebrafish with *Mgat1a* knocked out is already in the F1 generation phase. Since zebrafish have two copies of *Mgat1*, it will be interesting to see if both copies have a similar impact on larva development. The *Mgat1a* *-/-* fish will be crossed with the *Mgat1b* *-/-* fish to see if a total knockout of *Mgat1* is as lethal in zebrafish as it is in other vertebrate species, such as mice. Currently large numbers of the *Mgat1b* *-/-* population are being generated for dissection of brain and spinal cord. RT-PCR could be performed on the brain and spinal cord to determine if there is a differential expression of *Mgat1a* and *Mgat1b*.

In order to study the outcome of a change in glycan type on the Kv3.1 subunit, the different glycoforms could be injected into the *Mgat1b* *-/-* zebrafish line. Confocal

microscopy of the primary motor neurons and locomotor assays could be conducted. This would address whether the change in oligomannose glycan concentration and different Kv3.1b glycoforms affect neuron development.

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Appendix A



Animal Care and Use Committee
212 Ed Warren Life Sciences Building | East Carolina University | Greenville, NC 27834-4354
252-744-2436 office | 252-744-2355 fax

February 5, 2019

Ruth Schwalbe, Ph.D.
Department of Biochemistry
Brody 5E
East Carolina University

Dear Dr. Schwalbe:

Your Animal Use Protocol entitled, "Roles of N-Glycans and Potassium Channels in Zebrafish" (AUP #C065) was reviewed by this institution's Animal Care and Use Committee on February 5, 2019. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

www.ecu.edu

