REGULATORY MECHANISMS AND FUNCTIONS OF NEURAL SIALYLATION IN DIVERSE BIOLOGICAL SYSTEMS

A Dissertation

by

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ABSTRACT

Defects in sialylation cause numerous human pathologies including heart disease, cancer, immune system dysfunction, and neurological abnormalities. However, the pathological mechanisms of these disorders and regulation of sialylation remain poorly understood. I used the *Drosophila* model combined with genetic, neurobiological, and microscopy approaches, to investigate the function and regulation of sialylation in the heart and nervous system.

I found that defects in sialylation result in cardiac abnormalities, including a reduced heart rate. My results indicated that sialylation affects heart functions via regulation of the nervous system, suggesting that sialylation controls neurons that innervate the heart. I established *Drosophila* as a model for further research to elucidate genetic and molecular mechanisms of sialylation-mediated regulation of heart development and physiology, which will potentially shed light on analogous mechanisms that regulate human heart and contribute to etiology of cardiovascular diseases.

Previous experiments in our lab outlined a sialylation-mediated mechanism of glia-neuronal coupling. I tested this hypothesis and confirmed that CSAS, the sugar donor-synthetizing enzyme (CSAS) functions in glia. I further investigated another enzyme, sialic acid synthase (SAS), working upstream of CSAS in the sialylation pathway. SAS remains largely uncharacterized in animals, while understanding its functions could suggest approaches for therapies in pathological conditions. I revealed that *SAS* mutations result in neurological defects. My experiments indicated that SAS is required in glial cells,

and that its product, sialic acid can be transported between the cells. I found that sialic acid can be provided during a critical time in development as a dietary supplement to rescue *SAS* mutants. Intriguingly, my data also suggested that SAS has additional function outside of the canonical sialylation pathway. Taken together, my experiments provided the first detailed *in vivo* characterization of SAS and supported a bipartite regulation of neural sialylation. I established a useful model for further elucidation of SAS functions, which is expected to reveal novel biomedically relevant mechanisms.

DEDICATION

I would like to dedicate this work to my family for their support and encouragement throughout my graduate career. I would have not made it to this point in my life without your love and encouragement. Additionally, I would like to thank my loving husband for never giving up on me, and for showing me that there is a beautiful light at the end of the tunnel.

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NOMENCLATURE

CCAP	crustacean cardioactive peptide
CDG	Congenital Disorders of Glycosylation
CNS	Central nervous system
CSAS	CMP- Sialic acid synthetase
CST	CMP-Neu5Ac transporter
Dol-P	dolichol-phosphate
DSiaT	*Drosophila* Sialyltransferase
ER	endoplasmic reticulum
Eag	ether a go-go
GAG	glycosaminoglycan
Gal	Galactose
GalNAc	N-Acetyl-D-galactosamine
Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	N-Acetyl-D-glucosamine
GNE	UDP GlcNAc 2-epimerase
HIBM	hereditary inclusion body myopathy
IDD	intellectual developmental disorders
KDN	2-keto-3-deoxy-D-glycero-D-galacto-nononic
SAS	Sialic acid synthase

Man	mannose
MARCM	mosaic analysis with a repressible cell marker
NANS	N-acetylneuraminic acid synthase
NANSP	Neu5Ac-9-P phosphatase
NCAM	neural cell adhesion molecule
NeuAc	N-Acetylneuraminic acid
NPL	Neu5Ac pyruvate lyase
Para	Paralytic
PEP	phosphoenolpyruvate
PG	perineural glia
POMT	protein O-mannosyltransferase
SEG	subesophageal ganglion
Sei	seizure
SOHA	semi-automated optical heartbeat analysis
SPG	subperineural glia
TS	temperature-sensitive
VGIC	voltage-gated ion channel
VLM	ventral longitudinal muscle
VNC	ventral nerve cord
WT	wild-type

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This work was supervised by a dissertation committee consisting of Dr. Vlad Panin (advisor), Dr. Michael Polymenis, Dr. Lanying Zeng of the Department of Biochemistry and Biophysics at Texas A&M University, and Dr. Ginger Carney Dean of the College of Science at the University of Idaho.

All work conducted for the dissertation was completed by the student independently.

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1. INTRODUCTION

1.1 Introduction of glycosylation

Glycobiology is a rapidly emerging field that focuses on the study of carbohydrates and their structure, function and biology. Carbohydrates, also referred to as glycans, are present in every living organism making them interesting candidates of research in both biomedical and basic science. Proteins and lipids can be post-translationally modified by glycans to form glycoconjugates, such as glycolipids and glycoproteins. Glycoproteins fall into two different classes based on attachment site. N-linked glycans attach to proteins through a nitrogen atom, while O-linked glycans attach through an oxygen atom. These modifications can result in changes both intrinsically and extrinsically. Intrinsically, glycans can modify the properties of proteins affecting their stability and function. Glycans can also physically provide structural components to cell walls and matrices. Extrinsically, glycans can interact with lectins to mediate and modulate cell adhesion, signaling and trafficking.

Monosaccharides serve as the basic building blocks of glycans. Multiple monosaccharides can be linked together to form oligosaccharides or polysaccharides. Oligosaccharides describe glycans containing less than 20 monosaccharides, while polysaccharides describe any linear or branched polymer. Glycans can be referred to as simple or complex. Complex glycans contain more than one type of monosaccharide. Vertebrate glycoconjugates are typically composed of nine common monosaccharides: Glucose (Glc), Mannose (Man), Xylose (Xyl), Fucose (Fuc), N-Acetyl-D-glucosamine

(GlcNAc), Galactose (Gal), N-Acetyl-D-galactosamine (GalNAc), Glucuronic acid (GlcA), and N-Acetylneuraminic acid (NeuAc). Additionally, these nine monosaccharides can be further modified after incorporation to generate more structures. Bacteria, fungi and invertebrates use some of the same monosaccharides as vertebrates, however they can also contain unique species-specific sugar residues.

Every cell contains a glycocalyx, which is a dense coating of oligosaccharides and polysaccharides on the surface of the cell. Glycan-binding proteins such as lectins and glycosaminoglycan (GAG)-binding proteins can recognize certain types of glycans on the cell surface and potentiate multiple cell-cell, cell-matrix and cell-molecule interactions. Additionally, glycan-binding proteins can mediate interactions between species, for example in the case of pathogen-host interactions. Due to the vast nature of these interactions, there are many pathological and biological diseases linked to mutations in glycan-binding enzymes and modified glycan structures, some of which will be discussed in this document. (This section, along with 1.1.1 and 1.1.2, are reviewed in Varki et al., Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.)

1.1.1 N-linked glycosylation

All eukaryotic N-glycans inevitably begin with GlcNAc covalently attached to the amide nitrogen of an asparagine residue. This glycosidic linkage is abbreviated as GlcNAcβ1-Asn. In eukaryotes, N-glycan synthesis begins on a lipid-linked precursor oligosaccharide referred to as dolichol-phosphate (Dol-P) on the cytoplasmic side of the

endoplasmic reticulum (ER) membrane. During synthesis, the glycan structure is flipped in towards the lumen of the ER. Once synthesis is complete, the oligosaccharide is transferred *en bloc* to the protein. At this point, initial trimming of the glycan structure by glycosidases begins, removing some of the original sugar residues. Processing of the glycoprotein continues as it moves through the Golgi apparatus, undergoing more trimming, followed by addition of terminal sugar residues by glycosyltransferases. Resulting is a diverse glycoprotein with unique branching and structure that can now be delivered or secreted to the plasma membrane.

In order for a protein to receive an N-glycan, a series of proper conditions have to be met. Approximately 70% of the time, proteins can receive an N-glycan at one or multiple Asn-X-Ser or Asn-X-Thr sequons, where X signifies any amino acid except proline and Asn is the glycosylated residue. However, there are other rarer contexts where asparagine's can become glycosylated, such as in the case of an Asn-X-Cys sequon. In order for glycosylation to occur, these sequons must be located on the surface of proteins, due to conformational restraints and the hydrophobic nature of the protein interior when folded. Lastly, potential glycosylation sites must be accessible to the ER lumen in order to receive an N-glycan.

In general, there are three different classes of N-glycans: oligo-mannose, complex and hybrid **(Figure 1-1)**. All of these glycans contain the same common core consisting of $Man_3GlcNAc_2Asn$. Once past the core structure, N-glycans become further modified by glycosidases and glycosyltransferases to form a structure that can be classified into one of the above three classes. Understanding the regulatory mechanisms of these modifier

enzymes and their ability to selectively glycosylate proteins would provide a significant advantage in understanding mammalian glycobiology.

1.1.2 O-linked glycosylation

Commonly, O-linked glycans begin with GalNAc linked to the hydroxyl group of a serine or threonine residue. In mammals, there are four common core structures of O-glycosylation **(Figure 1-1)**. As with N-glycans, each core can be modified to include branching or lengthening of its structure by the addition of more glycans. Many enzymes involved in generating the structures of N-glycans are thought to be shared with O-glycans, due to the fact that many terminal structures are similar if not identical between these different types of glycosylation. Many different O-glycans exist in nature, greatly varying in structure and function. Unfortunately, this route of glycosylation is relatively unexplored when compared to N-linked glycosylation, leaving a gap in understanding when it comes to identifying the biological significance of this diversity.

Mucins and proteoglycans are the most heavily glycosylated proteins by O-linked glycans, both of which have important biological functions. Mucins are found throughout the body, particularly lining surfaces directly exposed to the environment. In the gastrointestinal, genitourinary, and respiratory tracts mucins retain water to shield these surfaces from damage and invasion by microorganisms. Clusters of sialylated glycans modify mucins allowing them to retain large amounts of water with their strong negative charge. In humans there are 20 MUC genes identified, each having its own distinct set of properties.

Proteoglycans also bind water, but in contrast to mucins they provide structure. One example of a specific function for proteoglycans is their ability to provide resistance to cartilage when compressed. The cartilage proteoglycan, aggrecan, contains over 100 O-linked chondroitin sulphate chains, each reaching a length of about 100 sugar resides. The large number of sulfate resides and sugars allow for the binding of water, and as a result a highly hydrated state is obtained that provides resilience to cartilage.

1.1.3 Biological roles of glycans

The biological roles of glycans are remarkably diverse. Briefly, glycans can physically provide structural components to cell walls and extracellular matrices. Additionally, they can modify the properties of proteins by affecting their solubility and stability. Extrinsically, glycans can interact with lectins to mediate and modulate cell adhesion and signaling. Lastly, glycans can direct the trafficking of both intracellular and extracellular glycoconjugates. Here I will highlight some examples of each of these classes of biological roles.

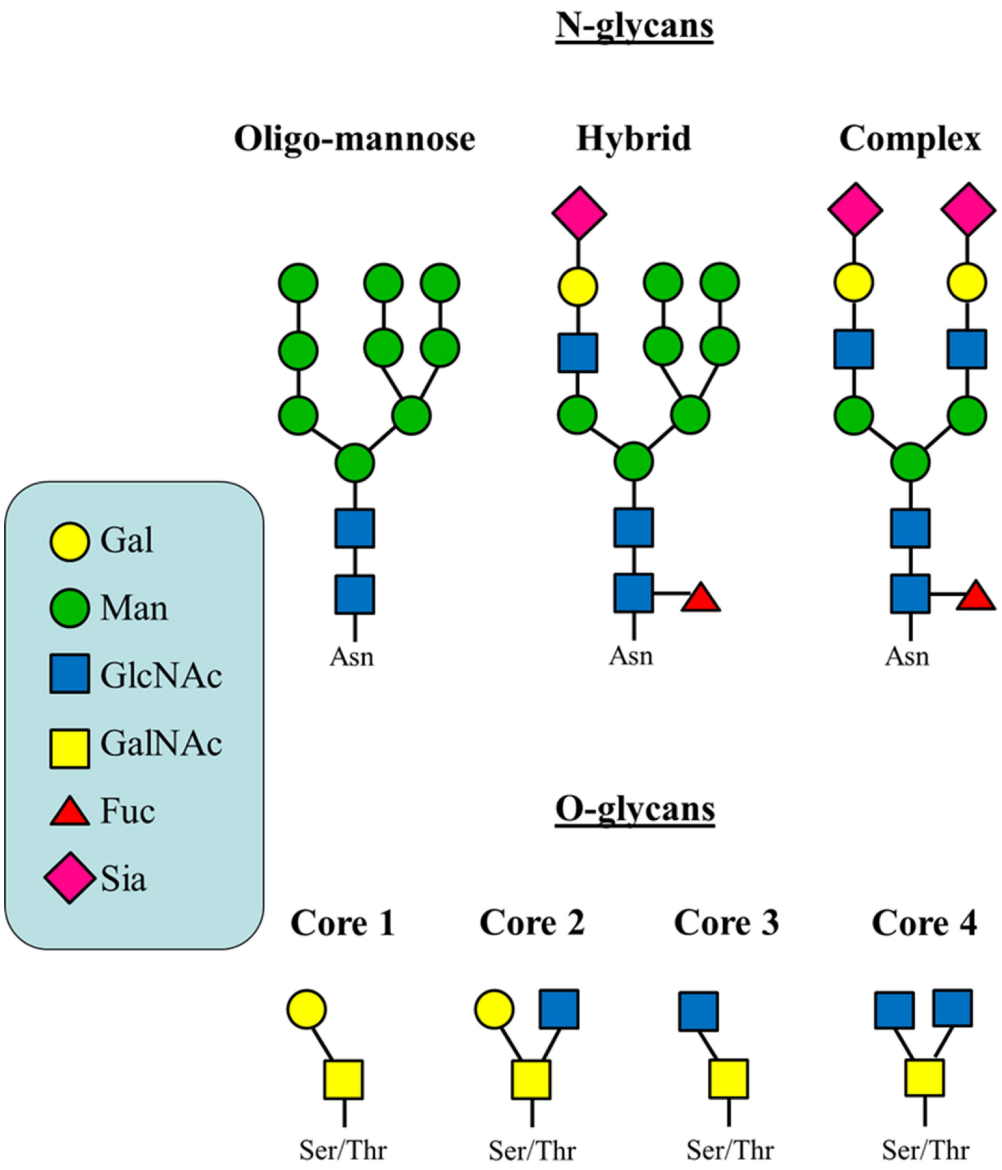


Figure 1-1 Classes of N-glycans and core structures of O-glycans. Examples from the three classes of N-linked glycans attached to an asparagine residue and the four core structures of mammalian O-linked glycans attached to serine or threonine residues. Shapes and colors represent different sugar moieties as demonstrated in the key.

Up until now, I have discussed glycosylation in relevance to vertebrates. Not surprisingly, glycans are also present in plants, fungi, invertebrates and microorganisms. These organisms have been found to not only contain all or most of the common monosaccharides found in humans, but they also have many unique residues specific to each group. Plants and fungi rely heavily on cellulose for the strength and rigidity of their cell walls[73]. Cellulose is an organic glycan polymer consisting of β-linked homopolymers of glucose. Bacteria also secrete cellulose, in a slightly modified form, to make biofilms that are resistant to antibiotic penetration. Chitin is another example of a diversified glycan polymer that provides strength to cell walls, as well as exoskeletons beaks and fish scales. Discussed earlier, mucins form a protective physical barrier to prevent microorganisms from invading and destroying epithelial surfaces. In vertebrates, glycan polymers make up the basement membranes and cartilage structure of the extracellular matrix by forming larger aggregates with specific proteins. There are numerous other examples of organizational, protective and structural roles for glycans in nature[115].

Glycans can affect the structure and function of proteins by the types and numbers of glycans present. The proteins that make up viral envelopes are heavily glycosylated to protect them from host immune recognition[122]. The large number of glycans present on the surface of the virus often disrupt recognition by T-cell receptors in vertebrates. Additionally, glycan structure and branching can affect the function of glycoproteins. For example, cytokine receptors are regulated by glycan structure and branching which modulates endocytosis rates[82]. The modification of individual glycans can also prevent or enable the binding of certain proteins. One example, is the modification of Neu5Ac by O-

acetyl groups to prevent the binding of certain viruses[54, 74]. Another common modification is the addition of O-GlcNAc to nuclear and cytoplasmic proteins[40]. O-GlcNAc can act as a molecular switch by preventing or enabling the phosphorylation of proteins, thereby altering their function. For example, O-GlcNAc modifications are thought to regulate gene expression by affecting the functions of histone proteins, transcriptional and epigenetic regulators[36, 65].

It is not surprising that glycans play a large role in cellular interactions given the fact that they densely coat the surface of every cell found in nature. Many cellular interactions, such as cell adhesion and signaling, are mediated through lectin binding of specific glycans present on glycoproteins. In the case of cell signaling, macrophages have mannose receptors on their cell surface that can recognize and bind mannose and GlcNAc residues present on the surface of microorganisms[94, 105]. Binding of the macrophage mannose receptor signals for phagocytosis of the microorganism. In the case of cell adhesion, selectins (a type of lectin), mediate the rolling of leucocytes in circulation within the blood vessels allowing them to transiently interact with glycans present on the endothelial cell surface[95]. Once the leucocyte-endothelium interaction is formed, leucocytes can migrate through the endothelium and travel to sites of inflammation.

Lastly, I will touch on how glycans can function in the trafficking of glycoproteins. Both intracellular and cell surface lectins have the ability to recognize and bind specific glycans thereby affecting the trafficking of glycoproteins. A well-known example involves the lysosomal enzyme trafficking pathway. In the 1980s while characterizing a human genetic disorder referred to as I-cell disease, a mannose-6-phosphate recognition

marker was identified to be selectively expressed on lysosomal enzymes[62]. Specific receptors were found to recognize the mannose-6-phosphate residue and direct or traffic these lysosomal enzymes to the correct location within the lysosome. This finding held great biological significance as it provided the first evidence that the act of recognition of a specific glycan could function as valuable biological role. Another example is the membrane-bound lectin, ERGIC-53, and its ability to transport glycoproteins with mannose-containing glycans from the ER to the Golgi[46]. Mutations in human *ERGIC-53* result in a blood clotting deficiency, due to improper transport of the highly glycosylated coagulation factors V and VIII through the secretory pathway.

1.1.4 The role of glycosylation in human disease

Since the 1980s, the number of disorders identified to be associated with glycosylation defects has grown substantially[115]. Several examples of glycosylation disorders have already been mentioned above, demonstrating how essential glycosylation is to human health, as well as the health of other organisms. The occurrence of human disease states as a result of abnormal glycosylation can potentiate from multiple different avenues. One possibility is that the glycosylation machinery could be defective, resulting in a number of glycoconjugates either having too much or too little glycosylation. Scenarios such as these greatly affect the regulation and function of many essential enzymes (i.e. dystroglycan, voltage-gated ion channels, etc.). Additionally, defective glycosylation machinery could lead to altered branching patterns and composition of sugar residues which could affect glycan recognition. Another possibility is that glycan-binding

proteins could be defective, resulting in misfunctioning cellular communication. Signaling between cells could become blocked or upregulated. The trafficking of proteins could become affected, making it difficult for many enzymes to reach their proper destinations within the cell where they pursue their specific functions. In this section I aim to stress the significance of glycosylation and its role in human disease. I will highlight a few examples which I find interesting and that have a significant impact on human health.

Congenital Disorders of Glycosylation (CDG) is the term used to describe any diseases associated with defects in enzymes involved in glycan biosynthesis, often resulting in under-glycosylation of glycoproteins. Most of these conditions are inherited and are considered to be rare, however as research in the field progresses more CDGs continue to be identified. CDGs can affect multiple systems within the human body, although interestingly most of these disorders have some neurological component. CDGs can be divided into two types. One set of syndromes consists a lack of N or O-linked glycans at all relevant glycosylation sites. The other set of syndromes results when all relevant glycosylation sites are being used, but the number of glycans present is reduced or lacking complexity. The most prevalent CDG, found to be affecting over 700 individuals, is a mutation within the *phosphomannomutase (PMM)2* gene[47]. Defects in PMM2 affect the synthesis of GDP-mannose in the mannose pathway. GDP-mannose is important for the assembly of the dolichol-pyrophosphate precursor in the ER, thus when affected it leads to the hypoglycosylation of many glycoproteins resulting in an array of phenotypes. Mutations in *N-acetylneuraminic acid synthase* (*NANS*), aka *sialic acid*

synthase (*SAS*), correspond to another example of a CDG that will be discussed in depth in Section 3.

Glycobiology has become a very important topic of study in cancer research given the vast nature of biological roles for glycans. Defects in sialylation, fucosylation, and glycan branching are some of the most common modifications observed in cancer cells[2, 15, 35]. Stomach, colon and ovarian cancer have all been directly linked to an excess of sialylation due to altered glycosyltransferase expression[66]. Additionally, polysialic acid is associated with several types of cancers, often presenting in tumors with increased expression[25, 108]. Polysialic acid can affect neural cell adhesion molecule1 (NCAM1), often resulting in an increased likelihood of cancer cell proliferation and spreading.

Genetic defects in O-mannosyltransferase 1 (POMT1) and POMT2, which mediate protein O-mannosylation, can result in severe muscular dystrophies and neurological abnormalities[28, 72]. Dystroglycan, a glycoprotein which links the extracellular matrix to the cytoskeleton, is a known target for the POMT1/POMT2 complex. Dystroglycan serves as an anchor for dystrophin, which is a cytoplasmic actin-binding protein that maintains the proper architecture of muscle fibers. Therefore, defective POMT1/POMT2 causes aberrant O-mannosylation of dystroglycan, preventing the protein from interacting with extracellular ligands such as dystrophin, thereby resulting in muscle weakness[30].

Identifying glycoproteins prevalent in human disease and further elucidating the mechanisms of these proteins can help guide the way to discovering effective therapeutic treatments. In fact, dietary supplements have already been shown to effectively treat some

CDG. Unfortunately, other CDG involve much more complex pathways and need to be studied in great detail before finding an effective treatment. Interestingly, changes in glycosylation have been identified as a useful biomarker tool to detect certain cancers. For example, the mucin, MUC-1, is expressed on the surface of breast epithelial cells. In tumor cells, the O-linked glycosylation sites on MUC-1 are occupied by core 1 structures instead of the core 2 structures found in non-cancerous cells[109]. This change results in shorter O-glycan chains and an increased exposure to the immune system through a lack of proper shielding. These tumor epitopes can then be recognized using immunotherapy to identify cancerous cells. Additionally, the level of MUC-1 expression has been found to be directly correlated with cancer aggressiveness[44]. Overall, the future for glycobiology looks promising in regards to making therapeutic advancements that can help treat CDGs and other disease phenotypes correlated to glycosylation changes.

1.2 Sialylation

Sialylation is a type of glycosylation in which sialic acids are transferred to the terminal ends of glycoproteins, glycolipids and secreted glycan molecules. Sialic acids are a family of diverse negatively charged, nine-carbon sugars derived from neuraminic acid[93]. Although they are primarily found in deuterostomes, sialic acids are also present in microbial pathogens and serve as a form of molecular mimicry to invade vertebrate hosts[118]. In this section, I will discuss the biosynthetic machinery necessary for vertebrate sialylation, while also shedding light on aspects of sialyation in other organisms. Then, I will focus on a biological role for sialylation in the nervous system, particularly its ability

to regulate membrane excitability through modifying voltage-gated ion channels, due to its relevance to this project. I will finish this section by tying together the role for sialylation in vertebrate excitability to the current findings reported in *Drosophila* sialylation.

1.2.1 The key players

The vertebrate sialylation pathway is comprised of many key players. Each of these players performs important enzymatic steps in sialic acid metabolism. The biosynthesis of sialic acids begins with the bifunctional enzyme UDP GlcNAc 2-epimerase/ManNAc kinase (GNE) converting UDP-GlcNAc into ManNac-6-phosphate[87] **(Figure 1-2)**. GNE catalyzes the rate-limiting step of sialic acid biosynthesis. GNE defects result in detrimental effects to human health and development. Human mutations in GNE are responsible for a recessively inherited disease referred to as hereditary inclusion body myopathy (HIBM) [13, 78, 79]. Additionally, the autosomal dominant disorder sialuria is linked to GNE mutations, in which young children and infants experience delayed development, liver and spleen enlargement, frequent upper respiratory infections and microcytic anemia[24, 63, 100, 107]. As the children get older, they may also have an onset of learning disabilities and seizures. Mice deficient in GNE are embryonic lethal[97]. Bacteria have a NeuC gene homologous to the vertebrate GNE, however this enzyme lacks kinase ability and therefore results in a product of unphosphorylated ManNac[75, 89]. Surprisingly, there has yet to be a homologous *GNE* gene discovered in *Drosophila*. However, *de novo* sialylation does happen in insects based on the presence of other functional homologs in

the pathway. Therefore, how sialylation biosynthesis begins in *Drosophila* remains unclear.

Following this first two enzymatic steps, Neu5Ac-9-P synthase (NANS) then converts ManNac-6-phosphate into phosphorylated N-acetylneuraminic acid (Neu5Ac-9-phosphate) through a condensation reaction with phosphoenolpyruvate (PEP). Additionally, human NANS can yield 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic (KDN), by using the widely available Man-6-P in a condensation reaction with PEP to produce KDN-9-P[60]. NeuB, a well-characterized bacterial Neu5Ac synthase, uses ManNAc instead of ManNAc-6-P to yield Neu5Ac (aka sialic acid (Sia)). Similar to humans, the *Drosophila* homolog of NANS, SAS, produces phosphorylated sialic acids and exhibits multi-functional substrate activity using both ManNAc-6-P and Man-6-P[55]. ManNAc-6-P condenses to become Neu5Ac, and Man-6-P becomes KDN. Human patients with hypomorphic NANS mutations display defects in development and growth, presenting with short limbs and craniofacial defects[113]. Additionally, these patients also have intellectual disorders with most patients not ever acquiring speech. Consistent with the phenotypes in humans, knockdown of *nans* in zebrafish also results in abnormal skeletal development and a small head. So far, *Drosophila SAS* mutants were not characterized.

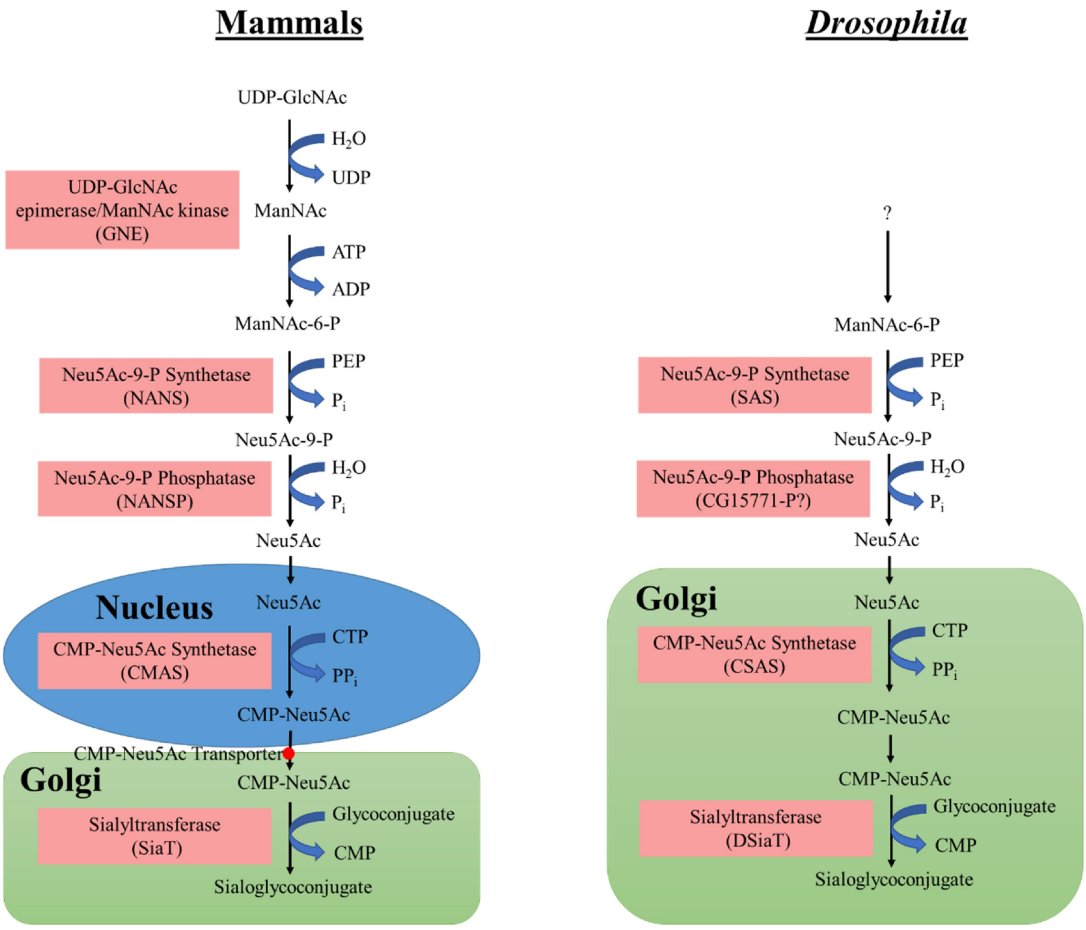


Figure 1-2 Comparison between biosynthetic pathways of sialylation in mammals and *Drosophila* (Adapted from Koles et al. 2009). *Question marks* represent unknown/hypothetical steps or enzymes. In *Drosophila*, it is unclear how ManNAc-6-P is being synthesized given that the *Drosophila* genome lacks a UDP-GlcNAc 2-epimerase/ManNAc kinase gene. CG15771-P denotes the protein product of the *D. melanogaster CG15771* gene. See text for discussion.

The next step in the biosynthetic pathway of sialic acids is the dephosphorylation of Neu5Ac-9-P by Neu5Ac-9-P phosphatase (NANSP) to yield Neu5Ac[70]. KDN-P can also be used as a substrate for NANSP[114]. Interestingly, NANSP activity was found to not

be essential for de novo sialic acid production as demonstrated by normal levels of CMP-sialic acid and unaffected cell surface sialyation in NANSP KO CHO cells[129]. These results suggest a bypass mechanism, potentially another phosphatase, in the pathway. The sialylation pathway in bacteria does not contain this step because Neu5Ac is synthesized directly without being phosphorylated. *Drosophila* on the other hand, contains a *CG15771* gene that encodes a protein homologous to the vertebrate NANSP enzyme. This gene and its product have yet to be characterized.

Produced only in the presence of Neu5Ac, Neu5Ac pyruvate lyase (NPL), also referred to as sialic acid aldolase, can mediate the conversion Neu5Ac into ManNAc and pyruvate. NPLs were initially detected and studied in bacteria, where their main purpose is to regulate sialic acid catabolism by controlling intracellular sialic acid levels[42, 76]. By doing so, NPLs prevent accumulation of toxic levels of sialic acid within the cell[119]. Vertebrate NPLs contain many properties similar to the bacterial enzymes. Like in bacteria, multiple vertebrate tissues (human liver and erythrocytes, pig and beef kidney) have confirmed aldolase activity in the cytosol[9, 102, 103]. Additionally, vertebrate and bacterial NPLs share similar kinetic properties and substrate preferences[9]. Currently, there has not been a homologous NPL gene identified in *Drosophila*.

Continuing on with the biosynthetic pathway, CMP-Neu5Ac synthetase (CMAS) then takes the substrate, Neu5Ac, and produces the sugar donor molecule, CMP-Neu5Ac. CMAS is highly conserved across many bacterial and animal species. Interestingly, the subcellular localization of CMAS varies amongst organisms. In vertebrates, CMAS localizes to the nucleus, due to the presence of nuclear localization signal motifs.

However, the homologous *Drosophila* enzyme, CSAS, localizes to the Golgi compartment[120]. CSAS functions in the nervous system and is believed to regulate neural excitability through modulation of voltage-gated ion channels[45]. Mutations in *CSAS* result in severe neurological defects. Most prominent phenotypes include decreased longevity, temperature-sensitive (TS) paralysis, locomotor defects, and defects of neural transmission at neuromuscular junctions.

In vertebrates, after CMP-Neu5Ac is synthesized in the nucleus, it diffuses into the cytosol so that CMP-Neu5Ac transporter (CST) can pump it into the Golgi compartment to be used in the final step of the pathway. The *Drosophila* sialylation pathway does not need this step, due to the fact that CMP-Neu5Ac is already generated in the Golgi by CSAS. However, Nue5Ac appears to be synthesized in the cytosol (Section 3) and there has yet to be an explanation for how it gets into the Golgi compartment to be used by CSAS. Both bacteria and vertebrates have a variety of sialic acid transporters, some of which share homology with *Drosophila* enzymes. For example, sialin, a human lysosomal sialic acid transporter has multiple *Drosophila* homologs[117]. It is possible that one of these homologous enzymes functions as a Neu5Ac transporter in the *Drosophila* sialylation pathway.

Sialyltransferase enzymes mediate the final enzymatic step in the sialylation pathway by transferring Neu5Ac from CMP-Neu5Ac to glycoconjugates. Twenty different sialyltransferases have been identified in mammals, each of which can be classified into one of four groups (β-galactoside α2,3; β-galactoside α2,6; GalNAc α2,6; and Ac α2,8) based on substrate and linkage specificity[37]. Several sialyltransferases have

been identified in bacterial species, and structural information has been obtained for some of them[14, 77]. The bacterial enzymes have a broader specificity than the vertebrate enzymes and they lack significant sequence similarity to their human counterparts. The *Drosophila* sialylation pathway includes only one sialyltransferase, DSiaT. DSiaT is most closely related to the human ST6Gal family of sialyltransferases, and therefore falls within the β-galactoside α2,6 group[88]. In the last decade, we have obtained enzymatic and functional *in vivo* data for DSiaT, providing new insights into the roles of animal sialyltransferases. DSiaT was discovered to be crucial to the nervous system, as mutations in this enzyme resulted in severe neurological phenotypes and reduced longevity[58, 88]. Additionally, DSiaT was demonstrated to have an important biological function in neural excitability by mediating regulation of voltage-gated ion channels, which was suggested to be an evolutionarily conserved function between flies and mammals.

Sialidases, also referred to as neuraminidases, cleave sialic acids from glycoconjugates. There are four sialidase *NEU* genes in eukaryotes (*Neu 1-4*), that function in different compartments of the cell to regulate sialic acid metabolism. Each sialidase has its own unique set of enzymatic properties. Some sialidases have high substrate specificity, while others act broadly. Interestingly, there are examples of sia linkages that resist sialidase cleavage, such as those modified by O-methylation and O-acetylation, and are thought to have some biological significance. Bacterial and viral sialidases typically aid in host pathogenesis, making them interesting drug targets[106, 118]. Although one or more sialidases potentially exist in *Drosophila*, they have yet to be identified.

1.2.2 Voltage-gated ion channels and membrane excitability

In vertebrates, glycans have been demonstrated to affect the function of multiple voltage-gated ion channels (VGICs). VGICs are responsible for the generation and propagation of action potentials which drive electrical signaling in neurons, cardiac tissue and muscles. Within this family of large, transmembrane proteins, channels specific to sodium (Na_v), potassium (K_v), and calcium (Ca_v) have all been shown to be directly affected by N-glycans[4, 31, 50, 86, 98, 125, 126, 128, 132]. More specifically, not only are these channels heavily glycosylated, they are commonly heavily sialylated. Current models postulate that presence of numerous sialic acid residues can affect the electrostatic mechanisms of channel gating, due to their negative charge[22, 99].

Significant progress has been made in the last three decades in determining how sialic acid modulates the activity of Na_v (reviewed in Ednie and Bennett, 2012)[22]. We now know that the removal of sialic aids, has no significant effect on channel structure or stability. However, sialic acids have been found to directly affect channel function. Bennett et al. demonstrated how reducing sialylation through various avenues led to depolarizing shifts in channel gating[3]. These depolarizing shifts were attributed to an increase in divalent cation concentration that is usually dampened in the presence of negatively charged sialic acids. Thus, sialic acids prime VGICs by slightly depolarizing the surrounding membrane so that a smaller shift in membrane potential is required to activate channel gating.

Interestingly, VGIC isoforms are affected differently by sialic acids. Nine Na_v isoforms have been characterized in mammals, each of which serve a unique purpose

based on the cell type in which they reside. Each isoform is differentially glycosylated based on the number and location of glycosylation sites present, leading to different severities of gating phenotypes. For example, the human skeletal muscle Na_v (hSkM1) has higher levels of glycosylation and sialylation than the human cardiac Na_v ($Na_v1.5$), and therefore displays large depolarizing shifts in voltage-conductance when desialylated[4]. $Na_v1.5$ voltage-conductance was essentially unaffected by desialylation. These results suggest a direct correlation between the level of sialylation and channel gating.

Similar results have been observed when modifying levels of sialylation on voltage-gated K^+ channel (K_v) isoforms. More than 60 different K_v isoforms have been discovered in mammals, all of which are developmentally and spatially regulated within different cell types. In 2007, a group of scientists used a novel approach to examine the role of sialylation on the $K_v1.2$ isoform expressed predominantly in the rat brain[127]. In this study, they introduced two new glycosylation sites to $K_v1.2$ expressed in cell culture, which resulted in a shift in voltage-conductance to a hyperpolarized state. Additionally, these channels displayed an increased rate of activation and inactivation. These results confirm a mechanism by which sialylation influences the divalent cation concentration. Due to their molecular diversity, the effect of sialylation on voltage-gated Ca^{2+} channels are still poorly understood. With reduced complexity of the nervous system and decreased genetic redundancy (e.g. there is only one *Na_v* gene in fruit flies, called *paralytic* (*para*)) invertebrate model organisms, like *Drosophila*, may prove useful in elucidating the mechanisms by which sialylation is regulating VGICs, provided the current advancements

in genetic approaches and the widely available tools to study neurobiology and cardiobiology.

1.2.3 Regulation of neural excitability in *Drosophila*

As mentioned above in section 1.2.1, *Drosophila* has many of the same sialylation pathway enzymes as vertebrates. Many of these enzymes are highly conserved, and therefore are believed to serve similar functions in vertebrates. *Drosophila* provides many advantages to studying sialylation, one of which is the lack of functional redundancy of sialylation pathway enzymes in these organisms. Thus, this model provides the ability to examine roles and mechanisms of sialyation that would have otherwise been very difficult to elucidate in a vertebrate model. Mutations in *CSAS* and *DSiaT* have demonstrated a role for sialylation in the regulation of neural excitability. Phenotypically, both *CSAS* and *DSiaT* mutations result in locomotion abnormalities, reduced longevity and TS paralysis[45, 88]. All of these phenotypes indicate that there are severe defects present within the nervous systems of these mutants.

In addition to the phenotypes listed above, defects of neuromuscular junctions in DSiaT mutants, provide evidence that neural excitability may be decreased in these mutants. The decreased number of synaptic boutons and branches are comparable to the reduced NMJ synapse growth observed in *nap* mutants where para activity is reduced[7, 133]. Interaction experiments further confirmed that *DSiaT* and *para* have strong synergistic interactions. Together, these results indicate that DSiaT is likely regulating the function of Para, and that the decreased neural transmission observed in *DSiaT* mutants is caused

by an action potential defect. These hypotheses were further supported through the observation that paralysis phenotype in *CSAS* mutants improved upon the introduction of an extra functional copy of *para*, thus suggesting that sialylation can control the amount of sodium channels involved in neural transmission. As it currently stands, this mechanism needs to be further confirmed. Our laboratory is currently working on addressing this hypothesis by analysis of Para expression in sialylation mutants.

1.3 *Drosophila* as a model system to study heart and nervous system functions

Drosophila melanogaster has proved useful in modeling many human diseases. In addition to their short life cycle and their ability to produce large numbers of progeny, *Drosophila* have many genetic techniques available, such as variety of tools to manipulate gene expression *in vivo* in time and tissue-specific manner, that provide an advantage when trying to elucidate molecular and cellular mechanisms underlying human disease. Although humans and *Drosophila* are very different morphologically, a lot of the molecular pathways involved in human brain and heart diseases are highly conserved. In this section I will briefly discuss the functional morphology of both the circulatory and nervous systems in *Drosophila*. I will follow this with the discussion of advantages and limitations of *Drosophila* model system for studying genes involved in human diseases.

1.3.1 The *Drosophila* heart

The *Drosophila* heart is built early during embryogenesis, where 104 cardiomyocytes are arranged symmetrically into two rows to form a luminal space[92]. Each cardiomyocyte becomes differentiated into different cell types based on the identity genes (e.g. T-box genes, Ladybird, Tinman) that are expressed within each cell. Some cardiomyocytes become ostia, others become intracardiac valve cells, and the rest become contractile cardiomyocytes. Near the end of embryogenesis, the cardiomyocytes start to contract leading to the flow of hemolymph within the cardiac lumen.

The larval heart is a linear vessel running the entire length of the dorsal midline[19]. The vessel is composed of a thoracocephalic aorta and an abdominal heart. Comparable to vertebrates, the abdominal heart is multichambered and is defined by three pair of valves. During metamorphosis, the larval heart undergoes drastic changes resulting in an adult heart with new morphological and physiological properties **(Fig. 1-3A, B)**. A new anterior chamber, referred to as the conical chamber, forms in the abdominal heart and serves as the retrograde pacemaker. Additionally, the abdominal heart gains a layer of longitudinal muscle fibers, called the ventral longitudinal muscle (VLM), that run segmentally between segments 3-5 along the heart tube providing support. Pericardial cells flank the abdominal heart tube and make up the excretory system of the fly. Recent studies have demonstrated a role for pericardial cells to act as nephrocytes to filter the hemolymph blood as it flows in through the incurrent ostia of the heart tube. Pericardial cells can also act as sensors of oxidative stress.

Interestingly, the adult abdominal heart presents with substantially more innervation compared to the larval heart, which only possesses minor innervation of the aortic tissue. Symmetrical transverse nerves directly innervate the vessel, forming unique branches within the heart tissue. The transverse nerves arise from the segmental nerves that run horizontally along each segment terminating on surrounding muscles. In the posterior abdominal segments, the transverse nerves do not meet. However, in segments 1 and 2 the transverse nerves from the left and right sides converge forming the transverse bridge through the conical chamber.

In the past couple of decades, the *Drosophila* heart has emerged as an exceptional human disease model to study cardiac disease. Arrhythmias, cardiomyopathies, cardiac decline through ageing, obesity and diabetes are only a few of the cardiac disorders successfully modeled using this system[11, 34, 38, 41, 56, 81, 84, 134]. Many genes important for cardiac function and formation of the heart are highly conserved between *Drosophila* and humans. A recent screen using a gel-LC-MS/MS approach identified 1293 heart-associated peptides using dissected adult fly hearts that had not been previously identified in a recent *Drosophila* peptide screen[10]. Analysis of the *Drosophila* cardiac proteome uncovered that 82% of the protein family domains discovered were conserved in the mouse heart, demonstrating the potential of the system to provide useful information on vertebrate cardiobiology. Much criticism has been directed at the fly heart as a model, pointing out that it will yield few insights that are translatable to human disease. However, many recent studies have counteracted this statement by demonstrating that genes and

enzymes involved in *Drosophila* cardiac function and physiology display similar roles and mechanisms to their vertebrate counterparts[20, 21, 29, 51, 69, 71, 80, 81, 85, 110, 124] .

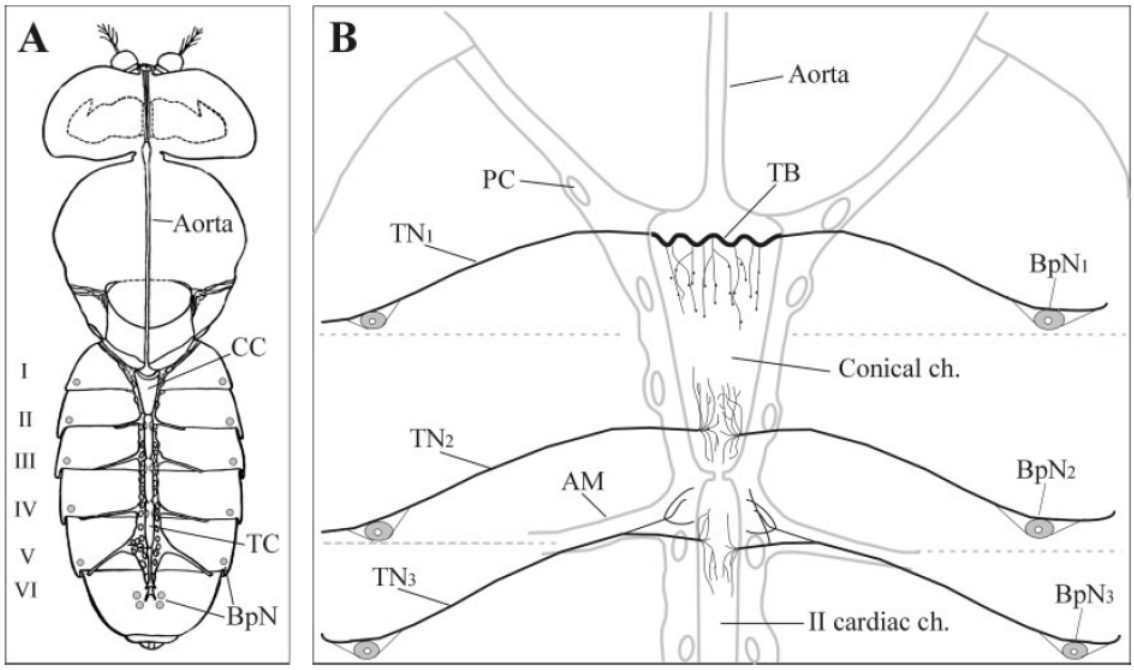


Figure 1-3 Schematic of adult *Drosophila* heart and its innervation. A) *Drosophila* heart is composed of an anterior aorta, running through the head and thorax regions, and a posterior abdominal heart. The abdominal heart initiates beats from either of two chambers, the conical chamber (CC) or the terminal chamber (TC). Heart supported by alary muscles running from abdominal segments 2-5. Bipolar neurons (BpN) are located in each segment along the edge of the abdomen. **B)** Transverse nerves (TN) innervate the heart in each abdominal segment. In the first segment, symmetrical TNs merge to form the transverse bridge (TB), which runs through the conical chamber and initiates retrograde beats. Pericardial cells (PC) flank both sides of the dorsal vessel and filter hemolymph as it flows throughout the abdominal cavity. (Figure modified from Dulcis and Levine, 2003)[19].

1.3.2 The *Drosophila* nervous system

During embryogenesis, the *Drosophila* central nervous system (CNS) consists of a subset of primary neurons and glial cells that differentiate to form the larval brain. The *Drosophila* larval CNS is composed of a brain and ventral nerve cord (VNC). The VNC contains both thoracic and abdominal neuromeres that later transform to form the thoracic ganglion in the adult CNS. The brain is composed of two hemispheres and a central subesophageal ganglion (SEG) that connects directly to the VNC. Together the central brain and the SEG house a majority of neuroblasts[111]. These neuroblasts proliferate throughout the larval stage resulting in clusters of immature, postmitotic neurons that are thought to have no function in the larval CNS. Glial cells form the scaffolding around the neuroblasts and neurons, which is used to stabilize them in the cortex[83]. Several subtypes of glia can be found in larvae and adults[27]. The perineural glia (PG) secrete a dense carbohydrate-rich cover that protects the CNS and peripheral nerves from external factors, such as chemicals, that may be harmful. The subperineural glia (SPG) reside under the PG glia and cover the entire surface of the CNS. In conjunction with the PG, the SPG make up the blood-brain barrier. Cortex glia, ensheathing glia and astrocytes all reside deeper in the CNS and are associated with neurons.

The *Drosophila* CNS undergoes dramatic changes during metamorphosis. During this period some larval neurons die resulting in degeneration mainly in the abdominal regions of the CNS. Approximately 12 hours after pupation, the SEG and thoracic portions of the brain become separated by a constriction and the morphology of the brain morphs to resemble a shape characteristic of the adult[111]. During the next 24 hours, cells grow and

differentiate into nurites and axons, elaborate processes are formed, and the brain and thoracic neurophils expand. Resulting is an enlarged thoracic region containing three neuromeres and a minimized abdominal CNS.

The *Drosophila* brain has been used to study many aspects of vertebrate neurobiology ranging from neuron development and circuit formation[104] to neurodegenerative and neurological disease[48]. Although the adult brain is very complex housing somewhere between 200,000-300,000 neurons, sibling neurons that derive from the same parent neuroblast can be group together into lineages making manipulation and study more manageable[27, 104]. The UAS/Gal4 system[6] and genetic techniques, such as flip-out clones[39] and mosaic analysis with a repressible cell marker (MARCM)[61], are some of the approaches that have been useful in the visualization and identification of individual neural lineages. In addition to the above benefits, the *Drosophila* brain is easy to access through simple dissection and flies can reproduce quickly to allow for high-throughput pharmacological screens and behavioral assays. Many behavioral assays that have already been established in the *Drosophila* model to assess neurological phenotypes such as TS paralysis, negative geotaxis, and righting assays[1, 131]. Taken together, a wide variety of *Drosophila* brain models exist, and by using the tools available within this system we can detect genes modulating disease processes relevant to humans.

1.3.3 Limitations and challenges

Although *Drosophila* is a convenient model to study human disease, there are some disadvantages to using this system. First, one must keep in mind that *Drosophila*

morphology and physiology can be quite different from that of vertebrates. For example, an obvious difference is that *Drosophila* possess an open circulatory system void of any closed blood vessels. This could make something like a brain hemorrhage, a type of stroke where blood burst from the arteries, difficult to study because *Drosophila* do not have arteries. Another example is the mechanism of vertebrate cardiac looping, which cannot be adequately studied using the linear dorsal vessel of *Drosophila*. Second, not all genes are conserved between fruit flies and humans. Therefore, some disease processes may be vertebrate-specific and may not be able to be properly demonstrated using a *Drosophila* model. For example, multiple sclerosis is a vertebrate immunological disease that is difficult to recapitulate in flies. Proteomic techniques can also be challenging. Although *Drosophila* do produce large numbers of progeny, collecting a sufficient amount of protein from these animals can be tedious to say the least. Nevertheless, new tools and information are constantly emerging enabling the potential for some of these obstacles to be overcome.

1.4 Studying sialylation using a *Drosophila* model

In many of the previous sections of this document I have briefly touched on the advantages to using *Drosophila* as a model organism to study human pathologies. To reiterate in more detail, *Drosophila* have a short life span (approximately 7 weeks), female flies can lay up to 100 eggs per day for up to 20 days[49], and many genetic tools have been established in this organism to aid in the investigation of target genes and their underlying mechanisms. In this section I would like to expand on these advantages while discussing how they tie in to using *Drosophila* as a model to study sialylation.

In the last two decades, we have gained a significant amount of knowledge on the sialylation pathway in *Drosophila*. The release of the complete sequencing of the *Drosophila* genome in the year 2000 led to the identification of several sialylation pathway genes orthologous to vertebrate enzymes, suggesting that the sialylation pathway is conserved between flies and mammals **(Figure 1-2)**. So far, Neu5Ac phosphate synthase (*SAS*), CMP-Neu5Ac synthase (*CSAS*), and sialyltransferase (*DSiaT*) genes have all been discovered[55, 57, 120]. Study of the sialylation pathway in vertebrates poses many obstacles such as limitations of genetic approaches and complexity of glycosylation. *Drosophila* contain fewer genes than in humans and have overall less genetic redundancy making the pathway easier to study in these organisms. Inactivated sialylation pathway by means of targeted mutagenesis has demonstrated a role for *DSiaT* and *CSAS* in the regulation of the nervous system[45, 88]. Sialylation is also a fundamental process necessary for the proper development and function of the vertebrate nervous system, therefore it is likely that many of the genetic and biochemical mechanisms are conserved between these species. Additionally, several genetic interactions that have been established with vertebrate sialylation pathway genes have also been confirmed in *Drosophila*. A relevant example, would be the well-known interactions with VGICs[45, 88]. Using *Drosophila* to screen for other genes coding for proteins which are sialylated is a definite possibility and could help in the understanding of genetic and biochemical mechanisms of sialylation in vertebrates which still remain poorly understood.

Drosophila genetic approaches, such as the UAS/Gal4 system, have enabled the identification of tissue-specific and developmental requirements of sialylation, which

would have otherwise been difficult to study in vertebrate models. Using this approach in addition to molecular and immunolabeling techniques, we can visually examine how sialylation affects the localization and expression of glycoproteins throughout development. Another advantage of the UAS/Gal4 system is the ability to study toxic or lethal gene products by restricting expression to non-essential tissues. Pharmacological approaches in *Drosophila* are relatively straightforward and free of any ethical dilemmas that may be problematic in vertebrate models. Drugs can be administered indirectly through feeding or directly through injection or dissection of the tissue. In fact, the direct exposure of the beating *Drosophila* heart to different drugs and inhibitors lead to the identification of some essential VGICs and neurotransmitters [29, 51, 69, 71]. Similar approaches could be used for investigation of VGICs and how they are affected in the hearts of *Drosophila* sialylation mutants. These studies could potentially be useful in identifying mechanisms that may be exploited in treatment of cardiac abnormalities. Interestingly, Neu5Ac was delivered indirectly via tank water to zebrafish deficient in *NANS*, resulting in a partial rescue of skeletal defects[113], which further highlights the importance of model systems. In section 3, I discuss my results that demonstrated a full rescue of neurological defects upon the feeding of Neu5Ac to *SAS* mutants. This raises the question of whether Neu5Ac can be orally administered to humans deficient in NANS in order to treat neurological and skeletal defects. Taken together, the advantages discussed here have demonstrated that *Drosophila* is emerging as a powerful model system to study sialylation. This approach is expected to reveal genetic and molecular mechanisms that will suggest strategies for treatment of diseases caused by sialylation defects.

1.5 Main aims

The main goal of this research is to elucidate mechanisms and functions of sialylation, in hopes to better understand how we can treat and cure congenital disorders of glycosylation and other vertebrate pathologies linked to this metabolic pathway. In the current research, I aimed to: 1) investigate the role of sialylation in *Drosophila* heart physiology and function. I approached this aim by using established methods to assess cardiac functions and morphology in sialyation pathway mutants. 2) Determine the mechanism by which sialylation is regulated in the nervous system. I explored this aim using genetic and immunolocalization/ microscopy approaches, while mainly focusing on characterization of *SAS*, which up until now has remained largely unexplored. I also investigated relationships between *SAS* and other sialylation pathway genes, as well as VGIC genes.

2. FUNCTION OF GLYCOSYLATION IN REGULATION OF *DROSOPHILA* HEART FUNCTIONS

2.1 Introduction

Cardiovascular disease poses a major threat in the United States, ranking as one of the world's deadliest diseases. An estimated 70 million people die of cardiovascular disease each year (World Health Organization). As such, cardiovascular research is on the rise, as many researchers aim to discover new cardiac genes that may help unravel pieces to the very complex web of mechanisms responsible for human heart pathologies. In the past couple of decades, significant progress has been made through the study of cardiac physiology in model organisms. A study done in a mouse model for heart failure demonstrated the necessity for post-translational modifications, specifically sialylation, in the event of cardiac excitability[112]. In this study, voltage-gated Na^+ channels were discovered to be sialic acid-deficient in muscle LIM protein knockout mice, leading to arrythmias and heart failure. This finding was confirmed a few years later in a study examining cardiac phenotypes in ST3Gal4-deficient mice[23]. Notably, *ST3Gal4*$^{-/-}$ mice had reduced sialylation of ventricular Kv4.2 and Kv1.5 isoforms, thus affecting the generation of action potentials and ventricular repolarization. *ST3Gal4*$^{-/-}$ mice are also linked to dilated cardiomyopathy and stress-induced heart failure[18]. Although the main focus in recent studies of heart sialylation has been placed on voltage-gated channels, rat hearts metabolically labeled with azidosugars conjugated to fluorophores aided in the identification of more than 200 cardiac proteins modified with sialic acids[90]. Their roles

in heart regulation remain largely unknown. Furthermore, the genetic and biochemical mechanisms by which sialylation influences the heart remain poorly understood. Here we aim to elucidate pathological mechanisms that underlie the connection between abnormal sialylation and heart disease using a more malleable model system, *Drosophila melanogaster*. This model provides many advantages, such as the availability of non-invasive imaging techniques for analyses of heart physiology, superb genetic approaches, and simplified glycosylation pathways compared to vertebrates.

Our laboratory previously characterized *Drosophila* sialyltransferase (DSiaT), an enzyme modifying glycoproteins with sialic acids[57, 88]. While human organisms possess twenty sialyltransferases, *Drosophila* has only one sialyltransferase, which makes *Drosophila* a convenient model for studying sialylation. DSiaT represents the final enzymatic step in the sialylation pathway, where it is preceded by CMP-Neu5Ac synthetase (CSAS)[58]. CSAS provides the sugar donor molecule CMP-Neu5Ac to DSiaT. In *Drosophila*, much focus has been placed on the role of sialylation in the nervous system. DSiaT mutations cause an array of neurological phenotypes including TS paralysis, locomotion abnormalities, and reduced longevity[88]. Additionally, electrophysiological analyses revealed that DSiaT regulates neuronal excitability and affects the function of the voltage-gated Na^+ channel Paralytic (Para). The *Drosophila* heart is in fact innervated and also relies heavily on voltage-gated ion channels (VGICs)[29, 51, 80, 81, 85]. Therefore, there is a high likelihood for involvement of sialylation in the cardiac system. Until now, the role of sialylation in *Drosophila* heart physiology and function was unknown.

The larval *Drosophila* heart is a linear vessel running the entire length of the fly body. Three pair of ostia divide this tube into chambers, and facilitate the inward flow of hemolymph into the heart. Although the larval heart does possess innervation of the aortic tissue, rhythmic activity of the larval heart is believed to be myogenic in nature[52]. During metamorphosis, the larval heart undergoes huge morphological and physiological change. The heart tube becomes separated into an anterior aorta running the length of the head and thorax and a posterior abdominal heart. The abdominal heart contains a newly formed conical chamber that is uniquely innervated by a pair of glutamatergic neurons referred to as the transverse bridge[19] **(Fig 1-1A, B)**. The conical chamber initiates retrograde beats referred to as cardiac reversal and its activity has neurogenic properties[20]. The caudal chamber contains four large crustacean cardioactive peptide (CCAP)-positive neurons responsible for initiating anterograde beats. CCAP is a neurotransmitter found in insects that behaves as a cardioaccelerator. In addition to the transverse bridge, the abdominal heart is segmentally innervated by transverse nerves originating from the midline of the thoracic ganglion. The transverse nerves innervate both sides of the heart tube, however symmetrical nerves never merge, as is the case with the transverse bridge.

Here we investigate a role for sialylation in heart function using loss-of-function alleles, $DSiaT^{L22}$ and $DSiaT^{S23}$. We show that deficient sialylation results in bradycardia which can be attributed to longer diastolic and systolic intervals. However, larval hearts are unaffected by a lack of sialylation. This is likely because of the changes that occur in the heart during development, and specifically, due to differences in heart innervation at

these stages. Our results uncover sialylation-mediated neural control of heart function and defective innervation of the conical chamber in DSiaT mutants.

2.2 Results

2.2.1 *Drosophila DSiaT*$^{-/-}$ adults display a decreased heart rate relative to wild-type

Previously, our laboratory characterized the role of DSiaT in the regulation of neural transmission; however, the effect of sialylation on the heart has never been studied in *Drosophila*. To test if DSiaT is important for *Drosophila* heart physiology and function, we performed an intact heart rate assay on the *DSiaT* loss-of-function allele, *DSiaT*^{L22}. Using semi-automated optical heartbeat analysis (SOHA) software, we observed DSiaT mutants have a significantly reduced heart rate when compared to wild-type (WT) flies (bradycardia) **(Fig. 2-1A)** Additionally, we quantified the intact heart rate in *CSAS* mutants to determine if other sialylation pathway enzymes also have an effect on heart function. Indeed, *CSAS*^{221} homozygous mutants resulted in a similar bradycardia phenotype. To investigate the cause of bradycardia in more detail, we used SOHA to generate an m-mode trace, which shows the movement of the heart edges through alignment of 1-pixel-wide, vertical strips from successive movie frames, of the semi-intact beating heart for both wild-type and mutant flies over a 10 second interval **(Fig. 2-1B)**. The m-modes show that both wild-type and *DSiaT*$^{-/-}$ hearts are very rhythmic, yet *DSiaT*$^{-/-}$ flies have an increase in both diastolic and systolic intervals leading to an overall larger heart period. Upon quantifying this finding, we determined that DSiaT$^{-/-}$ hearts have significantly longer

average diastolic intervals **(Fig. 2-1C, D, I)** as well as systolic intervals **(Fig. 2-1E, F, I)**. When combined, these effects led to a significantly longer heart period and thus slower beats **(Fig. 2-1G, H, I)**. In *Drosophila*, the larval heart is very different from the adult heart. During metamorphosis, the larval heart undergoes a drastic cardiac rearrangement leading to many morphologic and physiological changes. Therefore, we examined the heart rate in larval *DSiaT*$^{-/-}$ mutants to see if they presented with the same phenotype. Interestingly, we did not notice a difference between wild-type and *DSiaT*$^{-/-}$ larval heart rate **(Fig. 2-1J)**. These results suggest that proper sialylation is necessary specifically for the function of the adult heart.

2.2.2 Nervous system sialylation-mediated control of heart function in *DSiaT* $^{-/-}$ mutants

It is known that sialylation pathway genes *CSAS* and *DSiaT* genetically interact with voltage-gated ion channel genes such as *paralytic* (*para*) and *seizure* (*sei*) in the brain. Glycan modifications can strongly influence the neural excitability of these ion channels by affecting the generation and propagation of action potentials[22, 23, 98, 99, 112, 125-128, 132]. Action potentials are also responsible for initiating contractions of the heart and thus can influence pace and rhythmicity. Therefore, we hypothesize that sialylation is influencing the heart rate by affecting neural excitability and that glycoproteins are involved in regulation of action potentials either directly in the heart tissue or indirectly through the nervous system. In order to elucidate if sialylation is working directly in the heart tissue to influence its function, we initially tried to utilize the UAS-Gal4 system[12] to carry out

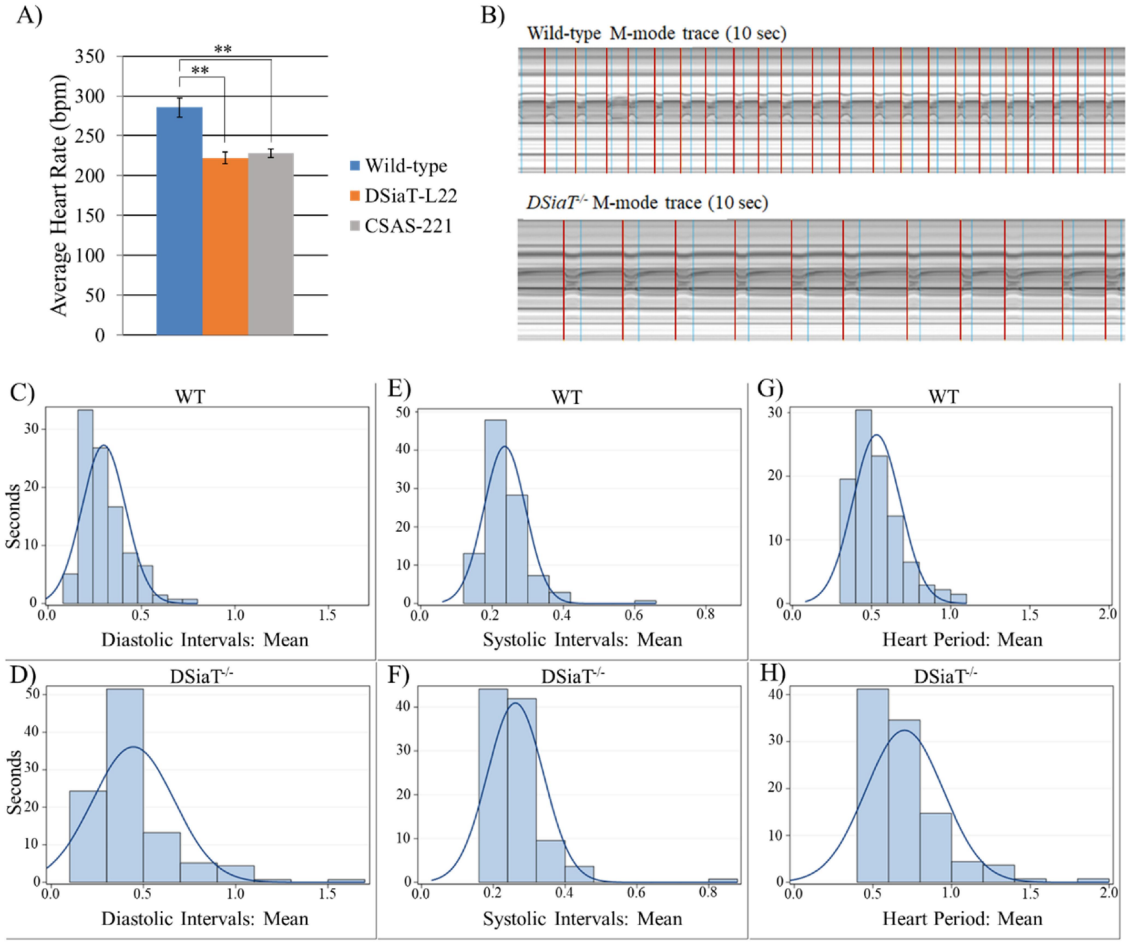


Figure 2-1 Sialylation affects heart function in adults, but has no effect on larval hearts. A) Adult sialylation mutants have significantly slower heart rates than WT control. Intact heart rate assay was performed on homozygous mutant *DSiaT* and *CSAS* five-day old male flies (males were used to reduce heart variability). Each fly was subjected to 3 trials, by which all values were averaged together to obtain the number of beats per minute for each fly. Each genotype contains a sample size of 10. **B)** Representative m-mode traces demonstrate semi-intact *WT* and *DSiaT* mutant hearts beating over a 10 second period. Vertical red lines indicate the end of each diastolic event and the beginning of each systolic event. Vertical blue lines indicate the beginning of each diastolic event and the end of each systolic event. *DSiaT* mutant hearts display longer intervals that are rhythmic and a slower beating heart. Histograms display mean diastolic interval for **(C, D)**, mean systolic interval **(E, F)**, mean heart period **(G, H)** for WT and *DSiaT* mutants.

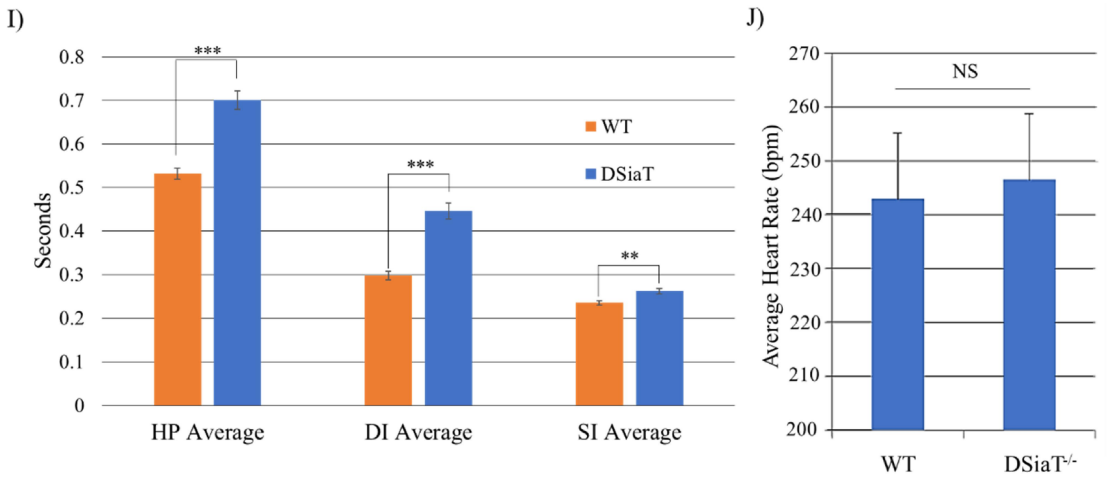


Figure 2-1 Continued. I) *DSiaT* loss-of-function causes a significant increase in heart period (HP), and systolic (SI) and diastolic intervals (DI) compared to WT controls. **J)** *DSiaT* mutant larvae do not have the reduced heart rate phenotype as displayed in adults. Intact late-3rd instar *DSiaT*$^{-/-}$ larval hearts were not significantly different from WT hearts, suggesting the heart rate phenotype occurs after metamorphosis. For each genotype, 10 male larvae were assayed. NS- not significant. Error bars represent SEM. Asterisks indicate statistically significant differences (*t*-test, **p<0.01, ***p<0.001).

our tissue-specific rescue experiments. However, all our transgenic insertions of *UAS-DSiaT* construct were able to rescue DSiaT mutant phenotypes without the presence of a Gal4 driver. This result indicated that UAS-DSiaT constructs have "leaking", driver-independent expression, possibly due to some internal enhancer-like sequences. To overcome this obstacle, I utilized the UAS-GAL4 system in conjunction with the FLP-FRT system. To this end, a UAS construct containing a series of three stop codons placed between two FRT sites located upstream of DSiaT-GFP coding region was generated by molecular cloning techniques and transgenically introduced in flies. This FRT-flanked fragment can be flipped out when acted upon by the FLP site-specific recombinase (aka

"Flippase"), thereby leading the expression of GFP-tagged DSiaT **(Fig. 2-2A)**. Without the presence of Flippase, there should be no leaking expression of DSiaT-GFP.

To test that expression system, I carried out a series of immunostaining experiments using late third instar larval brains and hearts to analyze the expression of DSiaT-GFP with or without a driver, which confirmed that the system was working as expected **(Supplemental Fig. A-1)**. Using this tripartite system, I drove the expression of *DSiaT* using different tissue-specific promotors and assayed flies for rescue of the heart rate phenotype in a *DSiaT* homozygous mutant background **(Fig 2-2B)**. Without the presence of a tissue-specific promotor we did not observe a rescue of the heart rate thereby further confirming that our expression system is working properly. As expected, when we used the ubiquitous promotor, *Actin-Gal4*, there was a full rescue of heart rate phenotype. Surprisingly, when we used the heart-specific promotor, *tinC-Gal4*, to drive the expression we did not see a significant difference from the control. However, when we used the neuronal-specific promotor, *C155-Gal4*, we again saw a full rescue of the heart rate phenotype. These results suggest that DSiaT is working in the nervous system to influence *Drosophila* heart function indirectly.

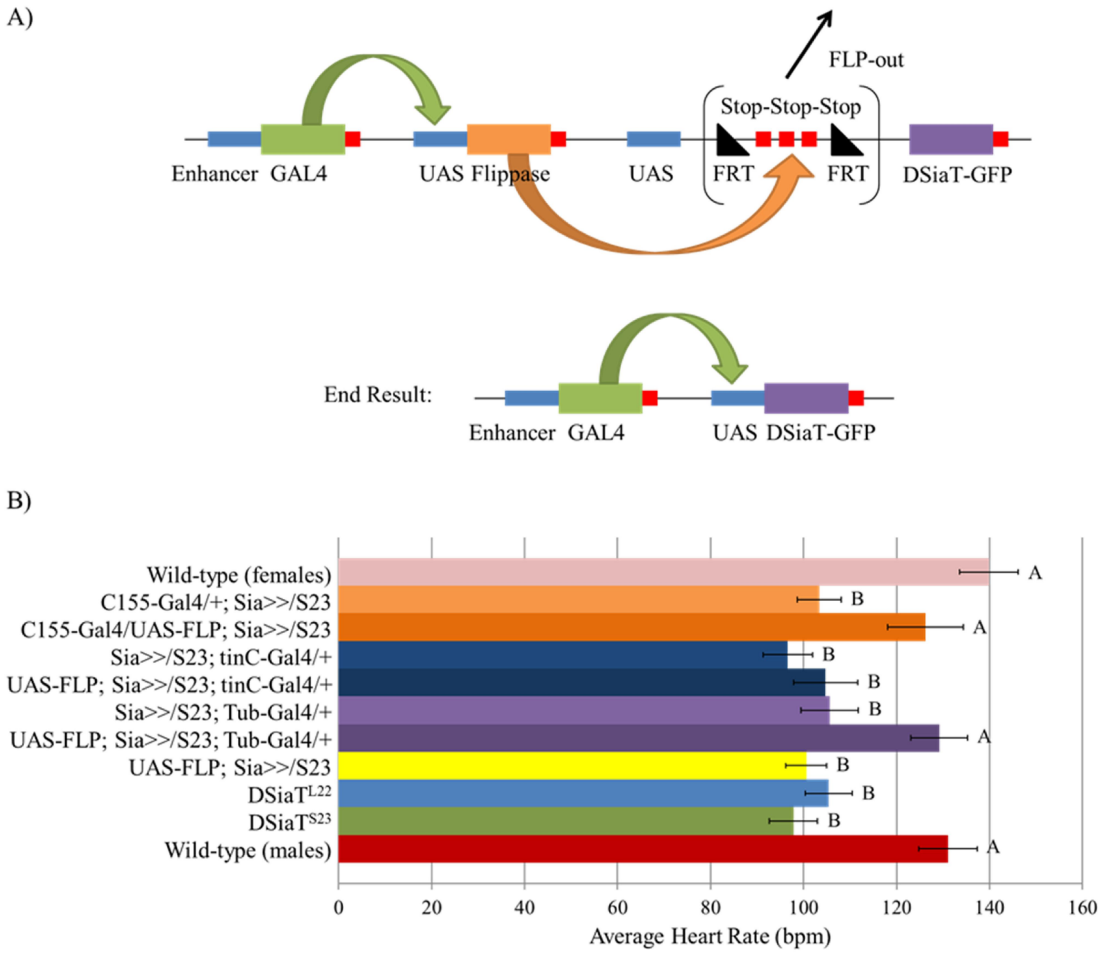


Figure 2-2 Adult heart rate phenotype is due to nervous system sialylation-mediated control of heart function. A) Schematic of FLP/FRT strategy. Rescue was performed using FLP/FRT system that contains a UAS construct containing a series of three stop codons placed between two FRT sites located upstream of DSiaT-GFP coding region (Sia>>). **B)** Semi-intact heart rate assay displaying tissue-specific rescue of heart rate phenotype in *DSiaT* mutants. Adult *DSiaT* mutant heart rate can be rescued upon expression of DSiaT in the nervous system. There is no rescue of heart rate when DSiaT is expressed in the adult mutant heart. For each genotype, 20-30 male flies were assayed, except for genotypes containing the *C155-Gal4* driver in which cases I used female flies. Wild-type females were added as a control. Statistical analysis was performed using one-way ANOVA (Tukey-Kramer). Letters indicate statistically significant differences of $p<0.05$. Error bars are SEM.

2.2.3 The adult *Drosophila* heart lacks endogenous expression of DSiaT

To determine if there is endogenous expression of DSiaT in the heart, we used an HA-tagged DSiaT transgenic BAC construct previously generated in our lab that reflects the natural distribution of DSiaT (see Materials and Methods). Third-instar larval brains were used as a control for immunostaining, in which case we expect to see expression of DSiaT in neurons **(Fig. 2-3A, B)**. Upon analyzing expression of DSiaT in the adult heart tube, we did not detect DSiaT-HA signal above background using a wild-type genotype (lacking the transgenic BAC) as a negative control. **(Fig. 2-3C, D)**. These data suggest a lack of endogenous expression of DSiaT in the adult heart.

2.3 Discussion

This work has provided new evidence supporting a role for sialylation in regulation of *Drosophila* heart function. Our results demonstrated a significant reduction in heart rate in the absence of sialylation, which can be attributed to a lengthening in both diastolic and systolic intervals. Deficient sialylation in vertebrates leads to arrhythmogenesis which can eventually result in heart failure[112]. Although we did not report an arrhythmia phenotype here, we did see a significant increase in arrythmia index in $DSiaT^{S23}$ homozygous mutants **(Supplemental Fig. A-2)**. However, arrhythmia index was normal in $DSiaT^{L22}$ mutants placed within the same genetic background as $DSiaT^{S23}$, suggesting some difference between functional properties of these two loss-of-function alleles. The nature of this difference is unclear at the moment, and further studies are necessary to verify the arrhythmia phenotype and reveal its mechanism.

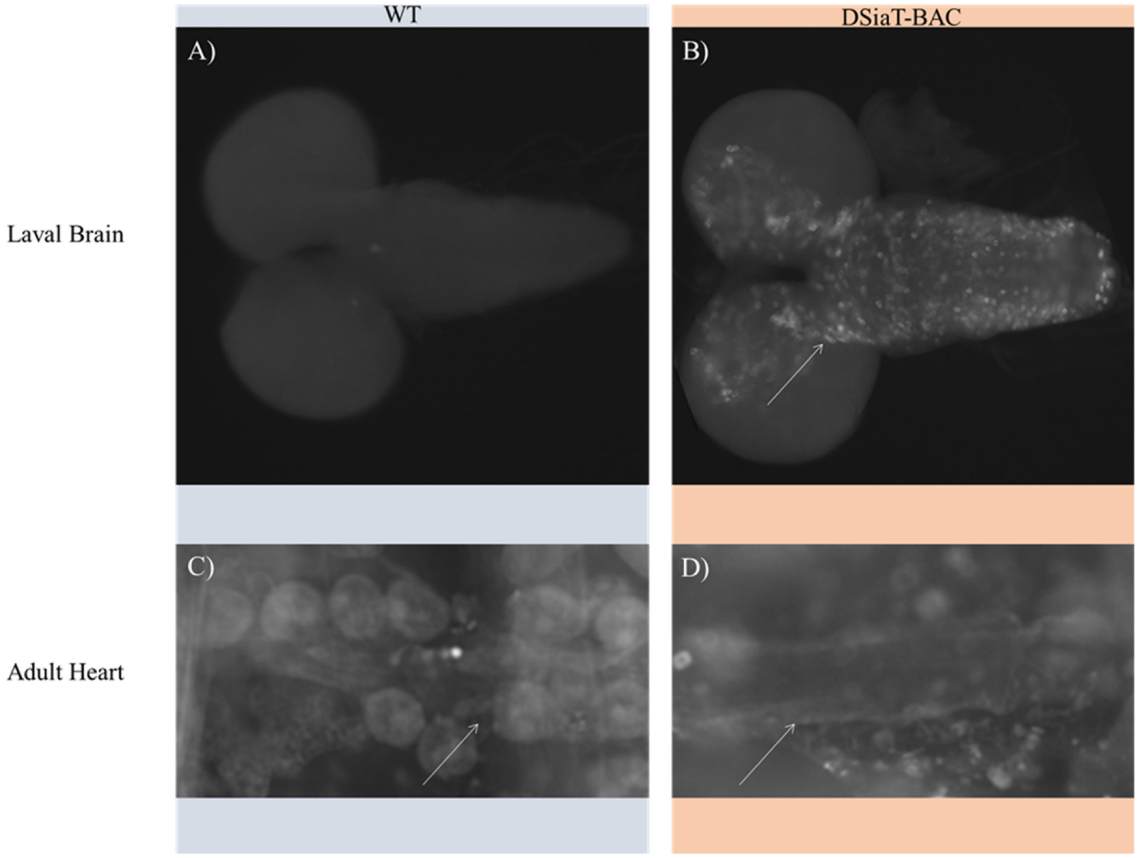


Figure 2-3 Adult *Drosophila* heart lacks endogenous expression of DSiaT. A) Negative control, WT 3rd-instar brain with no DSiaT expression. **B)** BACSia-3xHA 3rd-instar brain showing endogenous expression of DSiaT in neurons. Arrow indicates an example of a neuron were BACSia-3xHA was expressed. **C)** Negative control, WT heart of 5-day old adult fly. **D)** Adult BACSia-3xHA 5-day old heart. BACSia-3xHA expression could not be distinguished from background levels, indicating a lack of endogenous DSiaT expression in the adult heart. Arrows in **C** and **D** indicate regions where fluorescent intensity was measured using ImageJ software for comparison. Hearts and brains were stained with rat anti-HA (1:1000) and goat anti-rat Alexa488 (1:125). All images were taken using a 10X objective. Exposure times were the same for larval brains (400 ms), and adult hearts (699 ms).

It has been shown that voltage-gated ion channels not only play a large role in the vertebrate heart, but also are major contributors to *Drosophila* heart physiology and

function. Mutations in the ERG voltage-gated K^+ channel, *seizure (sei)*, causes bradycardia and arrhythmias[81]. Additionally, *sei* mutants display reduced heart contractility. Mutations in *KCNQ1*, a gene encoding the α subunit of another type of voltage-gated K^+ channel in *Drosophila*, can result in prolonged contractions and cardiac fibrillations similar to Torsades des Pointes arrhythmias[80]. "OPQ-type" $Ca2^+$ channels are critical for the *Drosophila* heart as demonstrated by cardiac arrest in pupae treated with channel blockers such as omega-Conotoxin MVIIC[51]. These pharmacological analyses also suggested that Na^+ and Cl^- channels are not essential for *Drosophila* cardiac function, although these experiments were done during the initial stage of pupariation (P1) prior to cardiac rearrangement and may not accurately represent the function of the adult heart possessing more intricate innervation. Interestingly, when we examined *DSiaT*$^{-/-}$ larval hearts we did not see a significant difference in heart rate compared to wild-type. This result supports the notion that sialylation can have different roles post-cardiac rearrangement. Therefore, this should be considered when studying other pathways relevant to cardiac function and physiology.

Until now, *Drosophila* sialylation studies have focused primarily on the nervous system, where aberrant sialylation results in defective synaptic transmission and neural excitability, and was shown to affect voltage-gated ion channels[45, 88]. This poses the question of whether sialylation influences heart function indirectly through its role in the nervous system. To explore this possibility, we used the FLP/FRT system under the control of the UAS-Gal4 system. Using this genetic approach, we ectopically expressed *DSiaT* in a tissue-specific manner to obtain functional data through rescue of the heart rate

phenotype. Our results indicate that there is sialylation-mediated neural control of heart function based on the observation of a full rescue of heart rate phenotype when DSiaT was expressed in neurons, but not the heart of *DSiaT* mutants. These results are interesting considering we used the semi-intact preparation method of dissection, which removes the head and a large portion of the thorax that contains the thoracic ganglion, and therefore neural control of the heart was largely eliminated during our assays[121]. There are two possible explanations for this result: 1) Mutations in DSiaT result in a developmental defect that affects the heart rate. 2) There could be an effect through innervating neurons that are still present in the semi-intact preparations. These possible scenarios could be investigated through careful examination of the morphology of mutant hearts and innervating neurons using microcopy and immunostaining techniques. In addition, a pharmacological approach could be employed on wild-type semi-intact flies to remove sialylated glycans (e.g. by treatment with a sialidase or N-glycanase) to analyze if heart phenotypes resemble what is observed in sialylation mutants.

The abdominal heart is innervated in all segments of the abdomen by symmetrical transverse nerves that originate from the ganglion[19]. In segments 2-5 these nerves never touch, yet in the first abdominal segment arborizations merge to form a glutamate-immunoreactive synaptic structure referred to as the transverse bridge. The transverse bridge runs through the conical chamber, which has been demonstrated to function as the retrograde pacemaker[20]. We hypothesize the innervation though the conical chamber is altered in *DSiaT* mutants, resulting in the observed bradycardia phenotype. Notably, the vertebrate heart is modulated by the autonomic nervous system. Stimulation of the

sympathetic and vagal nerves can increase or decrease heart rate and affect conduction velocity[33]. This data demonstrates a role by which the autonomic nervous system influences heart rate, and therefore highlights a need to further explore heart innervation in sialylation mutants.

2.4 Materials and methods

2.4.1 *Drosophila* strains and transgenic insertions

We obtained w^{1118} flies from Bloomington *Drosophila* Stock Center. The $DSiaT^{S23}$ loss-of function allele was generated by an ends-in gene-targeting approach as previously described[88, 91]. The $DSiaT^{L22}$ loss-of function allele was generated with an ends-out targeting technique as previously described[32, 88]. $CSAS^{221}$ null mutation was generated as described previously[45]. Act-Gal4 and C155-Gal4 lines were from the Bloomington Stock Center (Indiana University). TinC-Gal4 was from Dr. Matthew Wolf (Duke University). All *Drosophila* strains were reared in a controlled environment incubator (25°C, 35% humidity, 12 h light/darkness) on standard cornmeal-malt-yeast medium.

CSAS-LexA BAC and *DSiaT-HA BAC* transgenic insertions were generated by φC31 integrase-mediated transgenesis using BAC constructs produced in the lab by recombineering [refs: PMID 17360644, 18676454]. *CSAS-LexA BAC* construct was based on a clone with ~25 kb genomic region surrounding the *CSAS* gene. This construct includes an insertion of *nlsLexA::p65*-encoding sequence within the last exon of *CSAS*.

DSiaT-3HA BAC transgenic construct was generated using similar strategy. This construct is based on a BAC clone with an ~24 kb genomic region centered on the *DSiaT* gene. A 3xHA tag sequence was fused to the last codon of *DSiaT* within this BAC by recombineering. Functionality of *CSAS-LexA BAC* was confirmed by rescue experiments (it was used to express transgenic *CSAS* in *CSAS* null mutants). We also confirmed that *DSiaT-3HA BAC* recapitulates the endogenous expression pattern of *DSiaT* by immunostaining[88].

2.4.2 Heart rate assay

Both intact and semi-intact preparations were used throughout. Intact flies were collected on the day of eclosure (day 0), and aged for 5 days on standard food at 25°C. Flies were transferred to fresh food on day 3 and all vials were reared in a Percival incubator on a diurnal 12/12 dark-light cycle maintained at 25°C. Intact mid-third instar larvae were collected from the sides of developing vials and placed in an empty petri dish for examination. Intact adult flies were placed in an empty polystyrene vial on day 5 and were anesthetized for 1'30" with 0.5 ml of Flynap. Flies were mounted individually on small glass cover slips, dorsal side up, in a thin layer of petroleum jelly. To avoid suffocation, each fly head was turned to the side. The beating adult heart was visualized through the cuticle of the first abdominal segment. The intact larval heart was visualized through the dorsal side of the epidermis.

Semi-intact preparations were dissected as previously described[121]. Briefly, we collected adult flies on the day of eclosure (day 0), separated them by gender and then

aged them on standard food at 25°C to 5 days old. Vials were transferred to fresh food on day 3, and all vials were reared in a Percival incubator on a diurnal 12/12 dark-light cycle maintained at 25°C. 6-10 individuals were placed in each polystyrene vial and were anesthetized for 1'30" with 0.5 ml of Flynap. Flies were then mounted individually, ventral side up, on small glass coverslips covered with a thin layer of petroleum jelly. Semi-intact dissection was performed in a temperature-controlled room maintained at 21°C. Dissected flies were then left alone for 25 minutes so that hearts could stabilize before recording. A Physitemp TS-4MP thermoplate was setup on the microscope stage where coverslips were placed during all recordings, and maintained a temperature of 21°C. Recordings were taken on a Zeiss Examiner.D1 microscope with a 10X water immersion lenses using a high-speed ORCA-flash4.0 Hamamatsu camera. We routinely did adult heart counts by visually counting each heart five times for 10 seconds and then averaging the five counts to generate the individual heart rate. We then averaged the heart rates for all individuals to create the values represented for particular genotypes. SOHA analysis software was used for analysis of videos to obtain m-modes and heart rate parameters with the exclusion of rate[26].

2.4.3 Immunofluorescent staining and microscopy

Larval brains were dissected in ice-cold Ringer's solution, washed, and fixed in fresh fixative solution (4% paraformaldehyde) for 20 minutes at room temperature with gentle agitation. Approximately ten adult hearts consisting of an equal ratio of males and females were dissected using the semi-intact method in ice-cold hemolymph, and fixed in

fresh fixative at room temperature for 15 minutes on the dish and then placed in a microcentrifuge tube with 1 ml of fixative to continue fixation for 15 minutes with gentle agitation. Fixed tissues were analyzed by immunostaining and microscopy. For endogenous expression experiments we used rabbit anti-HA (1:1000) from Jackson ImmunoResearch, and goat anti-rabbit Alexa488 (1:125) from Molecular Probes. For DSiaT-GFP expression experiments we used mouse anti-GFP 8H11 (1:80) from DSHB and goat anti-mouse Alexa488 (1:125) from Molecular Probes. For experiments looking at innervation of the adult heart we used rabbit anti-HRP (1:250) from Vector. As secondary antibodies we used goat anti-rabbit Alexa488 (1:125) and Alexa594 Phalloidin (1:1000). All brains and hearts were mounted in anti-fade Vectashield medium from Vector. Immunofluorescent staining was examined using a Zeiss Axioplan 2 microscope with 10x objective. We used ImageJ software to quantify fluorescent intensity.

2.4.4 FLP/FRT system

pUAST>stop>DSiaT-GFP construct was generated by standard molecular biology techniques using pUAST>stop>mCD8-GFP (Addgene ID 24385, Potter et al Cell. 2010;141(3):536-48) as a backbone plasmid. DNA region encoding mCD8 was replaced in that plasmid with DSiaT cDNA, which created in-frame fusion of the 3'-end of DSiaT-coding sequence with GFP-coding region, referred to as Sia>> in this paper (Lyalin D., unpublished results). Upon FLP-mediated excision of FRT-flanked expression termination region (>stop>), the construct starts to express DSiaT-GFP under control of UAS regulatory elements.

2.4.5 Statistical analyses

For comparison of multiple groups, one-way ANOVA (Tukey-Kramer) was used to identify significant differences in mean. For comparison of two groups of data, unpaired two-tailed t test was used.

3. REGULATORY MECHANISMS CONTROLING SIALYLATION IN THE *DROSOPHILA* NERVOUS SYSTEM

3.1 Introduction

Sialic acids are a family of negatively charged monosaccharides consisting of a relatively large 9-carbon backbone. Through the process of sialylation, sialic acids are terminally added to the ends of glycan chains modifying glycoproteins and glycolipids. The study of sialylation in mammals is fascinating from the perspective of its involvement in diverse biological processes. For example, sialic acids act as recognition sites to aid in cell-cell communication; therefore they play major roles in host-pathogen recognition, regulation of immune response and cancer progression[116]. Sialylation has also been shown to be a necessity for the proper development and function of the mammalian nervous system as demonstrated by the need for polysialylation of neural cell adhesion molecule (NCAM), which is important for migration and differentiation of neural progenitor cells and regulation of axonal growth and targeting[17, 43]. Although much headway has been made in the exploration of roles for sialylated glycans in the mammalian nervous system, mechanistic understanding of their functions is commonly lacking. The established *Drosophila melanogaster* model of neural sialylation has provided us with many useful tools to examine genetic and molecular mechanisms in great detail. Additionally, the *Drosophila* glycosylation pathways lack functional redundancy of enzymes and intricate regulation like in mammals, thereby simplifying genetic approaches. Studies investigating sialylation pathway enzymes, *Drosophila* cytidine monophosphate-sialic acid synthetase

(CSAS) and *Drosophila* sialyltransferase (DSiaT), have suggested a functional role for sialylation in the regulation of neural excitability where sialylation is thought to affect the gating properties of voltage-gated channels[45, 88]. Here we aim to look deeper into the mechanism by which sialylation is regulated in the nervous system through the examination of *Drosophila* sialic acid synthase (SAS), a minimally explored upstream enzyme of the sialylation pathway.

SAS was identified and initially characterized in vitro in 2002, and it became the first cloned *SAS* gene in proteasome organisms[55]. SAS and its human counterpart N-acetylneuraminic acid synthase (NANS) function similarly in terms of substrate specificity where SAS can use both N-acetylemannosamine-6-phosphate (ManNAc-6-P) and mannose-6-phosphate (Man-6-P) in a condensation reaction with phosphoenolpyruvate (PEP) to create a phosphorylated product of either N-acetylneuraminic acid (Neu5Ac) or 2-keto-3-deoxy-D-glycero-D-nononic acid (KDN)[55] **(Fig. 3-1A)**. Following the condensation reaction, there is a dephosphorylation step with Sia-9-P-phosphtase to obtain Neu5Ac. CSAS then uses the dephosphorylated Neu5Ac product to generate the sugar donor molecule CMP-Neu5Ac that can be used by DSiaT in the final enzymatic step of the sialylation pathway. Similar to CSAS and DSiaT, SAS is expressed during all *Drosophila* developmental stages, although whether SAS expression and function is restricted solely to the nervous system remains unclear[45, 55, 88]. Interestingly humans hypomorphic for *NANS* not only have intellectual disorders due to impaired brain function but they exhibit defects in skeletal development and growth, suggesting a role for SAS outside of the nervous system[113]. Additionally, knockdown of *nans* in zebrafish resulted

in abnormal skeletal development and pericardial edema[113]. In the present study, we characterized SAS function *in vivo* using genetic and neurobiological approaches. In addition, we examine the subcellular localization of SAS in different tissues and explore interactions between *SAS* and other genes known to be involved with the sialylation pathway. We tested SAS functional requirements in different types of cells. We compared it to the requirement for CSAS, which shed light on a bipartite mechanism that regulates neural sialylation in the CNS.

3.2 Results

3.2.1 Characterization of *Drosophila SAS^{RFP}* allele

Phenotypes of *CSAS* and *DSiaT* mutants have been previously characterized in *Drosophila*[45, 57, 58, 88]. Mutations in these genes result in early onset temperature-sensitive (TS) paralysis, locomotion defects and reduced lifespan, all of which are indicative of neurological impairment. In line with these findings, human patients hypomorphic for *NANS* are found to have intellectual developmental disorders (IDD) along with skeletal dysplasia[113]. Currently, there is no behavioral, functional or localization data available on *SAS* mutants, thus we obtained and characterized a *SAS* mutant allele from the *Drosophila* Genomics Resource Center that was generated in a *piggyBac*-based insertional mutagenesis screen[96]. The *SAS* allele, which we denote in this paper as *SAS^{RFP}*, is composed of an insertion located in the intron between the first and second exons of *SAS* **(Fig. 3-1B)**. Here we use the *SAS^{RFP}* allele to elucidate the function of SAS *in vivo*. We

started by outcrossing the SAS^{RFP} allele eight times to a partially isogenized wild-type *Drosophila* strain (MH2Jw-). The outcrossed flies are homozygous viable but very weak and difficult to collect upon enclosion. In fact, most of the flies die if the vial is kept upright in the incubator. Therefore, in order to obtain SAS^{RFP} homozygous mutants, we kept the line balanced over TM6 carrying the Tb mutation that can be used as a morphological marker at larval and pupal stages. We collected long, late pupae (non-TM6) that should be homozygous for SAS^{RFP}. The long pupae were placed on the wall of a new food vial and the vial was kept on its side until flies hatched. We did not observe any obvious morphological defects. However, many SAS^{RFP} homozygous mutants were observed to be stuck three-quarters of the way out of the pupal case where they eventually died **(Fig. 3-1C)**. Due to the strong neurological defects reported in other sialylation mutants and an observed overall weakness of SAS^{RFP} homozygous mutants, we hypothesized the cause of failed enclosure to be neurological in nature. To test our hypothesis, we examined SAS^{RFP} mutant legs under high magnification to look for signs of any morphological defects. Upon comparison to wild-type legs, we did not see any morphological differences **(Fig. 3-1D, E)**. These observations suggest that this phenotype is likely neurological in nature, which led us to focus on behavioral assays assessing nervous system function.

3.2.2 Behavioral assays uncover severe neurological phenotypes in *SAS^{RFP}* homozygotes

To determine the allelic strength of *SAS^{RFP}*, we combined one copy of the *SAS^{RFP}* allele with *SAS* deficiency (*SAS^{Df}*). Upon subjecting flies to elevated temperature and assaying TS paralysis, we found that the *SAS^{RFP}* allele behaves indistinguishably from a null allele **(Fig. 3-2A)**. Thus, we continued to use it as a null/loss-of-function in all of the following behavioral assays. Comparable to what was previously published for *CSAS* mutants, *SAS^{RFP}* mutants demonstrate an early onset of TS paralysis within approximately 350 seconds **(Fig. 3-2B)**. This is a significantly shorter onset of paralysis than *DSiaT* mutants (approximately 550 seconds), suggestive of the previously noted independent roles for sialylation enzymes in the *Drosophila* nervous system[45]. Additionally, *SAS^{RFP}* mutants showed a significant decline in negative geotactic ability **(Fig. 3-2D)**. Out of the twenty 5-day old flies tested, all *SAS^{RFP}* mutants took longer than 60 seconds to walk up the vial to the 5 cm mark after being tapped down. Specifically, 13 out of 20 mutants reached the mark within three minutes, while the other seven took over ten minutes. Wild-type flies took less than ten seconds on average to reach the mark. Similar to the neurodegeneration phenotype seen in *DSiaT* mutants, geotaxis and paralysis phenotypes significantly increased in severity with age for *SAS^{RFP}* mutants **(Fig 3-2E, F)**. Only 5% of 1-day old *SAS^{RFP}* mutants took longer than 60 seconds to climb to the mark after being tapped down, compared to the 65% of 10-day old flies. In further support of this data, 10-day old *SAS^{RFP}* mutants had a significantly earlier onset of paralysis compared to 1-day

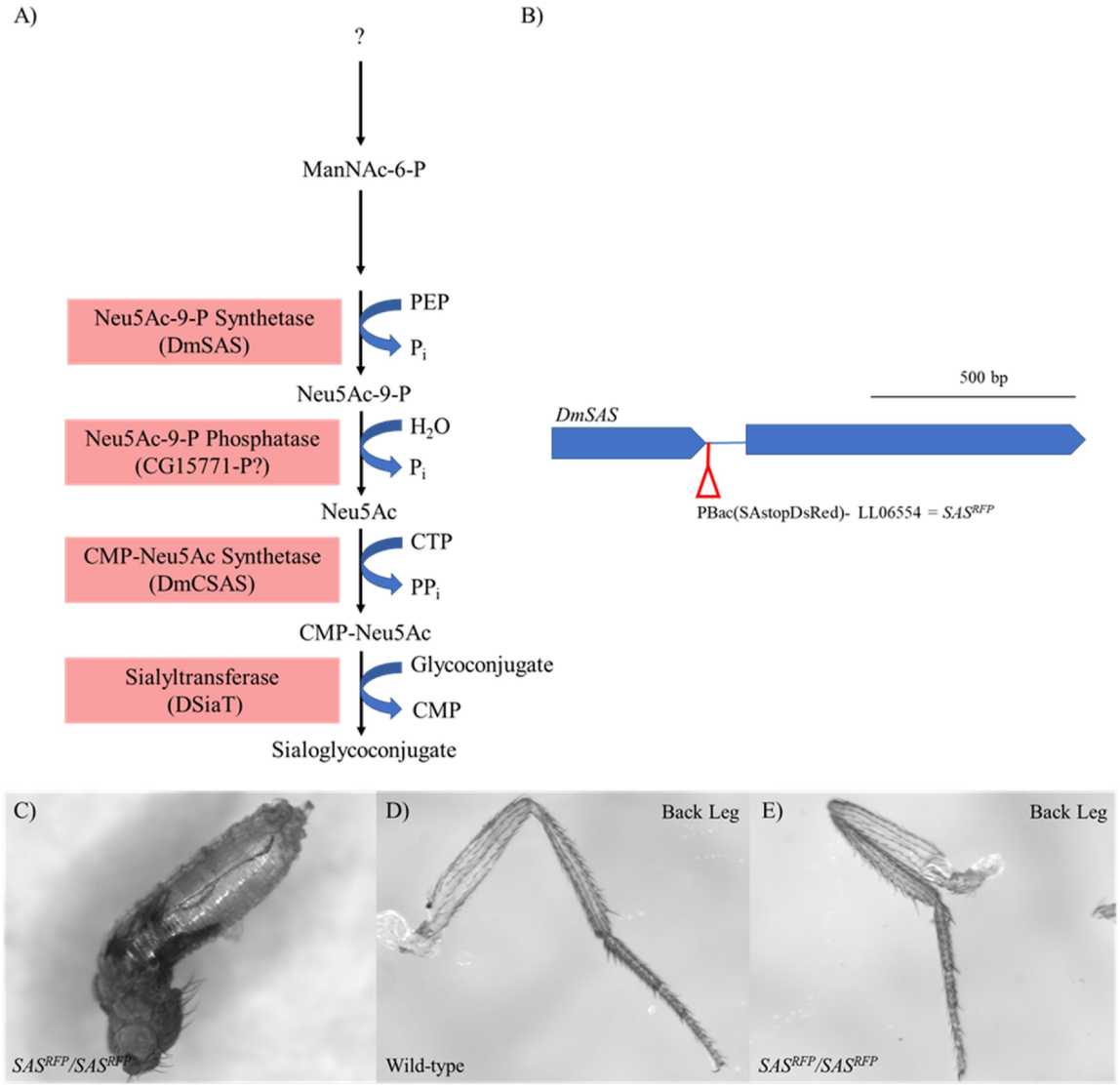


Figure 3-1 Schematic of *SAS*^{RFP} mutants and general signs indicative of neurological impairment. A) *Drosophila* sialylation pathway. **B)** *SAS* gene region indicating the location of the piggy-bac insertion (red triangle) in the *SAS*^{RFP} allele. Blue regions indicate exons. **C)** Representative photo of a dead homozygous *SAS*^{RFP} mutant stuck in the pupal case upon eclosion. **D, E)** Comparison of *SAS*^{RFP} and WT back legs. There are no morphological defects observed, suggesting that *SAS*^{RFP} mutants fail to hatch due to a neurological impairment.

old mutants. SAS^{RFP} heterozygotes were not significantly different from wild-type, indicating that removing one copy of the SAS^{RFP} allele is not sufficient to compromise the pathway.

Upon examining longevity, we found the lifespan of SAS^{RFP} homozygotes to be decreased by 73% compared to wild-type flies **(Fig. 3-2G)**. On average, SAS^{RFP} mutants lived approximately eleven days compared to the forty-one days seen in wild-type flies. To confirm that the *SAS* loss-of-function mutation was responsible for the observed longevity phenotype, we expressed an untagged full-length SAS protein, UAS-SAS, using the UAS-Gal4 system. Upon expressing SAS in the neurons (*C155-Gal4*) or glia (*Gli-Gal4*), we obtained a partial rescue of longevity. Overall, these results indicate that *SAS* mutants, like other sialylation pathway mutants, display defects in neurological function that increase in severity with age resulting in a decreased lifespan.

Previous works provided evidence that neural phenotypes seen in *DSiaT* mutants may be due to abnormal (decreased) excitability in neurons. In order to test this hypothesis, I subjected flies to diethyl ether, which has been shown to induce hyperexcitable phenotypes in some mutants. I developed a system to judge over-excitability verses under-excitability, by scoring the number of observed hyperexcitable phenotypes (i.e. leg shaking, abdomen twitching, head bobbing, wing fluttering, rapid spinning) that presented in each fly during a 5-minute period after subjection to diethyl ether. Genotypes obtaining a score significantly lower than wild-type are deemed under-excitable, while scores significantly higher than wild-type are considered to be over-excitable. Flies with a mutation in *ether a go-go* (*eag*), a voltage-gated potassium channel, served as our positive

control. *Eag* mutants have been previously shown to display over-excitability phenotypes[53, 123]. Our scoring system resulted in *eag* mutants possessing the highest score, as predicted **(Fig. 3-2C)**. Interestingly, all sialylation pathway mutants tested also resulted in a score significantly higher than that of wild-type, indicating an overall over-excitability in these flies. *Para^{ts1}* mutants obtained a score significantly less than wild-type, suggesting under-excitability in these mutants. These results indicate, at least at the organismal level, that sialylation pathway mutants are over-excitable.

3.2.3 *CSAS* and *SAS* function in glial cells to influence neurological behavior

Previous studies in our laboratory indicated that *CSAS* is expressed in the nervous system, however, the expression pattern of *CSAS* has not been characterized at the resolution of individual cells. Intriguingly, the distribution of CSAS mRNA revealed by *in situ* hybridization shows a diffuse web-like pattern at the surface of larval brain lobes, indicating a possibility that CSAS is expressed in glial cells[45]. Preliminary data from my collaborator (Hilary Scott, Massachusetts Eye and Ear) also suggested that *CSAS* functions in glial cells. To test this hypothesis, I used a transgenic reporter based on a *CSAS-LexA BAC* construct (see Materials and Methods). The expression of this reporter is expected to recapitulate the endogenous *CSAS* expression, which was confirmed by rescue experiments using *CSAS-LexA*-driven expression of transgenic *CSAS* cDNA in *CSAS* mutants (Hilary Scott, personal communication). In collaboration with Hilary Scott, I analyzed *CSAS-LexA*-driven mCD8-GFP and GFP-nls reporters to reveal the expression of *CSAS* at different developmental stages. Using markers for glial cells, double-staining

experiments revealed that *CSAS* is expressed only in glial cells throughout development **(Fig 3-3A)**. To test if *CSAS* function is required in glial cells, but not in neurons, we carried out rescue experiments using cell-specific GAL4 drivers to induce expression of transgenic *UAS-CSAS* in *CSAS* mutants. Glial-specific expression of *UAS-CSAS* was able to rescue TS paralysis phenotype of *CSAS* mutants, while expression in neurons did not result in rescue, which indicated that *CSAS* functions in glial cells. To further confirm this result, I performed *CSAS* knock-down experiments using an RNAi approach. My data revealed that glial-specific knock-down produced a strong TS paralysis phenotype, while *CSAS* knock-down in neurons did not result in TS paralysis **(Fig 3-3B)**.

My further experiments were focused on elucidation of *SAS* functions. To examine cell-specific requirements of *SAS*, I employed an *UAS-SAS* construct in combination with different tissue-specific drivers to perform rescue using the UAS-GAL4 system. Based on the evidence that *CSAS* is functioning in glial cells, we hypothesized that *SAS* also functions within the same cell type in order to provide the substrate Neu5Ac. Therefore, if we express *SAS* specifically in glial cells of *SAS* loss-of-function homozygous mutants, we would expect to see a rescue of the longevity phenotype. In fact, we observed close to a full rescue when expressing *SAS* in glial cells **(Fig. 3-2G)**. However, we also saw a similar rescue of phenotype when expressing *SAS* in neurons. To confirm that these results were an accurate portrayal of *SAS* functional requirement, we performed rescue using the TS paralysis assay. Interestingly, my results demonstrated that expression of *SAS* anywhere within the brain results in a full rescue of paralysis phenotype (with the exception of using a heart-specific driver which resulted in a partial rescue), suggesting

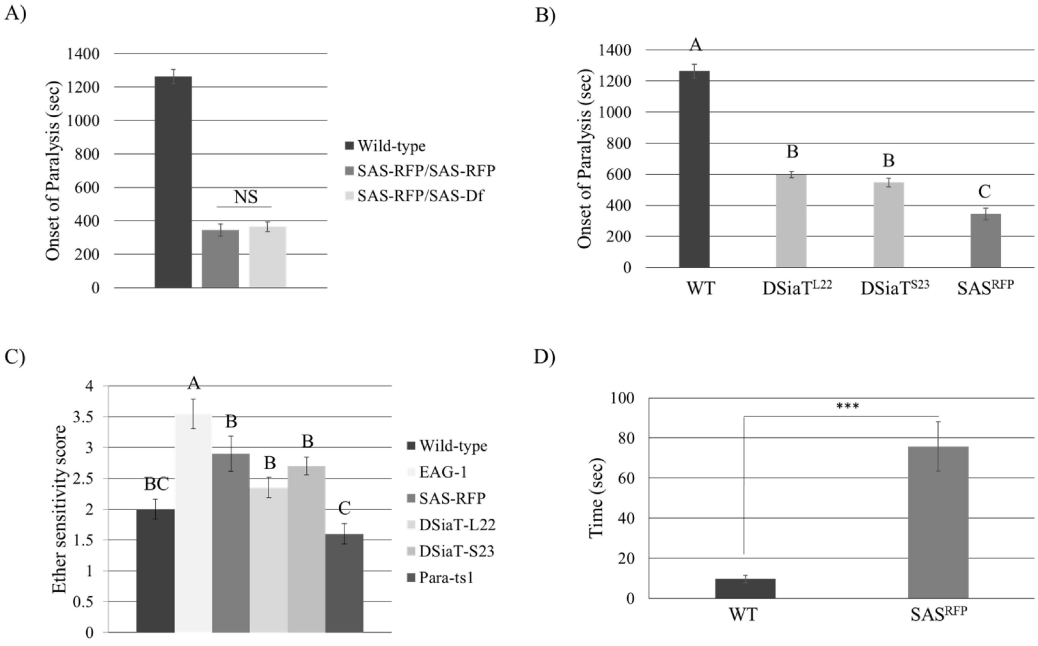


Figure 3-2 *SAS* mutations result in neurological phenotypes and decreased longevity. **A)** TS paralysis assay demonstrating that SAS^{RFP} behaves as a null allele. SAS^{RFP} homozygotes display the same onset of paralysis as SAS^{RFP} combined with *SAS* deficiency. N=40 Five-day old flies (20 males and 20 females) were assayed. **B)** TS paralysis assays revealed that SAS^{RFP} homozygotes have a significantly earlier onset of paralysis than *DSiaT* null mutants. **C)** Flies were exposed to 2.5 mls of diethyl ether for 1'30", and then their 'shaking' behavior was observed over a 5-minute period. **D)** Negative geotaxis assay demonstrating significant differences between wild-type and SAS^{RFP} mutants. Ten five-day old males were assayed per genotype. Each fly was assayed individually in two trials at which point the times were averaged together to represent the overall time of that fly.

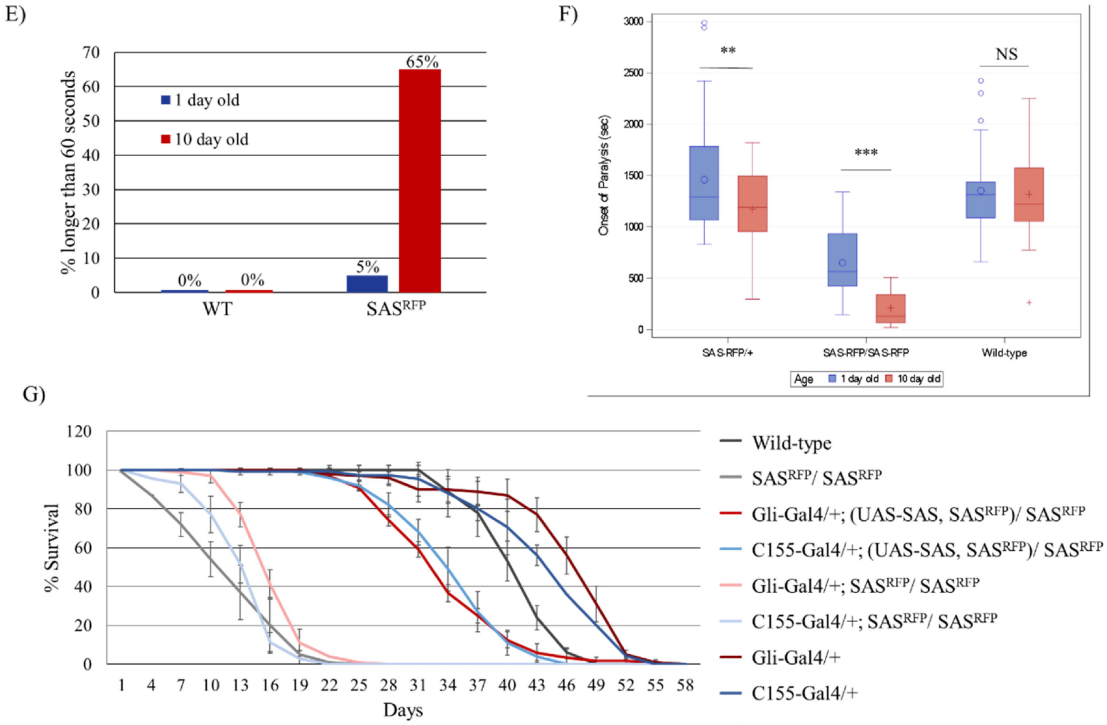


Figure 3-2 Continued. E) Negative geotaxis assay comparing the percent of flies that took longer than 60 seconds to reach the 5 cm mark. 65% of ten-day old *SAS*^{RFP} mutants took longer than 60 seconds to reach the mark versus the 5% of one-day old flies that did not reach the mark. This shows the increase in severity of phenotype with age. WT flies always reached the mark within 60 seconds in all assays. Each bar represents a total of 20 individual males. **F)** TS paralysis assay demonstrating how paralysis phenotype changes with age. 40 flies (equal number of males and females) were analyzed per genotype per age. **G)** *SAS*^{RFP} mutants have significantly reduced longevity. Graph shows survival curves for each genotype. 100 female flies were analyzed for each genotype. Error bars represent SEM. For **B** and **C**, letters indicate statistically significant groups ($p<0.05$). NS- non significant.

that the substrate of SAS, Neu5Ac, potentially has the ability to be secreted from one cell type to another **(Fig. 3-4A, Supplemental Fig. A-3)**.

To see if there is a correlation between the number of cells expressing SAS in the brain and the efficiency of rescue of paralysis phenotype, we dissected and immunostained

late-third instar brains of flies containing *UAS-GFPnls* in combination with each tissue-specific driver. Our results indicate that there is a direct correlation between expression level of SAS in the brain and the level of rescue **(Fig. 3-4B)**. Ubiquitous, neuronal and glial drivers express in a large subset of cells within the *Drosophila* brain, resulting in a full rescue of paralysis phenotype when driving the expression of *SAS* in *SAS* mutants.

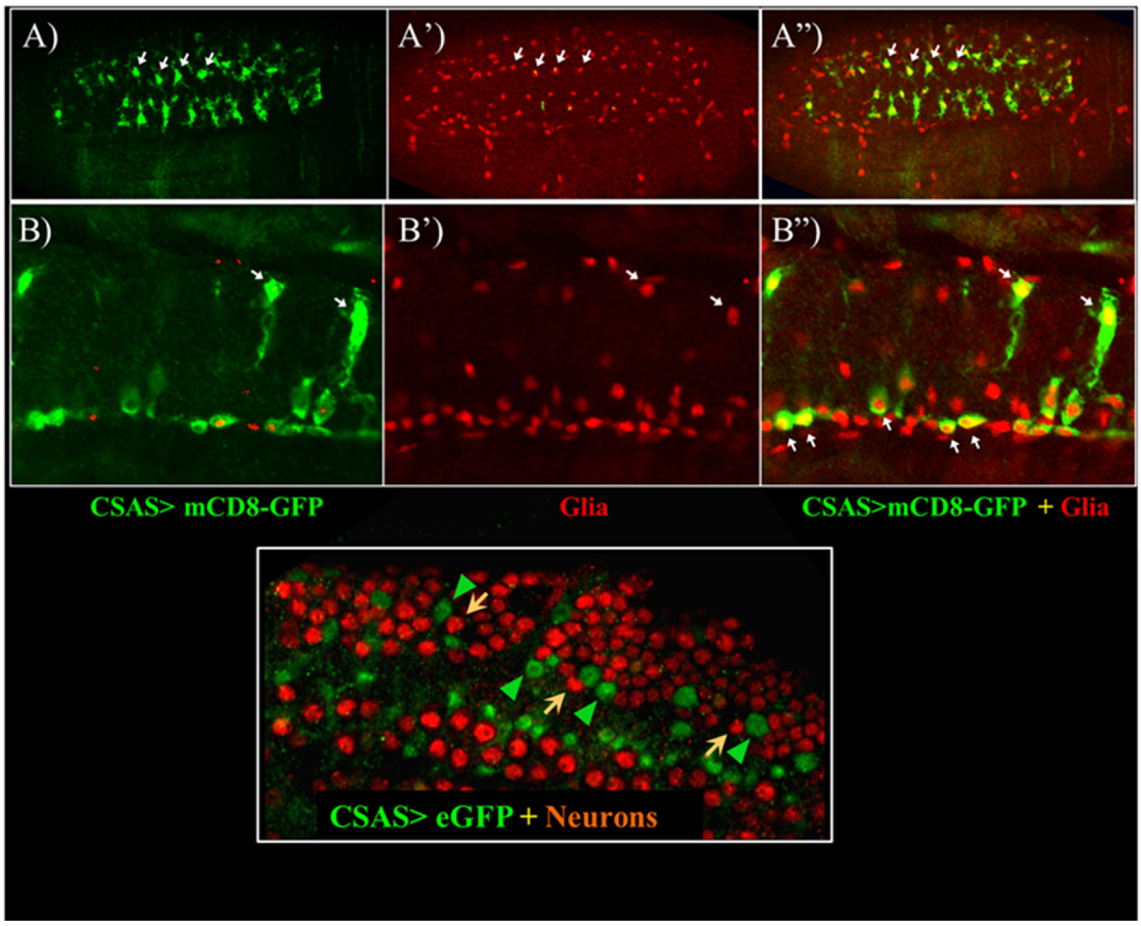


Figure 3-3 CSAS functions in glial cells. *CSAS-LexA*-driven mCD8-GFP and GFP-nls reporters reveal the expression of CSAS at **A)** embryonic and **B)** larval stages. CSAS colocalizes with glial cells, but not neurons.

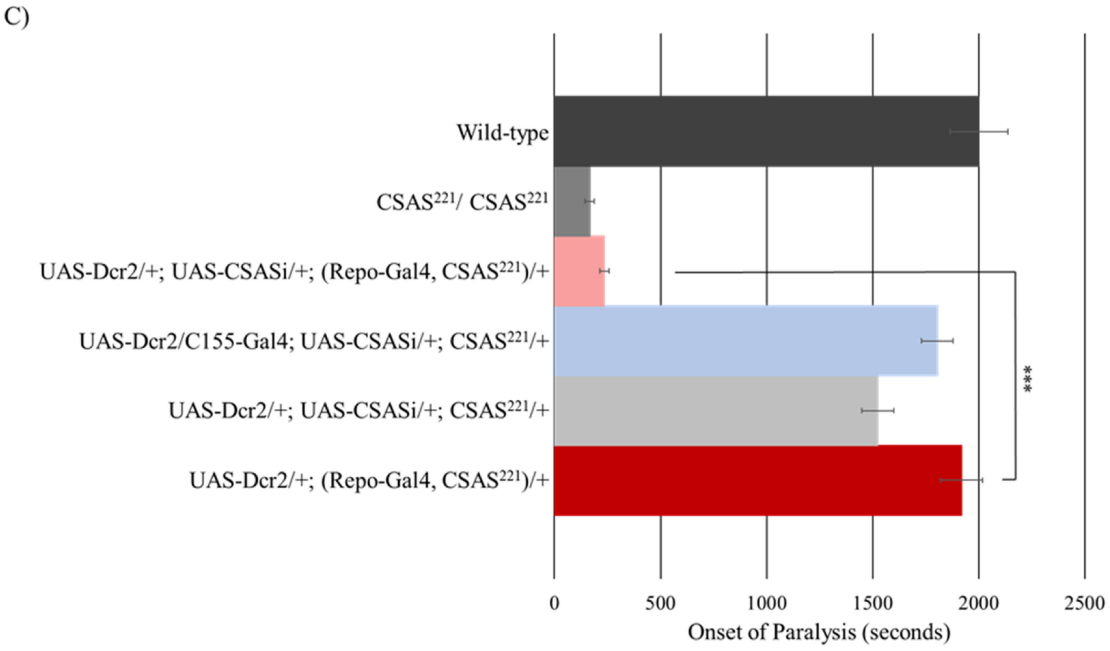


Figure 3-3 Continued. C) Endogenous CSAS functions specifically in glial cells to regulate neural function. Results of TS paralysis assays of genotypes with tissue-specific RNAi-mediated knockdown of *CSAS* are shown. *CSAS* knockdown was performed specifically in glial cells or in neurons by expressing *CSAS-RNAi* construct (*UAS-CSASi*) using glial specific (*Repo-Gal4*) or neuron-specific (*C155-Gal4*) drivers, respectively. Co-overexpression of Dicer-2 (*UAS-Dcr2*) and one copy of *CSAS* mutant allele ($CSAS^{221}$) were introduced in knockdown genotypes to facilitate efficiency of RNAi approach. 20 individual five-day old females were assayed per genotype. Statistical analysis was performed using one-way ANOVA (Tukey-Kramer). ***$p<0.001$. Error bars represent SEM.

The muscle-specific driver, Dmef-Gal4, is expressed in a smaller subset of cells in the brain which also resulted in a full rescue of paralysis phenotype, indicating that expression of *SAS* in these cells is sufficient for SAS function. However, the onset of paralysis was shorter than that observed using ubiquitous, neuronal or glial drivers for rescue. The heart-specific driver, Dhand-Gal4, expresses in only a few cells in the brain, resulting in only a

partial rescue of TS-paralysis. These results demonstrate that expressing *SAS* in more cells within the brain, leads to a higher level of TS-paralysis rescue.

In a further attempt to elucidate where endogenous SAS is functioning, we obtained a *UAS-SAS-RNAi* transgene (VDRC#103919) and combined it with either a glial (*repo-Gal4*) or neuronal-specific (*C155-Gal4*) drivers. To potentiate the effect of RNAi, a *UAS-Dicer* and one copy of the SAS^{RFP} allele were also introduced in assayed genotypes. As hypothesized, we saw an earlier onset of paralysis when we knocked down *SAS* in glial cells, but not in neurons **(Fig. 3-4C)**. Therefore, these results indicated that endogenous SAS functions specifically in glial cells, but transgenically expressed SAS can function in other tissue types by producing Neu5Ac that can be secreted, thus allowing CSAS to maintain its function in glial cells.

3.2.4 Feeding of sialic acid prior to pupation rescues neural phenotypes in SAS^{RFP} mutants

Previously, van Karnebeek *et al*. demonstrated a partial rescue of skeletal abnormalities in *nansa* knockdown zebrafish through the administration of exogenous sialic acid to the embryo tank water[113]. To further investigate the hypothesis that sialic acid can be delivered as a dietary supplement and then diffuse between different tissues, we fed flies sialic acid (Nu5Ac) in an attempt to rescue phenotypes seen of *SAS* mutants. Interestingly, when larvae were fed sialic acid at mid-third instar stage, a full rescue of paralysis phenotype was observed **(Fig. 3-5A).** In addition, we also observed a significant rescue of partial lethality during pupal stages as seen by an increase in survival upon

feeding of sialic acid **(Fig. 3-5B)**. To determine the minimum concentration of sialic acid required for rescue in mutants, we gradually decreased the dose of sialic acid until we no longer saw a rescue of paralysis. When we used 0.005 mM sialic acid, the phenotype was only partially rescued **(Fig. 3-5C)**. Survivability of mutants decreased in a stepwise fashion as concentration of dietary sialic acid was lowered **(Fig. 3-5D)**. These data indicated that sialic acid can be used as a dietary supplement to be delivered to the nervous system, possibly by diffusion, to correct defects in *SAS* mutants.

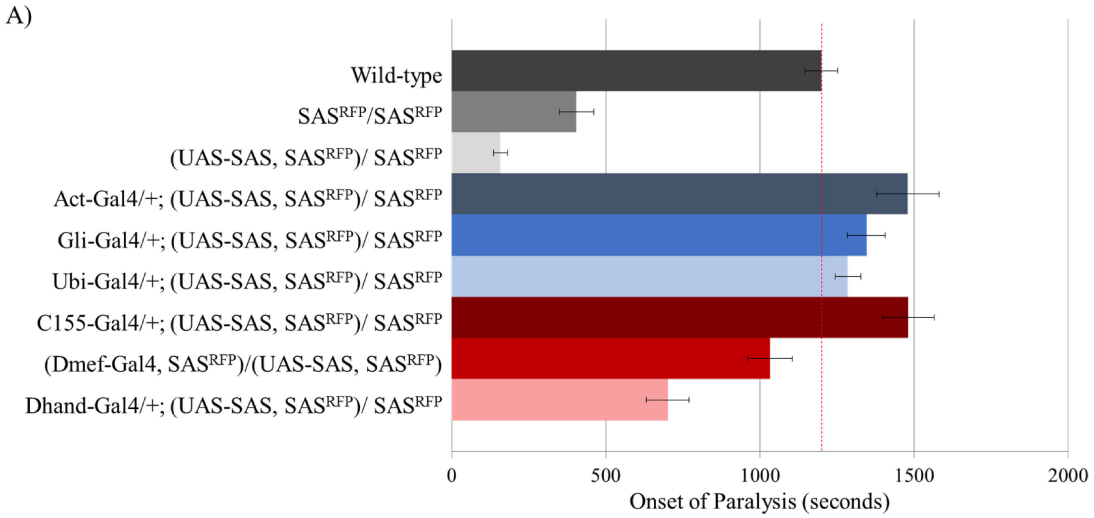
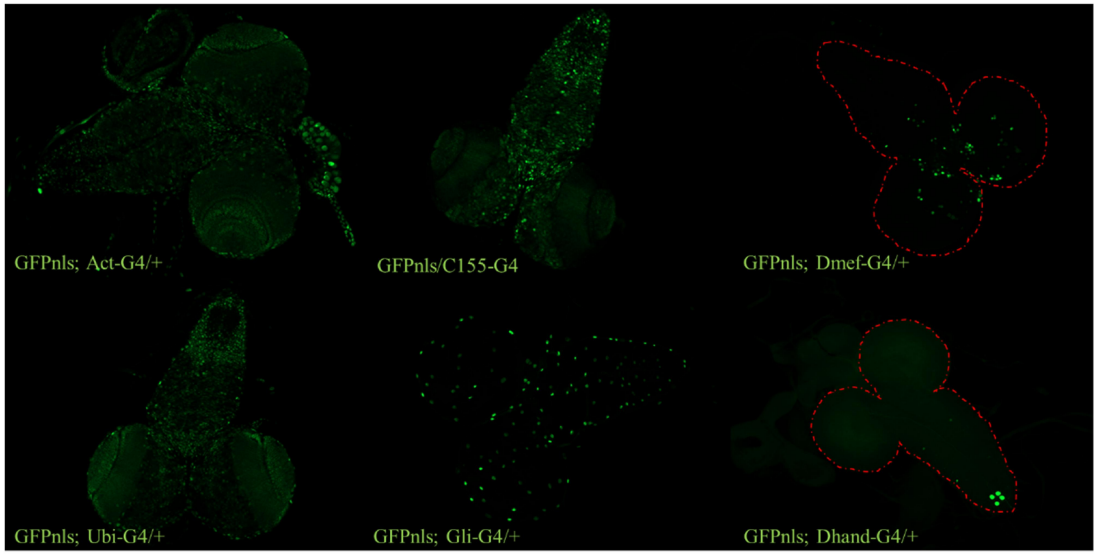


Figure 3-4 SAS functions in glial cells to regulate neuronal activity. A) Transgenic expression of *SAS* using the UAS-Gal4 system rescues paralysis phenotype. TS paralysis assay of flies with different combinations of transgenes. Controls are located in the Supplemental material. Red dashed line indicates the upper cutoff range of the assay at 38°C. 20 individual five-day old males were assayed for each genotype.

B)



C)

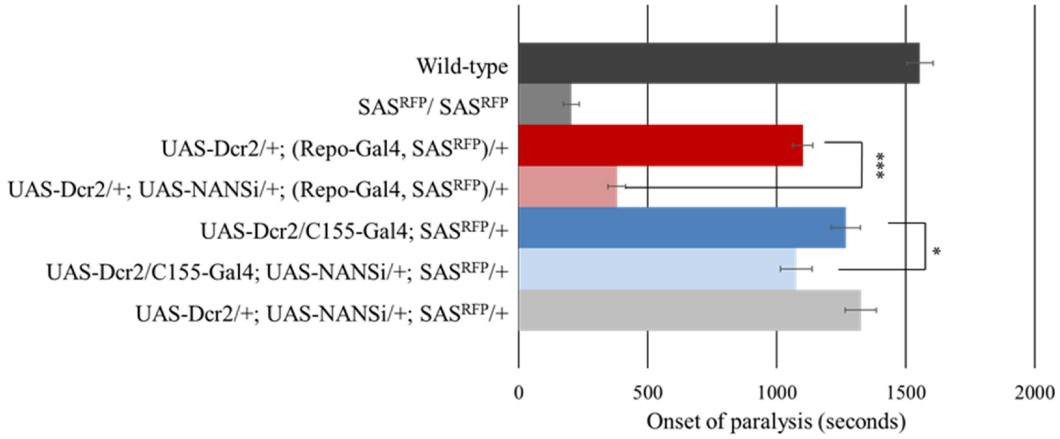


Figure 3-4 Continued. B) Number of expressing cells correlates to the level of rescue obtained during TS paralysis. Mid-third instar larval brains containing a *UAS-GFPnls* construct driven by different tissue-specific drivers were analyzed to asses driver expression patterns. Brains were immunostained using mouse anti-GFP (1:80) and goat anti-mouse Alexa488 (1:125).**C)** Endogenous *SAS* functions in glial cells to regulate neural function. TS paralysis assay of tissue-specific RNAi-mediated knockdown of *SAS*. 20 individual five-day old females were assayed per genotype. **A, C)** Statistical analysis was performed using one-way ANOVA (Tukey-Kramer). *p<0.05 and ***p<0.001. Error bars represent SEM.

In contrast to the rescue effect seen when developing larvae were fed sialic acid, feeding sialic acid to adult mutants was toxic **(Fig. 3-5E)**. When adult mutants were fed the same doses of sialic acid as used in experiments with larvae, a majority of flies did not live to day 5 to be assayed for TS paralysis. The few flies that survived to day 5 were extremely sick and unable to walk or right themselves. As we continued to try lower concentrations of sialic acid, we did not see a significant increase in survival until concentration was lowered to 0.0005 mM at which point ~50% of mutants survived till day 5, while the concentration of 0.0001 mM did not have a detectable effect on fly viability. These results suggested a need for sialic acid at a critical developmental time during late larval or pupal stages in order to develop a healthy functioning nervous system. These results are consistent with observations in zebrafish, where exogenous sialic acid added to a water tank with embryos could rescue *nansa* mutants only when administered before 24 hours post-fertilization[113].

To determine if the harmful effects observed when feeding adult *SAS* mutants with Neu5Ac could be attributed to sialylation, rather than possible toxic side effects of free sialic acid, we fed sialic acid to *CSAS* homozygous and *CSAS, SAS* double homozygous mutants and assayed their viability upon hatching over a 5-day period **(Fig. 3-5F)**. I found that $CSAS^{Mi}$ mutants were unaffected by the sialic acid. Interestingly, sialic acid was also not toxic for $CSAS^{Mi}$, SAS^{RFP} double homozygous mutants, suggesting that the effect on *SAS* mutant viability was in fact due to sialylation. Taken together, these results revealed a need for sialic acid in development prior time of eclosion, while showing that sialylation has detrimental effects in mutants after this developmental window.

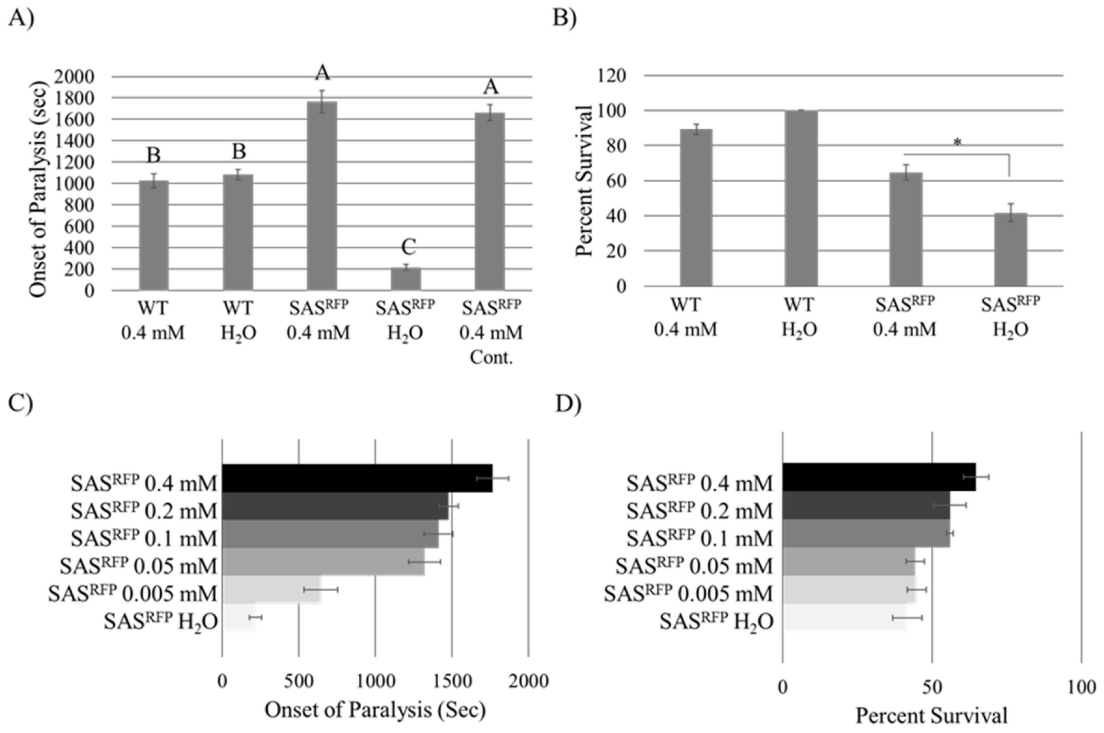


Figure 3-5 TS paralysis and survival are rescued by dietary sialic acid. A) Feeding of Neu5Ac to *SAS* homozygous mutants completely rescued paralysis phenotype. Mid-third instar larvae were fed H2O (control) or a final concentration of 0.4 mM Neu5Ac in 1 ml of food. Flies were collected on the day of enclosion and placed in a vial containing new food without Neu5Ac or placed in a new vial containing the same Neu5Ac concentration (continuous feeding). On day 5, individual flies were assayed for TS paralysis at 38^{o}C. N=20 for each genotype and condition. **B)** Survival of *SAS* mutants was significantly rescued upon feeding of 0.4 mM Neu5Ac. Percent survival is the percent of flies that reached eclosion. **C, D)** Level of rescue is dependent on dosage of Neu5Ac. 35-40 individuals were assayed for each condition, approximately equal numbers of males and females were assayed.

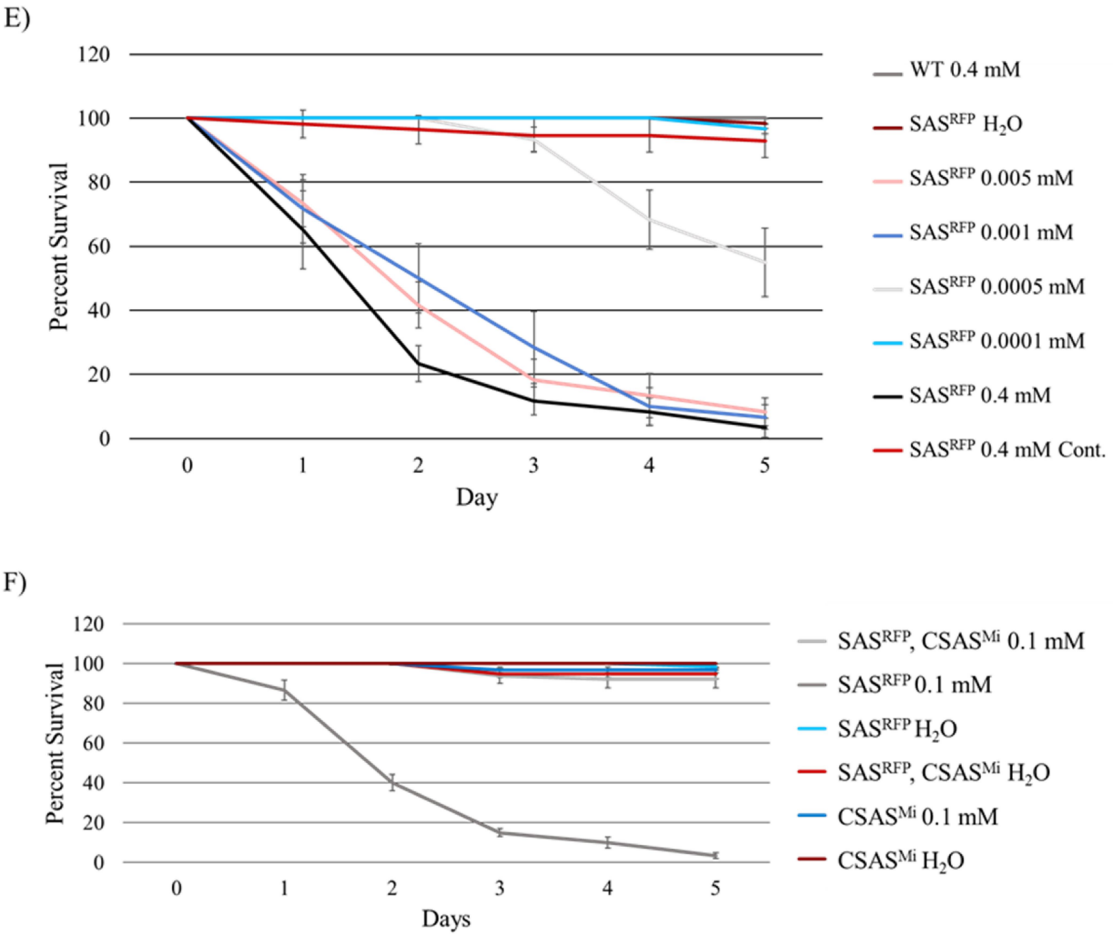


Figure 3-5 Continued. E) Feeding of Neu5Ac to adult *SAS* mutants results in toxicity. Adult flies were collected upon eclosion and placed in a vial with 1 ml of food containing indicated concentrations of Neu5Ac (water was added as a control). Percent survival was assayed every day over a five-day period. For continuous feeding, feeding began at mid-third instar stage and continued after eclosion to day 5. Flies were transferred to new food with Neu5Ac upon eclosion. N=60 flies per data point, approximately equal numbers of males and females were used. **F)** Feeding of Neu5Ac to adult *SAS* and *CSAS* single mutants, and *SAS, CSAS* double mutants. N=60 flies per data point, approximately equal ratios of males and females. Error bars represent SEM. NS- not significant.

3.2.5 *SAS* is essential for CMP-sialic acid biosynthesis in *Drosophila*

To investigate the activity of the sialylation pathway in SAS^{RFP} mutants, we analyzed the metabolite CMP-Neu5Ac by reverse-phase ion pairing chromatography coupled to triple quadrupole mass spectrometry in collaboration of Dirk Lefeber's group (Radboudumc, Holland)[8]. Before analysis, we separated parts of fly bodies to investigate differences in CMP-Neu5Ac levels in the head, thorax and abdomen. The highest level of CMP-Neu5Ac was detected in wild-type heads, which is consistent with the nervous system-specific expression of sialylation genes **(Fig. 3-6A, B)**. Wild-type abdomen and thoraces, although having significantly lower levels of CMP-Neu5Ac, also showed CMP-Neu5Ac expression. In contrast to wild-type flies, SAS^{RFP} homozygous mutants displayed no detectable CMP-Neu5Ac. These data indicated that *SAS* is necessary for CMP-sialic acid synthesis. These results are consistent with data on the sialic acid metabolite in mammalian *NANS* mutant tissue cultured cells[129].

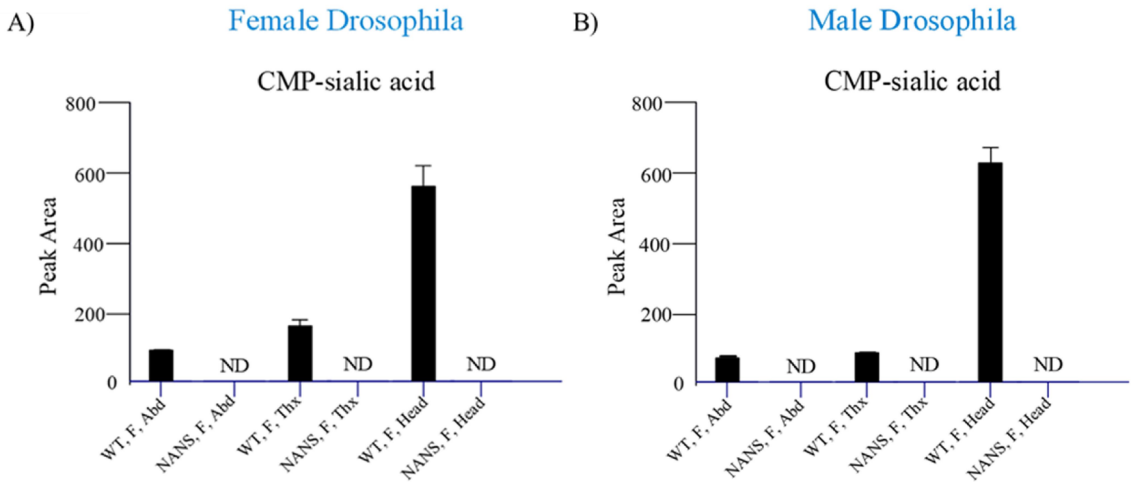


Figure 3-6 Metabolite levels of CMP-sialic acid biosynthesis in *SAS* mutant *Drosophila*. Levels of CMP-sialic acid in the abdomen, thorax and head of *SAS* mutant and WT *Drosophila* as measured by LC/MS. Figure represents **A)** female flies and **B)** male flies. Graphs represent mean peak area corresponding to equal amounts of protein of 3 independent measurements with error bars showing the standard deviation. Abd: abdomen, Thx: thorax. (Data obtained in collaboration with Dirk Lefeber)

3.2.6 Expression and subcellular localization of SAS protein

The expression pattern of SAS has yet to be characterized in *Drosophila*. The SAS protein is thought to be a cytosolic protein based on the evidence that the downstream N-acetylneuraminate-9-phosphate phosphatase has been localized to the cytosolic fraction in rat liver[114]. To examine the subcellular localization of SAS *in vivo*, we simultaneously expressed a full-length SAS protein with three consecutive C-terminal Myc epitope tags and a GFP-tagged nuclear protein, Koi-GFP, using the UAS-Gal4 system[59] **(Fig. 3-7A)**. The 3XMyc-tagged SAS was confirmed to be functionally active using rescue experiments, and it did not exhibit any detectable leaking expression **(Fig. 3-7B)**. Since

our functional data suggest that SAS is working in glial cells, we used Repo-Gal4 to drive the expression of *UAS-SAS-3xMyc* and *UAS-Koi-GFP* specifically in glial cells. Subcellular localization of SAS was then characterized by immunofluorescence using double staining of *Drosophila* 3rd-instar larval brains. In agreement with previous expectations, we observed diffuse cytosolic expression of SAS in glial cells **(Fig. 3-7C)**. Surprisingly, there were also some cells where SAS appeared to be nuclear localized.

To examine the subcellular localization of SAS in other tissues, we drove the ubiquitous expression of *UAS-SAS-3Myc* using Actin-Gal4, and added Hoechst as a nuclear stain. In all tissues, we observed diffuse expression of SAS within the cytosol **(Fig. 3-7D)**. Interestingly, we noticed that SAS can accumulate at the plasma membrane in some cells. In both salivary glands and fat cells, we found significant colocalization of SAS with the nuclear marker Hoechst. Taken together, these experiments indicated that SAS is localized primarily to the cytosol, however in some cells it can be nuclear. These data suggested that SAS may have another function independent of its role in the sialylation pathway. This result may also indicate that subcellular localization of SAS plays a role in SAS regulation.

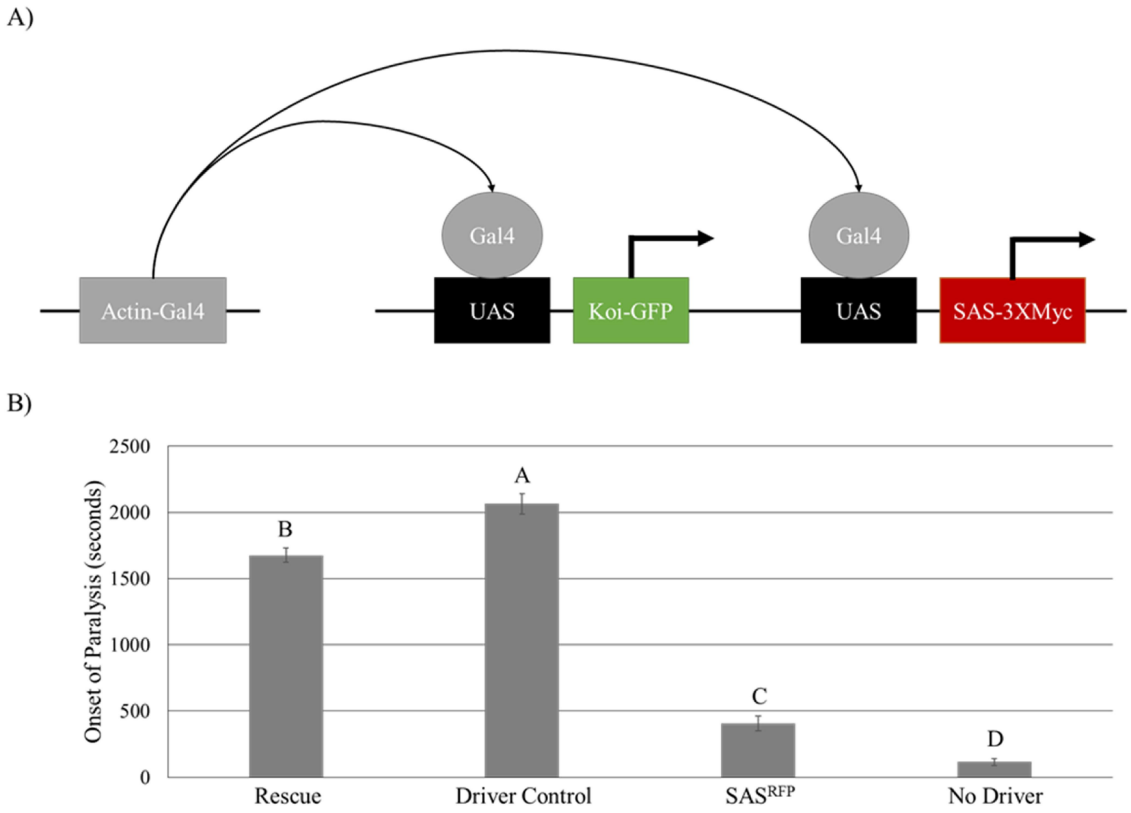


Figure 3-7 Expression and subcellular localization of SAS protein. A) Schematic representing ubiquitous driven expression of *UAS-SAS-3xMyc* and *UAS-Koi-GFP* to examine subcellular localization of SAS in larval brains. **B)** *UAS-SAS-3xMyc* construct was tested using TS paralysis assay. Construct exhibited rescue of TS paralysis phenotype and did not have any leaking expression (see no driver control). Error bars represent SEM. Letters represent statistically significant differences between groups ($p<0.05$). N~20 for each genotype consisting of five-day old males.

C)

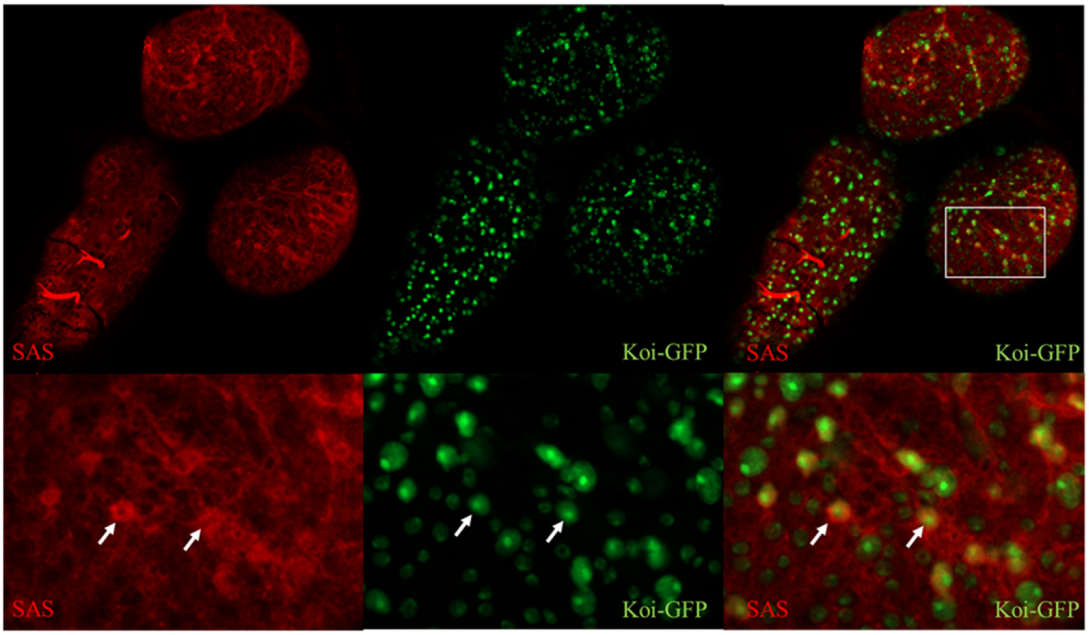


Figure 3-7 Continued. C) Double staining demonstrating SAS localizes to the cytoplasm in larval brains. White arrows mark cells where SAS localizes to the nucleus. Green channel indicates nuclear Koi-GFP expression. Red channel indicates SAS-3xMyc expression.

D)

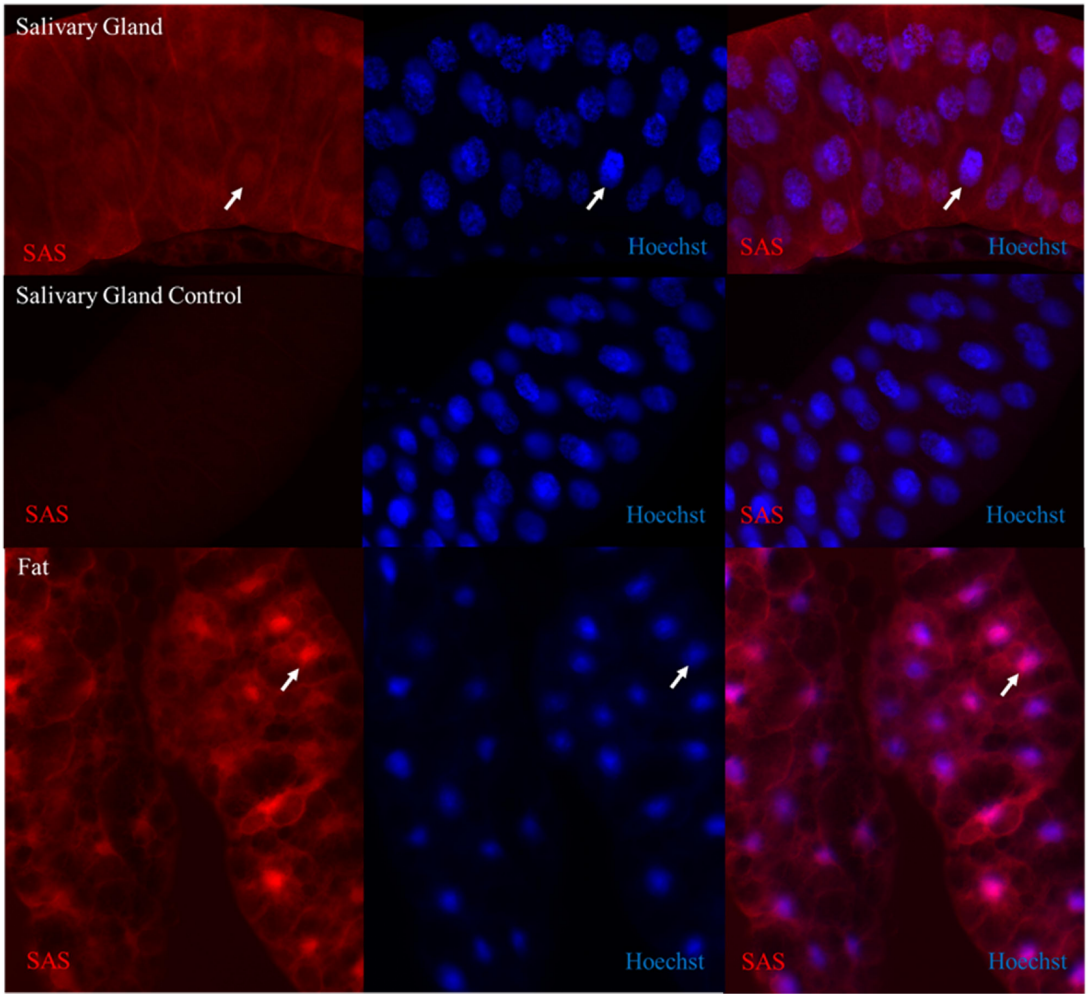


Figure 3-7 Continued. D) Double staining to examine SAS subcellular localization in salivary glands and fat body cells. SAS in expressed in the cytoplasm and the nucleus in some cells. White arrows mark cells where SAS localizes to the nucleus. Blue channel indicates Hoechst nuclear stain.

3.2.7 *SAS* interacts with voltage-gated ion-channel genes *para* and *sei*

DSiaT and *CSAS* both show genetic interactions with ion channel genes, supporting a prominent role for sialylation in the regulation of neural excitability[45, 88]. The voltage-gated Na^+ channel gene *paralytic* (*para*), is a major player in the neural excitability pathway in *Drosophila*. Therefore, we decided to test whether *SAS* and *para* interact. We combined the mutant temperature-sensitive allele $para^{ts1}$ with SAS^{RFP} to examine genetic interactions using the TS paralysis assay. As previously reported, $para^{ts1}$ heterozygous mutants were not paralytic, however a significant increase in phenotype severity was observed when one copy of $para^{ts1}$ was combined with *SAS* homozygous mutant genotype **(Fig. 3-8)**. This result suggests a functional synergism between *para* and *SAS*.

As compared to Na_v channels, voltage-gated K^+ channels usually play an opposing role in neural excitability by repolarizing the membrane during declining phase of the action potential[130]. Hence, we tested interactions between *SAS* and *seizure* (*sei*), a broadly expressed *Drosophila* voltage-gated K^+ channel gene. For this experiment we used the recessive loss-of-function allele sei^{ts1}. Homozygous sei^{ts1} flies are hyperactive and paralyze rapidly, similar to SAS^{RFP} homozygotes. Addition of one copy of sei^{ts1} to SAS^{RFP} homozygous mutants resulted in an increase in paralysis, indicative of a counteraction of excitability phenotypes. Thus, *SAS* and *sei* potentially display a genetic interaction which is working in an opposite mode then that between *SAS* and *para*. However, this interaction was not significant and needs to be further explored.

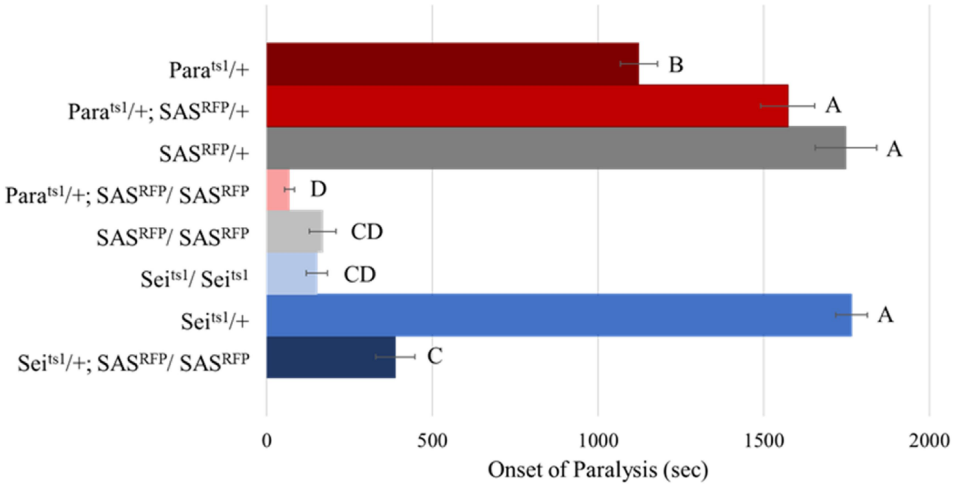


Figure 3-8 Genetic interactions between *SAS* and voltage-gated ion channel genes. Results of TS paralysis assay of flies with different combinations of *SAS*, *para* and *sei* mutant alleles. *SAS* and para exhibit a synergistic interaction when para is placed in a *SAS* homozygous mutant background. *SAS* and *sei* potentially interact as displayed by the alleviation of phenotype when one copy of sei is placed in a *SAS* homozygous mutant background. 20 five-day-old female flies were assayed for each genotype. Statistical analysis was performed using one-way ANOVA (Tukey-Kramer). Letters represent statistically significant differences between groups ($p<0.05$). Error bars are SEM.

3.2.8 Sialylation pathway genes may have some functions independent from the canonical sialylation pathway

Previously, we reported results of genetic interactions suggesting that *CSAS* and *DSiaT* may have functions outside of the sialylation pathway[45]. I decided to further investigate the functional relationship between sialylation genes, including *SAS*. To study the relationship between *SAS* and other sialylation pathway genes we created double homozygous mutants including different combinations of *SAS*, *CSAS* and *DSiaT* mutant alleles. Using TS paralysis assays, I observed a more severe, although not significantly

different, phenotype for SAS^{RFP}, $CSAS^{221}$ double mutants when compared to either single mutant **(Fig. 3-9)**. SAS^{RFP}, $DSiaT^{L22}$ double mutant phenotype was significantly worse than either single mutant. These results suggest that these genes can have some functions independent from participation in the canonical sialylation pathway. Alternatively, these results may indicate the pathway includes complex regulatory mechanisms that cannot be easily explained within a linear pathway.

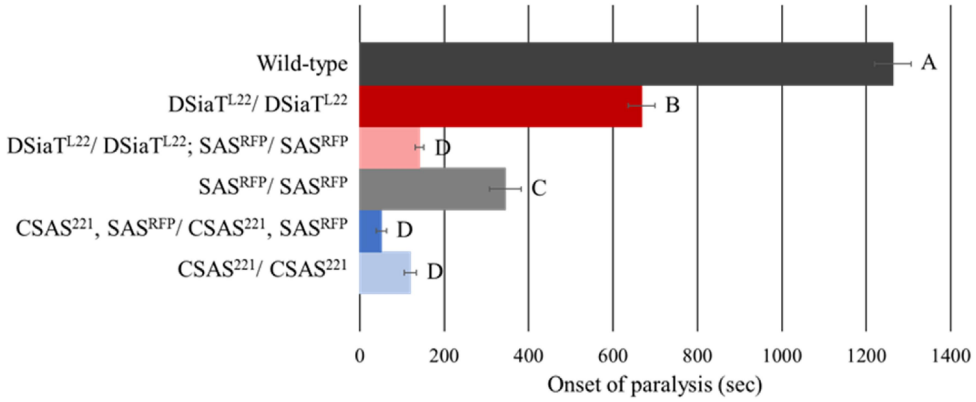


Figure 3-9 Genetic interactions between *SAS* and sialylation pathway genes. TS paralysis assays were used to analyze interactions between null alleles of *SAS*, *CSAS* and *DSiaT*. 40 five-day-old flies (20 males and 20 females) were analyzed for each genotype. Statistical analysis was performed using one-way ANOVA (Tukey-Kramer). Letters represent statistical significance between groups $p<0.05$. Error bars represent SEM.

3.3 Discussion

This work has provided further support for bipartite mechanism of regulation of neural transmission by sialylation **(Fig. 3-10)**. This mechanism underlies a novel model of glia-neuron coupling that regulates neural functions. Furthermore, this is the first investigation of SAS *in vivo* functions, and the first detailed analysis of biological functions of *SAS/NANS* in metazoan organisms. I found that SAS is required primarily in glial cells, and that its deficiency results in neurological abnormalities, decreased lifespan and TS paralysis. These neurological impairments in *SAS* homozygous mutants are comparable to phenotypes seen in *CSAS* and *DSiaT* mutants. These phenotypes become more severe with age **(Fig. 3-2)**. Interestingly, significantly shortened lifespan, has also been observed in *Drosophila* mutants with neural excitability problems, such as eag^{123}. Notably, *CSAS* and *DSiaT* mutants are believed to have decreased excitability based on the diminished evoked EJP amplitudes observed in electrophysiological assays[45, 88]. However, this method may not inform on the level of excitability at the organismal level assayed at different conditions, such as uncontrolled locomotion induced by ether.

To shed light on ether-induced excitability phenotypes at organism's level, we used ether assays and assigned a phenotype score based on different presentation of the phenotype **(Fig. 3-2C)**. We scored phenotypes of leg shaking, rapid wing fluttering, head bobbing, rapid twitching of the abdomen, and uncontrolled wing beating that resulted in spinning of the fly. *Eag* mutants served as a positive control for hyperexcitability, which is well documented in these mutants[123]. Surprisingly, all sialylation mutants obtained an ether sensitivity score higher than that of wild-type, with SAS^{RFP} homozygotes obtaining the

highest score. *Para*^{ts1} homozygotes showed an overall decreased excitability in our assays, which is consistent with ubiquitously reduced excitability at the level of individual neurons well-characterized in these mutants. These data validated our scoring system, and support the notion sialylation pathway mutants can show an overall increased excitability of the nervous system when exposed to ether. To dig deeper into relationships between sialylation genes and other players of the neural excitability pathway, we tested genetic interactions between *SAS* and voltage-gated ion channel genes, *para* and *sei* using TS paralysis assays **(Fig. 3-8)**. Para is the only known voltage-gated Na^+ channel working ubiquitously in the *Drosophila* CNS. *Para* mutations have been shown to decrease excitability of neurons by affecting the generation of action potential[67, 130]. *Sei*, an erg voltage-dependent K^+ channel, works in opposition by causing neural hyperactivity[110, 124, 130]. Using onset of TS paralysis as a quantitative measure of interactions, we observed a strong interaction when one copy of *para*^{ts1} was placed in a *SAS*^{RFP} mutant background. Additionally, we detected an improvement in paralysis phenotype when one copy of *sei*^{ts1} was combined with *SAS*^{RFP} homozygous mutant background. These results suggest that SAS is modulating excitability by affecting expression or/and functional properties of these voltage-gated ion channels.

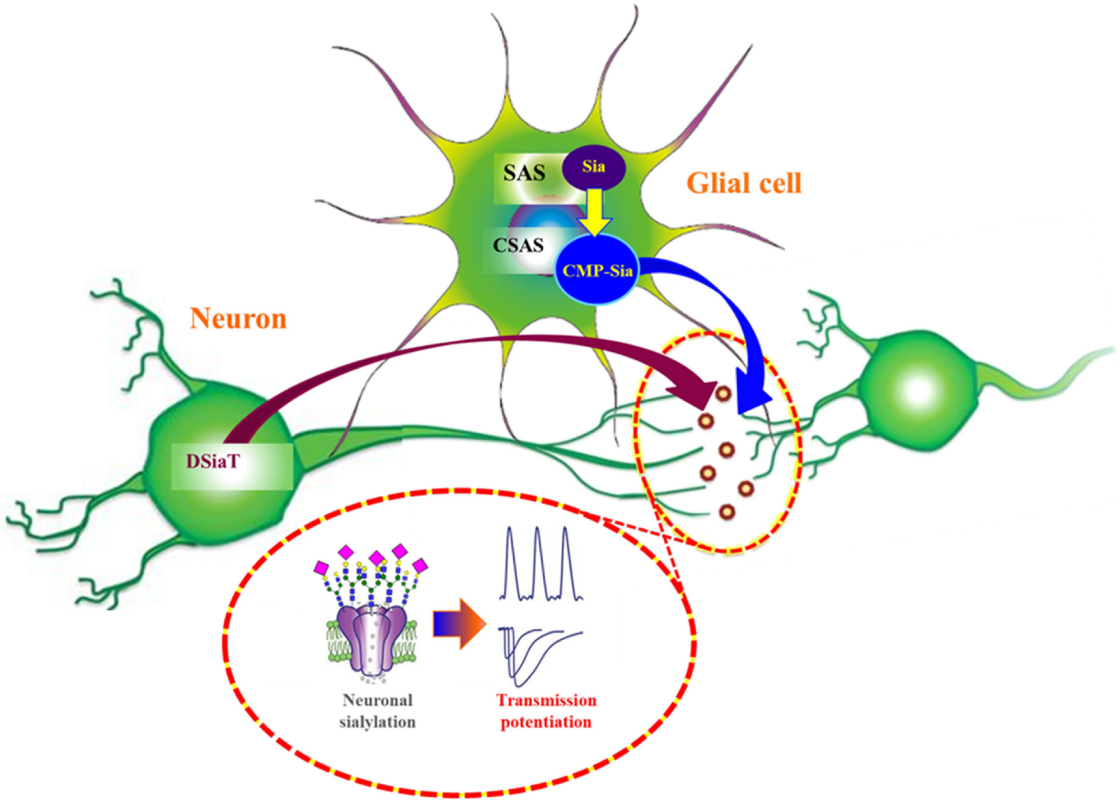


Figure 3-10 Bipartite mechanism of regulation of neural transmission by sialylation. SAS generates sialic acid (Sia) in glial cells. Sia is used as a substrate by CSAS in glial cells to make the nucleotide sugar CMP-Sia. CMP-Sia must then make its way to neurons for DSiaT-mediated sialylation of glycoproteins. Prospective targets of sialylation, such as VGICs, modulate the generation and propagation of action potentials, which is important for cellular excitability of neurons and therefore neural transmission.

Using tissue-specific RNAi mediated knockdown, we found that SAS is functioning primarily in glial cells **(Fig. 3-4C)**. This result comes as no surprise considering that SAS provides the substrate for CSAS which is also required in glial cells **(Fig. 3-3)** Upon knocking down SAS in neurons using C155-Gal4 driver, we also observed a small but statistically significant decrease in onset of paralysis. This result can be

potentially explained by the fact that C155-Gal4 driver may be also expressed in some glial cells and early neural progenitor cells, some of which may develop into glial cells. Alternatively, there is also a possibility that SAS functions in both glia and some neurons. Development of an antibody or a construct to examine endogenous expression of SAS in the CNS could test these alternative possibilities while providing new mechanistic insight.

In an attempt to uncover tissue-specific requirements of SAS, we stumbled upon the notion that the product of SAS could be secreted amongst different cells **(Fig. 3-4A, B)**. Our results demonstrated that we could express SAS anywhere in the brain and still obtain rescue of paralysis phenotype. We obtained the lowest level of rescue when SAS was transgenically expressed using the heart-specific Dhand-Gal4 driver, which correlated with the fact that this driver is only expressed in few cells within the CNS. Notably, during this experiment we were not able to obtain any progeny in which expression of SAS was driven by a strong glial-specific driver, repo-Gal4, which is expressed in a larger subset of glial cells then Gli-Gal4. Based on our functional data showing a requirement for SAS in glial cells, we hypothesized that transgenic expression of SAS led to a gain-of-function phenotype resulting in detrimental effects.

Working off of the hypothesis that the substrate of SAS can be secreted amongst different cells, we decided to inject Neu5Ac into the abdomens of SAS^{RFP} homozygous mutants to test if it could navigate to the correct tissue and rescue behavioral phenotypes. After multiple attempts at different concentrations, we could not get this method to work as it often resulted in dead flies. Therefore, we decided to deliver sialic acid as a dietary supplement **(Fig. 3-5)**. Our results indicated a full rescue of TS paralysis and a significant

increase in percent survival when mutants were fed a concentration of Neu5Ac between 0.05-0.4 mM. Importantly, *SAS*^{RFP} mutants fed continuously throughout development also displayed a full rescue of paralysis phenotype. However, feeding of similar concentrations of Neu5Ac to day 0 adult flies led to toxicity and an early death. These results indicate a specific requirement for SAS early in development.

Up until now, there have been no reports on localization of SAS/NANS in animal organisms. Based on the evidence that N-acetylneuraminate phosphatase is subcellularly localized to the cytosol in vertebrates, we hypothesize a similar localization for SAS[114]. Here we characterized the subcellular localization of the SAS protein *in vivo* using a *UAS-SAS-3xMyc* construct generated in our lab and expressed transgenically using the UAS-Gal4 system **(Fig. 3-7)**. Our results demonstrated cytosolic expression, as well as some nuclear localization. To ensure that this was not an artifact, we analyzed SAS subcellular localization in different tissue types (brain, fat, salivary glands). In all these different types of cells, we also found examples of cytosolic and nuclear localization of SAS-3xMYC. Interestingly, we also observed an increased level of SAS at the cell membrane of some cells. These differences in subcellular localization suggest that SAS function can be regulated by some factors that affect its subcellular localization. Whether the subcellular localization pattern of SAS is conserved in other species remains to be investigated.

Lastly, we investigated the relationship between *SAS*^{RFP} and other sialylation pathway genes using TS paralysis assays, to better understand the regulatory mechanisms of *Drosophila* sialylation **(Fig. 3-9)**. Onset of paralysis was significantly decreased in double mutants when compared to single mutants, suggesting that these enzymes have

additive effects on neural function. These results are consistent with previous data on interaction between *CSAS* and *DSiaT*, further supporting a possible scenario of complex regulations within the *Drosophila* sialylation pathway[45]. Additionally, these results may suggest an independent role for SAS outside of the sialylation pathway. More recently, a *Drosophila* RNAi screen for autophagy genes was performed in primary cultured muscles[135]. Intriguingly, this screen identified SAS as a positive regulator of autophagy, as knockdown of *SAS* led to increased autophagosome formation. Testing the hypothesis that SAS can function as an autophagic regulator may explain complex interactions between sialylation pathway genes and shed light on novel functions of these genes independent from their roles within the canonical sialylation pathway.

3.4 Materials and methods

3.4.1 *Drosophila* strains

$NANS^{DF}$ and *UAS-GFPnls* were from the Bloomington Stock Center (Indiana University). *yw; SAS^{RFP}/TM6, Tb* (stock #141875) was from Kyoto Stock Center. *$Para^{ts1}$* (hypomorph, conditional temperature-sensitive (TS) allele) and *sei^{ts1}* (null, TS paralytic allele) were received from Barry Ganetzky (University of Wisconsin- Madison). All *Drosophila* strains were reared in a controlled environment incubator (25°C, 35% humidity, 12 h light/darkness) on standard cornmeal–malt–yeast medium.

3.4.2 Behavioral assays

Behavioral assays were performed essentially as described previously[88]. For longevity assays, adult female flies were kept individually and transferred to a new vial with fresh food every three days. Dead flies were counted every day. For paralysis, locomotion and geotaxis assays, flies were collected on the day of enclosure and placed in groups of 5-8 flies per vial. Flies were then aged for 5 days and transferred to fresh food on day 3. In the temperature sensitive paralysis assay, individual flies were transferred to empty vials and the temperature was shifted to 38^{o}C by submerging vials in a temperature-controlled water bath. We defined paralysis as a condition when a fly lies on the bottom of a vial and is unable to stand and walk for at least one minute. Unless otherwise indicated, males and females were used in equal ratios for all experiments.

3.4.3 Feeding experiments

Mid-third instar larvae were collected in cold 25% sucrose and placed in a vial containing 1 ml of food with a final concentration of 0.4 mM, 0.2 mM, 0.1 mM, 0.05 mM, 0.005 mM Neu5Ac or distilled water. Twenty larvae were placed in each vial carefully using fine forceps. Vials containing larvae were then placed upright in a 25^{o}C incubator with 35% humidity and a 12-hour light/dark cycle until they reached enclosure. Flies were collected on the day of enclosure and placed in a new food vial without any sialic acid and aged for another 5 days. All flies were transferred to fresh food on day 3 and assayed for TS paralysis in a 38^{o}C water bath on day 5. The percent survival was calculated by averaging the percentage of flies that reached enclosure.

For continuous sialic acid feeding experiments, mid-third instar larvae were collected and assayed in the same manner as mentioned above except on the day of eclosion flies were placed in a new 1 ml food vial containing a desired final concentration of sialic acid. For this experiment flies remained on the sialic acid food for all five days of adulthood without being transferred.

For adult feeding assays, groups of ten flies were collected on the day of enclosure and placed in a 1 ml food vial containing the desired final concentration of sialic acid and placed in a 25^{o}C incubator. Flies remained on the sialic acid food for all five days without being transferred and were assayed for TS paralysis on day 5 if they were alive. Percent survival was measured by tracking the number of flies that were alive in each vial over a 5-day period. Percentages were then averaged for each day.

3.4.4 Mass spectrometry

Zero to two-day-old flies were sorted by sex and body fraction (head, thorax, abdomen) and stored in microcentrifuge tubes at -80^{o}C. Fly parts continued to be collected and stored until three tubes of 50 were obtained for each body fraction for each sex. Frozen samples were then lyophilized on Labconco FreeZone lyophilizer before biochemical analyses. Sample preparation and LC/MS measurements were based on previously described methods with some minor modifications[8]. Briefly, metabolites were extracted from *Drosophila* body fractions and homogenized on ice in 75mM ammoniumcarbonate buffer (pH 7.4) using a glass pestle. The homogenate was centrifuged and protein concentration was determined in the supernatant using BCA assay (Pierce, ThermoFisher).

A volume of supernatant corresponding to 100µg of protein was subjected to metabolite extraction in triplicate by adding 4 volumes of 1:1 acetonitril:methanol (-20°C). Samples were incubated at -20°C for 5 minutes. All samples were centrifuged at 16000g for 3 minutes followed by drying of the supernatant in a vacuum centrifuge at RT.

Samples were dissolved in 50-100µL LC/MS grade water. Samples were analysed using reverse-phase ion pairing chromatography (Agilent Technologies 1290 Infinity) coupled to a triple quadrupole mass spectrometer operating in negative ion mode (Agilent Technologies 6490 Triple Quad LC/MS). Metabolites were separated on an Acquity UPLC column (Waters, HSS T3 1.8µm, 2.1x150mm) using a gradient of solvent A (10mM tributylamine in water) and solvent B (10mM tributylamine in methanol). Flow rate was 0.25 ml/min and injection volume was 2 µl. Temperatures of the autosampler and column compartment were kept stable at respectively 7°C and 35°C. Metabolites were analysed using the MRM transition CMP-Neu5Ac (613.14->321.9 m/z). Peak areas were normalized over the sum of the peak area of AMP, ADP and ATP in cell experiments. MRM transitions of these compounds were: AMP (346.0-79.0 m/z), ADP (426.0->159.0 m/z) and ATP (506.0->159.0 m/z). All peaks were annotated after comparison with peaks from the commercial standard for CMP-Neu5Ac and its naturally occurring isomers: CMP-Neu5Ac.

3.4.5 RNAi experiments

SAS RNAi line (VDRC #103919) was obtained from the phiC31 RNAi Library (Vienna, Austria). Tissue-specific RNAi-mediated knockdown of SAS was obtained by

use of the UAS-GAL4 system. In short, flies containing the RNAi construct, tissue-specific driver of expression and one copy of *SAS*^{RFP} allele were reared in a 25^{o}C incubator. Flies were then collected on the day of eclosion and placed with 5-8 individuals per food vial. All flies were transferred to new food on day 3, and assayed for TS paralysis in a 38^{o}C water bath on day 5.

3.4.6 Expression constructs

Construct used for rescue (designated *UAS-SAS*) encodes *Drosophila SAS* cDNA sequence subcloned into pUASTattB. Protein was prepared by: (i) PCR amplification of *SAS* cDNA from pOT2-SAS vector where XhoI and AvrII sites were introduced before first codon and after last codon of *SAS* sequence. (ii) *SAS* cDNA fragment was digested at XhoI and AvrII, and pUASTattB vector was digested at XhoI and XbaI. (iii) *SAS* cDNA fragment was then ligated in-frame with the digested attB vector. Several transgenic *Drosophila* lines were generated by phiC31 integrase-mediated incorporation of the construct into VK31 (3L)62E1attP site on the third chromosome.

To generate the construct expressing SAS with an N-terminal 3X-Myc-tag (designated *UAS-SAS-3X-Myc*), I generated an ultramer set of primers that we ligated together to produce 3 Myc sequences flanked by KpnI and BamHI. I then removed the snap-tag region out of a previously generated *UAS-SAS-Snap* construct (unpublished) using KpnI and BamHI enzymes and ligated 3X-Myc tag encoding fragment in frame. Several transgenic *Drosophila* lines were generated by phiC31 integrase-mediated incorporation of the construct into VK31 (3L)62E1 attP site on the third chromosome.

3.4.7 Subcellular localization of SAS-3XMyc

SAS cDNA plasmid was generated by standard molecular cloning techniques. To examine SAS expression, we engineered a tag consisting of three Myc repeats and fused it in frame with the SAS reading frame. The SAS-3X-Myc construct was then expressed simultaneously with UAS-Koi-GFP (nuclear marker) in *Drosophila* neurons and glial cells as described previously using the UAS-GAL4 system[5, 45]. Briefly, late-third instar larval brains were dissected in Ringer's solution and then fixed for twenty minutes in fresh fixative solution (4% paraformaldehyde, 1M NaOH, 10X PBS) on a nutator at room temperature. Fixed tissue was washed with PBT (PBS + 0.1 % Triton X-100) and then stored in 100% methanol until enough brains were obtained for the experiment. Brains were then analyzed by immunostaining and epifluorescent microscopy. SAS-3X-Myc was detected using rabbit anti c-Myc primary antibody (1:200). Brains were incubated with secondary antibodies for 90 minutes at room temperature as described previously[68]. Secondary antibodies used were goat anti-rabbit Alexa-546 (1:125) (Molecular Probes). Brains were then dissected off of cuticles and mounted in anti-fade Vectashield medium (Vector Laboratories) and analyzed using Zeiss Axio Imager M2 with ApoTome module. For salivary gland and fat body staining, mouse anti c-Myc (DSHB 9E10) was used as the primary antibody at a concentration of (1:100) and goat anti-mouse Alexa-546 was used as the secondary at a concentration of (1:125). Hoechst was added in the final wash at a concentration of (1:1000) to visualize call nuclei.

3.4.8 *In vivo* rescue experiments

An untagged SAS rescue construct, denoted UAS-SAS, was generated by standard molecular cloning techniques and expressed in *SAS* loss-of-function homozygous mutants using the UAS-GAL4 system[5, 45]. TS paralysis phenotype was assayed as described above in the behavioral assays section. UAS-GFPnls was used in combination with the UAS-GAL4 system to examine in which cells SAS expression was being driven. For these experiments, late-third instar brains were dissected as mentioned previously and immunostained using mouse anti-GFP (DSHB 8H11) (1:80) as the primary antibody and goat anti-mouse Alexa-488 (1:125) as the secondary antibody. Brains were then mounted in antifade Vectasheild and imaged using Zeiss Axio Imager M2 with ApoTome module.

3.4.9 Statistical analyses

For comparison of multiple groups, one-way ANOVA was used to identify significant differences in mean. For comparison of two groups of data, unpaired two-tailed t tests were used.

4. SUMMARY

This work has provided new insights into the regulatory mechanisms that control neural sialylation. Through examining the role of *DSiaT* in the *Drosophila* heart, I uncovered a mechanism by which sialylation works in the nervous system to indirectly influence heart physiology and function. This finding not only demonstrates the significance of neural sialylation, but it brings to light the ability for sialylation defects to affect communication between organ systems. In humans, sialic acids levels can be quantified directly from the ventricular myocardium[16], suggesting that sialylation machinery is likely present in the heart tissue. In this respect, the mechanism of sialylation differs between humans and *Drosophila*, for I did not find any expression of *DSiaT* in the heart tissue. This result is consistent with rescue experiments that indicated that DSiaT function is required in the nervous system for normal heart physiology. It is known that the vertebrate heart receives neuronal input from sympathetic and parasympathetic nerves of the autonomic nervous system, which modulates the heart rate and force of contraction through controlling cellular excitability[101]. The function of heart-innervating neurons is dependent on VGICs which are known targets of sialylation enzymes[3, 4, 22, 23, 31, 45, 88, 125-127]. Therefore, changes in neural sialylation are expected to affect human heart function. This function of sialylation is revealed by my data. My project validated a model system that provides an opportunity to study the mechanisms by which neural sialylation influences the heart. My data opened new avenues to explore heart-controlling mechanisms in further detail, including the effects of altered sialylation on heart

innervation. For example, the pattern of innervation may be different in sialylation mutants. Additionally, it would be interesting to investigate if VGICs and neurotransmitter functions are affected in heart-innervating neurons of the mutants. Lastly, it would be important to characterize heart-specific phenotypes caused by aberrant sialylation in more detail (e.g. conduction velocity, contractility, development, morphology). This analysis could point at specific molecular pathways that are affected by sialylation and potentially suggest which glycoproteins are targets of sialylation.

The results of Repnikova et al. and Islam et al. have suggested a bipartite mechanism by which CSAS and DSiaT function in different cell types to regulate neural sialylation[45, 88]. While previous studies in our laboratory established that DSiaT functions in neurons to regulate neural transmission, functional requirements of CSAS were not elucidated. In collaboration with other members of the laboratory, I demonstrated that CSAS is specifically expressed and required in glial cells, which provided essential evidence for a novel bipartite mechanism of neural sialylation **(Fig. 3-10)**. My work on SAS has helped to further elucidate this mechanism by demonstrating glial-specific function of SAS, that provides the Neu5Ac substrate for CSAS to create CMP-Neu5Ac, an activated sugar donor for DSiaT-mediated sialylation Importantly, my work has uncovered that Neu5Ac can move amongst different tissues until it reaches glial cells, where it is used as a substrate for CSAS. DSiaT then uses CMP-Neu5Ac in neurons to modify glycoproteins, such as VGICs. Although mechanisms of Neu5Ac and CMP-Neu5Ac transport between the cells remain to be elucidated, genetic evidence suggests that Drosophila has several genes encoding putative transporters that can potentially

mediate these mechanisms. Further studies will need to elucidate these processes. The bipartite mechanism of neural sialylation described here provides a novel important addition to other mechanisms of glial-neuronal coupling that mediate essential regulation in the nervous system. It is tempting to speculate that this novel regulatory pathway is likely conserved in mammals, however further studies will be required to test this hypothesis.

My work on SAS provided not only new knowledge on the regulation of neural sialylation, but it suggested a means by which defects of sialylation can be treated therapeutically. My results demonstrated that feeding of Neu5Ac to *SAS* mutants in early development resulted in the full rescue of neurological phenotype (TS paralysis) and a partial rescue of survival. Similar experiments were done in a vertebrate model (zebrafish), showing a partial rescue in skeletal development in *nans* knockout zebrafish when given exogenous sialic acid[113]. However, these zebrafish experiments did not directly address the possibility of phenotype rescue using dietary sialic acid, and the effect of exogenous sialic acid on the nervous system or viability of mutants was also not investigated. My results suggested a possibility for dietary sialic acid supplementation to lessen, if not cure, some neurological symptoms in human patients. Supplementation could also result in a better prognosis and a longer life expectancy for *NANS* deficient patients. Of course, the mechanisms behind rescue of phenotypes using dietary sialic acid still need to be hashed out in detail before proceeding to human trials. Experiments using mammalian models and clinical studies would need to clarify many important questions about dosage, possible

side effects, and necessary chemical modification of sialic acid, etc., in order to determine therapeutic potentials of that approach.

Another significant finding that was highlighted in this work, and studies of others[45, 88], is the possibility that sialylation pathway enzymes can have some functions independent from their participation in the canonical sialylation pathway. As of now, these alternate roles have not been elucidated. Interestingly, *SAS* was recently identified as a positive regulator of autophagy (meaning knockdown caused reduced autophagosome formation) during a *Drosophila* RNAi screen in primary cultured muscles[135]. In line with this finding, I discovered that SAS is localized primarily in the cytoplasm, which is where autophagosome engulfment of damaged cell organelles or unused proteins occurs[64]. The potential role of *SAS* as an autophagic regulator is exciting, however this role needs to be confirmed though further *in vivo* experiments. Our lab is currently working on exploring the role for *SAS* as an autophagic regulator in the CNS. We hypothesize that *SAS* mutants will have reduced autophagosome formation in the brain, which could potentially explain some neurological phenotypes we see in these mutants. Autophagy is believed to serve as a protection mechanism due to the observed accumulation of autophagosomes in the case of many neurodegenerative diseases (e.g. Alzheimer's disease, Huntington's disease, etc.)[64]. If our hypothesis is accurate, it is possible that there is a buildup of damaged or misfolded proteins in the SAS mutant brains, which could eventually result in cell death. Determining where SAS is localized in the brain could give us more insight into which cells might be affected. My experiment using RNAi-mediated knockdown of *SAS* in different tissues suggests a functional role for SAS in glial cells. We are currently working

on generating a construct to visualize the endogenous expression pattern of SAS in the brain, which could further contribute to this research.

Overall, my work has expanded upon our current knowledge of the functional mechanisms of neural sialylation. Based on significant evolutionary conservation of the sialylation pathway between *Drosophila* and mammals, it is likely that these functional mechanisms are also conserved. Ultimately, our goal is to use the *Drosophila* model system to study human disease states. We hope that our efforts will contribute to uncovering new targets that will lead to the design of pharmaceutical drugs and treatment plans to cure disorders of glycosylation.

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APPENDIX

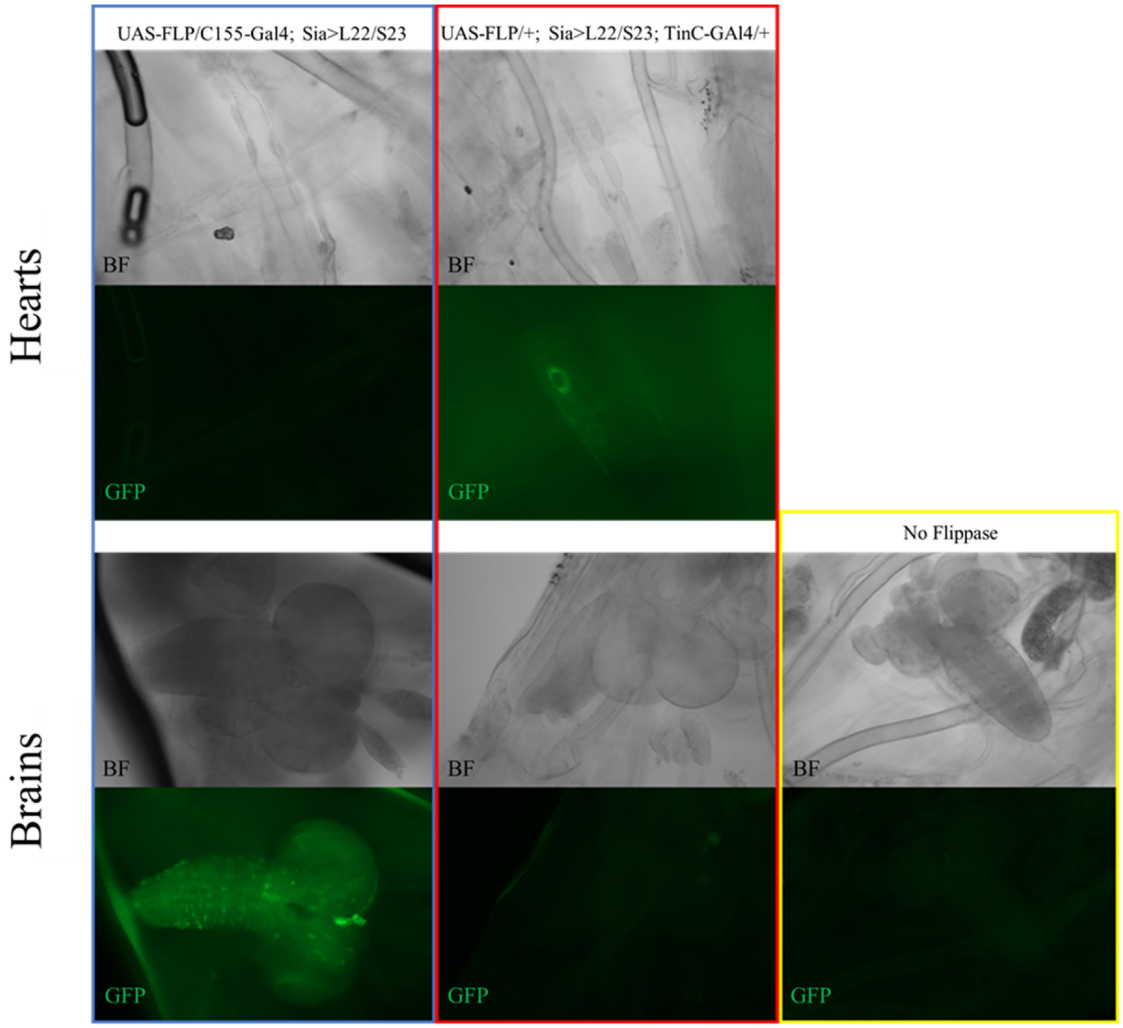

Figure A-1 Test of FLP/FRT system in larval brain and heart. Larval fillets were stained for GFP, indicative of DSiaT expression, using mouse anti-GFP (1:80) and goat anti-mouse Alexa488 (1:125). Upon driving expression of UAS-Flippase and UAS-DSiaT in neurons using C155-Gal4, we observed GFP expression in larval brains but not larval hearts. Results for this genotype are displayed in the blue box. When driving the expression of UAS-Flippase and UAS-DSiaT in the heart using TinC-Gal4, we saw GFP expression in the heart but not the brain. The red box displays the results for this genotype. The yellow box shows that the FLP/FRT system does not work without UAS-Flippase, as seen by a lack of GFP expression in the brain when using the C155-Gal4 promotor.

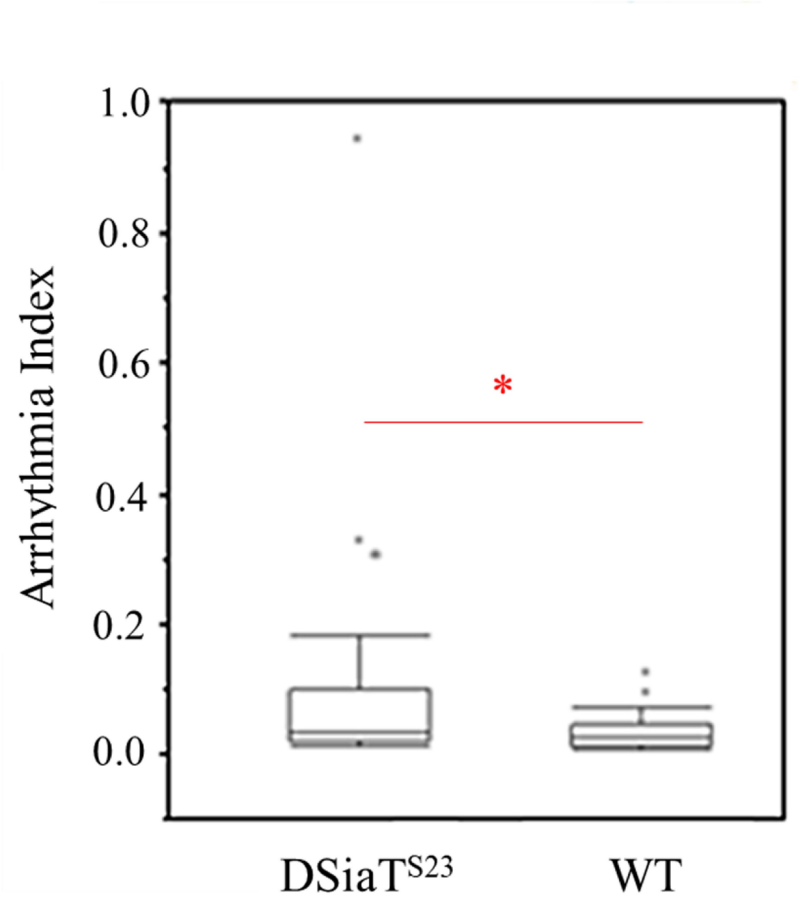

Figure A-2 *DSaiT^{S23}* homozygotes have arrhythmic hearts. Quantification of arrhythmia phenotype displaying *DSiaT^{S23}* homozygous mutants have significantly more arrhythmia than WT control. N=20 male five-day old flies assayed for each genotype. Asterisks indicate statistically significant differences (t test, *p<0.05).

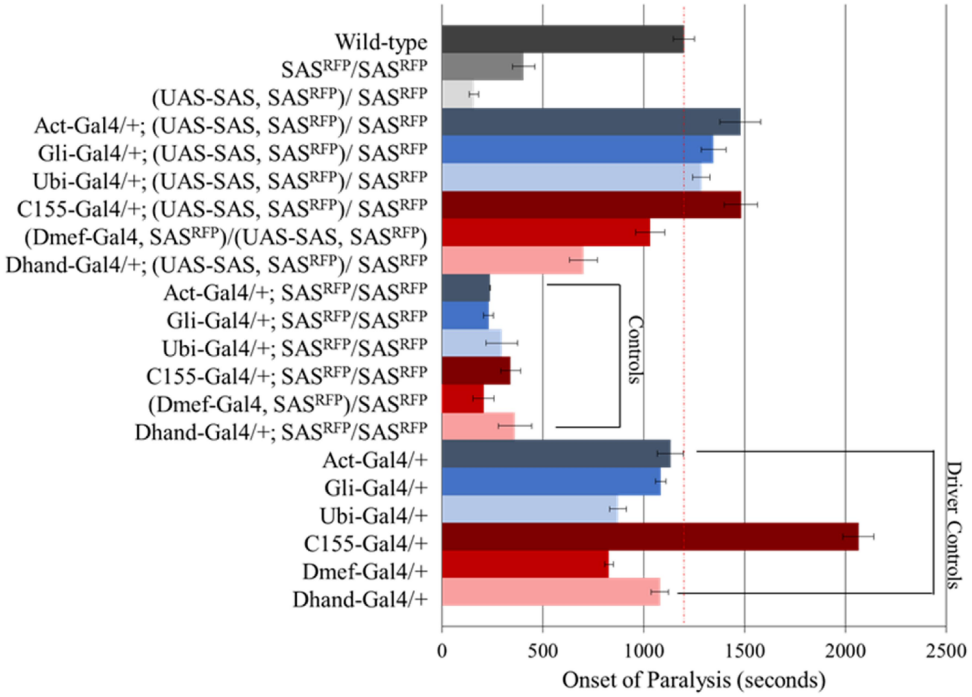

Figure A-3 Rescue controls for TS-paralysis. A) Transgenic expression of *SAS* using the UAS-Gal4 system rescues paralysis phenotype. Temperature-sensitive paralysis assay of flies with different combinations of transgenes. Different controls are indicated in the brackets. Red dashed line indicates the upper cutoff range of the assay at 38°C. 20 individual five-day old males were assayed for each genotype. Error bars represent SEM.