REGULATION OF SIALYLATION IN DROSOPHILA NERVOUS SYSTEM

A Dissertation

by

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ABSTRACT

Sialylation is a common post-translational modification in animal cells, yet its molecular and cellular regulation is poorly understood. It is involved in many vital functions in vertebrates, while perturbations in sialylation have been implicated in severe human pathologies such as mental impairment, epilepsy and heart failure. However, functional studies of sialylation have been hampered due to the pleiotropic effects on the organism as well as the complexity of the sialylation pathway in vertebrates. To overcome these problems, we use Drosophila as a model organism to reveal mechanisms of neural sialylation. Our experiments characterized key genes in *Drosophila* sialylation pathway, including sialyltransferase (DSiaT) and CMP-sialic acid synthetase (CSAS), and shed light on significant conservation between these genes and their mammalian counterparts. We found that *DSiaT* and *CSAS* function within a unique pathway that modulates neural transmission and relies on interactions between neurons and glia. Using a series of cell specific rescue studies, we have found that *CSAS* functions in glial cells, while *DSiaT* function is required in neurons. Analyses of the expression of these genes supported these conclusions, while revealing that CSAS and DSiaT are expressed in different types of cells in a non-overlapping manner. Our results demonstrate that sialylation is coordinated between glial cells and neurons to modulate neural transmission suggesting a role in glia-neuron coupling. Additionally, we found that DSiaT can operate cell non-autonomously to regulate neural excitability. Ectopic expression showed DSiaT can bypass CSAS taking advantage of previously unknown

sialyl-donors to modify glycoproteins in non-canonical sialylation. Collectively, this work provides novel mechanistic insights into the role of sialic acids in regulation of neural excitability in Drosophila. Our results also suggest that these mechanisms can be evolutionarily conserved in mammals, which potentially unveils a novel paradigm relevant for regulation of the nervous system in normal and pathological conditions in humans.

DEDICATION

This work is dedicated to my friends and colleagues at Texas A&M University that were a constant source of inspiration and knowledge. I would like to recognize Courtney Caster, Sandra Truong, Ryan McCormick and Ishita Chandel for their guidance and support. I would also like to dedicate this to my family for their love and patience during this quest for science.

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Contributors

This work was supervised by a dissertation committee consisting of Professor Vlad Panin and Professor Gary Kunkel of the Department of Biochemistry and Biophysics and Professor Paul Hardin and Professor Mark Zoran of the Department of Biology.

The work for this dissertation was completed by Hilary Scott in collaboration with Courtney Caster, and Ilya Mertsalov as well as co-authors on the manuscripts cited in part within.

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NOMENCLATURE

CMP-Neu5Ac	Cytidine Monophosphate N-acetylneuraminic acid
CMP-Sia	CMP-sialic acid
CSAS	CMP-Sialic acid synthetase
DTT	Dithiothreitol
DSiaT	Drosophila sialyltransferase
HPAEC	High-performance anion-exchange chromatography
HPLC	High performance liquid chromatography
KDN	2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
KDO	2-keto-3-deoxy-D-mannooctanoic acid
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
PPi	Inorganic pyrophosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SAS	Sialic acid synthetase
Sia	Sialic acid
UAS	Upstream activation sequence
VGIC	Voltage-gated ion channel

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1. INTRODUCTION

Most animal cells are covered with the glycocalyx, a dense layer of carbohydrates that extends out from the surface and functions as an interface between the cell and its surroundings. This glycocalyx is formed mainly through glycosylation of proteins and lipids. The most common type of post-translational modification on glycoproteins, glycosylation, typically creates glycan chains of monosaccharides linked by either N- or O-glycosidic bonds. N-glycans include N-acetyl glucosamine (GlcNAc) at their reducing end that is attached to an asparagine residue. The majority of membrane and secreted proteins in humans are N-glycosylated. Glycan modifications can have intrinsic functions, e.g. function as structural components in cell wall and the extracellular matrix (ECM), or function as modifiers of biophysical properties of proteins, such as solubility or stability. Glycosylation can also have extrinsic functions, including effecting the trafficking of glycoproteins, as well as modulating cellular interactions like adhesion and signaling events [4]. The structure of glycans is ultimately determined by the action of glycosidases, glycosyltransferases, and other enzymes that can modify glycan chains. Over 1% of our genome is dedicated to the regulation of glycosylation pointing to the importance of its role in biology [5, 6]. This importance is demonstrated through experiments showing that abolishing the biosynthesis of complex or hybrid N-glycans is tolerable in cell culture, however loss of these structures during mammalian development results in embryonic lethality [7].

Sialylation is mediated by the action of sialyltransferases decorating the termini of glycoproteins with sialic acid (Fig. 1.1), a large sugar with a nine-carbon backbone that is ubiquitous in vertebrates and essential for many biological processes [7-9]. Sialic acids are instrumental in neural development and physiology. Gangliosides are sialylated glycosphingolipids that are abundant in neurons. They are involved in axon-myelin interactions, axon stability and regeneration [10]. Polysialic acids (PSA) consisting of long homo-polymers of sialic acid in α2,8 linkage is highly enriched in the nervous system. Their role in neural development is widely documented. PSA regulates cell adhesion through modification of Neural cell adhesion molecule (NCAM) in axonal pathfinding [11, 12]. Electrophysiology experiments using cell cultures demonstrated that sialic acid can directly affect excitability through interactions with voltage gated channels, including sodium voltage channels. These channels are heavily sialylated, with up to 30% of carbohydrates being sialic acid in vertebrates. Sialic acid has a

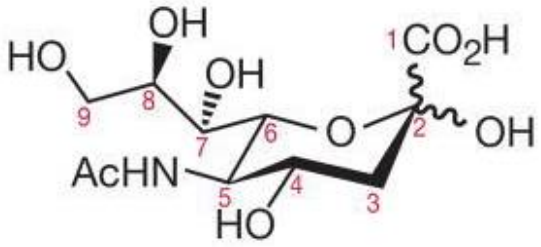

Figure 1.1. Diagram of N-acetylneuramic acid. The most common type of sialic acid in humans decorated the termini of glycoproteins. Typically sialic acid is attached to underlying glycans through glycosidic linkages at C2 or C6. Neu5Ac is a 9-carbon monosaccharide with an acetyl group at C5 that is negatively charged at physiological pH due to the carboxyl group at C1. Further modifications are possible at C4,7-9 increasing diversity of overall glycan chains. Modified from Varki et. al. 2009, Essentials of Glycobiology.

carboxyl group on C1 that at physiological pH carries a negative charge (Fig. 1.1). Glycans coupled with the large amount of PSA on sodium voltage gated channels may

alter gating properties due to electrostatic effects [13]. Moreover, sialylation appears to be regulated both developmentally and in cell-specific manner, which results in expression of different channel glycoforms with distinct gating properties in the heart and neural tissues, suggesting a role in tissue-specific modulation of cellular excitability [14-16]. Loss of sialylation in cultured myocytes results in excitability defects, while abnormal sialylation in the heart can lead to arrhythmia and heart failure [2]. Analogous effect of sialic acid removal in the brain was shown to result in defects of network excitability in epilepsy kindling models in rats [1, 17]. This provides mounting evidence to suggest that sialylation can directly influence neural excitability. Yet the mechanism underlying this function remains uncharacterized because of difficulties with direct experimental approaches due to the complexity of sialylation in the mammalian nervous system. Mammalian sialyloglycoconjugates are synthesized by the temporally and spatially regulated expression of twenty sialyltransferases with different specificity in linkages and substrates [18-20]. Moreover, in vivo analysis is confounded by pleiotropic effects of mutations in a single sialyltransferase and the lethality of mutants lacking activity of the sialylation pathway. These overwhelming obstacles demonstrate the need for a genetically amenable model organism to investigate cellular and molecular mechanisms of sialylation-mediated regulation of neural excitability in vivo.

While sialylated glycans are abundant in mammalian organisms, they represent less than 0.1% of the total content of N-linked carbohydrate modifications in fruit flies. Protostomes were thought to lack the ability to form sialylated structures given the difficulty in detection, as well as the absence of the bifunctional enzyme that provides

ManNAc-6-P, the main precursor in the biosynthesis of sialic acids. The presence of sialylation in *Drosophila* was confirmed by mass spectrometry [21, 22], while several genes of sialylation pathway were identified and enzymatic activity of their products was confirmed by in vivo and in vitro assays [23-26]. In contrast to mammalian sialylation, *Drosophila* has only one sialyltransferase, DSiaT. This enzyme attaches sialic acid to the underlying galactose of the termini of N-glycans in an α-2,6 linkage. DSiaT displays

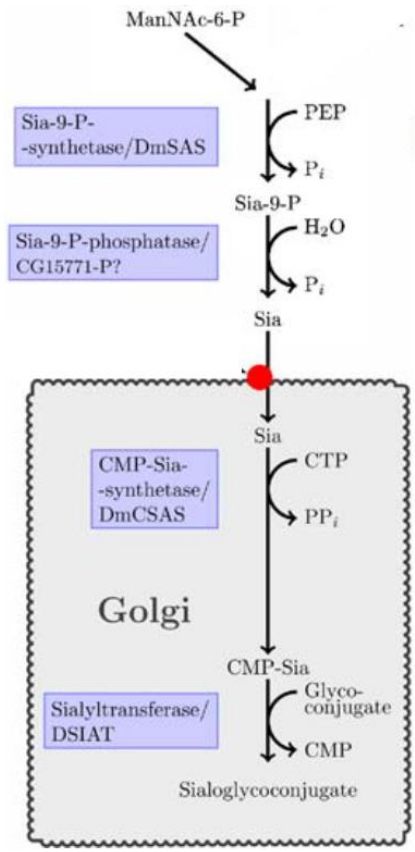

Figure 1.2 Diagram of Drosophila de novo sialylation pathway. The pathway shows all steps in the biosynthesis of sialylated glycans that have been characterized in vitro. Figure modified from [3].

significant sequence similarity to human sialyltransferases of the ST6Gal family, suggesting that the function of this enzyme is evolutionarily conserved [27]. Drosophila

CMP-sialic acid synthetase, DmCSAS, synthesizes the donor nucleotide sugar, CMP-Sia, necessary for DSiaT-mediated sialylation, and thus CSAS functions directly upstream of DSiaT in the biosynthetic pathway generating sialylated glycoproteins (Fig. 1.3). Detailed investigation of DSiaT and CSAS mutants in our laboratory revealed that loss of sialylation results in a significantly reduced life span, locomotor defects, temperature sensitive (TS) paralysis, and decreased evoked excitatory junction potential (EJP) at neuromuscular junctions (NMJs), which points to essential roles of sialylation in modulation of neural transmission and excitability [24]. *DSiaT* and *CSAS* interact with certain voltage gated channel genes known to affect cellular excitability, such as the potassium channel *seizure (sei)* and the sodium channel *paralytic (para)*, which suggests that there is a specific functional pathway that regulates neural transmission via sialylation [24, 28-31]. *CSAS* is the proposed constriction point within the sialylation pathway in both vertebrates and invertebrates, however experimental evidence of this scenario is missing. In vertebrates, the majority of nucleotide sugar synthetases function in the cytoplasm, however mammalian CSAS (aka CMAS) functions in the nucleus, and cells utilize a specific transporter to deliver CMP-Sia to the Golgi [32]. One likely reason for this unique intracellular separation of sequential steps in the sialylation pathway has been postulated to establish another layer of regulation. The sequestration of CMP-Sia biosynthesis in the nucleus perhaps also protects CMP-Sia from processing by cytoplasmic hydrolases, prevent a feedback inhibition of GNE, an upstream enzyme in the sialylation pathway, and could reflect an optimal condition due to greater abundance of CTP, a substrate of CMP-Sia Synthetases, in the nucleus [29].

Drosophila CSAS appears to have unique characteristics compared to its vertebrate counterparts, including its intracellular localization and possibly cell-specific expression. DmCSAS is a glycosylated protein localized predominantly in the Golgi (Fig. 1.2), which reveals a remarkable evolutionarily shift in intracellular localization of a protein with conserved function [29]. *DmCSAS* transcript has been shown to be present in astrocytes in adults [33], which suggests another unique characteristic of this enzyme that may shed light on putative cellular mechanisms that control network excitability by sialylation (Chapter 3).

Currently the genetic and molecular mechanisms that regulate sialylation in vivo remain largely unknown. The goal of this work is to elucidate these mechanisms through the investigation of the biochemical activity of CSAS (Chapter 2), revealing *CSAS* endogenous expression pattern within the nervous system (Chapter 3), as well as investigating cellular mechanisms that underlie the function of sialylation in the regulation of neural transmission (Chapter 3-4). By gaining a greater understanding of the regulation of the sialylation pathway in the Drosophila nervous system, conserved mechanisms that underpin the control of neural transmission may be uncovered, which is expected to shed light on analogous mechanisms that operate in mammals, including humans.

2. CHARACTERIZATION OF DROSOPHILA CMP-SIALIC ACID SYNTHETASE REVEALS UNUSUAL ENZYMATIC PROPERTIES[*]

2.1 Overview

CMP-sialic acid synthetase is a key enzyme of the sialylation pathway. CSAS produces the activated sugar donor, CMP-sialic acid, which serves as a substrate for sialyltransferases to modify glycan termini with sialic acid. Unlike other animal CMP-Sia synthetases that normally localize in the nucleus, Drosophila melanogaster CSAS (DmCSAS) localizes in the cell secretory compartment, predominantly in the Golgi, which suggests that this enzyme has properties distinct from those of its vertebrate counterparts. To test this hypothesis, we purified recombinant DmCSAS and characterized its activity in vitro. Our experiments revealed several unique features of this enzyme. DmCSAS displays specificity for N-acetylneuraminic acid as a substrate, shows preference for lower pH and can function with a broad range of metal cofactors. When tested at a pH corresponding to the Golgi compartment, the enzyme showed significant activity with several metal cations, including Zn2+, Fe2+, Co2+ and Mn2+, while the activity with Mg2+ was found to be low. Protein sequence analysis and site-specific mutagenesis identified an aspartic acid residue that is necessary for enzymatic activity and predicted to be involved in coordinating a metal cofactor. DmCSAS

[*] Reprinted with permission from "Characterization of Drosophila CMP-sialic acid synthetase reveals unusual enzymatic properties" by Ilya Mertsalov, Boris Novikov, Hilary Scott, Lawrence Dangott and Vladislav Panin, 2016. Biochemical Journal, 473(13), 1905-1916. Copyright 2016 by Portland Press.

enzymatic activity was found to be essential in vivo for rescuing the phenotype of *DmCSAS* mutants. Finally, our experiments revealed a steep dependence of the enzymatic activity on temperature. Taken together, our results indicate that DmCSAS underwent evolutionary adaptation to pH and ionic environment different from that of counterpart synthetases in vertebrates. Our data also suggest that these adaptations due to its sub-cellular environment may play a role in the regulation of DmCSAS within the pathway.

2.2 Introduction

Cytidine monophosphate sialic acid synthetases are evolutionarily conserved enzymes mediating a key step in the biosynthetic pathway of sialylation. They generate the activated sugar donor, CMP-sialic acid (CMP-Sia), from free sialic acid (Sia) and cytidine triphosphate (CTP) [34]. The product of CSAS, CMP-Sia, is utilized by sialyltransferase enzymes to modify the termini of glycans attached to glycoproteins and glycolipids and produce various sialoglycoconjugates [1]. These sialylated molecules are eventually delivered to the cell surface or secreted to extracellular milieu, where they mediate a plethora of crucial biological functions [2, 3]. While organisms can possess multiple sialyltransferases (e.g., humans have twenty different sialyltransferase enzymes with distinct linkage and substrate specificities), there is usually only one CSAS (aka CMAS in vertebrates) enzyme responsible for the sialic acid activation step in all known organisms mediating de novo sialylation, except for some fish species that have two CSAS genes due to a presumptive gene duplication [4].

CMP-Sia synthetases are unique members of the family of sugar-activating enzymes because they generate an unusual nucleotide sugar donor, CMP-Sia, that represents a nucleoside monophosphate diester, as compared to other activated sugars having a diphosphate diester form. Furthermore, unlike other nucleotide-sugar synthetases that use phosphorylated sugar molecules as substrates, CMP-Sia synthetases can utilize non-phosphorylated sialic acid [5]. Finally, while other sugar-activating enzymes are normally found in the cytoplasm, CSAS localizes to the nucleus in vertebrates, with one reported exception of a zebrafish CMP-Sia synthetase, DreCMAS2, that can be also found in the cytoplasm [6].

Recent studies identified CSAS in Drosophila [7] and found that its function is important for the control of neural transmission [8]. CSAS mutations in Drosophila cause defects in neural excitability and locomotion, while also resulting in temperature-sensitive paralysis and significantly shortened life span [8]. Surprisingly, the DmCSAS protein was found to be localized to the secretory pathway compartment and present mainly in the Golgi when it was expressed in the Drosophila nervous system in vivo or heterologously expressed in mammalian cell cultures [7, 8]. Furthermore, recent experiments revealed that DmCSAS is a glycoprotein modified with N-linked glycans (Fig. 1.2, [8]). Thus, DmCSAS represents the first example of CMP-Sia synthetases with Golgi localization, which unveils an unprecedented example of a radical evolutionary change in subcellular localization of a metazoan enzyme that occurred within a family of orthologous proteins with conserved function. The pH and ionic environments of the Golgi and nuclear compartments differ significantly, and thus the unusual subcellular

localization of DmCSAS suggests evolutionary adaptation of the enzyme to a distinct molecular environment, which poses important questions about properties of the Drosophila enzyme in comparison to the properties of vertebrate counterparts. CMP-Sia synthetase activity of the Drosophila CSAS protein was detected in cell lysates when this protein was heterologously expressed in mammalian tissue culture [7]. However, Drosophila CSAS was not purified and its enzymatic properties have not been characterized. In our work, we purified the DmCSAS protein from Drosophila cultured cells, characterized its enzymatic properties in vitro, and compared them to those of its human orthologue assayed under the same conditions. Along with demonstrating general similarity between enzymatic activity of the Drosophila enzyme and its vertebrate counterparts, our analyses also revealed crucial differences between them. Our results suggested that insect and vertebrate CMP-Sia synthetases are regulated by different molecular and cellular mechanisms, which shed light on evolutionary adaptation of these proteins to distinct subcellular environments.

2.3 Materials and Methods

2.3.1 Materials and reagents

Sugars, nucleotides, mouse anti-FLAG antibody and FLAG affinity beads were purchased from Sigma. EnzCheck pyrophosphate assay kit was obtained from Invitrogen. PNGaseF enzyme was from NEB. Rabbit GM130 antibody was from Abcam. Drosophila strains with GAL4 drivers were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN). DmCSAS mutants and

the FLAG-tagged DmCSAS construct were previously described [8]. GFP-tagged human CMP-sialic acid synthetase construct was a gift from Michael Betenbaugh (The Johns Hopkins University).

2.3.2 Expression constructs, site-directed mutagenesis and cloning

The UAS-CSAS-FLAG expression construct for in vivo expression was described previously [8]. For expression in Drosophila tissue culture cells, the CSAS-FLAG coding region from UAS-CSAS-FLAG was sub cloned under control of a metallothionein promoter between Bam HI and Spe I restriction sites of pMK33 vector using regular molecular cloning techniques. For generating the CSAS-DA mutant, we used a PCR-based site-specific mutagenesis protocol [9] and changed the codon 228, GAC to GCC, which resulted in D228A mutation in the CSAS protein. The final expression plasmids for CSAS-FLAG wildtype and DA mutant constructs were confirmed by sequencing and then used in transfection experiments with Drosophila S2 cells to generate stable lines.

2.3.3 Cell culture

Drosophila S2 cells were grown in M3 complete medium supplemented with 10% FBS, bactopeptone (2.5 g/L), yeast extract (1 g/L), penicillin/streptomycin solution (100u/100ug per ml) and fungizone (0.25 µg/ml). Cells were maintained at 25˚C in a low-temperature incubator without CO2 control. For transient and stable transfections, we used Ca2+-phosphate protocol as previously described [10] or Effectene reagent (Qiagen) according to manufacturer's protocol. Polyclonal stable lines were generated using hygromycin selection (200 µg/ml) for 4-6 weeks. Expression of recombinant

constructs was induced from metallothionein promoter with 0.7 mM CuSO4 in cultures with cell density of 80-90% confluency. After 20 hours of induction, the cells were collected by centrifugation, washed in ice-cold phosphate-buffered saline, and then processed for western blot analyses and protein purification.

2.3.4 Protein purification

Drosophila CSAS proteins. The cells with induced protein expression were washed twice with 1xTBS buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl) and then resuspended in 10 volumes (w/v) of Lysis buffer (50mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton-X-100, 1 mM PMSF, and cOmplete protease inhibitors cocktail (Roche)). The cells were lysed by sonication, using Branson Sonifier® 150. Lysis was completed by rocking vials on nutator for 20 min. Non-soluble material was removed from lysates by centrifugation at 14,000g for 20 min. To isolate FLAG-tagged proteins, 20 µl of FLAG-affinity beads (Sigma), prewashed in Lysis buffer, were added per 1 ml of lysate. After incubation on a nutator for 4-8 hours, the beads were collected by centrifugation, washed 4 times in Lysis buffer, and then stored at 4oC in Storage buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton-X100) until used in assays. All sample manipulations were performed on ice or at +4˚C. Concentration and purity of isolated protein was determined by densitometric analysis of proteins separated on 12% SDS-PAGE gels and visualized by Coomassie G250 staining with BSA as quantification standard. Quantification of digitized gel images was performed using TotalLab Quant software.

Human CSAS protein. GFP-tagged human CSAS protein [7] was expressed in Drosophila using UAS-GAL4 expression system [11] with tubP-Gal4 driver [12]. Flies were homogenized in Dounce grinder using 1.5 ml of Lysis buffer per 100 flies. The homogenized tissue was collected and sonicated by Branson Sonifier using four series of 5-second pulses. The lysis was completed by rocking on nutator for 20 min. The lysates were pre-cleared by centrifugation at 25,000g for 30 min, and then incubated with GFP-affinity beads (Trap-A, Chromotek) prewashed 3 times in Lysis buffer, using 25 µl of beads per 1.5 ml of lysate. After 4-8 hours nutator incubation, the beads were washed 4 times in Lysis buffer and then stored at 4˚C in Storage buffer. All manipulations with samples, starting from tissue homogenization, were performed on ice or at +4˚C. Protein concentration was determined as described above.

2.3.5 CMP-Sia synthetase in vitro assays

Unless stated otherwise, CSAS assay was performed in Reaction buffer (100 mM Tris-HCl, pH 8.0, 20 mM MgCl2, 0.2 mM DTT) containing 3 mM Neu5Ac and 5 mM CTP. The reaction was started by mixing 200 µl of the reaction mixture with CSAS purified on beads. The reaction was mixed, centrifuged immediately for 30 secs at 3,000 rpm, and then 100 µl of supernatant was withdrawn and kept at -20˚C as a sample corresponding to zero-time point. The remaining 100 µl of reaction mixture containing CSAS beads were resuspended and incubated in 0.2 ml PCR tubes with gentle rotations while submerged in a water bath with controlled temperature at 37˚C for 1h. The concentration of the Drosophila CSAS protein was typically 200-300 ng per 100 µl of the reaction mixture. Reaction was stopped by transferring reaction tubes on ice for 5

min, followed by centrifugation through 10K Amicon Ultra spin filter (Merck Millipore) to remove beads and protein content. Filtered reactions could be stored at -20° C for up to 6 months without noticeable product degradation. Studies at various pH were performed using two buffers to insure the stability of pH during assays. Cacodylate buffer was used in assays with pH ≤ 7.4 (50 mM sodium cacodylate, 20 mM MgCl2, 0.2 mM DTT), while Tris buffer (see above) was used for assays with pH ≥7.4. The pH conditions of the assays were also confirmed by measuring pH after addition of substrates, as adding substrates could substantially affect the final pH of the reaction buffer.

The amount of CMP-Sia was quantified using two approaches. In the first one, CMP-Sia was detected by high-performance anion- exchange chromatography (HPAEC) essentially as described previously [13]. Briefly, components of reaction mixture were separated on a 4 x 250 mm CarboPac PA-1 column (Dionex) using Ultimate3000 HPLC system (Thermo) equipped with variable wavelength detector VWD-3100. Column was equilibrated with 5 mM NaOH (eluent A) and elution was performed at flow rate 1 ml/min, using a gradient of 1 M sodium acetate in 5 mM NaOH (10%, 8 min; linear gradient 10-50%, 16 min, linear gradient 50-100%, 6 min; 100%, 12 min). Detection of eluted components was performed at 271 nm. Commercially available CMP-Neu5Ac (Sigma-Aldrich) was used as a standard for quantification.

The second approach was based on enzymatic detection of inorganic pyrophosphate (PPi) by EnzCheck kit (Invitrogen) using manufacturer's instructions with modifications. In a routine experiment, 5-20 µl from standard CSAS assay were

added to EnzChek reaction mixture in total volume 100 µl (50 mM Tris HCl pH 7.5, 1 mM MgCl2, 0.2 mM MESG substrate, 1 U purine nucleoside phosphorylase and 0.05 U inorganic pyrophosphatase) and incubated at 22 oC for 1 hour. The concentration of the product of enzymatic conversion of MESG substrate was detected with Ultrospec 2000 spectrophotometer (Pharmacia Biotech) at 360 nm. PPi concentrations were calculated with calibration curve plotted using PPi standards.

We tested the effect of different components of CMP-Sia synthetase reaction on EnzChek-mediated detection and found that Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} and Fe^{2+} could significantly affect PPi measurements. Thus, we resolved to use the HPAEC-based detection of CMP-Sia for CSAS assays in the presence of these ions. We also found that the EnzCheck method can be vulnerable to changes in pH. To overcome this problem, the EnzCheck reactions were adjusted to 150 mM Tris-HCl pH 7.5, which improved accuracy of PPi quantification.

The HPAEC and EnzCheck – based methods of CMP-Sia quantification were found to produce consistent results and have comparable level of sensitivity over a broad range of substrate concentrations that we tested (~ 3 – 1,000 µM). With exceptions described above, these two methods were used interchangeably in our experiments.

2.3.6 Kinetic analyses

Kinetic parameters were determined for Neu5Ac, Neu5Gc and CTP at saturating concentration of one of the substrates and varied concentration of the second substrate. Assays were conducted at 37°C in 100 µL of reaction mixture consisting of 100 mM Tris-HCl pH 8.0, 20 mM MgCl2, 0.2 mM DTT and 100 ng of Drosophila CSAS. Rate of

reaction was determined using EnzCheck kit as described above. Kinetic parameters (Vmax and Km) were determined by fitting experimental data to the Michaelis-Menten equation, using SigmaPlot v.10 software package (Systat Software, Inc.).

2.3.7 PNGase F treatment

Purified CSAS proteins were eluted from beads by incubation with Glycoprotein Denaturing buffer (0.5% SDS, 40 mM DTT) at 95˚C for 10 min. The reaction was briefly chilled, beads were removed by centrifugation, and the eluted protein was diluted 2x by adding H2O, 1% NP40 and 50 mM sodium phosphate pH 7.5. PNGase F (NEB) was added to 500u per 20 µl of reaction, and the reaction mixture was incubated at 31˚C for 2 hour. For the mock control, the protocol was repeated with all ingredients except for PNGase F.

2.3.8 Subcellular localization of CSAS-DA

The CSAS-DA mutant protein was expressed in Drosophila larval neurons essentially as described previously [8]. Briefly, the expression was induced in the brain using UAS-GAL4 system [11]. Third instar larval brains were dissected in ice-cold Ringers solution, rinsed and fixed in fresh Fixative solution (4% paraformaldehyde, 50 mM NaCl, 0.1 M Pipes pH 7.2) for 20 minutes at room temperature on nutator. Fixed tissue was washed with PBT (PBS pH 7.5, 0.1% Triton X100, 1% BSA, 0.01% sodium azide) and analyzed by immunostaining and epifluorescent microscopy. CSAS-DA-FLAG was detected using mouse anti-FLAG M2 (Sigma) primary antibody (1:2,000). Rabbit GM130 antibody (Abcam) was used at 1:100 dilution. Incubation with fluorescent secondary antibodies was carried out essentially as described previously

[14]. The following secondary antibodies and corresponding dilutions were used: goat anti-mouse Alexa-546 and goat anti-rabbit Alexa-488, 1:250 (Molecular Probes). Immunostained brains were mounted in Vectashield medium (Vector Laboratories) and analyzed using Zeiss Axioplan 2 microscope with ApoTome module for optical sectioning.

2.3.9 In vivo rescue experiments and paralysis assays

For rescue experiments, *CSAS* wildtype and *CSAS-DA* were expressed in *CSAS* loss-of-function homozygous mutant Drosophila [8] using UAS-GAL4 expression system [11]. Temperature-sensitive (TS) paralysis phenotype was assayed essentially as described previously [15]. Briefly, Drosophila strains were maintained using regular cornmeal–malt–yeast food in temperature, humidity and light-controlled environment (25°C, 38% humidity, 12 h light/darkness). For TS-paralysis assays, individual flies were collected on the day of eclosure and aged individually for 5 days. During this period, they were transferred once, on day 3, to a fresh-food vial. Five days old flies were transferred to empty vials and temperature was raised to 38˚C by submerging vials in a controlled-temperature water bath. We defined paralysis as a condition when a fly was paralyzed and unable to walk or keep standing position for at least 1 minute.

2.3.10 Protein sequence alignment and phylogenetic analysis

Protein sequences were aligned using Clustal Omega server at EMBL-EBI [16]. Only N-terminal parts of sequences corresponding to the CMP-Sia synthetase domain were used for enzymes with bipartite domain architecture [4]. Phylogenetic tree was assembled by ClustalW2 program using neighbor-joining algorithm [17].

2.3.11 Statistical analyses

Unless indicated otherwise, all data points show average values obtained from at least three independent experiments. Error bars indicate standard errors (SEM). For multiple-groups comparison (TS paralysis assays), one-way ANOVA (Kruskal-Wallis test) was used, followed by a post-hoc Wilcoxon Each Pair nonparametric test, to assess differences between the groups.

2.4 Results

2.4.1 Expression, purification and in vitro assays of DmCSAS

DmCSAS was previously expressed in vivo as a FLAG-tagged construct that was found to be active based on its ability to rescue the phenotype of *DmCSAS* mutants [8]. We used that construct in purification experiments. To this end, the CSAS-FLAG protein was expressed in Drosophila S2 cell culture and purified using FLAG-affinity beads. The amount and purity of CSAS was evaluated by Coomassie staining of purified proteins separated by SDS-PAGE. The identity of CSAS bands on Coomassie-stained gels was confirmed by western blots (Fig. 2.1A). Purified CSAS was present on the gel as two bands corresponding to the two glycoforms of the protein that were previously detected in vivo [8]. We could consistently isolate up to 0.5 µg of CSAS per 1 ml of culture with a purity of about 90%, as estimated by the relative amount of CSAS compared to non-specific proteins co-purified on FLAG-affinity beads.

Purified DmCSAS protein has a stable CMP-sialic acid synthetase activity. Enzymatic activity of purified CSAS was assayed in vitro using CTP and Sia as substrates (see

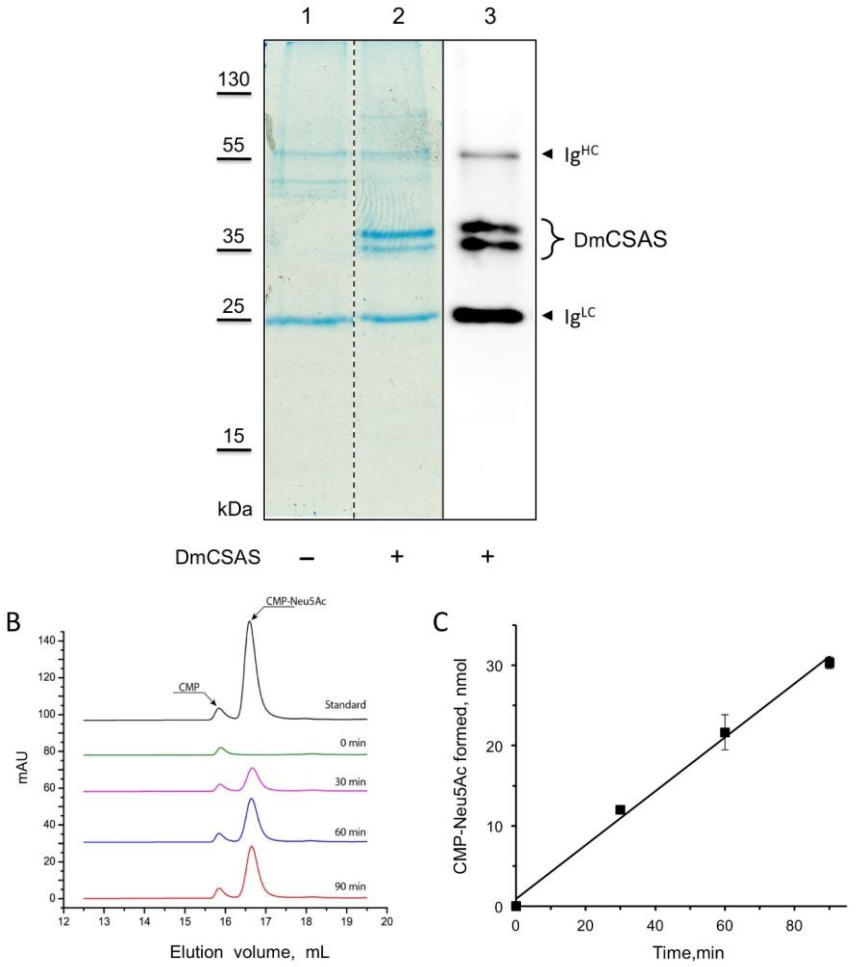

Figure 2.1. Purification of Drosophila CSAS protein and its in vitro enzymatic activity. **A**, DmCSAS-FLAG protein purified on FLAG-affinity beads and analyzed by Coomassie staining of SDS-PAGE gels and western blot. Lane 1, non-specific proteins purified from S2 cells without DmCSAS-FLAG expression. Lane 2, DmCSAS-FLAG purified from S2 cells. Lane 3: western blot analysis of purified DmCSAS (same sample as on lane 2). The positions of two DmCSAS glycoforms, as well as heavy and light chains of FLAG antibody leached from the beads are indicated. **B**, HPLC chromatograms illustrating the detection of CMP-Neu5Ac. The top trace shows CMP-Neu5Ac acid standard as a control. Traces below show the gradual increase of CMP-Neu5Ac product over a period of 90 min in the assay with purified DmCSAS. **C**, Purified DmCSAS can stably produce CMP-Neu5Ac for up to 90 min. The assays were carried our at standard reaction conditions (100mM Tris-HCl pH 8.0, 20mM $MgCl_2$, 0.2 mM DTT, 3 mM sialic acid, 5.5 mM CTP, and 100 ng DmCSAS) at 40°C.

Experimental Procedures for details). We quantified the product of catalytic activity, CMP-Sia, by HPLC, as well as by the enzymatic detection of pyrophosphate, a byproduct of the CMP-Sia synthesis reaction (see Experimental Procedures). These two

assays were compared in several experiments, which indicated that the assays produce consistent results and have similar sensitivity. Thus, we used the two assays interchangeably in our further experiments.

In our initial assays, we tested whether CSAS has a stable CMP-synthetase activity in vitro. Our experiments indicated that the purified enzyme was active and able to generate CMP-Sia in vitro with constant productivity for up to 90 minutes at 40˚C (Fig. 1B-C).

2.4.2 Effect of temperature and pH on DmCSAS activity

We characterized the dependence of DmCSAS activity on temperature and pH. We found that the activity steadily increases with temperature from 20˚C to 45˚C by approximately an order of magnitude (Fig. 2.2A). The activity declines dramatically at temperatures above 45˚C, which probably reflects the decrease of the protein stability at higher temperature. The analysis of the dependence of DmCSAS activity on pH revealed that the enzyme has a relatively narrow optimum around pH 8 (Fig. 2.2B). In

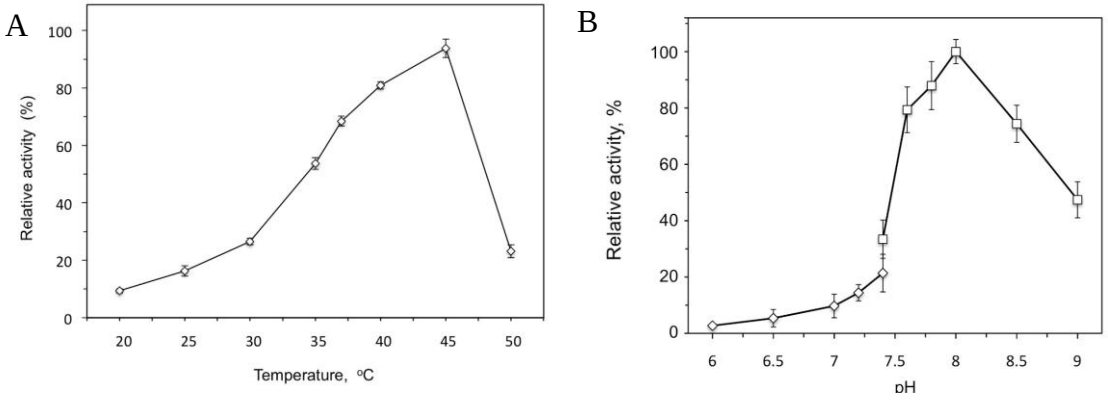

Figure 2.2. Effect of temperature and pH on DmCSAS activity. A, The dependence of enzyme activity on temperature. B, The dependence of enzyme activity on pH. Unless indicated otherwise, assays were performed at standard reaction conditions in the presence of 20 mM Mg^{2+} at 37°C, pH 8. Cacodylate buffer was used in assays with pH ≤ 7.4 (diamonds) and Tris-HCl buffer was used at pH ≥ 7.4 (squares).

standard assay conditions, DmCSAS activity declines precipitously at lower pH, dropping to 20% around pH 7.4 and becoming practically undetectable below pH 7.0. This result was surprising considering that DmCSAS localizes predominantly to the Golgi compartment that has estimated pH in the range of 6.2-6.7 [18, 19]. Above the optimal pH, the DmCSAS activity also declines substantially but more gradually, loosing ~ 50% at pH 9. Thus, in comparison to its mammalian and bacterial counterparts that generally have a broader optimum of activity at more basic pH conditions (pH 8.5-10 [20-22]), the Drosophila enzyme demonstrates the pH dependence with a relatively sharp optimum that is shifted toward lower pH.

2.4.3 Substrate specificity of DmCSAS

We further characterized the activity of DmCSAS by assessing its specificity. We measured its kinetic parameters for numerous substrates, including several types of sialic acid and structurally similar molecules found in various organisms. The apparent Km values were 410 uM and 450 uM for N-acetylneuraminic acid (Neu5Ac) and CTP, respectively (Fig. 2.3 A-B). Estimated Vmax for these substrates was in the range of 3.4-3.6 umol/min/mg. Other nucleoside triphosphates, including ATP, GTP and UTP, along with CDP and CMP, were also tested as potential substrates. However, one of them could be utilized by the enzyme (data not shown). We also tested N-glycolylneuraminic acid (Neu5Gc), another common sialic acid variant present in vertebrate species [1]. In comparison to Neu5Ac, Neu5Gc was found to be a relatively inferior substrate. The kinetics for Neu5Gc showed no saturation, so we could only estimate that the Km for Neu5Gc was > 3.5 mM (Fig. 2.3C). Considering that the

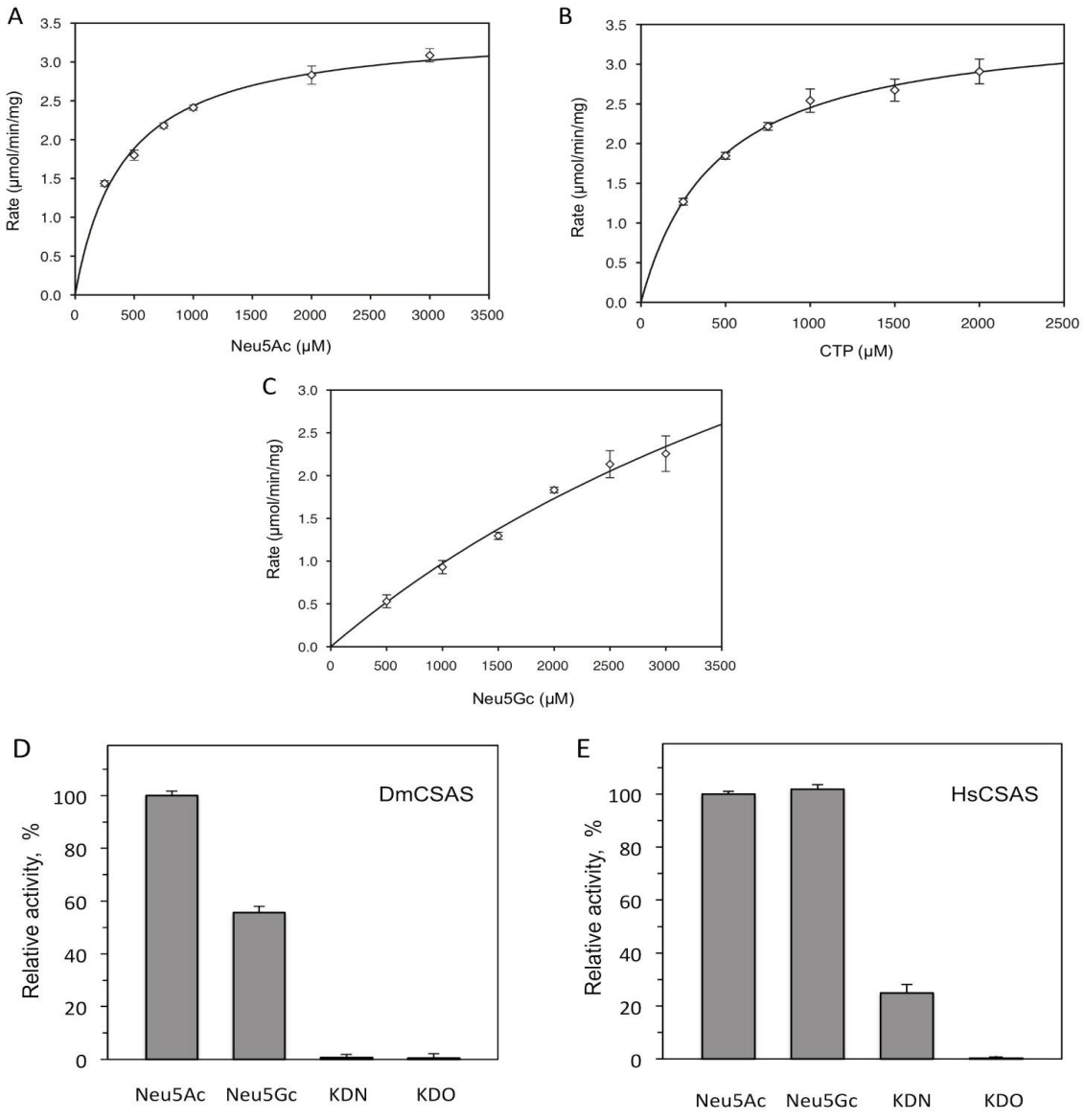

Figure 2.3 Kinetic analysis of DmCSAS and comparison of substrate specificities of Drosophila and human CMP-Sia synthetases. **A-C**, Graphs show kinetics for CTP (A), Neu5Ac (B), and Neu5Gc (C). D-E, Substrate specificities of purified DmCSAS (D) and HsCSAS (E) with Neu5Ac, Neu5Gc, KDN and KDO as tested substrates. Unless indicated otherwise, assays were performed using standard reaction conditions that included 100mM Tris-HCl pH 8.0, 20mM $MgCl_2$, 0.2 mM DTT, 3 mM sugar substrate, and 5.5 mM CTP. *Drosophila* and human enzymes were used at 100 ng and 30 ng per reaction, respectively. Reactions were incubated for 1 hour at 37°C.

structural difference between these two sialic acid variants is relatively small, consisting

of just the hydroxylation of the 5-N-acetyl group in Neu5Gc, this result suggests that the N-acetyl group of sialic acids plays a key role in CSAS substrate recognition.

In addition, two other structurally related sugars were tested as substrates, 2-keto-3-deoxynononic acid (KDN) and 2-keto-3-deoxyoctonic acid 3-deoxy-D-manno-2-octulosonic acid (KDO). The former is a common type of sialic acid abundantly present in simpler vertebrate species [23], while the latter is an eight-carbon sugar that is structurally similar to sialic acids and commonly found in Gram-negative bacteria and higher plants [24, 25]. We found that CSAS has a nearly undetectable activity toward KDN, while no activity was detected with KDO (Fig. 2.3D). To compare the activity of Drosophila CSAS to a mammalian counterpart assayed in similar conditions, we expressed human CMP-Sia synthetase (HsCSAS) in Drosophila and purified it on affinity beads (see Experimental Procedures). This recombinant human enzyme was found to be significantly more active when compared to DmCSAS, having ~ 20 times higher activity at 37˚C in the same assay conditions. The relative preference for sialic acid substrates was very different for HsCSAS which showed equal activities toward Neu5Ac and Neu5Gc, and lower but still significant activity toward KDN. Similar to the Drosophila enzyme, HsCSAS could not utilize KDO as a substrate (Fig. 2.3E).

2.4.4 Requirement for divalent metal cations

Previous studies revealed that divalent metals, such as Mg^{2+} or Mn^{2+}, are essential for the activity CMP-Sia synthetases as these metal ions bind near the enzyme active site and facilitate the orientation of substrates and catalysis [4]. Mg^{2+} is thought to be the preferred metal cofactor for CMP-Sia synthetases, and it was used in the majority

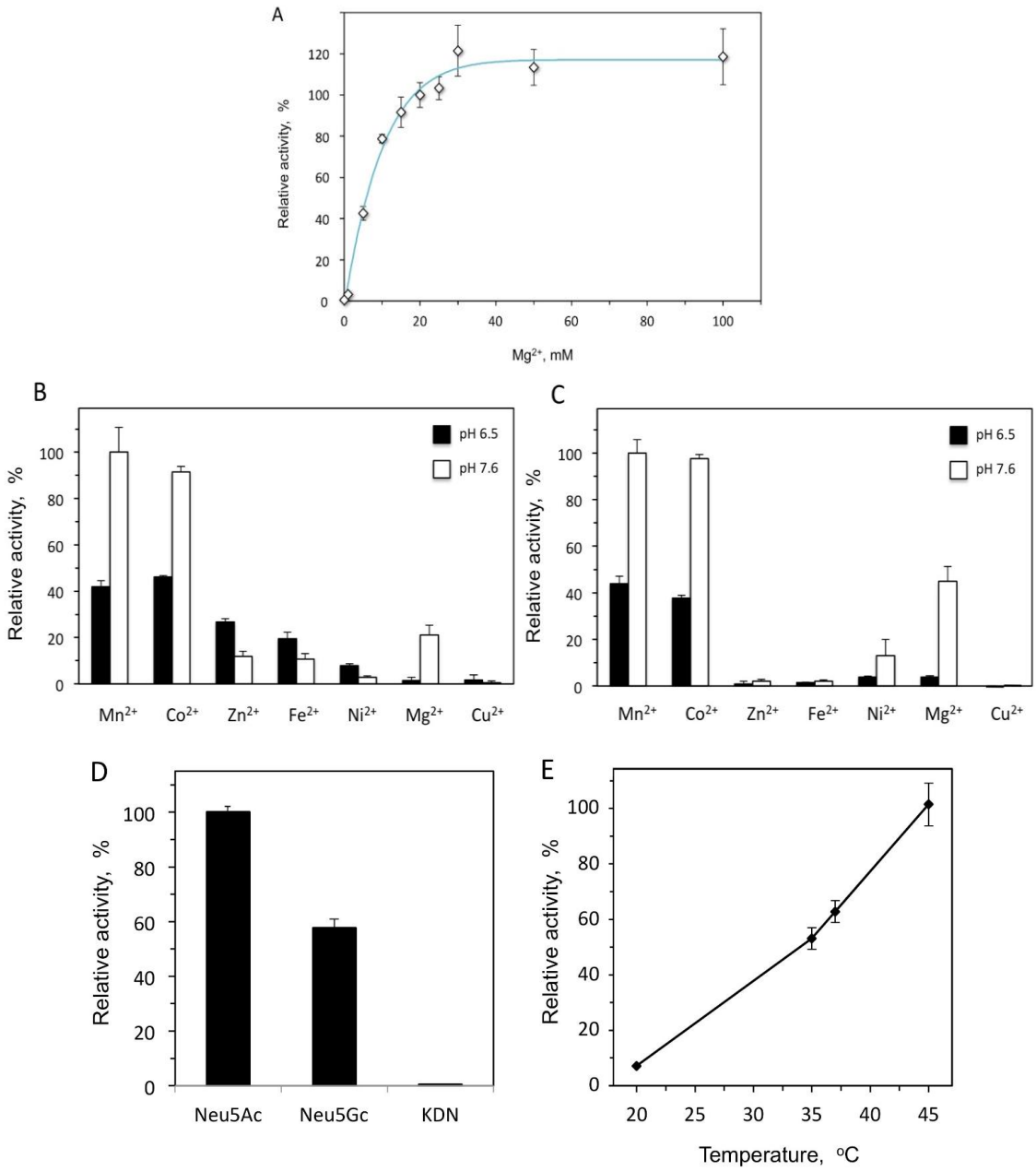

Figure 2.4. Effect of metal ions on CMP-Sia synthetase activity. **A**, DmCSAS requires the presence of divalent metal cations for its activity. The enzyme was assayed at different concentrations of Mg^{2+}. **B**, DCSAS can utilize different metal cofactors, while metal cofactors have differential effects on DmCSAS activity at distinct pH. **C**, Human CMP-Sia synthetase assayed with different metal ions at the same reaction conditions as the *Drosophila* enzyme in B. **D-E**, Substrate specificity (D) and temperature dependence (E) of DmCSAS activity in presence Mn^{2+} as a cofactor at pH 7.6. Unless indicated otherwise, assays were performed in standard reaction conditions using corresponding metal cofactor at 5 mM**.**

of previous studies on in vitro activities of the synthetases. Hence, we first examined the

requirement for the metal ions for DmCSAS activity by varying the concentration of Mg^{2+} in the reaction buffer. The enzyme was not active without a metal cofactor, and CSAS activity reached saturation at ~20-30 mM Mg^{2+} (Fig 2.4A). Interestingly, a previous study found that using Mn^{2+} instead of Mg^{2+} caused a shift of pH optimum of the bovine CMP-Sia synthetase to more neutral values, from ~ 9.5 to 8.0 [26]. Thus, we decided to test whether DmCSAS could utilize divalent metal cations other than Mg^{2+}, and whether these other metals could activate Drosophila CSAS at pH 6.5 corresponding to the endogenous environment of this synthetase in the Golgi compartment. We examined the activity of DmCSAS at pH 6.5 and 7.6 in the presence of different divalent metal cations (Fig. 2.4B). The effect of cations was analyzed at 5 mM concentration (to avoid precipitation of the reaction mixture that was observed at higher concentrations for some ions, such as Mn^{2+} and Cu^{2+}). For comparison, the activity of human CMP-Sia synthetase was also analyzed in parallel experiments using the same conditions (Fig. 2.4C). We found that DmCSAS exhibits significant activity at pH 6.5 in the presence of Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+}. All metals yielded a higher activity than Mg^{2+} at pH 6.5, while the activity with Mn^{2+} and Co^{2+} surpassed that with Mg^{2+} at pH 7.6 as well. These two preferred metal cofactors were also found to be the best cofactors for the human synthetase at the both pH conditions. However, the human enzyme was inactive in the presence of Zn^{2+} and Fe^{2+}, irrespectively of pH (Fig. 2.4C). These data suggest that Drosophila CSAS can endogenously utilize a metal ion other than Mg^{2+} while working in a lower pH environment inside the Golgi compartment.

To test whether different metal cofactors may influence substrate specificity of DmCSAS, we assayed the enzyme in presence of Mn^{2+} with different types of sialic acid. We found that DmCSAS substrate preference was essentially the same with Mn^{2+} as with Mg^{2+} (Fig. 2.4D vs. 2.3D). Utilization of Mn^{2+} instead of Mg^{2+} somewhat enhanced the dependence of enzyme activity on temperature, which was found to increase 14-fold between 20˚C and 45˚C in presence of Mn2+ (as compared to10-fold increase in presence of Mg^{2+} (Fig. 2.4E vs.2. 2A)). Taken together, these results

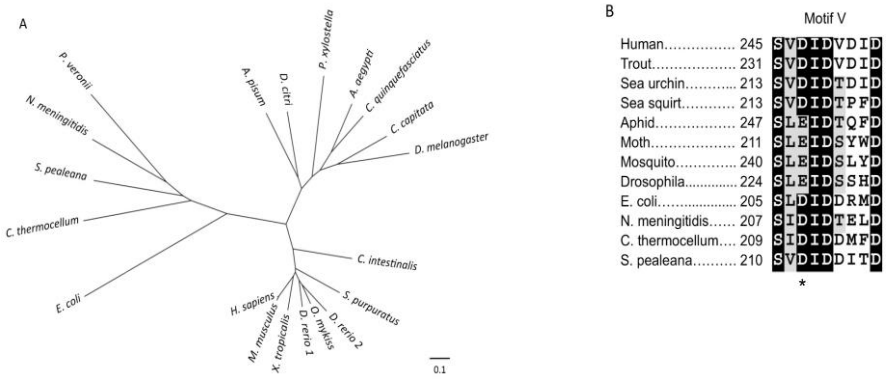

Figure 2.5. Phylogenetic analysis and metal binding site comparison between CMP-Sia synthetases from different species. A, Phylogenetic tree illustrating evolutionary relationship between CMP-Sia synthetases from bacteria, insects and complex animals (deuterostomes). The tree was built by ClustalW2 program using neighbor-joining algorithm. The accession numbers of bacterial sequences: *Pseudomonas veronii*, WP_017848838.1; *Shewanella pealeana*, CP000851.1; *Clostridium thermocellum*, CP002416.1; *Neisseria meningitidis*, AAB60780.1; *Escherichia coli*, J05023.1. The accession numbers of insect proteins: *Acyrthosiphon pisum* (pea aphid), NP_001156112.1; *Diaphorina citri* (Asian citrus psyllid), XM_008472953.1; *Plutella xylostella* (moth), XM_011559466.1; *Aedes aegypti* (yellow fever mosquito), XM_001662967.1; *Culex quinquefasciatus* (southern house mosquito) XP_001842321.1; *Ceratitis capitata* (Mediterranean fruit fly), XM_004522975.1. The accession numbers of deuterostome proteins: *Ciona intestinalis* (sea squirt), NM_001100127.1; *Strongylocentrotus purpuratus* (sea urchin) CSAS NM_001126308.1; *Danio rerio* (zebrafish) JQ015186.1, JQ015187.1; *Oncorhynchus mykiss* (rainbow trout), NM_001124190.1; *Xenopus tropicalis* (frog), NM_001097281.1; *Mus musculus* (mouse), NP_034038.2; *Homo sapiens* (human), NP_061156.1. **B**, The fragment of CMP-Sia synthetase multiple sequence alignment showing the conserved Motif V involved in metal cofactor coordination. Asterisk indicates the aspartic acid residue of the DID triad that is conserved in bacterial and vertebrate sequences but is substituted by glutamic acid in insect CSAS proteins. The multiple alignments were generated by Clustal Omega server at EMBL

suggested that substrate specificity and temperature dependence of DmCSAS activity do not significantly change when the enzyme utilizes different metal cofactors.

2.4.5 The role of conserved aspartic acid residues

Our phylogenetic analysis of CMP-Sia synthetase sequences from different species indicated that these enzymes in insects, more complex animals (such as tunicates and vertebrates) and bacteria represent three different well-separated clades (Fig. 2.5A). The clear phylogenetic separation between synthetases from insects and vertebrates presumably reflects the fact that these enzyme subfamilies evolved in distinct subcellular environments, as insect CSAS proteins are predicted to localize to the secretory compartment due to the presence of a putative signal peptide sequence, while the enzymatic activity. To test this hypothesis, we created a DmCSAS-DA mutant that has vertebrate enzymes are nuclear-localized proteins. Since the adaptation of insect synthetases to the milieu of the Golgi compartment is potentially associated with utilizing different metal cofactors (Fig. 2.4B), the insect enzymes may have evolved distinct interactions with metal cations. Thus, we decided to examine more closely the amino acid sequences of insect CSAS proteins, while focusing on the residues that are predicted to coordinate metal cations within catalytic centers. Two conserved aspartic acid residues in the motif V were proposed to coordinate metal cations at the active site of mammalian and bacterial CMP-Sia synthetase [27-29]. Interestingly, the sequence alignment of the motif V indicated that one of these aspartic acids, thought to be essential for coordinating the catalytic metal ion [29], is not conserved in insect enzymes

(Fig. 2.5B). This observation suggested that metal cofactors are bound differently in the i

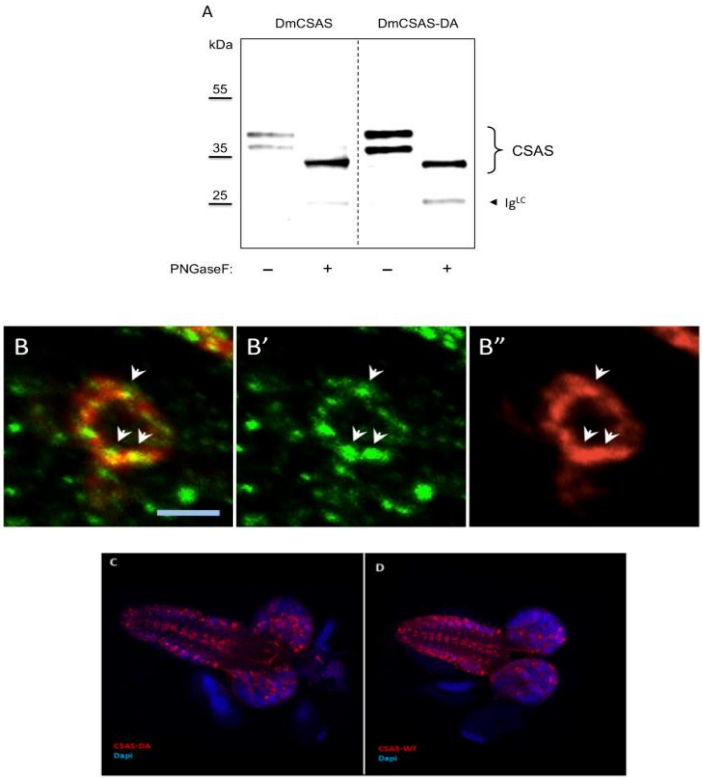

Figure 2.6. Analyses of the DmCSAS-DA mutant protein. A, DmCSAS-DA (DmCSAS with D228->A substitution) is properly glycosylated, as indicated by PNGase F treatment. DmCSAS wildtype protein was used as a control. Proteins were purified on FLAG affinity beads, treated with PNGase F, and analyzed by SDS-PAGE followed by western blot detection. Both proteins are expressed as two glycoforms represented by two bands on the gel. Upon PNGaseF – mediated removal of N-glycans, these bands collapse into one band of a lower molecular mass corresponding to deglycosylated protein. **B**, DmCSAS-DA is localized in the Golgi compartment, as reveled by double immunofluorescent staining with the GM130 Golgi marker [3]. B' and B" show single channel staining for GM130 (green) and DmCSAS-DA (red), respectively. B is the overlay of B' and B". Arrows point at examples of co-localization between GM130 and DmCSAS-DA. DmCSAS-DA was expressed in the CNS using UAS-GAL4 system. Images of fixed, dissected and stained brains were obtained using epifluorescent microscopy with optical sectioning. The image shows a confocal section through the cell body of a single neuron. Scale bar is 5 μm. **C-D.** DmCSAS-DA (red) and DmCSAS-WT (red) were expressed in the CNS as stated above. Dapi (blue) is a nuclear marker. Images are of fixed, dissected and stained brains from 3rd instar larvae. The image shows a confocal image through brain. Scale bar is 50um. Posterior is to the right. **E-F**, DmCSAS-DA and CSAS wild type CMP-Sia synthetase activity assays of DmCSAS-DA mutant at increasing concentrations of Mg^{2+}. DmCSAS wildtype protein was used as a positive control. No enzymatic activity of DmCSAS-DA was detected. **D**, DmCSAS activity was tested *in vivo* using a transgenic rescue approach. DmCSAS-DA and DmCSAS wildtype proteins were expressed in *DmCSAS* homozygous null mutants (*DmCSAS*$^{-}$*)* using UAS-GAL4 system. The rescue of *DmCSAS*$^{-}$ phenotype was analyzed by TS-paralysis assays. At least 20 flies were assayed for each genotype. **o** and *** indicate not statistically significant and highly significant differences, respectively. Error bars represent SEM in all panels.

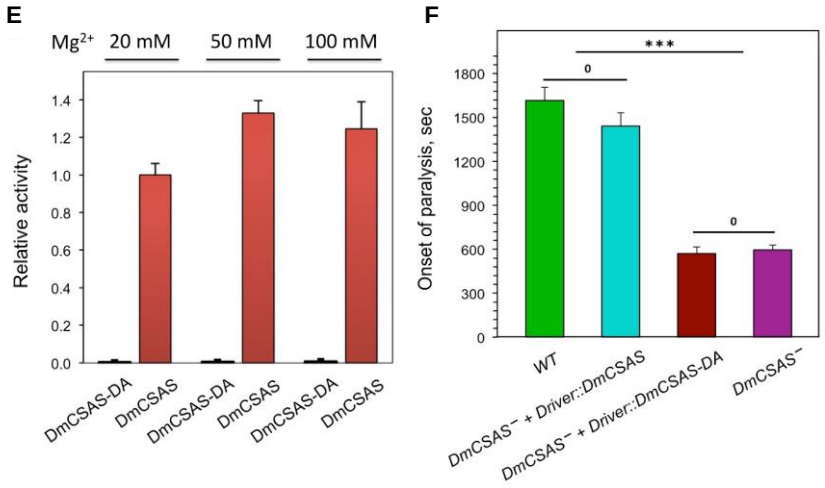

Figure 2.6 continued

insect enzymes, being primarily coordinated by one aspartic acid. Therefore, mutating the remaining conserved Asp residue is predicted to eliminate the D228A substitution. This mutant was expressed in Drosophila cells and its level of expression, subcellular localization and glycosylation were analyzed and compared to that of the wildtype DmCSAS protein. These experiments revealed no significant differences between DmCSAS-DA and wildtype DmCSAS, which indicated that the mutant protein was folded properly within the cell (Fig. 2.6A-B). At the same time, we could not detect any measurable activity of DmCSAS-DA when it was purified and assayed in vitro, even at increased Mg^{2+} concentration (Fig. 2.6C), the condition that was previously shown to elevate the activity of a bacterial CMP-Sia synthetase with the analogous mutation [29]. These results indicated that D228 is essential for DmCSAS catalytic activity and suggested that this residue plays a key role in coordinating metal cation in the enzyme active site. We also tested the activity of DmCSAS-DA in vivo using temperature-sensitive paralysis assays. DmCSAS mutant flies are paralyzed at elevated temperature

due to abnormalities in neural transmission. This phenotype can be rescued by transgenic expression of DmCSAS [8]. The DmCSAS-DA protein was unable to rescue the phenotype (Fig. 2.6D), which indicated that enzymatic activity is essential for DmCSAS function in vivo.

2.5 Discussion

Our work provided the detailed characterization of purified Drosophila CMP-Sia synthetase, the first purified and characterized member of the subfamily of CSAS proteins with unique secretory compartment localization. The phylogenetic comparison between insect and vertebrate CMP-Sia synthetases indicated that the insect enzymes belong to a separate clade of proteins (Fig. 2.5). This clear separation suggests that their evolution was significantly affected by differences in physiological environment, as insect CSAS proteins are predicted to work in the secretory pathway compartment, while vertebrate CMP-Sia synthetases are normally localized to the nucleus.

The phylogenetic separation between insect CSAS and their counterparts from more complex animals is consistent with unique enzymatic properties of the Drosophila CMP-Sia synthetase that were revealed in our experiments. Unlike most other characterized synthetases that prefer relatively basic pH conditions, the Drosophila enzyme shows preference for lower pH, with a sharp optimum around pH 8.0 in the presence of Mg^{2+} as a cofactor (Fig. 2.2B). Surprisingly, we found that the activity of the enzyme is negligible at the pH corresponding to the conditions inside the Golgi (6.3-6.7), the subcellular locale where the enzyme is predominantly found in vivo [8]. This

apparent conundrum can be resolved by the fact that the synthetase is able to efficiently utilize several metal cofactors beside Mg^{2+}, while showing significant activity with some of them at lower pH conditions. Remarkably, in the presence of Zn^{2+} and Fe^{2+} the activity is even higher at pH 6.5 than at 7.6 (Fig. 2.4B). On the other hand, our data on the recombinant human CMP-Sia synthetase, purified and assayed in vitro using the same conditions, indicate that fewer metals can activate the human enzyme (Fig. 2.4C). This observation is consistent with previously published data on other vertebrate CSAS orthologues [22]. The human CMP-Sia synthetase is unable to utilize Zn^{2+} and Fe^{2+}, while showing higher activity at pH 7.6, as compared to 6.5, with all tested cofactors (Fig. 2.4C). These differences between the human and Drosophila enzyme support the hypothesis of evolutionary adaptation of these enzymes to distinct subcellular environments. Interestingly, Zn^{2+} and Fe^{2+} are both present in Drosophila at relatively high levels [30]. They are essential for Drosophila development and physiology and known to be specifically transported to the Golgi compartment [31, 32], which indicates that these metal ions are reasonable candidates for endogenous cofactors of DmCSAS.

The presence of divalent metal cations is a strict requirement for the activity of CMP-Sia synthetase [4], including Drosophila CSAS (Fig. 2.4A). So far, however, no metal cofactors have been revealed on crystal structures of these enzymes [4]. The position of metal ions and the residues that coordinate them were proposed based on models using the structural information on bacterial CMP-Kdo synthetases, enzymes with similar fold architecture [33, 34]. These models suggested that the catalytic metal cation is coordinated by two aspartic acids within the conserved DID triad of the CSAS

motif V [4, 29, 33]. Surprisingly, our sequence alignments revealed that the first D in this motif is not conserved and is substituted with E in insect CSAS sequences (Fig. 2.5B). This data further supports the idea that the molecular evolution of insect CSAS enzymes resulted in metal cofactor interactions that are different from those of CMP-Sia synthetases localized outside of the secretory compartment. The importance of the remaining D residue conserved in insect enzymes was examined in our analyses of DmCSAS-DA, the mutant version of DmCSAS with D228A substitution. The level of expression, subcellular localization and glycosylation of this mutant were all similar to those of the wildtype protein, which strongly suggested that DmCSAS-DA was normally expressed and properly folded (Fig. 2.6A-B). However, we could not detect any enzymatic activity of the DmCSAS-DA protein. The analogous D211A mutation in the CMP-Sia synthetase from N. meningitidis also resulted in significant loss of activity, however some residual activity still remained [29]. Moreover, increased Mg^{2+} concentration was able to partially restore the activity of that mutant, while the mutation in the first D of the DID triad (D209 in the N. meningitidis sequence, Fig. 2.5B) caused a more dramatic loss of activity and was not restored by increased metal concentration [29]. Thus, the D209 of N. meningitidis appears to play a more important role in metal binding than D211, which is consistent with putative bidentate interactions of D209 with a metal cofactor proposed by analogy to CMP-Kdo synthetases [29, 33]. On the other hand, the activity of DmCSAS-DA was not detectable even at elevated metal concentrations (Fig. 2.6C), which suggests that the key role of metal cofactor coordination is “reassigned” in Drosophila CSAS to the last residue of the (D/E)ID triad

of the motif V. Taken together, our data suggested that the coordination of catalytic metal cofactors is different in insect CMP-Sia synthetases, as compared to vertebrate and bacterial counterparts. Our results also demonstrated that the aspartic acid residue of the EID triad is essential for the activity of DmCSAS and suggested that this residue plays similar role in other insect CSAS enzymes.

We also analyzed the activity of DmCSAS-DA in vivo using a transgenic rescue approach, which revealed that enzymatic activity of DmCSAS is essential for its biological function (Fig. 2.6D). Interestingly, previous genetic interaction experiments suggested that DmCSAS could potentially have some non-enzymatic functions in vivo, while studies in vertebrate systems have also led to speculations that CSAS proteins may have some yet unidentified role not associated with their CMP-Sia synthetase activity. Our experiments provided the first in vivo test for potential non-enzymatic functions of CSAS proteins. While it remains possible that DmCSAS has some in vivo functions not directly associated with its enzymatic activity, they are probably minor as our data demonstrated that DmCSAS enzymatic activity is essential for the main biological role of DmCSAS in maintaining normal neural transmission.

Our characterization of substrate specificity of Drosophila CSAS indicated that the enzyme is very selective in utilizing certain types of sialic acids. It shows the highest activity toward Neu5Ac, having Km value among the lowest reported for animal CMP-Sia synthetases (e.g., Km of the synthetase purified from rat, 0.72-1.3 mM; frog, 1.6 mM; hog, 0.8 mM; calf, 0.9-1.4 mM [20, 35-37]), which is consistent with the fact that the concentration of sialic acid in Drosophila is low [38, 39]. Despite subtle structural

differences between Neu5Ac and Neu5Gc (just a single hydroxylation of the 5-N-acetyl group in Neu5Gc [1]), the latter is a significantly poorer substrate for the Drosophila enzyme (Fig. 2.3). Mammalian CMP-Sia synthetases commonly do not discriminate between these sialic acid versions (Fig. 3E, [20, 22]), suggesting that the N-acetyl group plays a more important role in DmCSAS substrate recognition, as compared to mammalian CMP-Sia synthetases. This conclusion is consistent with the fact that KDN, a desamino form of sialic acid that carries a hydroxyl instead of the N-acetyl group [1], cannot be utilized by the Drosophila enzyme (Fig. 2.3D, 2.4D). KDN is a common type of sialic acid present in simpler vertebrates [23], and the possibility that Drosophila can also synthesize KDN-modified glycans was previously discussed [40]. Our results now indicate that this scenario is unlikely since the CSAS protein is thought to be the only enzyme that can activate sialic acids in Drosophila [8, 40].

Our analyses of DmCSAS activity at different temperature conditions revealed a remarkable stability of the enzyme and a robust increase of enzymatic activity at elevated temperatures up to 45˚C (Fig.2. 2A). For comparison, the counterpart enzymes from ectothermic vertebrates were found to reach optimum at 37˚C (frog CSAS [35]) or be unstable at a higher temperature (the trout CSAS is unstable above 25˚C [41]), whereas most bacterial synthetases have optimum at 37˚C [21]. Intriguingly, DmCSAS activity increases ~9 fold between 20˚C and 40˚C, within the range of temperature of Drosophila natural habitats. Drosophila is a poikilotherm, having organism's temperature regulated by ambient conditions. These data indicate that environmental temperature can potentially play an important role in the regulation of Drosophila

sialylation. Our previous experiments revealed that CSAS is essential for the regulation of neural excitability. In vivo analyses demonstrated that Drosophila CSAS mutants cannot maintain a sustainable level of neural activity and are paralyzed at elevated temperature (Fig.2. 6C, [8]). Thus, the significant temperature-dependent increase in DmCSAS enzymatic activity can potentially underlie a novel regulatory mechanism that controls the level of neural activity at different environmental temperatures, which is expected to have important implications for Drosophila neurophysiology and behavior.

3. GLIAL-NEURONAL FUNCTIONAL COUPLING MEDIATED BY SIALYLATION

3.1 Overview

Sialylation is known to play important roles in mammalian neural development, but its role in neural transmission is poorly understood. Studies in this area are hindered due to intricacies of sialylation and the complexity of the nervous system in mammalian organisms. Drosophila has emerged as an attractive model to uncover evolutionarily conserved mechanisms of sialylation-mediated regulation of neural excitability. Previously, we have shown that *DSiaT* and *CSAS* are expressed in the nervous system and function within a pathway that regulates excitability. Our current work focuses on the regulation of sialylation within the nervous system. We investigated cellular requirements of sialylation genes using genetic approaches, immunostaining, behavioral and electrophysiological assays.

Our results show that neural transmission is modulated through the coordinated function of DSiaT and CSAS in distinct cells within the nervous system. Immunostaining shows that *CSAS* is expressed in glial cells, while DSiaT is present exclusively in neurons. Rescue studies found that CSAS functions in a cell specific manner to maintain neural transmission. Moreover, CSAS modulates the level of excitability within the nervous system, and the enzymatic function of CSAS is required for this modulation (Fig. 2.6). Lowered levels of *DSiaT* by RNAi experiments demonstrated the necessity for *DSiaT* expression in neurons to maintain neural

transmission. Interestingly, the *ectopic* expression of DSiaT in either glial cells or neurons could rescue the phenotype, suggesting DSiaT-mediated sialylation of functionally important targets could occur extracellularly. Together, our findings suggest a novel bi-partite mechanism for the synthesis of sialylated glycoconjugates that modulates neural excitability. The coordinated function of CSAS and DSiaT, in glia and neurons, respectively, reveals a new form of sialylation- mediated glia-neuron coupling that regulates the nervous system in Drosophila and is potentially conserved in vertebrates.

3.2 Introduction

Sialic acids are large sugars with a negative charge that decorate carbohydrates on the outermost surface of cells. Sialylation occurs through post-translational modification of glycoproteins and of glycolipids [6, 18]. Sialylation is essential in human biology, and aberrant sialylation is implicated in heart disease, cancers, and neural dysfunction [5, 9]. While the role of polysialylation of NCAM (neural cell adhesion molecule) in neural development of mammals is well established, the mechanisms underlying function of sialylation in neuronal excitability remains unknown [35]. Sialic acids have been shown to directly influence excitability in the nervous system through effects on VGICs (voltage gated ion channels). Mammalian VGICs are heavily glycosylated with negatively charged poly sialic acid comprising up to 50% of glycans. Electrophysiological studies in cell culture demonstrated that glycans attached to pore-forming subunits modulate the gating properties of the channel, potentially

through electrostatic effects. Additionally, channel isoforms are distinctly modified to respond in a cell-specific manner to "functional" sialic acids [14]. Moreover, the level of sialylation affects surface charge of neuronal membranes which is crucial for excitability. Changes in surface sialylation have been shown to change network excitability and influence seizures in rat model of epilepsy [1]. Taken together, all these data highlight important roles of sialylation in neuronal excitability. Unfortunately, the study into these mechanisms have been hindered due to the pleiotropic effects on the organism as well as the complexity of the sialylation pathway in mammals.

The presence of sialic acids in *Drosophila* has been shown by mass spectrometry analyses [21, 22], while the sialylation pathway has been confirmed through a series of studies characterizing enzymes responsible for the biosynthesis of sialylated glycans [23, 24, 26, 29, 36, 37]. *Drosophila* sialyltransferase, *DSiaT*, is related to the human ST6Gal family of sialyltransferases, suggesting that this family represents the most ancient sialyltransferase enzymes with evolutionarily conserved roles in the nervous system. Mutations in key genes of the Drosophila sialylation pathway, *DSiaT* and *CSAS*, result in locomotor defects, temperature sensitive (TS) paralysis, and altered NMJ (neuromuscular junction) physiology. Mutant alleles of sialylation genes demonstrate interactions with a subset of *VGIC* genes, suggesting that a specific sialylation-controlled pathway modulates network excitability in the nervous system [24, 29]. Yet, complex genetic interactions between *DSiaT* and *CSAS* indicated that loss of *CSAS* results in a more dramatic effect at both organismal and cellular level, and it was postulated that *CSAS* may have some independent function within the nervous system

[29]. However recent studies of CSAS enzymatic mutant suggest that CSAS enzymatic activity is still required for this function (Fig. 2.6). CSAS-DA is expressed in a pattern similar to wild type CSAS suggesting CSAS-DA is localized and folded properly. Yet CSAS-DA is unable to rescue *CSAS* mutants (Fig. 2.6). This indicates that enzymatic activity is the primary function of CSAS in the maintenance of neural transmission. Recently, *CSAS* transcript was shown to be expressed in adult Drosophila astrocytes [33], opening up the possibility that the sialylation pathway may be functional in glial cells. *Drosophila* possess glial cells that are morphologically and functionally analogous to cells in humans, allowing for the investigation into the possible conserved biological role of sialylation in glia using Drosophila as a model system [38, 39].

Our goal in the present study was to uncover the cellular and molecular mechanisms that regulate sialylation in *Drosophila* and modulate network excitability within the nervous system. Our results suggest that sialylation is mediated by the coordination of gene activities that are separated between glial cells and neurons, and required for modulation of neural transmission, revealing a novel mechanism of glia-neuron coupling. Our model postulates that CSAS functions in glial cells, while DSiaT is expressed in neurons, and modifies glycoproteins inside the secretory compartment as well as in extracellular milieu. This represents a novel bi-partite mechanism of sialylation regulating the nervous system that could be evolutionarily conserved in mammals.

3.3 Materials and Methods

3.3.1 Drosophila strains

The following fly strains were used: *Canton-S wild type* (gift from Josh Dubnau, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), *repo-Gal4[40], MZ709-Gal4 [41], r182-Gal4 (gliotactin)[42], moody-Gal4 [43], Alrm-Gal4* [44], *elav-Gal4 , C155-Gal4, MJ85-Gal4, 1407-Gal4, UAS-FLp, gcmΔp1 ,UAS-CSAS15, UAS-CSAS111, $CSAS^{Mi}$* and *$CSAS^{21}$* [29], *$DSiaT^{L22}$, $DSiaT^{S23}$* [24], *UAS-FRT-Stop-FRT-Sia-GFP, LexOp-2xhrUAS-CD8GFP, LexOP-FlpL, LexOp-GFPnls, UAS-DSiaT-RNAi* (Bloomington Stock Center).

3.3.2 Generation of transgenic lines

HsCSAS was sub-cloned into pUAST by standard cloning methods. (cDNA was a generous gift from Dr. Michael Betenbaugh, John Hopkins University, MD). *BAC-CSAS-FLAG [2,3]* Recombineering-mediated tagging was performed as described [45, 46]. Briefly, attB-P(acman)-CamR BACmide BAC CH322-158A02 spanning 22,067 kb of genomic region that contains CSAS locus was co-transformed with a fragment containing a $kana^{r}rpsL^{+}$ cassette along with the CSAS gene and 50bp HA (homology arms) by electroporation into the DY380 *E. coli* cells to facilitate recombination using λ-prophage based Red recombination system. A positive/negative selection scheme with both kanamycin and streptomycin was used to screen for transformants. Colonies growing on kanamycin were selected and insert was confirmed by colony PCR. Next, the $kana^{r}rpsL^{+}$ cassette was "flipped" with a cassette containing *CSAS-FLAG* through recombination within HA. Selection was based on the loss of

streptomycin sensitivity achieved by kana^{r}rpsL^{+} cassette being successfully removed. Positive colonies were confirmed with colony PCR. The following primers were used to generate CSAS- kana^{r}rpsL^{+} and CSAS-FLAG: CSASkana-f PL452 (5'CGATCTGACTCTAGCCAAA TACATCTTAAGTAGTGAAA CAAAAACCGAGggtctgaagaggagtttacgtcc 3'), CSASkana-r PL452 (5'CAAGATCAATGAACTTTACACTTTCTTTGATGAATA GCATATC TATTTC aagggttccgcaagctctagtc 3'), CSASrpslkan a-f pSK+RpsL-kana (5' CGATCTGACTCTAGCCAAATACATCTTAAGTAGTGAAACAAAAACCGAG tgggcggttttatggacagca 3'), CSASrpslkan a-r pSK+RpsL-kana (5'CAA GATCA ATGAACTTTACACTTTCTTTGATGAATAGCATATCTATTTCgtgggcgaaga actccagcat 3'), CSASgnmc-f CSAS BAC (5' GAGTCCCATGACTGTGTTTTTGCC GCTAAGAGG 3'), CSASgnmc-r CSAS BAC (5'TGTGTGTCGCTCGGTTTGGCAG GACTTGCTTGG 3'). The resulting BAC-CSAS-FLAG was purified using Qiagen kit then transformed into EPI300 *E. coli* cells to induce increased copy number upon addition of 0.1% L-Arabinose. DNA was purified with Qiagen and sequenced (IDT, Coralville, IA). BAC-CSAS-FLAG was injected into fly embryos and incorporated into site VK31(3L,62E1) through Phi-C31 integrase-mediated recombination to generate BAC-CSAS-FLAG lines. (Rainbow Transgenics, Camarillo, CA).

BAC-CSAS-LexA Generation of this construction occurred in three sequential steps 1) site-directed mutagenesis of ATG sites in CSAS coding region 2) Generation of CSAS-LexA-LoxP-Kan plasmid construct through Gibson's assembly 3) recombineering to insert CSAS-LexA into BAC-CSAS. First, site-directed mutagenesis was accomplished

through PCR amplification. Second, the sequence for LexA-p65 hybrid transcription factor was inserted into the coding region of CSAS using Gibson assembly [47]. BAC-CSAS was used as a template to amplify 1.7kb fragments containing coding sequence with ATG 1,2 mutated. The following primers were used: Forward: CSAS-Gib-BamHI-Fd (5' GCC GCT CTA GAA CTA GTG CCA AAG GTATGC CAG TGT CCT TTA G-3'), Reverse: CSAS_dwn1(5' TTC ATgttgATCAATTCACCATCGACCACCTTCC-3'), CSAS_dwn2 (5' CGGTTTGGCAGGACTTGCTTGGT-3'). pBPnlsLexA--p65Uw was used as a template for a 1.46kb fragment containing the coding sequence of LexA-p65 hybrid transcription factor using the following primers: Forward: CSASLexA_up4 (5' GGTCGATGGTGAATTGATcaacATGAAGGCTCTCACGGCCCGAC-3'), Reverse: lexA-Gib-RI-rev (5' cgaagttatagaatcgTTAGG AGCTTATCTGGCTCAG CAAAG-3'), Forward: RI-Fd-Lox-Long (5' CGATTCTATAACTTCGTATAGCAT ACATTATACGAAGTTATTCA GAAGAACTCGTCAAGA-3'), Reverse: BamHI-PSK-Rev-Gibs (5' GATCCAC TAGTTCTAGAGCGGC-3'), BamHI-PSK-Rev-Gibs1 RR(5' CACTAGTTCTAGAGCGGCcgccac-3'). This resulted in the CSAS-kan-LoxP-pSK construct that was digested with EcoRI and BamHI and used as a template for amplification the fragment about 4,3 kb in length (DNA fragment 1). pBluescriptSK plasmid was digested with EcoRV in which 0,9 kb Kanamycin selective gene surrounded by LoxP sequences were subcloned (DNA fragment 2). A 262 bp fragment of 3' noncoding region of CSAS gene was subcloned into Kan-LoxP-pSK plasmid by HindIII and KpnI restriction sites (DNA fragment 3). Three fragments were ligated together according to the protocol of Gibson Assembly Cloning Kit (New England

Biolabs Inc., USA). Gibson ligation reaction was done in two steps. In first step fragments 1 and 2 were ligated together, then fragment 3 was added to ligation mixture to obtain final circular plasmid construct. The resulting CSAS-LexA-LoxP-Kan-CSAS construct was co-transformed into DH5α *E. coli* competent cells. Colonies were selected on kanamycin, then confirmed by restriction digest with AfeI and PsiI followed by sequencing. Third, recombineering-mediated tagging was performed as described [45]. Briefly, attB-P(acman)-CamR BACmide BAC CH322-158A02 spanning 22,067 kb of genomic region that contains CSAS locus was co-transformed with pBS- CSAS-LexA-LoxP-Kan by electroporation EL350 into cells. LoxP-Kan cassette was removed by induction of Cre recombinase using 0.1% Arabinose. The resulting BAC-CSAS-LexA was purified using Qiagen kit and then transformed into EPI300 cells to induce increased copy number. DNA was purified using Qiagen kit and sequenced (IDT). BAC-CSAS-LexA was injecting into fly embryos and incorporated into site VK27 (3L,89E11) through Phi-C31 integrase-mediated recombination to generate BAC-CSAS-LexA lines. (Rainbow Transgenics, Camarillo, CA).

BAC-Sia-3HA was created using recombineering-mediated tagging method described above. BAC containing genomic region of chromosome 2 including DSiaT locus was used for recombination with Sia-3HA sub-cloned into plasmid along with Lox-P sites flanking kanamycin cassette for selection and 100 bp HA both up-down stream of insert. All other steps were followed as above. (Rainbow Transgenics, Camarillo, CA)

3.3.3 Immunostaining and Imaging

Flies were dissected in ice cold Ringer's, washed, then fixed (4% paraformaldehyde, 2mM EGTA, 1mM $MgSO_4$, 0.1M PIPES) for 20 minutes at room temperature on nutator. Immunostaining was carried out with the following antibodies: mouse anti-Flag (1:2000, Sigma), mouse anti-GFP (1:5, DSHB), rabbit anti-GM130 (1:100, Abcam), rat anti-elav (1:10, DHSB), rat anti-HA (1:1000, Roche) mouse anti-repo (1:10, DHSB), mouse anti-elav (1:10, DHSB), mouse anti-nc82 (1:50, DSHB); secondary antibodies were goat anti-mouse Alexa 488, 546, 647 (1:250, Invitrogen). Fluorescent images were obtained using Zeiss confocal or Zeiss Axioplane 2 microscope with ApoTome module for optical sectioning. *Z*-projections were generated using Zeiss AxioVision and Zen software.

3.3.4 Rescue studies and Behavioral Assays

UAS-Gal4 binary system was used for cell specific expression of transgenic CSAS by crossing UAS-CSAS111 or UAS-CSAS15 in a CSAS mutant background to a Gal4 driver. BAC-CSAS-LexA rescue studies were carried out using a combination of LexA/LexOp and UAS-Gal4 expression systems to express UAS-CSAS15.

Locomotor Assays. Adult flies were collected on the day of eclosion then aged for five days, transferring to fresh food on day three. All flies were kept in a controlled environment at 25˚C in 60% humidity with 12-hour day/night light cycles. A minimum of twenty individuals were assayed for each genotype. Righting Assay: Individual flies were placed in a vial and allowed to acclimate for 10 minutes. Then tapped on a soft foam pad 5 times twice, the time they spent on their back was recorded. Two trials for

each fly with a 10-minute time between trials. Geotaxis Assay: Individual flies were placed in vials and acclimated for 10 minutes. The vials were banged five times on the counter and the time for the fly to crawl upwards 5 cm was recorded.

Temperature sensitive paralysis assay: Adult flies were collected and maintained as mentioned above unless otherwise noted for specific experiments. On day five, individual flies in vials were submerged in a temperature controlled water bath set to 38˚C and the paralysis time recorded. Paralysis is defined as the fly on its back unable to right itself at the bottom of the vial for one minute. A minimum of twenty individuals were assayed for each genotype and their mean taken as the value for that genotype.

3.3.5 Immunoprecipitation and Western blots

One day old adult flies were collected and homogenized in lysis buffer (50mM NaCl, 0.5% Triton X-100). For immunoprecipitation, the lysates were incubated with Flag conjugated agarose beads (M8823; Sigma, St. Louis, MO) at 4C for 30min. Lysate and beads were collected and boiled for 10min in SDS sample buffer. For all other experiments, whole lysate was used. Protein was loaded onto a 12% SDS-PAGE gel for separation, then transferred onto nitrocellulose membrane and stained using primary antibody mouse anti-flag 1:2000, secondary anti-mouse HRP 1: 10,000 (1706516; Biorad, Hercules, CA) and visualized by chemiluminescence kit (32106; Pierce Biotechnology, Inc, Rockford, IL).

3.3.6 Electrophysiology

Current clamp intracellular recordings were performed from muscles of dissected third-instar larvae as previously described [29]. Drosophila third instar larvae were

dissected in ice-cold Ca2 free HL3 solution and electrophysiological recordings were collected from abdominal muscle 6 at room temperature. The recoding solution was supplemented with CaCl for experiments as noted. Microelectrodes were filled with 3M KCl and had an input resistance of 8-16M. Membrane potentials were amplified by the Axoclamp 200B Amplifier, digitized and analyzed using pClamp10 software (Molecular Devices). Evoked EJP analysis was performed by averaging 10 events for each NMJ recording and considering the average amplitude as one measurements.

3.3.7 Statistical analysis

All rescue experiments were conducted with at least 20 individuals per genotype tested. Error bars indicate standard errors (SEM). For multiple-groups comparison one-way ANOVA (Kruskal-Wallis test) was used, followed by a post-hoc Wilcoxon Each Pair nonparametric test, to assess differences between the groups.

3.4 Results

3.4.1 CSAS is expressed in glial cells throughout all developmental stages

To elucidate the mechanism of regulation of *Drosophila* sialylation we sought to characterize the endogenous expression pattern of CSAS. We created BAC-CSAS-FLAG construct including a large genomic region of CSAS locus (~ 22kB), with CSAS coding sequence tagged with a short 3FLAG-encoding fragment (see Materials and Methods). We anticipated that this construct includes all essential regulatory sequences of the CSAS gene, and thus it should recapitulate the endogenous expression pattern of CSAS when transgenically introduced in flies. We found that BAC-CSAS-FLAG

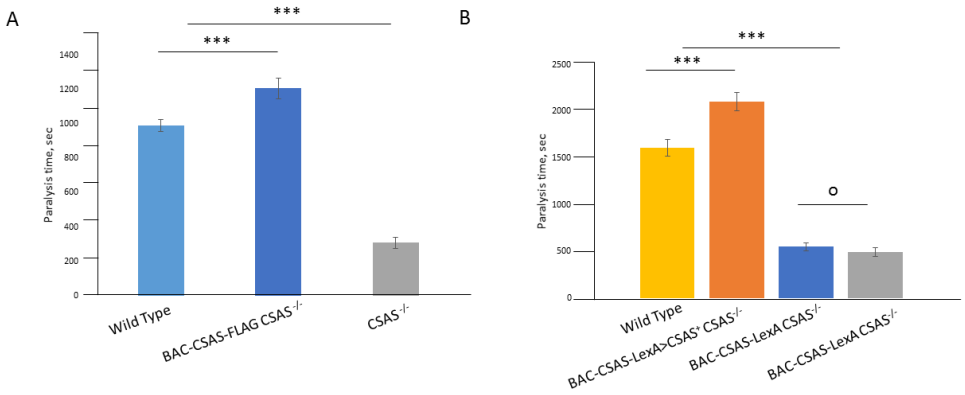

Figure 3.1 BAC-CSAS can rescue CSAS mutants. BAC-CSAS rescues the mutant phenotype indicating the insert transgene is functional. **A**. BAC-CSAS-FLAG has significantly higher paralysis time than CSAS null mutant. **B**. BAC-CSAS-LexA was tested for rescue by employing a a double binary strategy to allow LexA reporter which upon expression of LexOp-Flippase removed FRT sites flanking actin-Gal4 that drove expression of UAS-CSAS construct. This led to the rescue of CSAS mutant phenotype. Genotypes are as follows in order: **A** Wild type (Canton S, w^{1118}), BAC-CSAS-FLAG/+; $CSAS^{21}/CSAS^{21}$, (3) $CSAS^{21}/^{21}$. **B**. (1) Wild Type (Canton S, w^{1118}); (2) Ay-Gal4/LexOp-FLp; CSAS-LexA $CSAS^{Mi}$/ UAS-CSAS15 $CSAS^{Mi}$, (3) Ay-Gal4/+; CSAS-LexA, $CSAS^{Mi}$/UAS-CSAS15 $CSAS^{Mi}$ (4) LexOp-FLp/+; CSAS-LexA $CSAS^{Mi}$/ UAS-CSAS15 $CSAS^{Mi}$. Rescue is specific demonstrated by failure to rescue in controls that lack either CSAS-LexA or LexOp-Flippase. Flies were collected on day of occlusion and aged for 5 days, transferring to fresh food on day 3. Individual flies were assayed at 38C and paralysis. n=20 unless noted. ***, **, o. Highly significant $p<0.001$, significant $p<0.01$, not significant respectively.

transgene could rescue the TS paralysis phenotype of a *CSAS* mutant, thus demonstrating the activity of the construct (Fig3.1A). However, we were unable to detect the protein by immunostaining. This is most likely due to regulation of CSAS in which low expression levels are sufficient for function. To overcome this problem and analyze CSAS expression, we created a reporter construct using the same BAC that was used in BAC-CSAS-FLAG experiments. That reporter construct, called BAC-CSAS-LexA, includes LexA sequence under the control of CSAS genomic regulatory region, and thus it is expected to express LexA in the pattern of endogenous *CSAS* expression. Transgenic *CSAS* driven by BAC-CSAS-LexA rescues *CSAS* mutant phenotype, which verifies that BAC-CSAS-LexA is expressed in the spatiotemporal pattern that is

necessary for restoring CSAS function (Fig.3.1B). Furthermore, BAC-CSAS-LexA expression pattern visualized by a GFP reporter closely resembles *CSAS* mRNA expression previously revealed by in situ hybridization (Fig. 3.2A), which provides

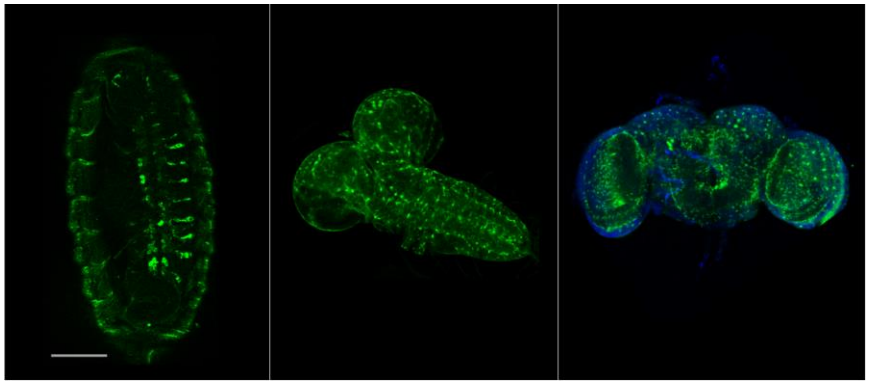

Figure 3.2 CSAS-LexA has nervous system-specific expression throughout development. A. CSAS-LexA is present in late-stage embryos (17-19h), B. The brain of 3rd instar larvae, C. Adult brains with expression throughout, including optic lobes, cortex, and olfactory bulbs. API (blue) was used to define brain architecture. All samples were dissected and fixed. Images were obtained with confocal microscope. A, ventral view, scale bar 20 um.B. dorsal view, scale bar 50um. C. dorsal view Images were taken with confocal microscope.

additional support to the conclusion that BAC-CSAS-LexA recapitulates the endogenous expression pattern of *CSAS* fairly accurately [29]. Thus, using CSAS-LexA-driven GFP reporter, we investigated in detail the expression pattern of CSAS at different developmental stages within the nervous system (Fig. 3.1). We carried out double staining experiments using cell markers for either glia or neurons. CSAS-LexA-induced GFP was found primarily colocalized with glial marker, Repo (Fig. 3.2A-B). Repo (*Reversed polarity)* is a transcription factor expressed in all glial cells (except some mid-line embryonic glia) [40]. Furthermore, our careful analysis of CSAS-LexA expression at embryonic, larval, late pupal and adult stages revealed that CSAS-LexA pattern does not overlap with that of Elav, a pan-neuronal marker which is expressed in all

differentiated neurons (Fig. 3.3C). Although we cannot completely exclude a possibility that rare examples of neuronal expression may exist, and a small number of neuroblasts could express CSAS at some developmental stages (which would be consistent with the fact that we found a few GFP-expressing cells that did not have Repo staining), our data

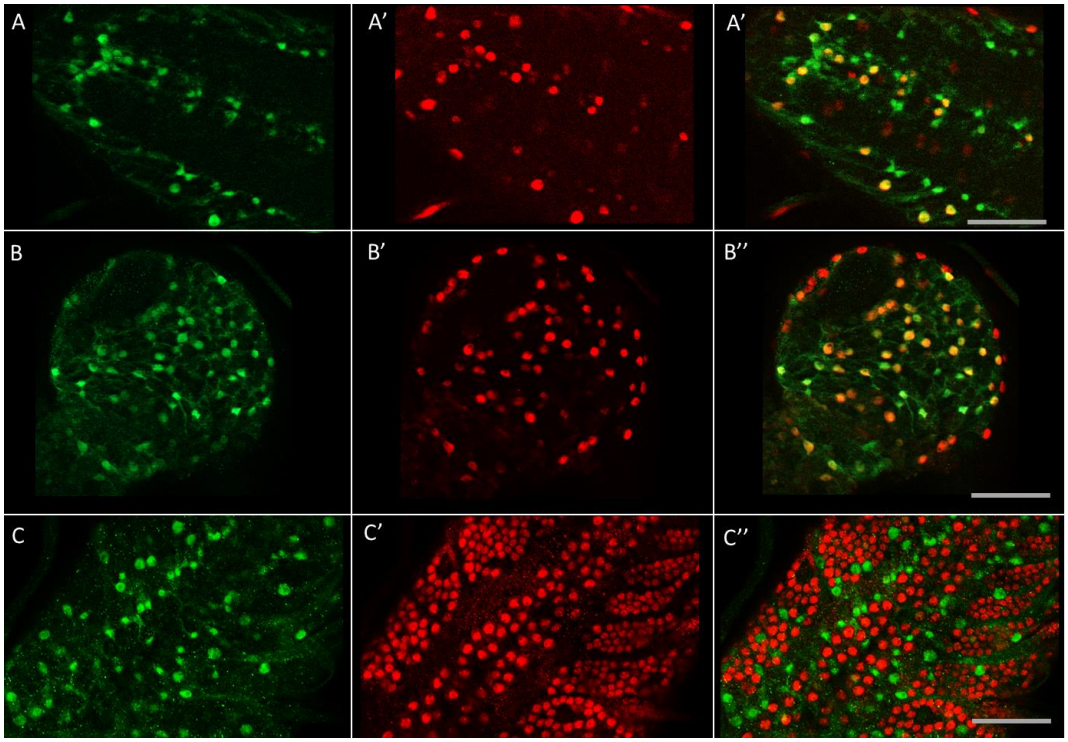

Figure 3.3 CSAS-LexA is expressed in glial cells but not in neurons. Double staining was carried out with CSAS-LexA expressing LexOp-GFP reporter with cell specific markers. A-A' show single channel images from ventral ganglion of 3rd instar larvae, A'' shows merge of both channels. CSAS-LexA (green) overlaps with glial marker, Repo (red), Ventral view, anterior to right. B-B' are single channel images from left hemisphere in 3rd instar larvae. B'' shows channels merged. CSAS-LexA (green) overlaps with Repo (red) at the surface of the hemisphere. Ventral view. C-C' show single channel images of ventral ganglion of 3rd instar larvae, C'' shows merged images. CSAS (green) shows no overlap with neuronal marker-Elav (red) indicating it is not present in neurons.Dorsal view, anterior to the left. Scale bar 50um.

clearly indicate that the vast majority of neurons do not express CSAS during development and in the adult brain.

3.4.2 CSAS expression is required in glial cells for function

The glial localization of CSAS prompted us to investigate the cellular requirement for CSAS function within the nervous system. *CSAS* mutants display locomotor defects, including a lack of coordination. These defects phenocopy abnormalities in *DSiaT* mutants which suggests that they are caused by the loss of sialylation, altering the function of PARA and resulting in a defect of excitability. [29]. To test the cellular requirements of CSAS, we expressed *UAS-CSAS* using either a pan-neuronal driver, (*C155-Gal4)*, or glial driver, (*Repo-Gal4)* in *CSAS* mutants and assayed flies for neurological phenotypes. Mutants with neuronal expression of transgenic *CSAS*

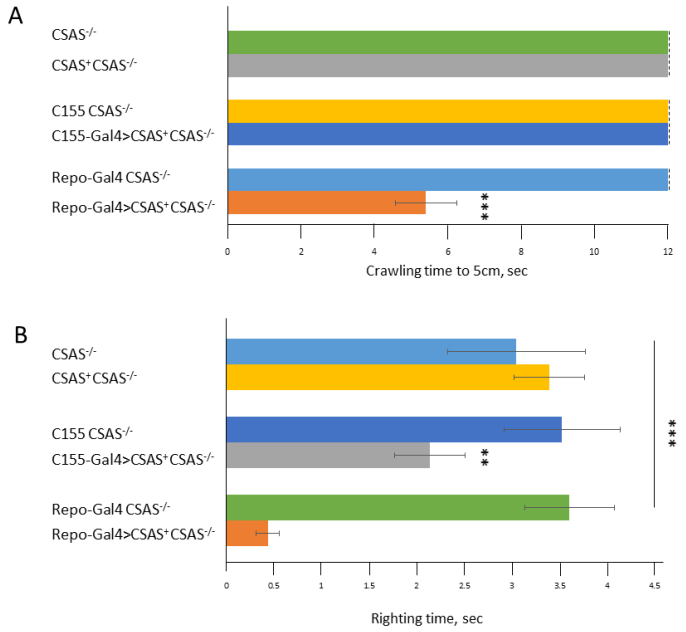

Figure 3.4. Glial expression of CSAS rescues locomotor defects. A. Geotaxis Assay, B. Righting Assay. A-B. CSAS was expressed in either glial cells, Repo-Gal4, or neurons, C155-Gal4, using UAS-CSAS111 in a mutant background. (see materials and methods for experimental details). Genotypes were the same for both experiments are listed in order as follows: $CSAS^{21}/CSAS^{21}$, UAS-CSAS111/+; $CSAS^{21}/CSAS^{21}$, C155-Gal4/+;$CSAS^{21}/CSAS^{21}$,C155-Gal4/CSAS111;$CSAS^{21}/CSAS^{21}$, Repo-Gal4 $CSAS^{21}/CSAS^{21}$, CSAS111/+; Repo-Gal4 $CSAS^{21}/CSAS^{21}$.***,**. Highly significant ($p>0.0001$), significant ($p<0.001$).

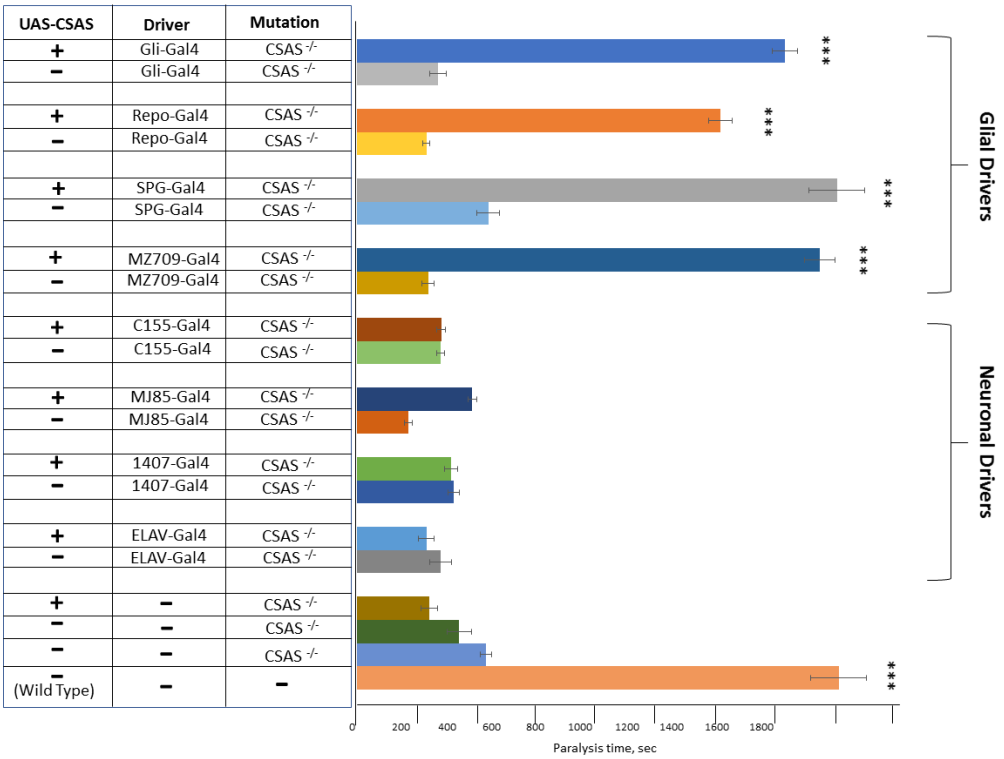

Figure 3.5. Glial expression of CSAS is required for neural transmission. Cellular requirements for CSAS function was determined through rescue studies using a panel of cell-specific Gal4 drivers with a UAS-CSAS construct in a CSAS null mutant background. Genotypes for rescue experiments are listed in order. Glial cell rescue were UAS-CSAS111/+;Gli-Gal4/+;$CSAS^{21}$/$CSAS^{/21}$, UAS-CSAS111/+; Repo-Gal4 $CSAS^{21}$/$CSAS^{21}$, UAS-CSAS111/+;SPG-Gal4/+;$CSAS^{Mi}$/$CSAS^{21}$, UAS-CSAS111/+;MZ709-Gal4$CSAS^{Mi}$/$CSAS^{21}$. Neuronal rescue genotypes were UAS-CSAS111/+;Elav-Gal4/+;$CSAS^{21}$/$CSAS^{21}$, UAS-CSAS111/+;1407-Gal4/+;$CSAS^{21}$/$CSAS^{21}$, UAS-CSAS111/MJ85-Gal4;$CSAS^{21}$/$CSAS^{21}$, UAS-CSAS111/C155-Gal4;$CSAS^{21}CSAS^{21}$. Controls included in rescue experiments were $CSAS^{Mi}$/$CSAS^{/21}$, UAS-CSAS111/+;$CSAS^{Mi}$/$CSAS^{21}$, $CSAS^{21}$/$CSAS^{21}$, Wild Type (Canton-S, W^{1118}). n≥20 for every genotypes. Statistical analysis performed using Anova, *** indicates highly significant differences ($p<0.001$). Experiments were performed in collaboration with Courtney Caster.

showed all characteristic phenotypes of sialylation mutants, including a defect in locomotion. They were unable to right themselves after gently disturbing the vial, and they show a defect in geotaxis (see methods and materials). *CSAS* mutants with glial expression of transgenic *CSAS* were fully rescued, as they show no significant difference from wild type flies in all assays (Fig.3.4). Sialylation mutants display TS paralysis

phenotypes, which indicates defects in neural transmission. evidenced by [24, 29]. Therefore, cell-specific requirement of CSAS was also investigated using rescue of this phenotype in *CSAS* mutants. We used a panel of neuronal and glial Gal4 drivers to induce transgenic *CSAS* in *CSAS* mutants to test for amelioration of TS paralysis phenotype. Drosophila possess diverse types of glia with unique morphology and functions that are similar to mammalian glial cells [39]. We sought to investigate

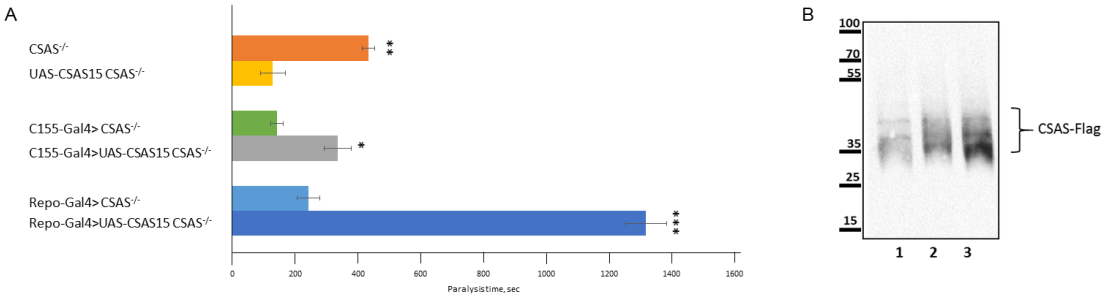

Figure 3.6. CSAS cell-specific rescue. **A**. CSAS rescues independent of transgenic insertion. *UAS-CSAS15* was expressed by pan-neuronal C155-Gal driver, and glial driver, Repo-Gal4, to insure cell specific rescue is not due to positional effects of inserts. Genotypes are listed in order: $CSAS^{21}/CSAS^{21}$, UAS-CSAS15/$CSAS^{21}$, C155-Gal4/+; $CSAS^{21}/CSAS^{21}$, C155-Gal4/+; CSAS15/$CSAS^{21}$, Repo-Gal4 $CSAS^{21}/CSAS^{21}$, Repo-Gal4 $CSAS^{21}$/UAS-CSAS15 $CSAS^{21}$. n=20 unless noted. Significance indicated as * $p<0.05$, **$p<0.001$, and *** $p<0.0001$. **B.** Western blot analysis of Flag-tagged UAS-CSAS lysate from Drosophila heads. Lane 1, Lysate from C155-Gal4/ UAS-CSAS. Lane 2, UAS-CSAS111/+;Actin-Gal4/+. Lane 3, UAS-CSAS111/+; Repo-Gal4/+. Positions of molecular mass markers shown on the left. Approximately equal amount of lysate was loaded on each lane.

whether CSAS is specifically required in certain types of glial cells. To this end, we used rescue approach with Gal4 drivers specific for main glial sub-types. *Repo-Gal4* was used for a global rescue of function in glia. *Moody-Gal4* (*SPG-Gal4*) is expressed in surface-associated glia that maintains the blood-brain barrier in Drosophila [43]. *Gli-Gal4* (*gliotactin*) is expressed in sub-perineurial glial cells [42]. *MZ0709-Gal4* is expressed in ensheathing glial cells which surround neuropil, providing trophic support

to neurons, mediating response to injury and modulating synaptic plasticity [41, 48]. *Alrm-Gal4* is expressed in astrocyte-like glial cells [44] that are present at synapses containing glutamate receptors and facilitate their uptake and recycling, which contributes to neural excitability through glia-neuron coupling [49]. Although specific glial drivers rescued to different degrees, all show a significant increase in onset of paralysis time as compared to *CSAS* mutants, including alleles with null ($CSAS^{21}$), strong hypomorphic ($CSAS^{Mi}$) mutations, and their heteroallelic combination ($CSAS^{Mi/21}$) (Fig. 3.5). Importantly, transgenic expression of CSAS could not restore CSAS function in mutants when expressed in neurons. These results recapitulate data obtained in like locomotor assays (Fig.3.4). To rule out the possibility of position effect of the insertion, we tested an independent transgenic insertion of UAS-CSAS using a pan-neuronal driver, *C155-Gal4*, and glial driver, *Repo-Gal4*, and found similar results (Fig.3.6A). Moreover, using western blot analysis, we confirmed that both of these drivers induce *UAS-CSAS* expression at comparable levels, which indicates that *C155-Gal4* failed to rescue the phenotype is not due to lack of transgenic expression of CSAS induced by this driver (Fig.3.6B). These results demonstrate that glial expression, but not expression in neurons, is vital for normal neural excitability, while transgenic *CSAS* can rescue the paralysis phenotype regardless of the glial sub-type in which it's expressed.

3.4.3 CSAS can potentiate neural excitability

Sialylation pathway genes *CSAS* and *DSiaT* have shown complex interactions, with *CSAS* having a more severe mutant phenotype both at the cellular and organismal level, which demonstrates that the level of *CSAS* expression is central to the regulation

of excitability by sialylation. If loss of *CSAS* results in lowered excitability, then perhaps overexpression of *CSAS* would result in increased excitability. Neural function

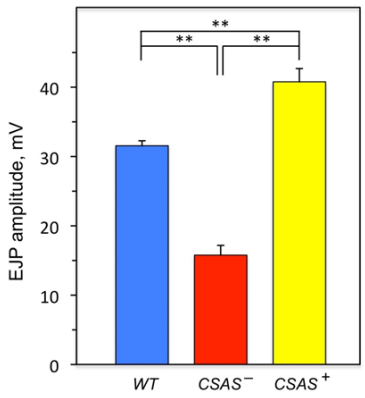

Figure 3.7 CSAS overexpression results in increased excitability over wild type. CSAS mutants display lowered excitability, while CSAS expressed with ubiquitous diver in wild type background resulted in higher excitability. Genotypes were Wild type (Canton S, w^{1118}), $CSAS^{Mi}/CSAS^{Mi}$, UAS-CSAS/+;Actin-Gal4/+. EJP analysis was performed 3rd instar larvae at muscle 6 NMJ. Statistical significance measured using student's t-test, $p<0.01$. Courtesy of Rafique Islam.

has been shown to be compromised in *CSAS* mutants at the level of individual neuromuscular junctions (NMJs) in third instar larvae [29]. To determine if elevated levels of CSAS could affect neural transmission at NMJs, we expressed *CSAS* in a wild type background using a ubiquitous driver, Actin-Gal4, and recorded evoked EJP response in the abdominal muscle MN6/7 innervated by motor neuron MN6/7-Ib. $CSAS^{+}$ increases the EJP response above wild type levels (Fig.3.7). This effect on EJP is specific to *CSAS* and is not seen when *DSiaT* is overexpressed (Personal communication with Vlad Panin), suggesting *CSAS* is the rate-limiting step that is responsible for setting the levels of excitability by sialylation at the cellular level. This result suggests that the level of CSAS activity is tightly regulated to maintain normal neural transmission, yet it

can dynamically respond to changes in the environment and the cellular requirements for modulation of excitability when needed.

3.4.4 DSiaT functions in a cell non-autonomous manner

We have previously shown that DSiaT is expressed in a subset of neurons [24], and biochemical characterization shows that access to CMP-sialic acid is necessary for DSiaT-mediated modifications [26]. Therefore, given the cellular requirements for

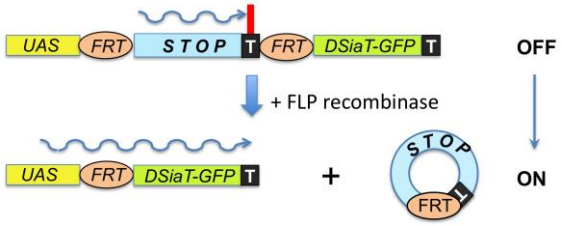

Figure 3.8 FLP-mediated control of DSiaT transgenic expression. *UAS>STOP>DSiaT-GFP* construct includes a transcription termination sequence (STOP) inserted between *UAS* activation sequences and DSiaT-GFP coding region, which prevents DSiaT-GFP expression. The STOP region is surrounded by FRT sites for FLP-mediated recombination, and upon induction of FLP, STOP is removed and *DSiaT-GFP* expression is activated. FLP recombinase can be induced by specific GAL4 drivers using *UAS-FLP* transgene.

CSAS, we wanted to determine if there is a cell-specific requirement for DSiaT function. *DSiaT* mutants display neurological defects and have a TS paralysis phenotype similar to *CSAS* mutants[24]. Revealing cell-specific requirement for DSiaT using rescue approach has been confounded by the fact that all *UAS-DSiaT* transgenes that we generated showed leaking expression sufficient to rescue mutants even without any GAL4 driver. We employed a new strategy to investigate the DSiaT cell-specific functions by "turning on" transgenic *DSiaT* in cell-specific manner in *DSiaT* mutants. We created a construct including a transcription termination sequence flanked by FRT sites and inserted upstream of a *GFP*-tagged *DSiaT* (Fig. 3.8). This would allow for the expression of *DSiaT-GFP* only when the sequence flanked by FRT sites was removed by

the Flp recombinase expressed by *UAS-FLP* under the control of a cell specific Gal4 driver. Both glial and neuronal drivers restored the function of DSiaT indicating that

Flippase	DSiaT	Driver	Mutation
+	ON	C155-Gal4	DSiaT $^{S23/L22}$
-	OFF	C155-Gal4	DSiaT $^{S23/L22}$
+	ON	Repo-Gal4	DSiaT $^{S23/L22}$
-	OFF	Repo-Gal4	DSiaT $^{S23/L22}$
+	ON	tinC-Gal4	DSiaT $^{S23/L22}$
-	OFF	tinC-Gal4	DSiaT $^{S23/L22}$
+	OFF	-	DSiaT $^{S23/L22}$
+	-	-	DSiaT $^{S23/L22}$
-	OFF	-	DSiaT $^{S23/L22}$

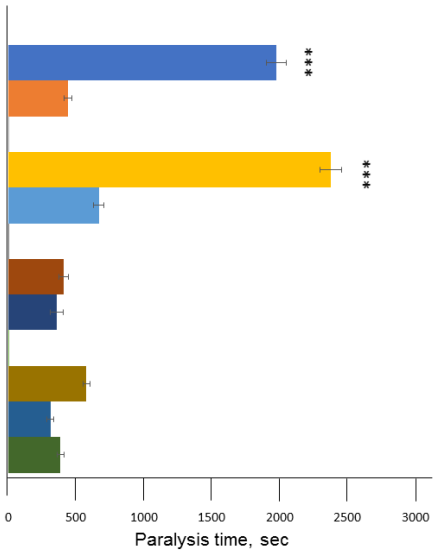

Figure 3.9 Neural expression of DSiaT is sufficient to rescue neural transmission defect. DSiaT expression was "turned on" in specific cells through UAS-FLP-mediate removal of stop codon flanked by FRT sites using Gal4 driver activation of UAS>STOP>DSiaT-GFP construct. DSiaT function is rescued by inducing FLp recombinase using either neuronal driver C155 or glial driver Repo, but not heart specific driver tinC-Gal4, demonstrating that neural expression is necessary for rescue. Genotypes are (in order from the top): *UAS-FLP/C155-Gal4;UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23},C155-Gal/+;UAS>STOP>DSiaT-GFPDSiaT^{L22}/DSiaT^{S23},UAS-FLP/+;UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23};Repo-Gal4/+,UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23};Repo-Gal4/+,UAS-FLP/+;UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23};tinC-Gal4/+, UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23};tinC-Gal4/+ and controls UAS-FLP/+;UAS>STOP>DSiaT-GFP DSiaT^{L22}/DSiaT^{S23},UAS-FLP/+;DSiaT^{L22}/DSiaT^{S23},UAS>STOP>DSiaT-GFP DSiaT ^{L22}/DSiaT^{S23}.* ***, highly significant differences ($p<0.0001$).

neural expression alone is sufficient to rescue the paralysis phenotype (Fig.3.9). The restoration of function was not due to leaking expression, as immunostaining showed the both *UAS-FLP* and the *C155-Gal4* driver were required for the rescue, as well as for DSiaT-GFP expression (Fig.3.10). Rescue by a pan-glial driver, Repo-Gal4 suggests that DSiaT may act cell non-autonomously, i.e. it could potentially be secreted into the extracellular matrix where it could modify glycoproteins extracellularly to maintain

neural excitability. This scenario is consistent with in vitro studies that showed that DSiaT is secreted as an active enzyme when expressed in cell culture [26]. Mammalian sialyltransferase ST6GalI has been shown to operate in a cell non-autonomous manner

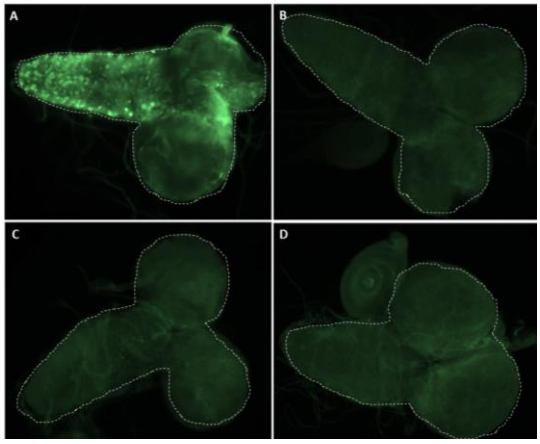

Figure 3.10 DSia-GFP requires both UAS-FLP and Gal4 driver for expression. A. Both a driver and FLP are needed to express the construct, as shown by positive DSiaT-GFP staining (green), *UAS-FLP/C155-Gal4;UAS>STOP>DSiaT-GFP DSiaT* ^{L22}*/DSiaT*^{S23}. **B,** No expression due to lack of flippase, *C155-Gal4/+;UAS>STOP>DSiaT-GFP DSiaT*^{L22}*/DSiaT*^{S23}. **C**, No expression due to lack of driver, *UAS-FLP/+;UAS>STOP>DSiaT-GFP DSiaT*^{L22}*/DSiaT*^{S23}. **D**, Control lacking all elements. Wild Type (Canton S, w^{1118}). Flies were dissected and flixed. Images were of 3rd instar larvae taken with Zeiss Axioplan 2 microscope with Apotome.

to sialylate glycoproteins on leukocytes [50]. This mechanism of extracellular sialylation may be potentially conserved in Drosophila. To test if DSiaT is secreted extracellularly *in vivo,* we used a ppk-Gal4 driver to ectopically express *DSiaT-HA* together with a membrane version of GFP (to visualize axonal termini). We performed double staining for DSiaT and GFP, focusing on ventral ganglion projections of ppk-positive neurons in 1st instar larvae. DSiaT appears both within and outside the boundaries of the axonal indicating that it can be secreted in vivo (Fig.3.11). Together, these results suggest that DSiaT can function in a cell non-autonomous manner to modify glycoproteins and regulate network excitability.

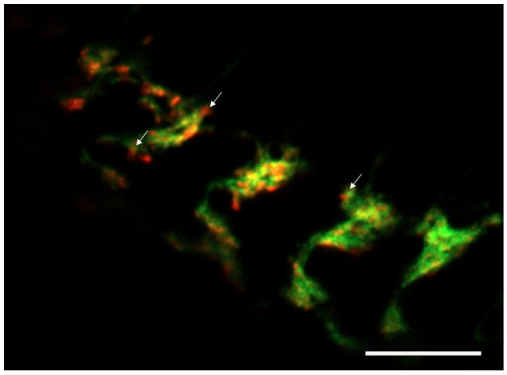

Figure 3.11 DSiaT is secreted in vivo. Ha-tagged UAS-DSiaT (red) and a membrane UAS-GFP (green) were expressed using a sensory neuron driver, ppk-Gal4. Double staining shows DSiaT is present within the axon termini as well as outside the membrane in some areas. Dorsal view of 1st instar larvae abdominal ventral ganglion. Arrows point to areas where DSiaT is seen outside of axon boundaries. Images are of single slices by optical sectioning with Zeiss axioplane 2 microscope with Apotome. Scale bar is 20 um. Provided by Ishita Chandel.

However, the effect of ectopic expression of DSiaT does not directly shed light on cell specific requirements for endogenous DSiaT. To elucidate these requirements,

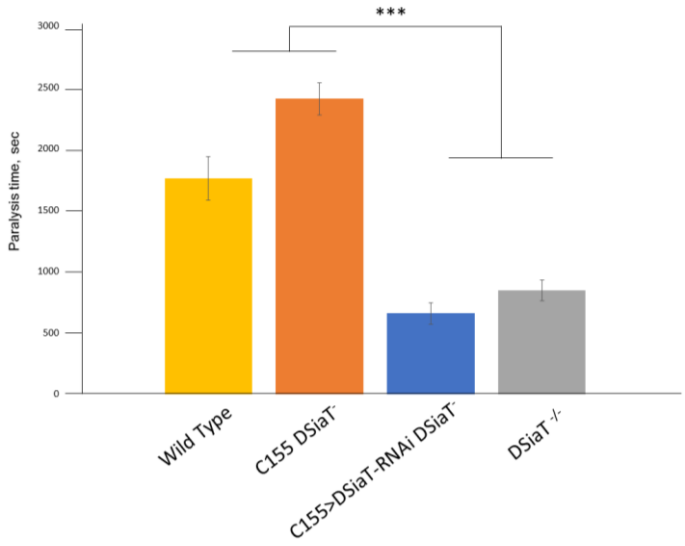

Figure 3.12 DSiaT function is required in neurons. Endogenous expression of *DSiaT* was reduced through *UAS-DSiaT-RNAi* in a cell specific manner using neuronal driver, *C155-Gal4*. RNAi significantly reduces paralysis time as compared to wild type flies and those without RNAi with no significant difference between RNAi and DSiaT null mutants. Flies were kept at 30˚C to increase efficiency of driver in addition to using lines heterozygous for DSiaT mutation. Genotypes are as follows: Wild type (Canton S, w^{1118}),*C155-GAL4/+; DSiaT*^{L22}*/DSiaT*^{S23}, *C155-GAL4/+;UAS-DSiaT-RNAi DSiaT*^{L22}*/DSiaT*^{S23}, *DSiaT*^{S23}*/DSiaT*^{S23}. ***, Highly significant ($p<0.0001$).

we downregulated endogenous DSiaT expression using RNAi approach. We induced expression of *UAS-RNAi* using a pan-neuronal Gal4 driver in otherwise wildtype flies. We found that loss of DSiaT in neurons results in TS paralysis phenotype similar to that of sialylation mutants (Fig. 3.12). Our results show that DSiaT is requisite in neurons to maintaining normal neural transmission.

3.4.5 Sialylation genes CSAS and DSiaT are expressed in distinct cells within the nervous system

Given that *DSiaT* and *CSAS* act in concert with one another to regulate excitability, we sought to determine the spatial relationship of their expression patterns

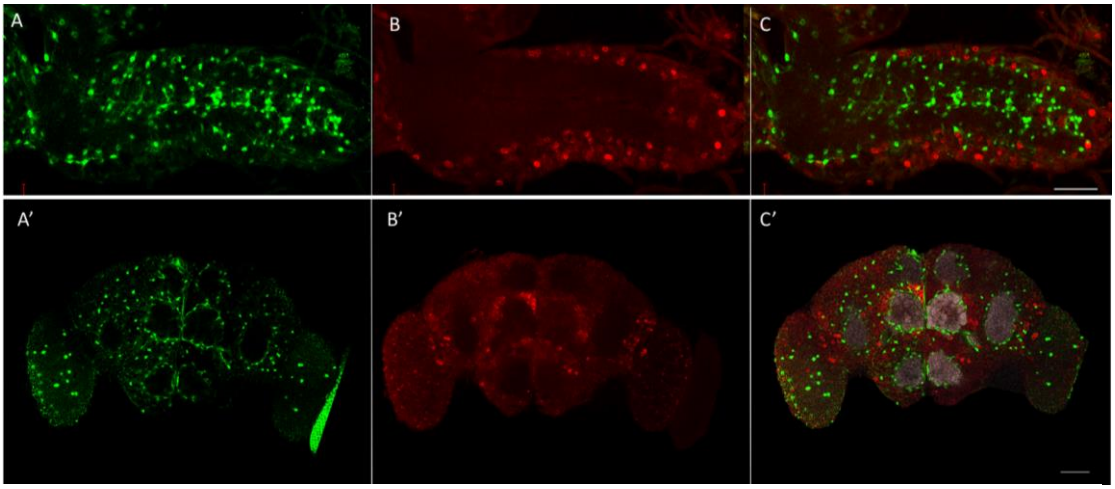

Figure 3.13. Sialylation genes CSAS and DSiaT display non-overlapping expression patterns. Endogenous expression patterns were visualized by double staining (A) LexA driven *CSAS* with a GFP reporter and (B) HA-tagged BAC-DSiaT. C. Merged channels. Results show no overlap between DSiaT (red) and CSAS-LexA (green), however some areas do show them in close proximity to one another suggesting their functional coordination in the brain. Flies were dissected and fixed. Dorsal view of 3rd in star larvae ventral ganglion. Imagees of single optical sections obtained with confocal microscope. Scale is 50um.

relative to each other, within the nervous system. Expression patterns of *DSiaT* and *CSAS* were analyzed by immunostaining for HA-tagged DSiaT expressed by BAC-

DSiaT-HA construct and by CSAS-LexA-driven GFPnls. Results show that *CSAS* and *DSiaT* have non-overlapping patterns (Figure 3.13), demonstrating that these genes are indeed expressed in distinct c ells within the nervous system in *Drosophila*. Thus, the biosynthesis of sialylated glycans is parsed between glia and neurons cells representing a striking new mechanism for regulation of the sialylation pathway.

3.4.6. Evolutionary conservation of CSAS function

Drosophila CSAS displays unique properties compared to its mammalian counterparts (Chapter 2, [37]). These unusual enzymatic properties can be attributed to its unique intercellular localization within the secretory compartment of the cell, primarily in the Golgi [29] , as compared to mammalian CMP-neuraminic acid synthetases that localize to the nucleus [34]. The function of the nuclear localization of mammalian CSAS is not known, but it has been suggested to play a role in the regulation of sialylation [32]. Experiments have been carried to investigate the importance of Golgi localization of CSAS for its function. To this end, the N-terminal part of *Drosophila* CSAS (which is responsible for CSAS secretory compartment localization) was swapped with that of human CSAS [23]. This swap construct was found to be inactive in cell culture, suggesting that Golgi subcellular localization is important for CSAS function [23]. However, the role of subcellular localization in CSAS function has not been elucidated *in vivo*. Thus, we decided to analyze the effect of localization on CSAS function in vivo, and to shed light on evolutionary conservation between functions of Drosophila and human CSAS proteins. To this end, we created a transgenic GFP-tagged *UAS-HsCSAS* for in vivo analysis. We found that *HsCSAS* rescues TS

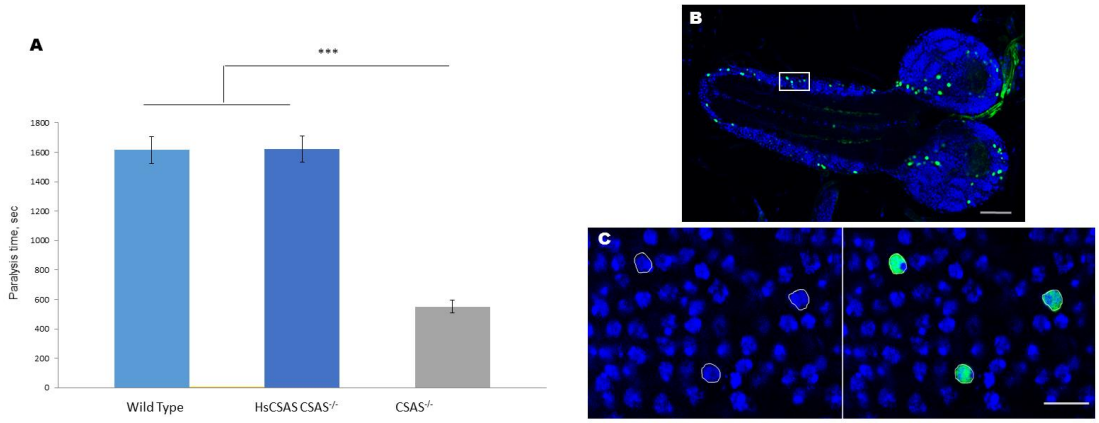

Figure 3.14 CSAS function is evolutionarily conserved despite localization differences. **A**. Human *CSAS* can fully rescue the paralysis phenotype of Drosophila *CSAS* mutant with no driver needed due to "leaking" expression level of *UAS-HsCSAS*. Genotypes were Wild type (Canton S, w^{1118}), *UAS-HsCSAS/+;*$CSAS^{21}/CSAS^{21}$, $CSAS^{21}/CSAS^{21}$. *** is highly significant. **B-C**. HsCSAS (green) is not present in the secretory compartment but, rather it localizes to the nucleus, overlapping with nuclear DAPI marker (blue). HsCSAS-GFP expression was induced using a weak neuronal driver (1407-Gal4) and visualized by anti-GFP staining of fixed larval brains. **B**, Dorsal view of 3rd instar larval brain (box framing inset in C). Scale 50um. **C**, Higher magnification of inset. Left panel features stained nuclei (blue). Right panel is a merged image of nuclei (blue) with HsCSAS staining (green). Scale 10um. Images represent single optical section taken by a confocal microscope.

paralysis phenotype of *CSAS* null mutants, demonstrating functional conservation between these proteins (Fig.3.14A). We also found that rescue can be mediated by a very low level of "leaking" expression that occurs without a GAL4 driver, suggesting that production of even a low amount of CMP-Sia is adequate for in vivo function of the sialylation pathway. A neuronal driver was required to detect HsCSAS at a level sufficient for immunostaining. We found that HsCSAS is not present in the secretory compartment and instead localizes mostly to the nucleus, yet is still able to rescue Drosophila CSAS mutants (Fig.3.14B). This result is consistent with recent data obtained with a version of Drosophila CSAS lacking a signal peptide sequence. This construct localizes in the cytoplasm but retains its in vivo activity, as assayed by rescue experiments (Boris Novikov, personal communication). Together, our results indicate

that the restriction of CMP-Sia biosynthesis to the Golgi is not important for Drosophila sialylation, suggesting that Golgi localization of endogenous Drosophila CSAS has some regulatory functions that remain to be elucidated.

3.5 Discussion

This work focuses on elucidating the cellular and molecular mechanisms of sialylation that impact neural excitability. Our results demonstrate that modulation of excitability occurs through the regulation of sialylation pathway genes *CSAS* and *DSiaT* that are expressed in glia and neurons, respectively. CSAS is present and functioning in glial cells, as shown by the expression of BAC-CSAS-LexA reporter and by functional studies. Double staining of BAC-CSAS-LexA with cell- specific markers show that the reporter expression colocalizes with Repo, a glial cell marker, while no overlap with Elav, a neuronal marker was detected, suggesting that CSAS is specifically expressed in glial cells (Fig.3.3). The glia-specific pattern of CSAS expression is consistent with our previous in situ results, as well as data on CSAS expression in adult astrocytes reported by another laboratory (Fig. 3.2, [29]). CSAS mutants display neurological defects including locomotor abnormalities and TS paralysis phenotypes. We found that glial expression of transgenic *CSAS* ameliorates these defects in *CSAS* mutants while neuronal expression is not sufficient to rescue these phenotypes (Fig.3.4-5). These surprising results expanded our focus into the role of sialylation pathway genes in glia. Drosophila possess glial cells that are morphologically and functionally similar to its mammalian counterparts [39]. Astrocytes in particular influence neuronal excitability through glia-

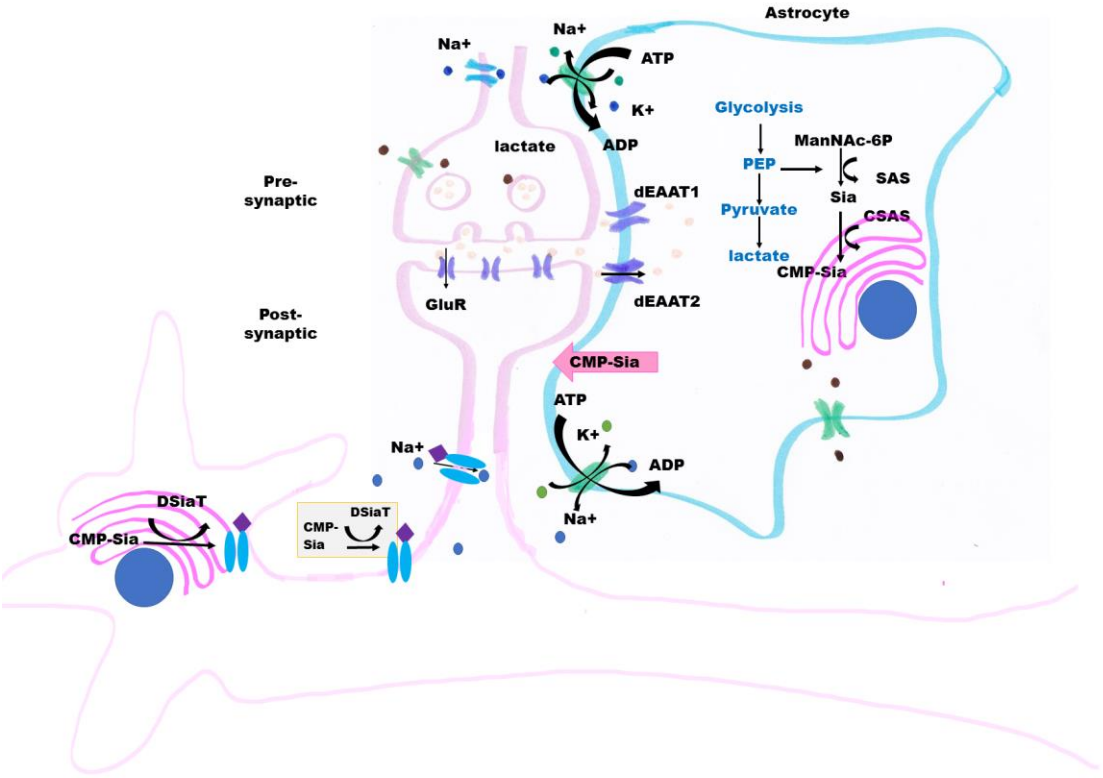

Figure 3.15. Model of bi-partite mechanism regulating sialylation. CSAS functions in glial cells creating the nucleotide sugar CMP-Sia required by DSiaT-mediated sialylation in neurons. Pre-synaptic terminals release glutamate which is taken up though excitatory amino acid transporters (dEAAT) along with Na+ ions. Glutamate is recycled by glutamate synthase to glutamine and returned to neurons, while Na+ ions are pumped out of the glial cell resulting in the hydrolysis of ATP. This reduction in ATP levels triggers glycolysis synthesizing pyruvate and leading to the production of lactate for the glia-neuron lactate shuttle [3]. In parallel, SAS utilizes PEP produced by glycolysis in a reaction with ManNAc-6P to create Sialic acid needed for CSAS-mediated synthesis of CMP-Sia. CMP-Sia makes its way from glia to neurons where DSiaT will use it in the modification of glycoproteins, including VGICs, inside and outside of the cell.

neuron coupling in both mammals and Drosophila [49, 51]. These cells are located in close proximity to synapses where they are involved in trophic interactions between glia and neurons, contributing to excitability [44]. Glutamate is taken up by glial cells via dEAAT 1-2 receptors (excitatory amino acid transporters) on the membrane along with Na+ ions. These ions are then transported out of the cell by Na+/K+ ion pumps, generating ADP, through the hydrolysis of ATP leading to an increase in glycolysis

[49]. While glia depends on glycolysis as the main source of energy production, the lactate created there is shuttled to neurons for use in mitochondria to create ATP through oxidative phosphorylation [52]. Studies have shown that oxidative phosphorylation is preferred while glycolysis is downregulated in neurons to alleviate oxidative stress to enhance neuronal homeostasis [53]. Meanwhile, the biosynthesis of sialic acid occurring in parallel requires PEP (phosphoenolpyruvate) in a reaction with ManNAc6-P mediated by SAS, which is an essential step in the sialylation pathway that produces sialic acid, the substrate for CSAS-mediated synthesis of CMP-Sia donor [36]. Thus, sialic acid biosynthesis would be favored in glia due to the availability of the substrate PEP in sufficient amounts via glycolysis, but is expected to interfere with oxidative phosphorylation in neurons (Fig. 3.13). As glial cells have diverse functions we sought to delineate the cell-specific requirement of CSAS in glial cells by expressing *CSAS* in several different glial sub-types. We found that CSAS could fully rescue mutant phenotypes regardless of sub-type, indicating that ectopic expression in any of the main subsets of glial cells that we investigated is sufficient for restoring sialylation function in the nervous system (Fig. 3.5). Neural excitability is very sensitive to level of *CSAS* with lowered levels resulting in reduced excitability, while overexpression causes an increase in excitability above the wild type level (Fig.3.9). Possible scenarios may include both DSiaT dependent and independent mechanisms that may occur in parallel to potentiate excitability. CSAS has been postulated to be the point of constriction in the sialylation pathway regulating DSiaT through substrate availability, however direct evidence for this claim remains elusive [54]. Overexpression of CSAS would lead to additional

CMP-Sia being delivered to DSiaT, effectively abolishing the bottleneck in the pathway. Presumably, this increase sialylation resulting in higher levels of excitability. Conversely, CMP-Sia may have an effect on excitability independent of glycan sialylation. Glia and neurons maintain very tight associations with each other as close as 0.5um or even direct contact between cells. The putative upregulation of negatively charged CMP-Sia may result in accumulation in the area between glia and neurons. This accumulation could produce a local electrostatic effect that may alter the gating properties of VGICs and modulate excitability in a DSiaT independent manner. Finally, it is conceivable that CMP-Sia could be used in neurons in the catabolic branch of the sialylation pathway, resulting in production of PEP in a N-Acetylneuraminate Lyase-type reaction providing pyruvate for the TCA cycle and stimulating energy production [55].

To shed light on the relationship between CSAS and DSiaT in regulation of neural excitability we examined the cellular requirements for DSiaT function. We found that downregulation of DSiaT in neurons is sufficient to cause TS-paralysis, indicating that DSiaT function is required in neurons (Fig.3.12). However, *ectopic* expression of DSiaT in either glia or neurons is sufficient for rescue, suggesting that DSiaT can function cell non-autonomously. (Fig.3.10). Studies in mammalian systems reveal that circulating HST6Gal-I extrinsically modifies erythrocytes using platelet-derived CMP-Sia [50, 56]. Similarly, DSiaT could potentially function outside of the cell, which is consistent with previous results demonstrating that secreted DSiaT in cell culture maintains enzymatic activity [26]. Immunostaining showed that ectopic DSiaT is

secreted in vivo in the termini of sensory axons in the larval brain (Fig. 3.11). DSiaT is expressed in MN1 at muscle 1 NMJ and electrophysiological studies have shown loss of sialylation leads to reduced EJP [24] indicating that experiments analyzing DSiaT expression at muscle 1 NMJ (e.g. using BAC-Sia3HA) may provide evidence of endogenous DSiaT secretion. These experiments are currently underway. Taken together, our results indicate that DSiaT can function in the nervous system in a cell non-autonomous manner to modulate neural excitability, potentially by modifying membrane glycoproteins in the extracellular milieu. Our results suggest that CSAS and DSiaT cooperate in the biosynthesis of sialylated glycans while working within distinct cells in the nervous system thus revealing a bi-partite mechanism regulating sialylation. (Fig. 3.15). This cellular regulation of sialylation provides a functional coupling between glia and neurons that contributes to neural physiology in novel way that may be conserved in mammals.

4. DROSOPHILA SIALYLTRANSFERASE DEMONSTRATES TRANS-SIALYLATION ACTIVITY IN VIVO

4.1 Overview

Evidence for de novo synthesis of sialylated glycoproteins *in vivo* has been provided through functional characterization of several sialylation pathway genes [24, 29]. Our lab has shown that sialylation is required for normal neural function in *Drosophila,* revealing a novel mechanism modulating excitability by regulation of sialylation (Chapter 2-3). The present work focuses on another aspect of pathway regulation associated with the ability of *DSiaT* to rescue *CSAS* mutants. We investigated the genetic interactions between sialylation pathway genes *SAS, CSAS and DSiaT* to shed light on the functional requirements of specific sialylation substrates provided by these enzymes in the regulation of sialylation-mediated neural excitability. Collectively, our in vivo experiments suggested that DSiaT can potentially bypass the requirement for CSAS-produced CMP-Sia and sialylate conjugates using an alternative source of sialic acid-donating molecules. One possible mechanism would involve a trans-sialylation-type activity using putative sialylated structures produced by a bacterial symbiont. This is the first study to reveal trans-sialylation activity of ST6Gal-type enzyme in vivo in a metazoan organism.

4.2 Introduction

Sialic acids are a family of nine carbon sugars that are ubiquitous on the cell surface of a wide range of organisms from bacteria to humans [6]. The addition of these

sugars residues onto non-reducing ends of glycans attached to proteins or glycolipids is mediated by sialyltransferases in the trans-Golgi [6]. In addition to this canonical pathway of sialylation, there are some examples of "non-canonical" sialylation that demonstrate that some sialyltransferase enzymes can show unusual activity relying on substrates other than CMP-Sia. These examples include the trans-sialidase activity and reversible sialylation [57, 58].

Trans-sialidases are found in Trypanosomes, protozoan parasites causing sleeping sickness and Chagas disease in humans. Trans-sialidase enzymes can utilize sialylated termini of host glycans as substrates to acquire sialic acid and sialylate glycans on Trypanosome cells, which facilitates invasion through receptor-mediated host cell targeting and protects parasites from immune responses [8, 19]. *T. cruzi* trans-sialidases do not require CMP-Sia as a substrate, but instead cleave sialic acid from the underlying lactose termini of host glycans and attach it directly to pathogen's cell surface [57].

Another example of CSAS-independent sialylation has been recently demonstrated in vitro in experiments that reveal the ability of a sialyltransferase to "reversibly" sialylate, i.e. transfer sialic acid from a donor structure onto 5' CMP, resulting in production of CMP-Sia that could be further used in a regular sialyltransferase reaction to create new sialylated glycans. This "reversible" activity was demonstrated for ST3GalII that normally mediates the attachment of sialic acid in an α2,3 linkage onto galactose residues at the termini of O-linked mucins. In some conditions, the "reversible" sialylation can be favored over the forward reaction [58].

Drosophila sialylation underlies novel regulatory mechanisms involved in modulation of neural excitability. We have shown that loss of sialylation severely impacts neural physiology, causing locomotor defects, TS paralysis, and reduced longevity [24]. *Drosophila* sialyltransferase, DSiaT, like most other sialyltransferase enzymes, utilizes a nucleotide sugar donor, CMP-Sia, as a substrate for the modification of sialylated glycoconjugates. CMP-Sia is normally provided by CMP-Sialic acid synthetase for de novo synthesis of sialylated glycoproteins in vivo. Surprisingly, our data indicated that ectopically expressed DSiaT can bypass the requirement for CSAS, suggesting that DSiaT could use sialic acid-donating substrates other than CSAS-producing CMP-Sia. We found that overexpression of transgenic *DSiaT* in a *CSAS* mutant can rescue TS paralysis phenotype, restoring normal neural transmission. This provides preliminary evidence of a non-canonical pathway for the biosynthesis of sialylated glycans in Drosophila. Several mechanisms can potentially explain these results, including reversible sialylation or trans-sialidase activity of DSiaT. Revealing these mechanisms will require further investigation.

4.3 Methods and Materials

4.3.1 Drosophila Strains

Wild-type control w^{1118} *Canton-S* was from Josh Dubnau (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), CSAS lines *UAS-CSAS111*, *UAS-CSAS-WT*^{15}, *MiCSAS* (strong hypomorph) and *CSAS*^{21} (loss of function allele) [29], DSiaT lines *UAS-*

Sia3HA, DSiaT L22, S23 (loss of function alleles) [24], *Actin-Gal4* (Bloomington Stock Center), *SAS-RFP* (loss of function allele, Kyoto Stock Center, DGGR).

4.3.2 Behavioral Assays

Temperature sensitive (TS) paralysis assays were performed as described previously. Briefly, adult flies were collected on day one and aged for five days, transferring to fresh food on day. Individual flies were placed in vials, then submerged in a controlled temperature water bath set at 38˚C. Paralysis time was recorded. Paralysis is defined as the time at which the fly falls on its back no longer able to right itself for a period on one minute. All experiments were conducted with a minimum of twenty individuals averaged to represent the paralysis time of that genotype. Rescue experiments using exogenous Sialic acid were performed by adding 0.2mM sialic acid (supplier) to standard cornmeal-yeast Drosophila fly food (recipe at Bloomington Stock Center). *SAS-RFP* third-instar larvae were transferred to sialic acid supplemented food and allowed to develop normally and adults were collected for assays as described above.

4.4 Results

4.4.1 DSiaT can bypass requirement for CSAS

We have previously shown that *CSAS* is required for neural excitability, and loss of CSAS activity leads to locomotor abnormalities, lowered excitability and reduced longevity, which essentially phenocopies defects caused by *DSiaT* mutations [24, 29]. These genes show complex interactions, which is unexpected, considering that they are

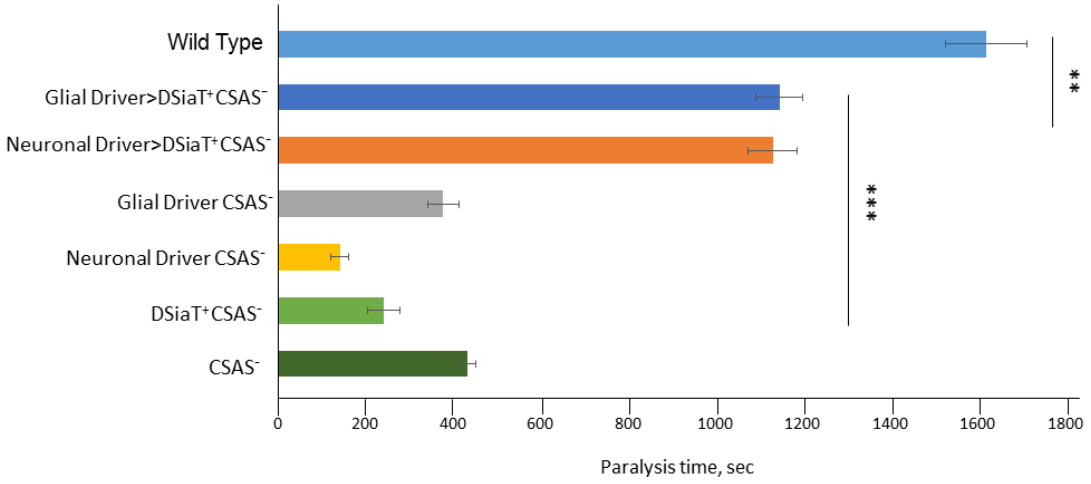

Figure 4.1 Ectopic expression of DSiaT can bypass the requirement for CSAS in maintaining normal neural transmission. *UAS-DSiaT* was overexpressed using glial and neuronal drivers in a *CSAS* mutant background. DSiaT rescues paralysis phenotype of CSAS mutants. Adult flies were assayed at 38˚C controlled temperature water bath. Genotypes are listed in order: Wild Type (Canton S, w^{1118}), *UAS-DSiaT/+;Repo-Gal4 CSAS^{21}/CSAS^{21}, C155-Gal4/+;UAS-DSiaT/+;CSAS^{21}/CSAS^{21}, Repo-Gal4 CSAS^{21}/CSAS^{21}, C155-Gal4/+;CSAS^{21}/CSAS^{21}, DSiaT/+;CSAS^{21}/CSAS^{21}, CSAS^{21}/CSAS^{21}*. *** and ** indicate highly significant ($p<0.001$) and significant ($p<0.01$) differences, respectively.

expected to work in a linear biochemical pathway of sialylation. To further clarify the functional relationship between CSAS and DSiaT in vivo, we overexpressed *UAS-DSiaT3HA* in a *CSAS*^{21} mutant background using both neuronal and glial Gal4 drivers. Sialylation is required for normal neural transmission; therefore, TS paralysis assay serves as an indicator of sufficient levels of functional sialylation. We found that overexpression of *DSiaT* could rescue the paralysis phenotype in *CSAS*^{21} mutants (Fig 4.1). Thus, *DSiaT* can maintain neural transmission without CMP-Sia provided by *CSAS*.

4.4.2 DmCSAS requires DSiaT activity for function

These overexpression data potentially uncovered novel function of DSiaT, therefore we sought to examine possible mechanisms that could underlie this function

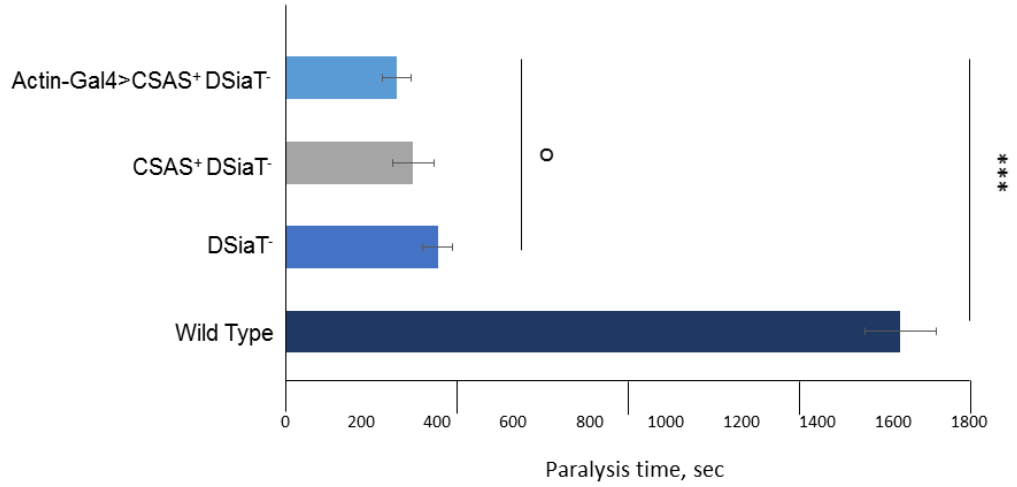

Figure 4.2. Ectopic expression of CSAS is insufficient to rescue DSiaT mutant phenotypes. To determine relationship between CSAS and DSiaT, *UAS-CSAS* was expressed using ubiquitous driver, *Actin-Gal4*. Genotypes are listed in order: *UAS-CSAS/+;DSiaT^{L22}/Actin-Gal4 DSiaT^{S23}*, *UAS-CSAS/+;DSiaT^{L22}/DSiaT^{S23}*, *DSiaT^{L22}/DSiaT^{S23}*. *** and o, highly significant ($p<0.001$) and not significant ($p>\ldots$) differences, respectively.

and explain our results. To this end, we carried out a series of interaction experiments using mutant alleles and transgenic overexpression conditions. Sialyltransferase activity is required to transfer sialic acid onto a glycoprotein and this modification is required for neural functioning in *Drosophila* [24, 26]. Since *DSiaT* is apparently the only Drosophila gene encoding a sialyltransferase, *DSiaT* mutations are expected to be epistatic to upstream perturbations at the level of CSAS-mediated step, and thus overexpression of CSAS is not expected to rescue neurological phenotypes of DSiaT mutants. To test this scenario, we overexpressed *CSAS* in a *DSiaT* null mutant background and analyzed phenotype using TS paralysis assay. We found that *CSAS* overexpression is unable to rescue *DSiaT* mutants at the organismal level. There is no

significant difference in paralysis time between rescue constructs expressing *CSAS* or *DSiaT* mutants alone (Fig. 4.2).

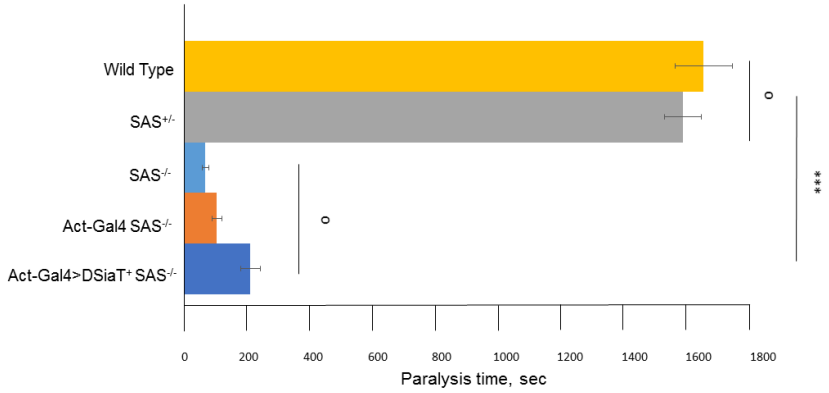

Figure 4.3. SAS activity is required to maintain neural transmission. SAS provides sialic acid intermediate necessary for DSiaT-mediated sialylation. A single copy of *SAS* is sufficient to function. *UAS-DSiaT* was unable to rescue *SAS* mutants. *UAS-DSia*T was expressed in a *SAS* mutant background using Actin-Gal4, a ubiquitous driver. Genotypes are listed in order: Wild type (Canton S, w^{1118}), *SAS-RFP/+*, *SAS-RFP/SAS-RFP*, *Actin-Gal4/+;SAS-RFP/SAS-RFP*, *Actin-Gal4/UAS-DSIAT3HA;SAS-RFP/SAS-RFP*. *** and o show highly significant ($p<0.001$) and not significant (p>…) differences, respectively.

4.4.3 SAS activity is required for DSiaT-mediated functions

N-acetyl neuraminic acid synthetase, SAS, synthesizes neuraminic acid, (in phosphorylated or non-phosphorylated form) from phosphoenolpyruvate and N-acetylmannosamine (or its phosphorylated form) [36]. This reaction provides the needed sialic acid for sialylation in de novo synthesis. Since flies are grown on a simple cornmeal and yeast food with no source of sialic acid (tested by mass spec, results not shown), SAS activity provides presumably the only source of sialic acid in flies reared in laboratory conditions. To test this assumption, we overexpressed *DSiaT* transgene in *SAS* mutants and assayed their paralysis phenotype. As *SAS* mutants have not been previously characterized, we examined both heterozygous and homozygous *SAS* mutants for

paralysis. We found that one copy of *SAS* is sufficient to maintain normal neural transmission, while flies homozygous for the mutation displayed a paralysis phenotype indicative of excitability defects seen in other sialylation pathway genes *CSAS* and *DSiaT* [24, 29]. Also, ectopic expression of *DSiaT* could not rescue *SAS* mutants (Fig. 4.3). These experiments supported the hypothesis that SAS activity provides the only endogenous source of sialic acid in Drosophila. The phenotype of SAS mutants also confirmed that neurological phenotype of DSiaT mutants is primarily due to the absence of their enzymatic activity and defect in sialylation of glycoconjugates.

4.4.4 Exogenous Sialic acid can restore neurological function

Exogenous sialic acid provided as a food supplement could potentially restore the sialylation pathway in *SAS* mutants if Drosophila has a mechanism of sialic acid uptake and metabolic incorporation. To test this hypothesis, we transferred *SAS* mutants as third instar larvae to vials containing food supplemented with 0.2mM sialic acid. These *SAS* mutants were then collected as adult flies and assayed for TS paralysis. We found that sialic acid supplements restored paralysis phenotype indicating that neural function was restored in *SAS* mutants (Fig. 4.4). This result demonstrated that sialic acid derived from an external source can be used by the sialylation pathway to maintain neural function within *Drosophila*. However, this result does not address the mechanism of how DSiaT can bypass the requirement for CSAS. To shed light in this mechanism, the feeding approach could be modified to include *SAS/CSAS* double mutants to investigate the requirement for CMP-Sia donor by testing rescue with CMP-Sia food supplementation. Mass spectral analyses have recently suggested that Drosophila

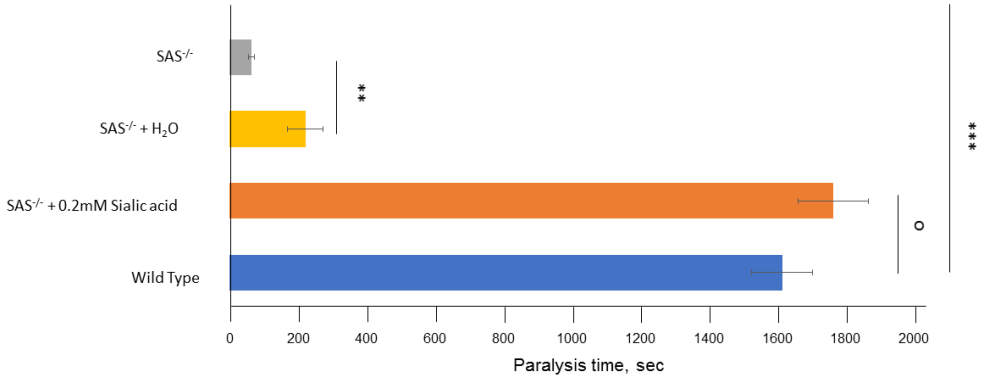

Figure 4.4. Exogenous sialic acid restores sialylation pathway despite lacking SAS activity. *SAS* mutant flies supplemented with sialic acid show no significant difference in paralysis time as wild type flies, while flies with only water added display paralysis time consistent with *SAS* mutants. Data provided by Brooke Howell. ***, ** and o show highly significant ($p<0.001$), significant ($p<0.01$) and not significant ($p>\ldots$) differences, respectively.

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possesses O-linked sialylated structures (Dr. Michael Tiemeyer, personal communication). Yet, the biochemical characterization of DSiaT shows that this enzyme can only modify of N-linked glycans. The presence of O-linked sialyloglycans could be potentially explained by presence of symbionts that synthesize these structures and may provide an additional source of sialic acid donors. However, further studies are required to test this hypothesis and to determine any relevant contributions these symbionts may have with regards to Drosophila sialylation.

4.4 Discussion

Sialylation accounts for a very small portion of all N-linked modifications in Drosophila, yet it is critical for neural development and function [22-25, 29, 36]. There is a single Drosophila sialyltransferase responsible for modifying conjugates with sialic

acid compared with 20 sialyltransferases in mammals [18]. Therefore, DSiaT-mediated sialylation can potentially perform many functions and we are just beginning to elucidate the unique mechanisms of DSiaT that fulfill these roles. In this study, we examined whether this extends to "reversible" or trans-sialylation activity. Our results show that *DSiaT* can maintain neural function even in a *CSAS* null mutant background. However, DSiaT does require a source of sialic acid whether provided through SAS during de novo sialylation or exogenous application.

DSiaT rescued TS paralysis phenotype of *CSAS* mutant despite the lack of CSAS activity that generates nucleotide sugar donor, CMP-Sia. This suggests that DSiaT can sialylate cell surface glycoproteins through a previously uncharacterized activity. The protozoan parasite, *T. cruzi* acquires sialic acid necessary for virulence from host glycoproteins using a bifunctional enzyme with combined neuraminidase and sialyltransferase activities. This trans-sialidase enzyme can cleave sialic acid from donor glycans and attach them to acceptor glycans [59-61]. By analogy, one may suggest that DSiaT can function as a trans-sialidase. However, DSiaT does not possess detectable sialidase activity (Fig. 4.5). One could also imagine that a separate neuraminidase may carry out removal of cell surface sialic acid, then DSiaT could transfer this free sialic acid onto glycoproteins. In this scenario, the only requirement for DSiaT-mediated trans-sialylation would be the presence of free sialic acid. In the case of *T. cruzi*, the energy required for attachment of sialic acid is provided by the breaking bonds on donor glycans when removing host sialic acid. If these two events are separated in Drosophila, the energy source for the subsequent modification becomes unexplained. An alternative

mechanism would be reversible sialylation that has been shown in mammalian ST3GalII. This enzyme is more closely related to DSiaT than trans-sialidases, having similar conserved sialyltransferase motifs (Fig. 4.5). St3GalII requires only 5'CMP and a sialyl glycan as a donor, and it does not generate free sialic acid as an intermediate [58]. Our results support this assertion since *DSiaT* was not able to rescue neural transmission in *SAS* mutant background. However, exogenous application of sialic acid could rescue *SAS* mutants. Lepidopteran insect cell lines do not have detectable levels of CMP-Sia, yet sialylated structures can be seen on cell surface when cultured in medium containing serum. These cells utilize bovine sialylated glycoproteins present in the serum as external sources of sialic for incorporation into their own glycoconjugates.

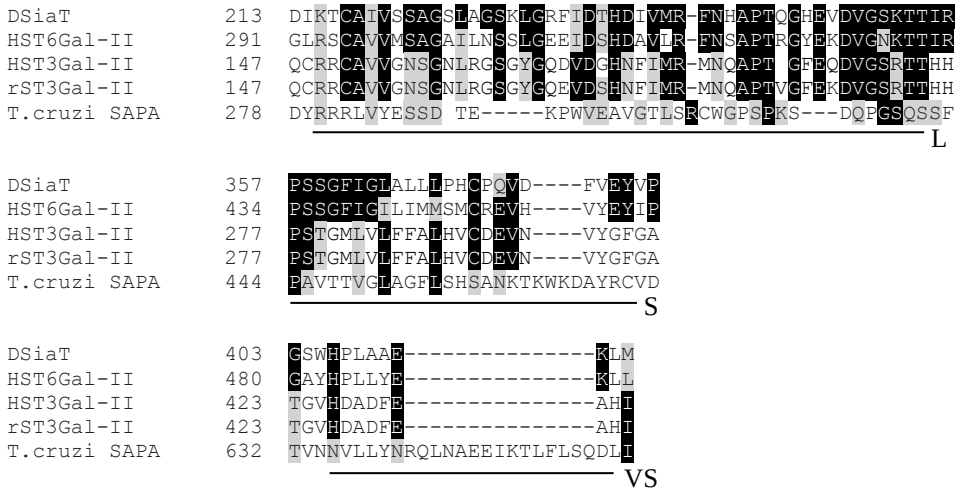

Figure 4.5 DSiaT is more closely related to ST3Gal-II sialyltransferases than T.cruzi trans-sialidase Alignment of protein sequences of Drosophila (DSiaT), human ST6-Gal-II, human ST3Gal-II, rat ST3Gal-II, and T. cruzi trans-sialidase, the acute phase antigen SAPA. Gene Bank™ Accession numbers are Q9W121, BAC2793.1, NP_00858.1, NP_113883.2, CAA40511.1 respectively. Conserved sialyl motifs are underlined and labeled (L) large, (S) short, (VS) very short. Areas of fully conserved residues are shaded black, while areas conserved with strong similarities are shaded in grey. Alignment was performed using Clustal Omega (1.2.4) server EMBL-EBI.

This salvage pathway has never been shown in Drosophila in vivo until now. In our feeding experiments, dietary supplementation of free sialic acid can rescue *SAS* mutants, however these mutants still have functional CSAS and therefore this result does not address whether CMP-Sia is an intermediate in the salvage pathway. Future experiments could include *SAS/CSAS* double mutants with normal or overexpressed levels of *DSiaT* to address more directly the mechanism by which DSiaT circumvents the requirement for CSAS. Although only ectopic DSiaT could bypass CSAS activity to restore normal neural transmission and rescue the paralysis phenotype, we cannot discount the possibility that endogenous DSiaT could mediate sialylation in some cells without CSAS activity (which is not sufficient to rescue CSAS mutants at organismal level). Our results have previously shown a cell non-autonomous function for DSiaT that may modify glycoproteins extracellularly (Chapter 3). A possible model then emerges whereby DSiaT utilizes sialylated glycans provided by symbionts as donor substrates for reversible sialylation that generates CMP-Sia. Then this nucleotide sugar donor can be used in canonical sialylation of functionally important Drosophila glycoproteins in the absence of CSAS activity. This hypothesis could be tested by several strategies, including, for example, rescue experiments with ectopic expression of *DSiaT* in *CSAS* mutants reared on food supplemented with O-linked sialylated glycans similar to those found by mass spectrometry. This would be a promising area of further study.

5. CONCLUSIONS

Sialic acids decorate the termini of glycoproteins participating in numerous biological processes in a broad spectrum of organisms, including bacteria, and animals. Recent studies have implicated sialylation as playing a role in neural excitability, however its functions and regulation in the nervous system, besides those of PSA modifications, remain largely unknown. This knowledge gap is mostly due to limitations of available approaches and the complexity of glycosylation pathways in mammals. The main goal of this work has been to gain mechanistic insights into the regulation of sialylation within the nervous system and to reveal how this regulation contributes to modulation of neural transmission. We approached these goals using advantages of Drosophila model system.

One specific focus of our project was on the regulatory mechanisms that control CSAS, an essential enzyme within the sialylation pathway. Our studies revealed that Drosophila CSAS has a unique sub-cellular localization. All mammalian CSAS proteins analyzed to date localize to the nucleus [62, 63]. While the reason for this localization is unknown, it has been postulated that the separation to distinct cellular compartments plays a yet unknown role in regulation of sialylation [64]. Drosophila CSAS is a glycosylated protein that is localized to the Golgi compartment [29]. Despite these differences in subcellular localization, the function of CSAS is functionally conserved between Drosophila and mammals, as we found that human CSAS can rescue Drosophila CSAS mutants. This result suggests that Golgi localization is not essential

for CSAS activity in vivo, and it probably plays some role in the regulation of CSAS and/or the sialylation pathway. Our in vitro studies revealed important differences between DmCSAS and its mammalian counterparts, including different preferences for metal cofactors and pH, revealing evidence of evolutionary adaptation of these enzymes to distinct subcellular environments.

Additionally, KDN has long been postulated to be a substrate for DmCSAS, which provided a putative explanation how insect cells could initiate the pathway without GNE, a key enzyme that generates ManNAc-6P from UDP-GlcNAc. This enzyme is missing in insects, and an alternative mechanism of synthesizing KDN, instead of NeuAc, from Man-6P was proposed to exist in insect cells. However, our in vitro results demonstrated that Drosophila CSAS has practically no activity with KDN as a substrate, while showing preference for Neu5Ac, the most abundant type of sialic acid in humans. These results indicate that KDN-modified glycans are unlikely produced by the sialylation pathway in Drosophila.

Intriguingly, we found that DmCSAS activity increases 10-fold with a temperature increase from 20˚C-40˚C, suggesting that this enzyme plays a role in adjusting neural transmission in response to temperature, and possibly mediating responses to environmental stresses.

We examined in detail the cell-specific expression and requirement of CSAS. Our results demonstrated a unique bi-partite mechanism that regulates neural transmission. That mechanism is dependent on the cooperation between sialylation genes in both glia and neurons. Cell-specific rescue studies indicated that CSAS functions in

glial cells, confirming results of analyses of CSAS cell-specific expression. A possible explanation for CSAS glial localization is the availability of phosphoenolpyruvate in glial cells, a substrate for biosynthesis of sialic acid, a substrate for CSAS. Glycolysis is the main energy source for glial cells. Glycolysis in glial cells generates lactate that delivered to neurons where it's used in oxidative phosphorylation to meet their energy needs. Similarly, we propose that CMP-Sia is provided by glial to neurons where it's used by DSiaT to modify glycoproteins that regulate neural excitability, such as VGICs, including the voltage gated Na+ channel PARA. RNAi mediated downregulation of DSiaT specifically in neurons results in excitability defects characteristic for loss of sialylation. Taken together, these data support our model of sialylation-mediated glia-neuron coupling (Fig. 3.15). Interestingly, ectopic expression of DSiaT in either glia or neurons is sufficient to rescue DSiaT mutants, suggesting a cell non-autonomous function of DSiaT. We continued the examination of cellular and molecular mechanisms of sialylation-mediated regulation of the nervous system, with main focus on the relationship between CSAS and DSiaT. Remarkably, we found that ectopic expression of DSiaT can rescue CSAS mutants, suggesting that DSiaT can bypass the requirement for CSAS activity by synthesizing sialylated glycans in the absence of CMP-Sia. It could be explained by a scenario in which DSiaT can utilize unknown sialyl-glycoconjugates as donors of siliac acid to sialylate functionally important acceptors and to maintain normal neural transmission at an organismal level. This non-canonical sialylation is reminiscent of "reversible sialylation" mediated by ST3Gal-II in vitro. DSiaT is closely related to ST6Gal family of mammalian sialyltransferases, and

thus it seems possible that this function is conserved between Drosophila and human sialyltransferases [24]. This is the first example of in vivo trans-sialylation occurring in a metazoan organism. This mechanism may have important implication for elucidating sialylation-mediated regulation of neural transmission in human brain.

Taken together, our data shed light in several novel aspects of neural regulation mediated by the sialylation pathway, including a bi-partite mechanism of sialylation that underlies functional glia-neuron coupling, cell non-autonomous function of DSiaT that can regulate neural excitability, and a unique trans-sialylation activity of DSiaT that can bypass the requirement for CSAS in neural regulation. These molecular and cellular mechanisms may be conserved in mammals, leading to a greater understanding of the role of sialic acids in the human nervous system.

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