ROLE OF SIALYLATION IN THE NERVOUS SYSTEM DEVELOPMENT OF *DROSOPHILA MELANOGASTER*

A Dissertation

by

ELENA ALEKSANDROVNA REPNIKOVA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Genetics

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Approved by:

Chair of Committee,	Vladislav M. Panin
Committee Members,	Bruce Riley
	Sumana Datta
	Ginger Carney
Intercollegiate Faculty Chair,	Craig Coates

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ABSTRACT

Role of Sialylation in the Nervous System Development of

Drosophila melanogaster. (August 2009)

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Chair of Advisory Committee: Dr. Vladislav M. Panin

The sialyltransferase family is a group of enzymes that transfer sialic acid from donor CMP-Neu5Ac onto suitable carbohydrate chains of glycoproteins and glycolipids. In vertebrates, sialylation is implicated in many physiological and pathobiological processes, including nervous and immune system development and functioning, pathogen-host interaction, cancer cell proliferation and apoptosis. However, the complexity of the sialylation pathway and limitation of genetic and in vivo approaches interferes with functional analyses in mammalian organisms. We use *Drosophila* because of its simplified physiology and reduced genetic redundancy to characterize the evolutionarily conserved function of sialylation and to reveal its relationship to the role of sialic acids in humans. This dissertation focuses primarily on *Drosophila* sialyltransferase, DSIAT, so far the only sialyltransferase described in protostomes.

Gene targeting of the DSIAT endogenous locus with a DSIAT-HA tagged version uncovered its remarkably dynamic stage- and cell-specific expression. I found that the expression of *DSIAT* is developmentally regulated and is restricted to motor neurons and

cholinergic interneurons within the central nervous system of *Drosophila*. To reveal the role of DSIAT in development and functioning of fly nervous system I performed characterization of neurological phenotypes of *DSIAT* knockout flies, also generated by gene targeting approach. I observed that *DSIAT* mutant larvae are sluggish and have abnormal neuromuscular junction (NMJ) morphology. Electrophysiological analysis of mutant larval NMJ showed altered evoked NMJ activity. It was also observed that *DSIAT* knockout adult flies are paralyzed when are exposed to higher temperatures. Longevity assays showed that *DSIAT* adult mutants have significantly reduced life span. I used genetic interaction analysis to identify possible sialylated targets in *Drosophila* and found that α-subunit of voltage gated sodium channel is a potential sialylated protein in the fly nervous system.

All these data strongly supports the hypothesis that DSIAT plays an important role for neural transmission and development in *Drosophila*. This research work establishes *Drosophila* as a useful model system to study sialylation which may shed light on related biological functions in higher organisms including humans.

DEDICATION

To all whom I love

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First of all, I would like to express sincere gratitude to my advisor, Dr. Vlad Panin for his excellent guidance, expertise, constant encouragement and support during my studies at Texas A&M University. It was he who gave me such a brilliant opportunity to dive into the fantastic area of neurobiology and genetics. I am also very thankful to Dr. Mark Zoran who introduced me to the magnificent subject of electrophysiology. Without his knowledge and expertise we wouldn't be have been able to uncover the mechanism that underlies sialyltransferase functioning in *Drosophila*.

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I would also like to express special thanks to my family and my husband's family for their endless support. Especially, I am deeply grateful to my husband, Konstantin for his enormous and unfailing help.

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CHAPTER I

INTRODUCTION*

Glycosylation is one of the most common forms of posttranslational protein modifications in eukaryotic cells. It involves transfer of carbohydrates onto lipids or proteins. Carbohydrates are collectively termed as "glycans". Glycan chains are extended by various glycosyltransferases. Depending on the amino acid of the protein to which the first sugar is transferred, glycosylation is divided into *N*-linked, *O*-linked or *C*-linked. *N*-linked glycosylation occurs on the asparagines in the consensus sequence Asn-X-Ser/Thr. *O*-linked glycosylation is usually an attachment of sugar to the peptide through the hydroxyl group of serine or threonine residue. This type of glycosylation does not require a consensus sequence and oligosaccharide precursor. *C*-linked glycosylation involves attachment of α-mannose to the C^2 atom of the idol ring of tryptophan.

Sialylation, which occurs on glycolipids and N- and O-glycans, stands out among the other types of glycosylation as it is typically found on the terminal positions of the glycan chains.

This dissertation follows the style and format of Neuron.

Unlike most other sugar residues, sialic acids are negatively charged, have a relatively large nine-carbon backbone, and can carry a variety of side group modifications. The combination of these features makes sialic acids uniquely suited to participate in molecular recognition and to regulate cellular interactions.

Sialic acids can be found at almost all evolutionary levels of life (reviewed in (Angata and Varki, 2002) . Although most abundantly present and best studied in deuterostomes (echinoderms, ascidians, and vertebrates), sialylation is also found in a number of microorganisms (reviewed in (Vimr et al., 2004)). Many of these microorganisms are pathogenic species able to synthesize sialic acids de novo or metabolize host sialic acids for their own cell surface epitopes to mimic the properties of host cells and to evade the host organism's immune response.

Through specific receptors, they can also recognize host sialylated structures and use them in the pathogenic process. While it has been originally suggested that the presence of sialylation pathway enzymes in these microorganisms is a result of horizontal gene transfer, in many cases an independent evolutionary re-occurrence of this pathway is a more likely explanation, especially since the corresponding bacterial genes often do not show a close evolutionary relationship to their animal counterparts (Yamamoto, 2006).

Great strides have been made over the past few decades in uncovering a large number of different sialylated structures and in cloning numerous sialyltransferases and other genes of the sialylation machinery from different species (for reviews see (Harduin-Lepers et al., 2005; Miyagi and Yamaguchi, 2007; Schauer and Kamerling, 1997). However, our knowledge about the structural diversity and widespread occurrence of

sialic acids is yet to be matched by the understanding of their biological function and underlying molecular mechanisms. Even though striking biological roles of sialic acids in key cellular and molecular interactions and developmental pathways have been revealed (Varki, 2007) (*e.g.*, virus-host interactions (Alexander and Dimock, 2002; Nilsson et al., 2008; Olofsson et al., 2005) cancer cell differentiation (Hedlund et al., 2008), immune (Crocker et al., 2007) and nervous system functioning and development (Isaev et al., 2007; Rutishauser, 2008), integrin-mediated adhesion (Seales et al., 2005), apoptosis (Malisan et al., 2002), TrkA tyrosine kinase receptor signaling (Woronowicz et al., 2004) etc.), these findings likely represent only the tip of the iceberg.

Gene targeting has recently become one of the most productive approaches for studying the biological functions of sialylation in animals, with sialyltransferase genes being the focus of these studies (Angata et al., 2007; Hashiramoto et al., 2006; Muramatsu, 2000; Oltmann-Norden et al., 2008; Santos et al., 2008; Weinhold et al., 2005). However, the genetic redundancy and limitations of genetic approaches can often interfere with the phenotypic analysis of mutations in mammalian organisms. Thus a simpler and more experimentally amenable organism like *Drosophila* represents an attractive model system to study the evolutionarily conserved functions of sialylation. However, sialylation in *Drosophila* and other protostomes (including annelids, arthropods, and mollusks) has not been extensively studied, and in fact some of these species appear to be devoid of biochemical potential for sialylation (Angata and Varki, 2002). Therefore, the possibility of using *Drosophila* for studying sialylation poses two important questions, both still awaiting a more comprehensive consideration. First, does

Drosophila indeed possess an efficient biosynthetic machinery to produce sialylated structures, or is it an "incomplete tool kit" (to paraphrase Angata and Varki (Angata and Varki, 2002))? Second, is the function of sialylation in *Drosophila* evolutionarily related to the roles of sialic acids in higher animals? In this chapter we have attempted to shed some light on these questions by discussing the current knowledge of the insect and, more specifically, the *Drosophila* sialylation pathway in relation to what is known about sialylation in other organisms, especially in mammals.

BIOSYNTHETIC PATHWAY OF SIALIC ACIDS

The vertebrate sialic acid metabolic pathway comprises several enzymatic steps (Figure 1). The biosynthetic branch specific for Neu5Ac begins with the synthesis of ManNAc-6-P from UDP-GlcNAc by a bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase, or GNE (Stasche et al., 1997). Point mutations in human GNE are associated with hereditary inclusion body myopathy (Eisenberg et al., 2001), while the lack of this enzyme leads to embryonic lethality in the mouse (Schwarzkopf et al., 2002), clearly demonstrating the essential role of sialic acids in higher animals. In bacteria, the corresponding step is mediated by a homologous enzyme (BLAST E-value 2e-20 for *E. coli* NeuC and rat GNE proteins (Ringenberg et al., 2001)), but it produces ManNAc instead of ManNAc-6-P, and the absence of the kinase activity is in agreement with the lack of the C-terminal kinase domain in the bacterial enzyme (Murkin et al., 2004; Ringenberg et al., 2003). At the same time, the bacterial enzymatic activity (GlcNAc-6-P 2-epimerase) that catalyzes the conversion of GlcNAc-6-P to ManNAc-6-P

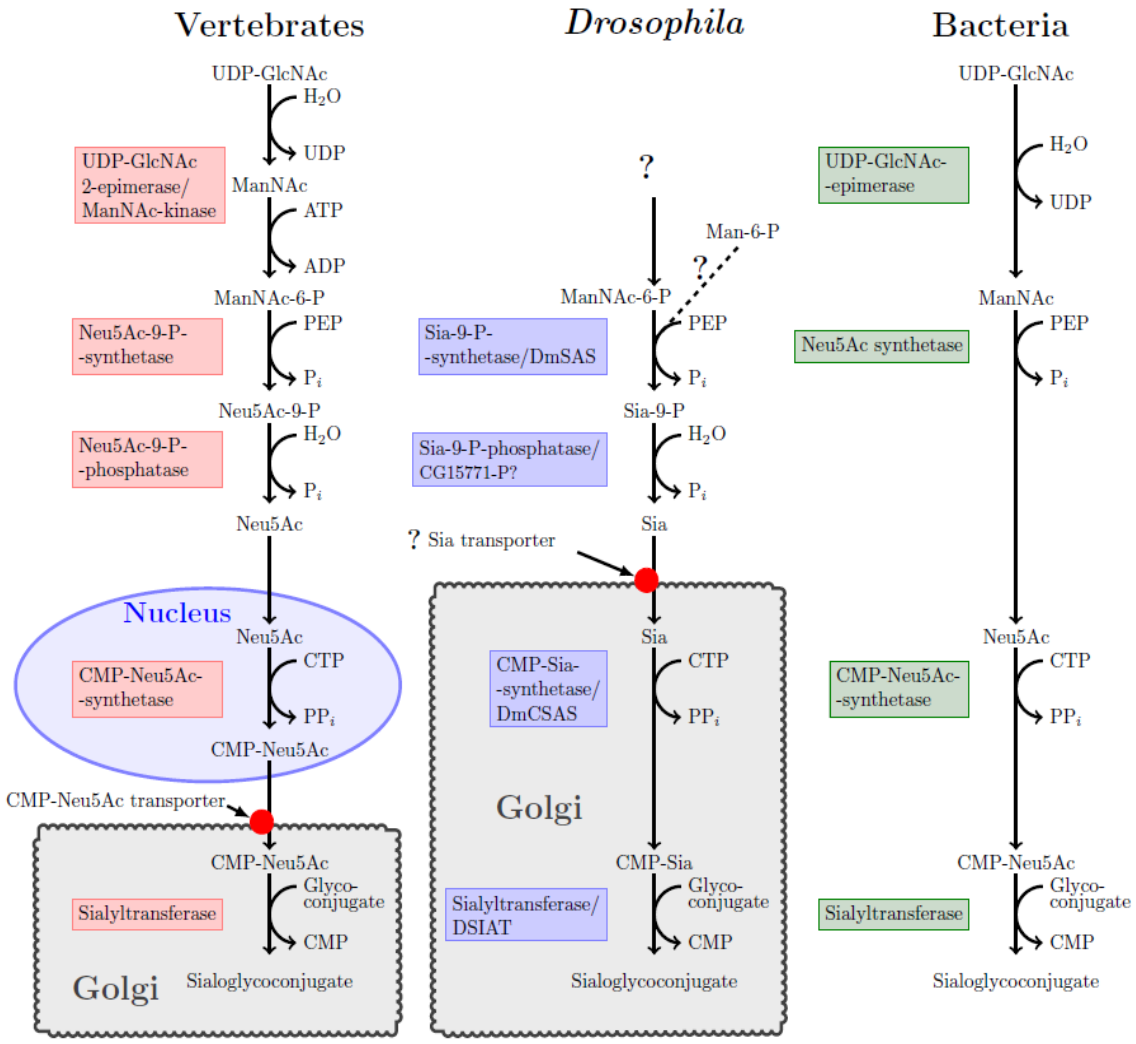

Figure 1. Comparison between the de novo biosynthetic pathways of sialylation in vertebrates, *Drosophila* and bacteria.
Question marks indicate unknown/hypothetical steps or enzymes. Thus, in *Drosophila*, given apparent absence of UDP-GlcNAc 2 epimerase/ManNAc kinase gene from the genome, it is unclear how ManNAc-6-P is being synthesized. The question mark next to Man-6-P indicates the hypothetical possibility that in *Drosophila*, similar to lower vertebrates, the KDN pathway may be initiated at this step, thus bypassing the requirement for the epimerase/kinase enzyme. Therefore we use "Sia" as a general term for any of the possible sialic acids in this context, *e.g.* Neu5Ac or KDN. CG15771-P denotes the protein product of the *Drosophila melanogaster CG15771* gene. See the text for discussion and the complete names of enzymes and other abbreviations.

is not linked to sialic acid biosynthesis (Ferrero et al., 2007). Interestingly, the genome of *Drosophila melanogaster* does not have a gene encoding a UDP-GlcNAc epimerase homologue, and only a very low level of UDP-GlcNAc epimerase activity was detected in lepidopteran Sf9 cultured cells (Effertz et al., 1999). In addition, these cells were found to have ManNAc kinase activity, which could come from an unrelated cytosolic kinase able to phosphorylate ManNAc (*e.g.*, GlcNAc kinase (Hinderlich et al., 2001)). This poses an intriguing question about the initiation of sialic acid biosynthetic pathway in insects, since, as discussed below, most other genes of the pathway are present and functional, which would suggest that insects are capable of de novo sialylation, but how they do it in the absence of UDP-GlcNAc epimerase remains to be revealed.

In the next step of the vertebrate biosynthetic pathway, ManNAc-6-phosphate is converted to Neu5Ac-9-phosphate through condensation with phosphoenolpyruvate (PEP) by Neu5Ac-9-P synthetase (Figure 1). In addition to the Neu5Ac pathway, the biosynthesis of KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic or deaminated neuraminic acid) has been established as a prominent sialylation pathway in lower vertebrates and some pathogenic bacteria (reviewed in (Inoue and Kitajima, 2006)). KDN is a unique form of sialic acid which is structurally very similar to Neu5Ac but it differs from it in having a hydroxyl group at C-5 instead of an aminoacyl group (Song et al., 1991). The de novo KDN biosynthesis is initiated by the condensation of Man-6-P with PEP to produce KDN-9-P, which is analogous to the condensation of ManNAc-6-P and PEP in the Neu5Ac pathway (Angata et al., 1999). Since Man-6-P is presumably available within the cell from several biosynthetic sources (Davis et al., 2002), this

initiation bypasses the step mediated by UDP-GlcNAc 2-epimerase/ManNAc-kinase in Neu5Ac pathway mentioned above, while the downstream part of KDN biosynthesis appears to follow the same steps as the biosynthesis of Neu5Ac in vertebrates (see below and (Inoue and Kitajima, 2006)). The enzymes of the KDN biosynthetic pathway have not yet been well characterized, although some of them are possibly shared between the Neu5Ac and KDN pathways (Go et al., 2007; Inoue and Kitajima, 2006; Lawrence et al., 2000; Nakata et al., 2000). Indeed, the human Neu5Ac-9-P synthetase can use Man-6-P instead of ManNAc-6-P as a substrate to produce KDN-9-phosphate (Lawrence et al., 2000). The *E.coli* sialic acid synthetase, neuB (Annunziato et al., 1995), shows significant similarity to the human enzyme (35% identity, 56% similarity of amino acid sequences (Lawrence et al., 2000)), however instead of ManNAc-6-P, neuB uses ManNAc in the reaction with PEP to yield Neu5Ac. Many bacterial Neu5Ac synthetases have been cloned and characterized (*e.g.*, *E. coli K1* (Vann et al., 1997), *Streptococcus agalactiae* (Suryanti et al., 2003), *C. jejuni* (Sundaram et al., 2004), and *N. meningitidis* (Gunawan et al., 2005)). *Drosophila* has a single homologue of this enzyme, DmSAS, the activity of which was confirmed by *in vitro* and cell culture assays (Kim et al., 2002). Interestingly, similar to the human but unlike the murine homologues (Chen et al., 2002; Nakata et al., 2000), DmSAS can also synthesize KDN-9-P using Man-6-P as the substrate (Kim et al., 2002). The expression of *DmSAS* is present at every developmental stage examined (Kim et al., 2002), however the pattern of expression within the tissues has not been characterized in detail and as of now, no data on the phenotype of *DmSAS* mutants is available.

In the following biosynthetic step, Neu5Ac-9-P is dephosphorylated by *N* -acetylneuraminate-9-phosphate phosphatase (Maliekal et al., 2006) to yield Neu5Ac (Figure 1). The enzyme has been localized to the cytosolic fraction in rat liver by subcellular fractionation and enzymatic assays, and presumably it can also use KDN-P as a substrate (Van Rinsum et al., 1984). A *Drosophila* homologue is encoded by the CG15771 gene on the X chromosome, but functional or phenotypic information is not yet available for this gene. Bacteria do not have this step in the pathway since Neu5Ac is synthesized directly, as opposed to its phosphorylated intermediate, in the upstream reaction.

The resulting Neu5Ac then becomes a substrate for CMP-Neu5Ac synthetase (CSAS) which produces the sugar donor molecule, CMP-Neu5Ac, necessary for the final enzymatic step of the pathway mediated by sialyltransferases. CMP-sialic acid synthetases were characterized and cloned from a number of bacterial and animal species (Munster et al., 1998; Tullius et al., 1996; Vann et al., 1997), reviewed in (Kean et al., 2004)). They all show significant amino acid sequence conservation within the five 'signature' domains of this enzyme family, with 41–47% overall protein sequence identity between the mammalian and bacterial enzymes (reviewed in (Munster-Kuhnel et al., 2004)). Genetic and biochemical data from a number of vertebrate species indicate that CMP-Neu5Ac synthetases have a relatively broad substrate specificity and can also synthesize CMP-KDN, although their efficiency for KDN activation varies significantly between different species (Inoue and Kitajima, 2006). The enzyme localizes to the nucleus in vertebrate cells due to the presence of nuclear localization signal motifs,

however the enzymatic activity and nuclear localization are separable features for the fish enzyme, and thus the functional importance of nuclear localization of vertebrate CMP-Neu5Ac synthetases is currently not known (Tiralongo et al., 2007). Nuclear localization of animal CMP-Neu5Ac synthetases originated relatively recently in evolution, early in the deuterostome lineage (in echinoderms), as the recently characterized *Drosophila* CMP-sialic acid synthetase, DmCSAS, unlike its vertebrate counterparts, localizes to the Golgi compartment (Viswanathan et al., 2006). This unusual localization of DmCSAS is likely due to the presence of an N-terminal hydrophobic domain that presumably functions as a signal peptide/anchoring domain. The Golgi localization of DmCSAS appears to be important for the functionality of this enzyme as a swap fusion of the *Drosophila* enzyme with the N-terminus (including nuclear localization signal) from the human counterpart was found to localize to the nucleus, while being enzymatically inactive (Viswanathan et al., 2006).

In vertebrates, the CMP-Neu5Ac activated donor synthesized in the nucleus diffuses to the cytoplasm, and then it is pumped into the Golgi compartment by CMP-Neu5Ac transporter (CST) that belongs to the family of nucleotide sugar transporters (reviewed in (Berninsone and Hirschberg, 2000)). The mammalian CMP-Neu5Ac transporter has 9 predicted membrane-spanning domains and localizes to the medial-trans Golgi (Eckhardt et al., 1996; Zhao et al., 2006). Two genes encoding close homologues of the mammalian CMP-Sia transporters are present in *Drosophila* genome, CG2675 and CG14040; their products have 40% identity/61% similarity and 25% identity/45% similarity to the human CST protein, respectively. However, predicting substrate

specificities of nucleotide transporters from protein sequence alone can be misleading, since even transporters with ~50% identical amino acid sequence can prefer distinct substrates, while homologues from different species may have only 20% sequence identity and show the same substrate specificity (Berninsone and Hirschberg, 2000; Martinez-Duncker et al., 2003). Thus, when assayed directly, the protein product of CG2675, the closest *Drosophila* homologue of mammalian CSTs, transported UDP-galactose but not CMP-sialic acid (Aumiller and Jarvis, 2002; Segawa et al., 2002). The product of CG14040, the more distant *Drosophila* CST homologue, has never been functionally characterized; curiously, it shows a closer phylogenetic relationship to the recently characterized CST from *Arabidopsis* (Bakker et al., 2008). However, considering that *Drosophila* CSAS possibly works in the Golgi (Viswanathan et al., 2006), perhaps the *Drosophila* sialylation pathway does not need a CMP-Neu5Ac transporter, since in this scenario the donor sugar would be synthesized directly within the Golgi compartment. However, the sialic acid would still need to be transported to the Golgi for the synthesis of the activated donor. Both bacteria and vertebrates have sialic acid transporters. In *E. coli*, it is the product of the *nanT* gene (Vimr and Troy, 1985); it has 14 membrane-spanning domains and shows little homology to any *Drosophila* protein. There are, however other, more general types of transporters in bacteria that are also involved in sialic acid uptake (for review see (Vimr et al., 2004)), and homologues of these transporters were noted in *Drosophila* (Viswanathan et al., 2006). Human sialin, a well characterized lysosomal sialic acid transporter implicated in the lysosomal free sialic acid storage disorders (Verheijen et al., 1999), has multiple *Drosophila* homologues that

have various developmentally regulated patterns of embryonic expression (Laridon et al., 2008). Thus, the possibility exists that some of these homologues function in the *Drosophila* sialylation pathway as transporters to deliver sialic acid to the Golgi.

A number of enzymes that are responsible for the generation of over 50 different sialic acid modifications (reviewed in (Angata and Varki, 2002)) have so far eluded molecular cloning in eukaryotic organisms, even though their biochemical properties have been assessed. The nature of some of these modifications can be subtle at the molecular level (*e.g.*, Neu5Gc *vs.* Neu5Ac, or the presence of an extra *O*-acetyl group) yet they have far reaching biological consequences, affecting a wide variety of cellular processes, like apoptosis, erythrocyte survival, immune system function, cancer prognosis, susceptibility to influenza virus, etc. (reviewed in (Angata and Varki, 2002; Tiralongo and Schauer, 2004)). In *Drosophila*, so far only Neu5Ac has been detected (Aoki et al., 2007; Koles et al., 2007; Roth et al., 1992), while nothing is known about the existence of other varieties. Given their widespread presence in both prokaryotes and deuterostomes, it is likely that they will also be identified in protostomes in the future.

The final step in the biosynthetic pathway of sialic acids is the transfer of sialic acids from CMP-Sia to the acceptor glycoproteins or glycolipids by sialyltransferases. Sialyltransferases all share similar architecture (Figure 2). There are twenty different sialyltransferases belonging to four main families (ST3Gal, ST6GalNAc, ST8Sia and ST6Gal) found and cloned in mammals (Harduin-Lepers et al., 2005). They synthesize different linkages (α2-3, α2-6, α2-8) and prefer different glycoprotein acceptors

(Harduin-Lepers et al., 2001; Krzewinski-Recchi et al., 2003b; Takashima et al., 2002a; Takashima et al., 2002b).

Sialidases, or neuraminidases, are responsible for the cleavage of sialic acids from glycoconjugates for catabolic purposes or for post-synthetic remodeling of glycan chains. Strictly speaking, they do not belong to the de novo biosynthetic pathway of sialic acids, but we mention them here because their functions can significantly contribute to the regulation of cellular sialylation. Mammalian sialidases fall into four general categories, based on their subcellular localization, enzymatic properties, and substrates; they have been implicated in the modulation of many important biological processes (reviewed in (Miyagi and Yamaguchi, 2007)).

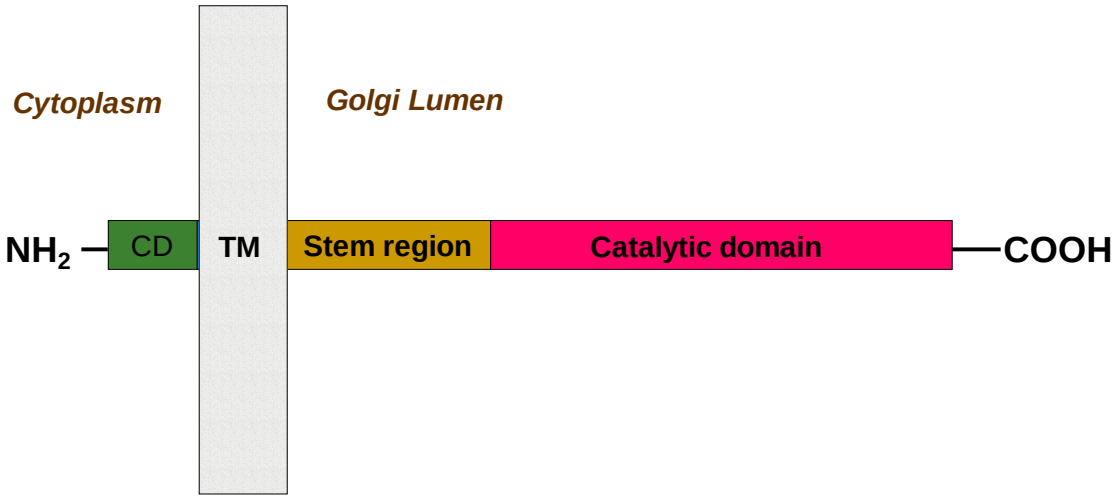

Figure 2. Typical structure of the sialyltransferase.
Sialyltransferases have a cytoplasmic tail (CD) located in the cytoplasm, short transmembrane domain (TM), relatively long stem region and conserved catalytic domain which resides in the Golgi lumen.

Sialidases are present in pathogenic bacteria and viruses where, besides catabolic functions, they are involved in pathogenesis (see reviews (Corfield, 1992; Vimr et al., 2004)). Protein sequences of animal and bacterial sialidases show some similarity, including the presence of specific motifs (such as FRIP and Asp-box) (Miyagi and Yamaguchi, 2007; Monti et al., 2002). Somewhat surprisingly, similar to the GNE enzyme, sialidase homologues have not yet been identified in insects, including *Drosophila*.

Thus, recent studies have provided compelling evidence that *Drosophila* has the genetic potential for de novo sialylation. Most *Drosophila* homologues of key enzymes in the sialylation pathway have been indentified and functionally confirmed *in vitro* and in cell culture assays. The components of the pathway characterized so far show close relationship to the sialylation pathway in vertebrates, suggesting their common evolutionary origin.

SIALYLTRANSFERASES

Sialyltransferases are the group of enzymes that transfer sialic acids from CMP-sialic acid donor onto the suitable carbohydrate of the glycoprotein or glycolipid. There are 20 mammalian sialyltransferases identified up to date. These sialyltransferases are grouped into four families according to carbohydrate linkage they synthesize (Takashima, 2008). Analysis of their amino acid sequence and acceptor specificities allows subdividing them further into seven subfamilies. Table 1 shows classification of vertebrate sialyltransferases, their expression and possible function.

Animal sialyltransferases are rather conserved and are all type II transmembrane proteins localized in the Golgi compartment. They have very common structure that includes a short N-terminal cytoplasmic tail, membrane-anchoring domain, and so-called 'stem region'. Their C-terminal part shows significant conservation and corresponds to a vaguely defined 'catalytic' domain. This domain contains highly conserved stretches of four "sialylmotifs", called *L*arge (L), *S*hort (S), motif III and *V*ery *S*hort (VS) (Datta and Paulson, 1997; Drickamer, 1993; Jeanneau et al., 2004); these motifs are present in all known metazoan sialyltransferases. The functional significance of these motifs has been assessed through mutagenesis studies, and the L-sialylmotif is thought to be involved in the binding of CMP-Neu5Ac (Datta and Paulson, 1995), whereas the S-sialylmotif binds both the donor and acceptor (Datta et al., 1998; Laroy et al., 2001). The VS sialylmotif has been suggested to be part of the active site and shown to be required for catalytic activity (Jeanneau et al., 2004; Kitazume-Kawaguchi et al., 2001). Motif III is thought to play an important conformational role and its integrity is also required for the enzymatic activity (Jeanneau et al., 2004). The molecular structure of animal sialyltransferases has not been solved, and so far detailed structural information is only available for some bacterial sialyltransferases (Chiu et al., 2007; Ni et al., 2007) and thus, they probably appeared independently during evolution. In addition, bacterial sialyltransferases have broader enzymatic specificities in comparison to the more strict enzymatic properties of most eukaryotic sialyltransferases. It is commonly accepted that sialic acids are not present in plants, however, sialyltransferase-like proteins with sialyl-like motifs have been identified in plant cells (Takashima et al., 2006).

Table 1.	Vertebrate sialyltransferases				
Sialyltransferase family	Linkage	Subfamily members	Substrate specificity	Phenotype	Proposed function
β-Galactoside *α*2,3-Sialyltransferase (ST3Gal)	α2,3	ST3Gal I	Gal*β*1,3GalNAc on *O*-glycan of glycoprotein/ glycolipid	apoptosis of $CD8^+$ T lymphocytes	$CD8^+$ T lymphocyte homeostasis
		ST3Gal II		Unknown	Biosynthesis of gangliosides GM1b, GD1a, GT1b
		ST3Gal III	Gal*β*1,3/4GlcNAc on glycoproteins	Unknown	Leukocyte trafficking and inflammation
		ST3Gal IV	Gal*β*1,4/3GlcNAc on glycoproteins	Deficiency in plasma VWF (von Willebrand factor). Thrombocytopenia	Modifier of plasma VWF levels
		ST3Gal V	Gal*β*1,4GlcNAc*β*1Cer	Seizures, diffused atrophy at older age	GM_3 ganglioside glycosylation
		ST3Gal VI	Gal*β*1,4GlcNAc	E- and P- selectin ligand formation	Synthesis of glycolipids
β-Galactoside *α*2,6-Sialyltransferase (ST6Gal)	α2,6	ST6Gal I	Gal*β*1,4GlcNAc	Severe immunosuppression	Immune system functioning
		ST6Gal II	GalNAcβ1,4GlcNAc/ Gal*β*1,4GlcNAc	Unknown	Unknown

Table 1.	Continued				
Sialyltransferase family	Linkage	Subfamily members	Substrate Specificity	Phenotype	Proposed function
GalNAc *α*2,6-Sialyltransferase (ST6GalNAc)	α2,6	ST6GalNAc	GalNAc, Gal*β*1,3GalNAc, Siaα2,3Galβ1,3GalNac on O-glycans of glycoproteins	Unknown	synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer
		ST6GalNAc II		Unknown	Synthesis of Sialyl-6T-T antigen
		ST6GalNAc III	Sia*α*2,3Gal*β*1,3GalNAc	Unknown	Protection of expressing cells from natural killer cell attacks
		ST6GalNAc IV		Unknown	Unknown
		ST6GalNAc V		Neurodegeneration	Synthesis of GD1alpha in the nervous tissues
		ST6GalNAc VI		Unknown	Synthesis of alpha-series gangliosides

Table 1.	Continued				
Sialyltransferase family	Linkage	Subfamily members	Substrate specificity	Phenotype	Proposed function
α2,8 Sialyltransferase (ST8Sia)	α2,8	ST8Sia I	Terminal sialic acid	Increased sensory responses to thermal and mechanical stimuli	GD3 ganglioside synthase development and maintenance of the sensory nervous system
		ST8Sia V		Unknown	Synthesis of GM1b, GD1a, GD3, and GT1b gangliosides
		ST8Sia VI		Unknown	Synthesis of the disialic acid motif on O-glycoproteins
		ST8Sia II		Befasciculated mossy fibers and ectopic synaptogenesis in the hippocampus	Memory formation and behavioral processes
		ST8Sia IV		Impaired long-term potentiation (LTP) and long-term depression	Regulation of synaptic plasticity in hippocampal CA1 synapses
		ST8Sia III	Siaα2,3Galβ 1,4GlcNAc	Unknown	Biosynthesis of the ganglioside G_{T3}

Bacterial enzymes show little sequence similarity to their animal counterparts, no sialylmotifs can be identified within their sequences (reviewed in (Yamamoto, 2006)),

In contrast to mammals, *Drosophila* has a sole gene encoding a vertebrate-type sialyltransferase, DSIAT. *DSIAT* has been identified by searching the *Drosophila* genome database with the known homology to other sialyltransferases (Koles et al., 2004). This enzyme has significant structural (38% and 41% amino acid sequence identity with human ST6Gal I and ST6Gal II, respectively, Figure 3) and functional (linkage and acceptor specificity, as assayed *in vitro*) similarity to ST6Gal family of vertebrate sialyltransferases (Koles et al., 2004).

DSIAT cDNA has been cloned and the biochemical activity of its protein product has been characterized. DSIAT is a type II transmembrane protein with a typical N-terminal domain residing in the Golgi compartment, transmembrane signal region, short 'stem' region, and C-terminal catalytic domain (Koles et al., 2004).

Biochemical characterization of DSIAT showed that it exhibits highest activity towards GalNAcβ1-4GlcNAc carbohydrate structures at the non-reducing termini of the oligosaccharides and glycoprotein glycans. It was also shown that glycolipids are not modified by DSIAT in vitro.

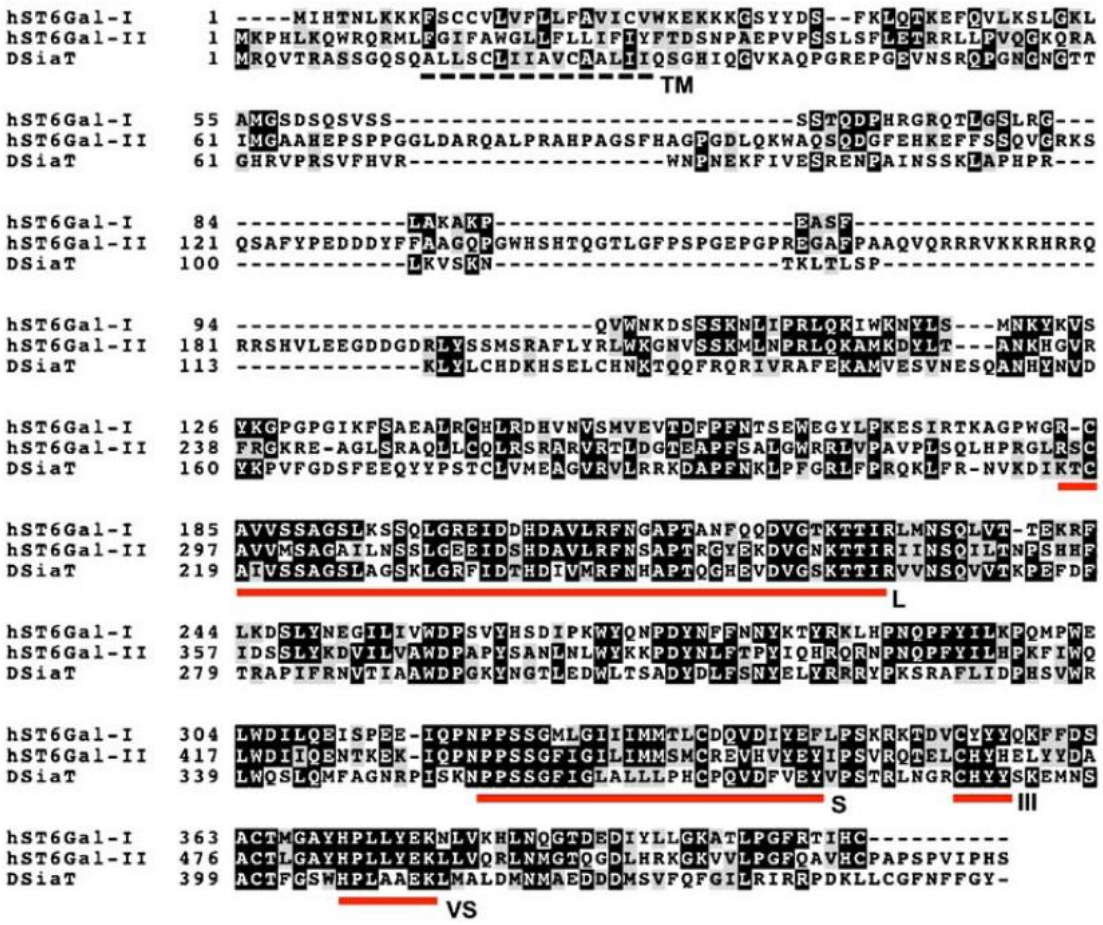

Figure 3. Multiple sequence alignment of protein sequences of human ST6Gal-I, ST6Gal-II, and *Drosophila melanogaster* DSIAT sialyltransferases.
Dashed underline indicates transmembrane/anchoring domain of DSIAT, solid underline indicates L, S, III and VS sialylmotifs. Alignment was performed by ClustalW2 program at http://www.ebi.ac.uk/Tools/clustalw2/index.html (Larkin et al., 2007).

BIOLOGICAL IMPORTANCE OF SIALYLATION

In vertebrates sialic acids are generally found at the terminal positions of N- or O-linked glycans attached either to the cell surface, secreted glycoproteins or glycosphingolipids (Varki, 2007). This diversity provides the basis for the broad range of biological processes they are responsible such as cell adhesion, cell signaling, target recognition, subcellular and extracellular trafficking and receptor modulation and activation.

Sialic acids provide negative charge and hydrophilicity to the cell surface, they primarily serve as receptors for pathogens, mask galactose residue from recognition by certain proteins (Bratosin et al., 1995) and can serve as a recognition target by certain proteins called lectins: sugar-binding proteins (Lehmann et al., 2006; Varki, 2007); . Viruses, bacteria and certain protozoa use lectins to recognize sialic acids attached via specific glycosidic linkage and infect the host cell. For example, influenza viruses A possess viral lectin haemagglutinin (HA) to recognize Neu5Acα2,6Gal structure of the non-ciliated cells of the human trachea and neuraminidase (NA), which contains sialidase activity that removes sialic acid from sialyloligosaccharides to evade the host cell, replicate inside it and infect other cells (Hughes et al., 2000).

Bacterial cell surfaces are covered with glycoconjugates. Bacterial specific lectins (adhesins) attach to the host cell surface via sialoconjugate ligands. For example, pathogenic *E.coli* expresses S-fimbriae, K99-fimbriae or F41 adhesin to bind to gangliosides that carry sialylated glycans (Hacker et al., 1993; Hanisch et al., 1993). Some bacterial pathogens express soluble lectins that bind cell specific surface receptors

via Sia containing oligosaccharide and alter protein synthesis, signaling and impede vesicular trafficking (Lehmann et al., 2006).

Sialic acid is also involved in regulation of cellular activation within the immune system. A good example is studies on molecules that belong to Siglecs (sialic acid binding Ig-like lectins): sialoadhesin (Siglec-1/CD169), a macrophage lectin-like adhesion molecule, which has high expression in tumor-infiltrating macrophages in breast cancer and Siglec-2 (CD22), a B-cell restricted member of the Ig superfamily (IgSF) that plays an important role in regulating B-cell activation (Crocker and Varki, 2001).

Sialic acids play an important role in nervous system development and functioning of vertebrates. It is worthy to mention that a large developmental role is attributed to polysialic acid (PSA) which is mostly found on neural cell adhesion molecule (NCAM). It is known from studies in mice that polysialic acids facilitate neural cell migration and neurite outgrowth (Angata et al., 2002). Interfering polysialyltransferase ST8SiaII/STX inhibits neuritic development in mouse hippocampus (Brocco and Frasch, 2006). Removal of two polysialyltransferases: ST8Sia-II and ST8Sia-IV leads to specific brain wiring defects, progressive hydrocephalus, postnatal growth retardation, and precocious death in mice (Weinhold et al., 2005). It has been also shown that in the developing nervous system polysialic acid is involved in motor neuron targeting (Rafuse and Landmesser, 2000), axon guidance and fasciculation (Angata and Varki, 2002).

DROSOPHILA AS A MODEL FOR STUDYING SIALYLATION

Historically, the occurrence of sialic acids in protostomes, including insects, has been somewhat controversial. While some early studies confirmed the presence of sialylation (including, endogenous sialyltransferase activity, or biochemical potential to provide sugar donor for sialylation (Hiruma and Riddiford, 1988; Hollister and Jarvis, 2001; Malykh et al., 1999; Roth et al., 1992; Vadgama and Kamat, 1969), other reports failed to detect sialylated glycans, CMP-Neu5Ac donor, or sialyltransferase activity in insect cells (Butters et al., 1981; Hollister et al., 1998; Hooker et al., 1999; Jarvis and Finn, 1996; Lopez et al., 1999; Marz et al., 1995; Tomiya et al., 2001). This controversy has now been partially resolved, as several recent studies have provided crucial evidence that endogenous sialylation does occur in *Drosophila*. For example, advanced mass spectrometry approaches have unambiguously revealed the presence of sialic acids in *Drosophila* at embryonic stages and in the adult heads (Aoki et al., 2007; Koles et al., 2007), although the detected amount of sialylation was significantly lower than reported previously (Roth et al., 1992). In contrast to the previously reported presence of α2–8 linked polysialic acid in cicada *Philaenus spumarius* and *Drosophila* (Malykh et al., 1999; Roth et al., 1992), our experiments failed to detect this structure in *Drosophila* at any developmental stage (K. Koles and V. Panin, unpublished data). The only sialylated structure so far reliably detected in *Drosophila* is Neu5Ac residues in α2–6 linkage on LacNAc termini of N-linked glycans (Aoki et al., 2007; Koles et al., 2007), which is in agreement with the in vitro characterized acceptor specificity of DSIAT (Koles et al., 2004). It is interesting to note that the acceptor preferred by DSIAT in in vitro assays, *i.e.*

LacdiNAc, has not yet been identified on *Drosophila* glycoproteins (Aoki et al., 2007; Koles et al., 2007; North et al., 2006), but it is abundantly present on *Drosophila* glycolipids (Seppo et al., 2000; Stolz et al., 2008a, b). However, sialylation has not been detected on *Drosophila* glycolipids (Seppo and Tiemeyer, 2000; Stolz et al., 2008a, b), which is consistent with the absence of in vitro activity of DSIAT towards embryonic glycolipid acceptors (Koles et al., 2004).

The apparent inconsistency of observations about the presence of sialylation in insect cells has previously led to the hypothesis that sialylation in *Drosophila* is a specialized process restricted to certain types of cells, possibly in a developmentally regulated manner (Angata and Varki, 2002; Marchal et al., 2001). The low level of sialylation detected in recent studies is also consistent with this hypothesis. Furthermore, it is supported by the data on the expression pattern of *DSIAT* and *DmCSAS* during development. The expression of both genes is not detectable early in embryogenesis, while they both become upregulated at later embryonic stages (Koles et al., 2004; Viswanathan et al., 2006). This late embryonic expression correlates with the development of the embryonic CNS, and *DSIAT* was previously shown to be specifically expressed there (Koles et al., 2004). To explore whether *DmCSAS* is expressed in the CNS, we assayed its pattern of embryonic expression by in situ hybridization and found that *DmCSAS* is also expressed within the developing CNS in a pattern similar to that of *DSIAT* (Chapter II and (Koles et al., 2009)). It has to be noted though that the spatial pattern of *DmCSAS* expression within the CNS appears to be somewhat different from that of *DSIAT*, and a more precise mapping of their expression awaits further

experimentation. Later in development, although detected in a broader domain of cells, the expression of *DSIAT* is still restricted to the CNS. The CNS-specific expression of *DSIAT* and *DmCSAS* during development suggests that the function of sialylation in *Drosophila* is mainly limited to the nervous system. This conclusion is consistent with locomotion abnormalities and defects in brain development of *Mgat1* mutant flies (Sarkar et al., 2006). These mutants are devoid of the activity of the *Drosophila* homologue of GlcNAcT-I involved in the biosynthesis of hybrid and complex type N-glycans, and thus *Mgat1* $^{-/-}$ flies presumably also lack any sialylated N-linked structures.

Humans, like other vertebrates, have two homologues of DSIAT, hST6Gal-I and hST6Gal-II, with hST6Gal-II being structurally more similar to DSIAT (Figure 3). Interestingly, hST6Gal-I is ubiquitously expressed in the organism, whereas hST6Gal-II has a more restricted pattern of expression with elevated levels in the fetal and adult brain (Krzewinski-Recchi et al., 2003a; Takashima et al., 2002b). In addition, both DSIAT and ST6Gal-II (but not ST6Gal-I) exhibit a peculiar substrate specificity revealed by *in vitro* assays, *i.e.* they both efficiently sialylate GalNAcβ1–4GlcNAc (LacdiNAc) termini (Koles et al., 2004; Rohfritsch et al., 2006). These structural and functional similarities between DSIAT and ST6Gal-II may reflect their common biological function, suggesting the existence of a conserved and ancient function for sialylation in a wide range of metazoan organisms, from *Drosophila* to humans.

NEUROMUSCULAR JUNCTION DEVELOPMENT AND MECHANISMS OF SYNAPTIC TRANSMISSION IN *DROSOPHILA MELANOGASTER*

Neuromuscular junction (NMJ) development and function has been extensively studied in flies during the last century. Simple neuromuscular architecture and well-studied NMJ signal propagation provided a good background for understanding how neuromusculuture drives movements.

During embryonic development 36 motor neurons derived from 15 to 30 neuroblasts extend axons along the main nerves (TN, SN and ISN) and defasciculate further to innervate corresponding muscle in each hemisegment (Landgraf and Thor, 2006) (Figure 4). Growth cones of these axons use different attractive signals (NCAM, IgG family molecules) and repulsive signals (Semaphorin family molecules) to initially select appropriate axon tracts. Targeting of the motor neuron to the specific muscle is then determined by complementary code of molecular cues (for example LIM homeobox genes) that enables a precise match during target recognition process. Different muscles also express unique combinations of cell surface glycoproteins (for example NCAM, Fasciclin III) which delineate the future site of the motor neuron entry into the myotube surface (Featherstone and Broadie, 2004).

When the motor neuron growth cone contacts the appropriate muscle target, the axon terminus transforms into branched presynaptic terminal with varicosities (also called as boutons), specialized sites of neurotransmitter release. The terminal size and its architectural complexity are determined by the degree of electrical activity.

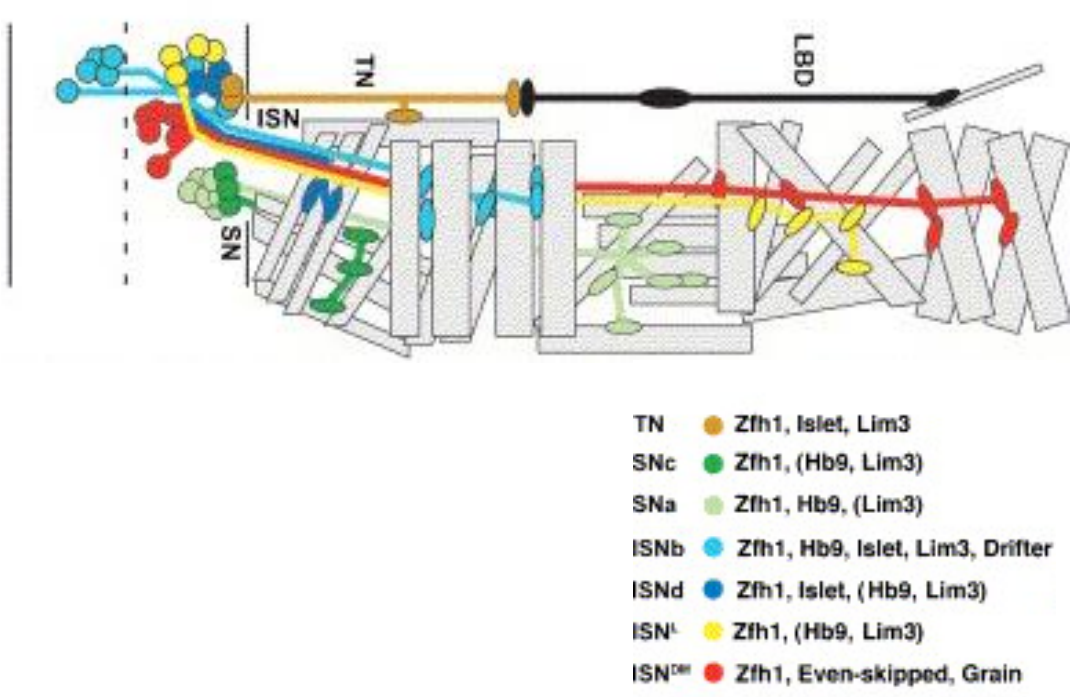

Figure 4. Specification of motor neurons and axonal projections in *Drosophila*. Diagrammatic representation of neuromuscular connectivity and motor neuron types. Based on the expression of even-skipped and Hb9 the ISN nerve can be further subdivided into ISN^{DM} (dorsal-most; red) and ISN^{L} (lateral; yellow). Genes are shown in parenthesis where their expression has not been unambiguously mapped to particular (sets of) motor neurons. One of the two motor neurons exiting in the TN appears to contact the lateral bipolar neuron (LBD), a specialized peripheral neuron that innervates the dorsal muscle. Modified from (Landgraf and Thor, 2006).

For example, hyperactive *eag* and *Sh* mutations (mutation in voltage-gated K^+ channel genes) lead to increased synaptic size and branch overgrowth (Budnik et al., 1990). It is also known that many other signaling molecules such as adhesion molecules, such as fasciclins and cell surface receptors such as Integrins can affect the structural complexity of *Drosophila* NMJ (Budnik, 1996; Otmakhov et al., 1997; Zhong and Shanley, 1995).

The pattern of connections is completely established by stage 16 of *Drosophila* embryogenesis and is maintained throughout larval life. There are three classes of motor neurons innervating *Drosophila* larval muscles: type I, II and III. Type I motor neurons extend thick terminal branches and use glutamate as the primary neurotransmitter to drive muscle contraction. They display enlarged type Ib (3-6 μm in diameter) and small type Is (1-3μm in diameter) NMJ boutons (Budnik et al., 1990). Type I boutons have typically dense pools of synaptic vesicles clustered around active zones (neurotransmitter release sites). Type II motor neurons have much longer and thinner branches with small (< 1 μm) synaptic boutons that use octopamine as a major neurotransmitter and serve modulatory function. Type III motor neurons are similar to type II neurons. They release peptide hormones to body muscles and are also thought to serve modulatory functions (Featherstone and Broadie, 2004).

As an example, longitudinal larval muscles 6 and 7 commonly used for NMJ functional and developmental studies are innervated by two motor neurons MN6/7-Ib and MNSNb/d-Is. These motor neurons typically contact both muscles in the central region of

the cleft between two muscles. MN6/7-Ib forms type Ib boutons and MNSNb/d-Is forms type Is boutons (Davis et al., 1997).

Dorsal longitudinal muscle 1 is innervated by three motor neurons: MN1-Ib, MNISN-Is and MNISN-II. A single type Ib axon (MN1-Ib) enters the synaptic site of muscle 1 on the proximal edge and then arborizes both anteriorly and posteriorly. The motor neuron axon MNISN-Is innervates muscle 1 at its proximal edge. Motor neuron MNISN-II within the ISN nerve branch innervates muscle 1 at its proximal edge and forms type II boutons (Hoang and Chiba, 2001).

Presynaptic mechanisms of neurotransmitter release from boutons are highly conserved among vertebrates and invertebrates. The process of communication between neurons can be broken into four major steps: propagation of action potential and arrival at the nerve terminal, release of neurotransmitters from presynaptic membrane into the synaptic cleft, recognition of neurotransmitters by receptors of the postsynaptic cell, inactivation of neurotransmitters, their further transport into the presynaptic cell and re-packaging.

The propagation of the action potentials along axons is the essential part of electrical signaling in the nervous system. It is dependent on movement of Na^+ and K^+ ions through voltage-gated sodium and potassium channels (Thackeray and Ganetzky, 1995). Initially, a neuron is at rest and it is more negatively charged from inside than from outside. Voltage-gated sodium channels (VGSC) abundantly present along the axon sense alteration of voltage by voltage sensors located within electric field. VGSC transits into the open state with Na^+ ions rushing inside the cell thus depolarizing the cell

membrane. When depolarization reaches a certain threshold the neuron fires an action potential. The action potential may have different size and/or shape in different neurons but it is always the same size within a single neuron. When the action potential reaches its maximum, voltage-gated potassium channels (VGPC) open and K^+ ions rush out of the neuron reversing depolarization. After that, channels close resulting in their inactivation and decay of sodium and potassium currents. During this refractory period, channels are unable to open again until the membrane returns to its resting state. Conductance of voltage-gated channels is restored only after membrane repolarization. In this case a sodium-potassium pump plays an important role in returning and maintaining the normal concentrations of ions across the neuronal membrane.

The second important step of synaptic transmission is arrival of the action potential at the synapse and release of neurotransmitters. When action potential arrives at the synapse Ca^{2+} flows in through N-type voltage-gated Ca^{2+} channels. Prior to Ca^{2+} ions entering the synapse, synaptic vesicles are held tight by the actin and cytoskeletal elements. Synapsin I, a synaptic vesicle protein is tightly bound to synaptic vesicle and actin filaments. It is phosphorylated by CaM kinase II upon Ca^{2+} influx, releases synaptic vesicles from the cytoskeleton and allows them to dock at the active zone sites. Following docking, vesicles fuse with the presynaptic membrane. Once vesicle fuses with the membrane it opens and empties neurotransmitters into synaptic cleft through the process called exocytosis. Finally, following exocytosis the nerve terminal recycles its vesicle pool for the new round of neurotransmitter release (Featherstone and Broadie, 2004).

Mutations in genes involved in the regulation of neural transmission often lead to temperature-sensitive paralytic phenotypes in *Drosophila*. Examples include: $para^{ts}$ mutants that are paralyzed at 30^0C (*para* - codes for voltage-gated Na^+ channel that is involved in action potential propagation), $shibire^{ts}$ mutants that are paralyzed at 28^0C (*shibire* codes for dynamin involved in synaptic vesicle recycling), cac^{ts2} mutants that are paralyzed at 38^0C (*cacophony* – codes for voltage-gated Ca^{2+} channel) etc.

The major attraction of the *Drosophila* NMJ is its simplicity and easy accessibility for electrophysiological and cell biological studies at the single cell level. Therefore we think that use of the *Drosophila* NMJ will serve a good model system to understand the role of DSIAT in neural transmission and CNS development of flies and higher organisms.

DISSERTATION OVERVIEW

To understand the role of sialylation in *Drosophila*, we focused our research on the *Drosophila* sialyltransferase gene, *DSIAT*, the first sialyltransferase gene discovered in the protostome lineage of organisms.

In Chapter II, I will characterize in detail DSIAT protein expression pattern at different developmental stages of *Drosophila*. Using a gene targeting approach, I will demonstrate that the expression of *DSIAT* is developmentally regulated and is restricted to motor neurons and cholinergic interneurons within the central nervous system of *Drosophila*. DSIAT expression is also detected in the projection neurons of the central brain and the medulla and lobula regions of the optic lobe in the adult fly.

In Chapter III, I will focus on detailed characterization of *DSIAT* mutant phenotypes. I detected several neurological phenotypes in *DSIAT* knockout mutants generated by gene targeting. *DSIAT* mutant larvae appeared to be sluggish and have abnormal neuromuscular junction (NMJ) morphology. Electrophysiological analysis of larval NMJs showed altered evoked NMJ activity. *DSIAT* knockout adult flies were found to be temperature sensitive and exhibit paralysis when exposed to higher temperatures. I also found that the *DSIAT* mutant phenotype strongly progresses with a fly age and performed longevity studies with *DSIAT* mutants.

In Chapter IV, I use genetic tools to identify potential sialylated targets in *Drosophila*. There are large numbers of proteins in vertebrates that are modified by sialyltransferases. I tested to see *Drosophila* counterparts of these proteins are sialylated in flies. The candidate targets are selected based on the genetic interaction studies and analysis of mutant phenotypes. Behavioral analysis shows that the *para* gene that encodes for *α*-subunit of voltage-gated sodium channel in *Drosophila* genetically interacts with *DSIAT*. Electrophysiological analysis of DSIAT mutant larvae shows their reduced sensitivity to tetrodotoxin (TTX), a pharmaceutical agent that strongly binds to the α-subunit of voltage-gated sodium channel, suggesting that PARA protein is a potential target for sialylation in flies.

CHAPTER II

CHARACTERIZATION OF *DROSOPHILA* SIALYLTRANSFERASE PROTEIN EXPRESSION PATTERN

INTRODUCTION

In vertebrates and bacteria, biosynthesis of sialylated glycoproteins involves several complex enzymatic reactions (see Figure 1). At the final step, the donor molecule, cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) is used by sialyltransferase enzyme for linkage-specific sialylation of glycoproteins.

The expression pattern of sialyltransferases strongly depends on acceptor specificity and type of linkage they synthesize between sialic acid and acceptor substrates (Harduin-Lepers et al., 2001; Koles et al., 2004). Additional level of complexity arises from various modification groups that these sugars can bear in different cells and species.

Earlier in situ hybridization data showed that *DSIAT* mRNA transcript is detected at late embryonic stages 16 and 17 within the ventral nerve cord and developing brain ((Koles et al., 2004) and Figure 5). Later in development, although detected in a broader domain of cells, the expression of *DSIAT* is still restricted to the CNS. In the adult head, *DSIAT* is expressed in a spatially restricted pattern in the optic lobes of the brain (Koles et al., 2009).

In order to understand the function of DSIAT we decided to investigate its expression pattern, by generating antibody for DSIAT protein. While the antibody could

visualize ectopically over expressed DSIAT, it was not useful for detecting endogenous protein (data not shown).

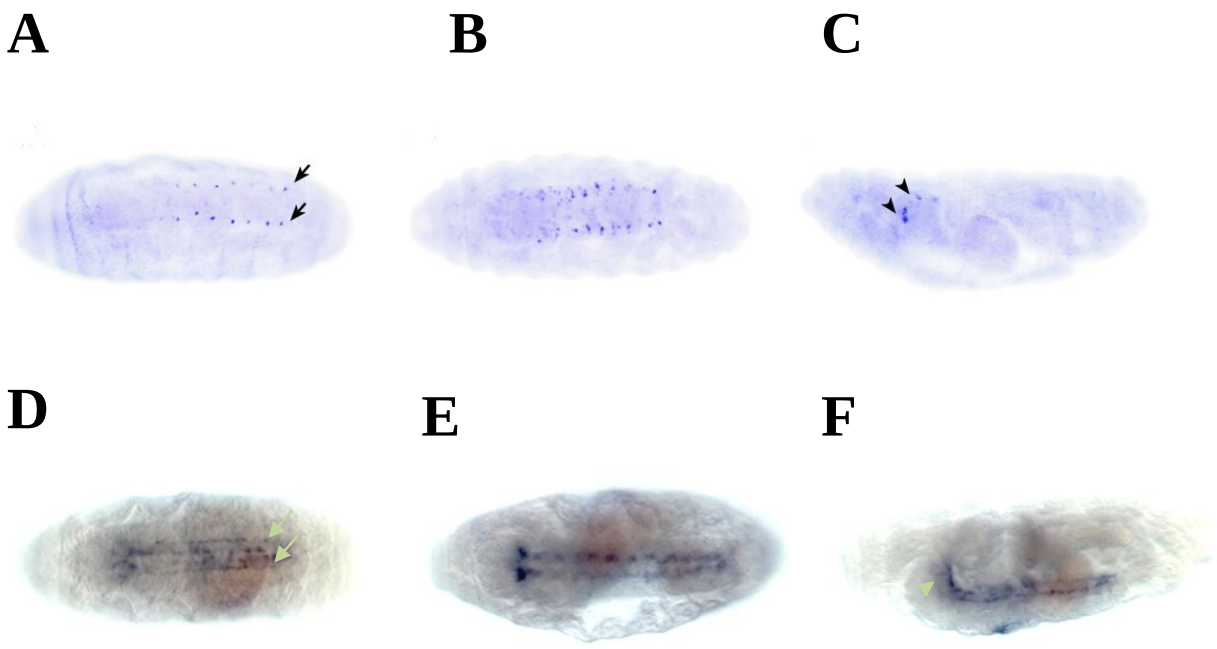

Figure 5. *DSIAT* and *DmCSAS* mRNA expression pattern at embryonic stages as revealed by in situ hybridization.
(A) Stage 16, ventral view. *DSIAT* is expressed in two rows of cells within the central nervous system (arrows).
(B) Stage 17, ventral view. More central nervous system cells begin to express *DSIAT*.
(C) Stage 17, lateral view. Expression of DSIAT in the embryonic brain (arrowheads).
(D) and (E) Stage 16, ventral view. *CMP-SAS synthetase* (*DmCSAS*) is expressed within ventral ganglion of the CNS (white arrows).
(F) Stage 16, lateral view. *CMP-SAS synthetase* is also expressed in the hemispheres of the CNS (white arrowheads).
In all images, anterior is to the left. (A), (B) and (C) are modified from (Koles et al., 2004)

Here we reveal the pattern of DSIAT protein expression by introducing a short sequence encoding two tandem HA epitopes into the wild type DSIAT locus. Not only have we determined the specific pattern of DSIAT expression, we also determined the identity of some of the DSIAT expressing neurons. Analysis of DSIAT pattern of expression brings us closer to understanding biological role of this enzyme in *Drosophila* and thus provides valuable data for functional importance of its vertebrate counterparts.

RESULTS

To understand the function of DSIAT during *Drosophila* development we decided to analyze the pattern of DSIAT expression during developmental stages.

Since earlier attempts to detect DSIAT protein in vivo using anti-DSIAT antibodies were not successful, we decided to use 'ends-in' targeting approach based on homologous recombination to introduce the HA-tag into the endogenous sialyltransferase locus and follow the expression of the tagged protein in vivo (Rong and Golic, 2001).

HA-tagged *DSIAT* construct has been designed in the way that HA-tag was placed in the stem region of the gene so that it doesn't interfere with the protein catalytic activity. Construct and targeting was accomplished according to (Rong and Golic, 2000). (See Figure 6 for schematic representation). Successful integration of *DSIAT-HA* construct in the *Drosophila* genome (fly line KI48) was verified by Southern blot analysis (Figure 7).

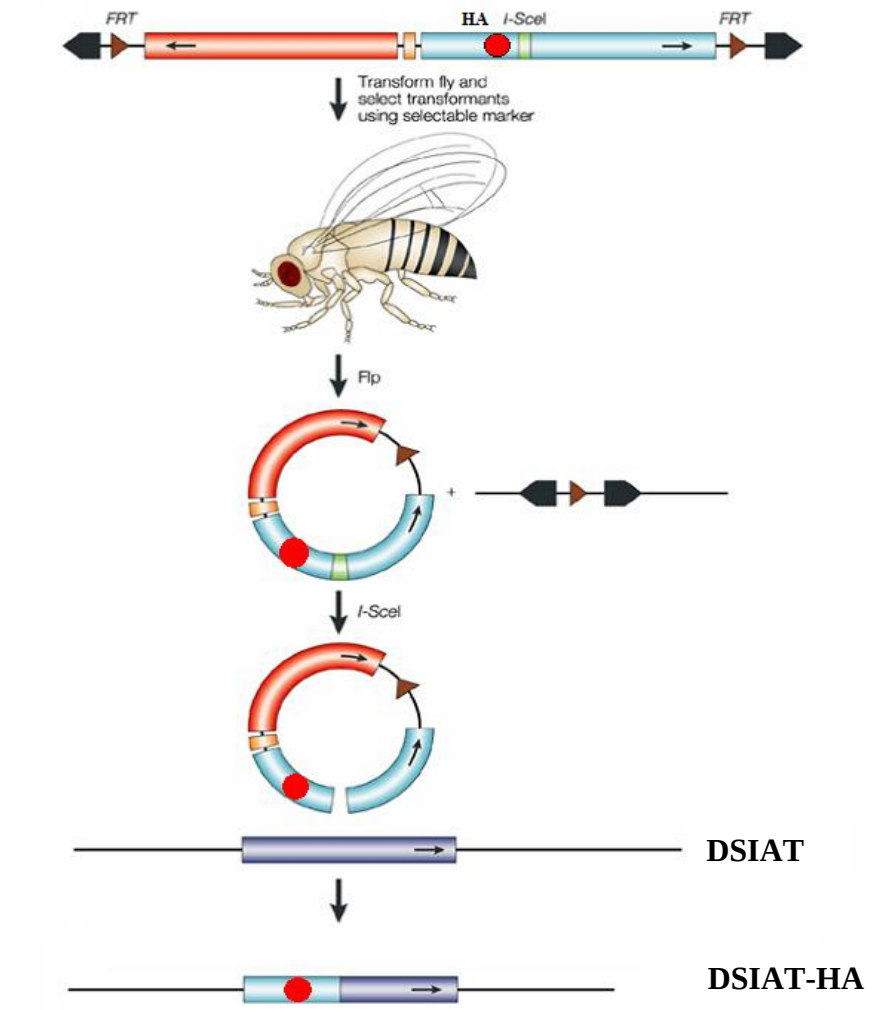

Figure 6. Gene targeting approach to introduce *HA*-tag into endogenous *DSIAT* locus. For generating *HA*-tagged version of DSIAT we used 'ends-in' targeting strategy. Briefly, we made vector-based donor construct including 8 kb *DSIAT* genomic region with *HA*-tag and I-SceI site in the middle of the *DSIAT* coding region. Construct was injected into flies and targeting events were induced after crossing transgenic flies carrying the donor construct to *70FLP 70I-SceI* flies with a heat-shock inducible flippase and I-SceI endonuclease. Analysis of several candidate knock-in lines revealed several targeting events with the duplication of 8 kb *DSIAT* gene region, with upstream copy including *HA*-tag. For the copy reduction step we used I-CreI mediated allelic substitution for one of the obtained targeted chromosomes and generated the *KI48* knock-in allele carrying *HA*-tag. Modified from (Adams and Sekelsky, 2002).

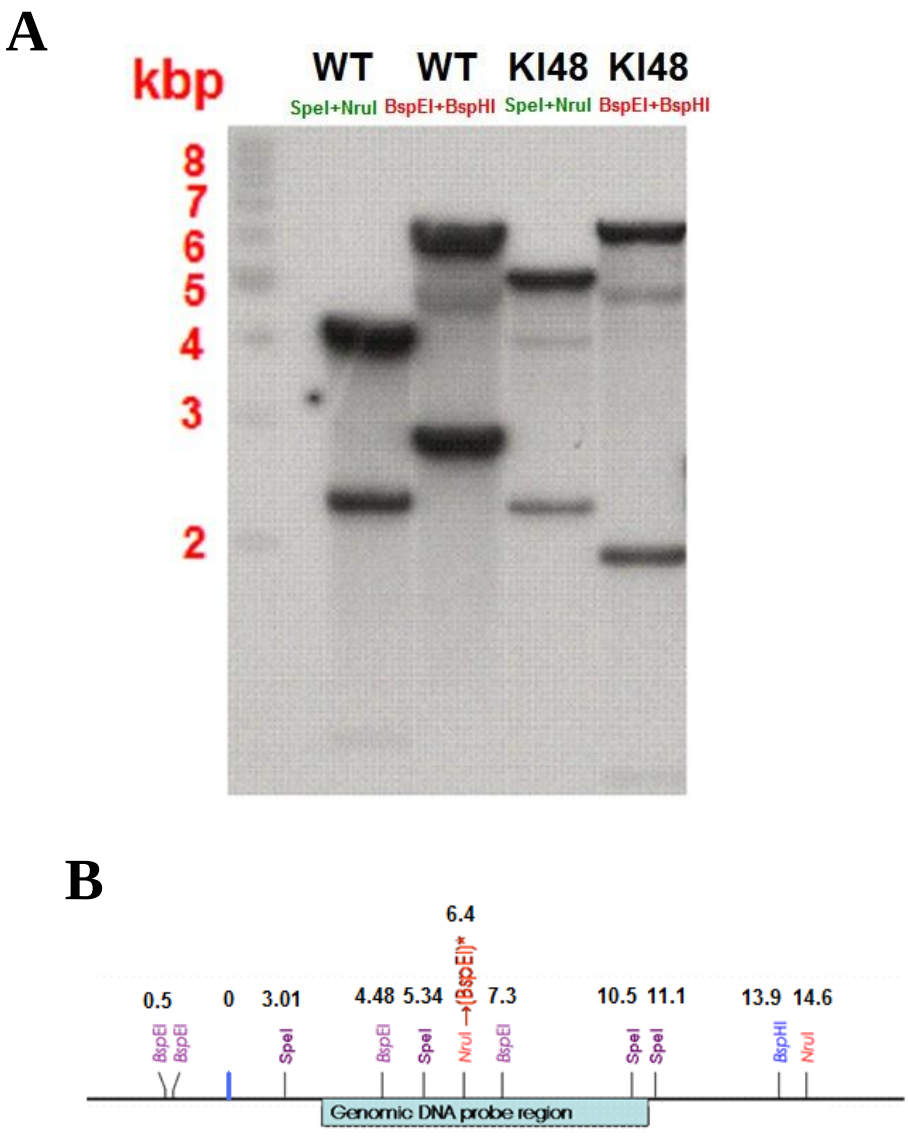

Figure 7. Verification of targeting.
(A) Genomic Southern blotting to verify targeted *DSIAT-HA*. Lane 1 contained SpeI- and NruI- double digested DNA isolated from wild type flies. Lane 2 contained BspEI- and BspHI- double digested DNA from wild type flies. Lane 3 contained SpeI- and NruI- double digested DNA from *KI48* fly line. Lane 4 contained BspeI- and BspHI- double digested DNA from *KI48* fly line. *NruI has been engineered into a BspEI site in *DSIAT-HA* knock-in flies.
(B) Schematic representation of expected DNA sizes produced by restriction enzymes of *DSIAT* and *DSIAT-HA* copies in wild type and knocked-in flies. Numbers indicate distance in kbp from marked 0 in the genomic region.

We found that embryonic expression of DSIAT protein is initiated at late stage 15 in two rows of cells (10±4 cells total) within the ventral ganglion, with the number of expressing cells approximately doubling at each of the following stages: 16 (20±4 cells) and 17 (40±8 cells). At first instar larval stage the number of cells expressing DSIAT-HA in the CNS reaches about 100 and approximately 400 in the third instar larval CNS (Figure 8). To determine the type of cells in the nervous system expressing DSIAT we did a double staining with cell specific markers: anti-ELAV (labels differentiated neuronal nuclei) and anti-REPO (differentiated glia cell marker). We found that DSIAT expressing cells are differentiated neurons and not glial cells (Figures 9, 10).

To determine type of neurons in the CNS expressing DSIAT, we performed double staining with a panel of different neuron-specific markers. Double staining for DSIAT-HA and anti-engrailed marker (labels early precursors for interneurons (Siegler and Jia, 1999)) showed that at embryonic stage none of the engrailed positive neuron expresses DSIAT (Figure 11). Similarly, double staining for DSIAT and anti-even skipped marker (labels early precursors for motor neurons and interneurons (McDonald et al., 1998)) showed no overlap between DSIAT and even-skipped expressing neurons (Figure 12).

We also used GAL4 lines to drive GFP expression in several types of neurons: DDC-GAL4 (drives expression in serotonergic and dopaminergic neurons), OK6-GAL4 and C380-Gal4 (drives expression in motor neurons (Sanyal et al., 2003), CHA-GAL4 (drives expression in cholinergic interneurons (Yasuyama and Salvaterra, 1999)).

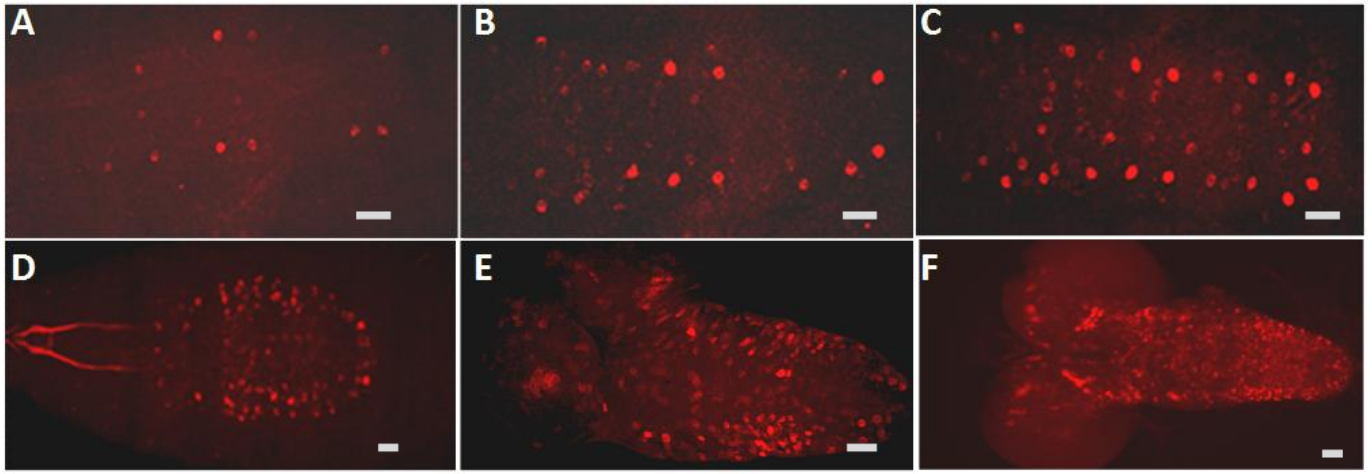

Figure 8. DSIAT stage specific expression in the CNS.
Embryonic and larval CNS in *KI48* transgenic fly line were stained with anti-HA antibody to reveal DSIAT expression.
(A) late stage 15 embryo, (B) stage 16 embryo, (C) stage 17 embryo, (D) first instar larva, (E) second instar larva, (F) third instar larva.
In all images, anterior is to the left. Embryonic and larval CNS was imaged at 20X magnification. Scale bar on every image corresponds to 20 μm.

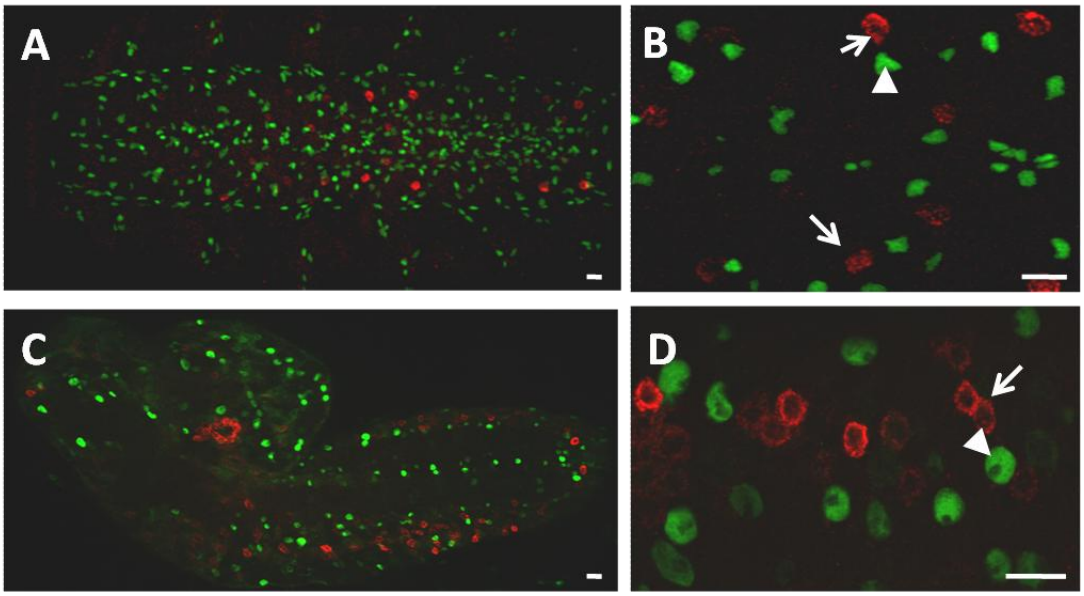

Figure 9. DSIAT is not expressed in glial cells.
Double staining of embryonic and larval CNS with anti-HA (red) and anti-REPO (green) antibodies to visualize DSIAT and REPO (glial cell marker) expression.
(A)Ventral view of stage 16 embryo (B) magnified dorsal medial section of the 16 stage embryonic CNS. (C) shows ventral view of second instar larval CNS, (D) shows magnified portion of the ventral ganglion of the larval CNS.
(A) and (C) Images were obtained using 20X objective magnification. (B) and (D) images were obtained using 63X objective magnification.
Arrows show DSIAT-expressing cells. Arrowheads label REPO-expressing (glial) cells.
In all images anterior is to the left. Scale bar corresponds to 20 μm.

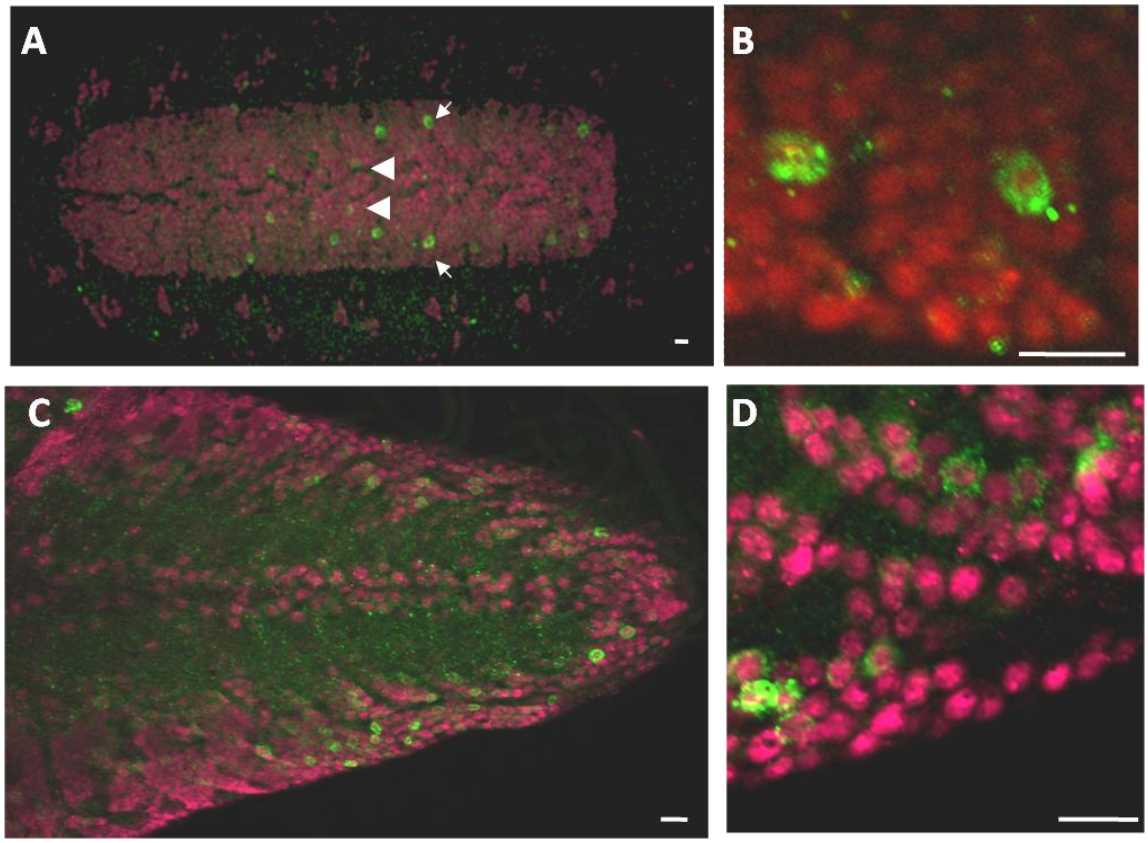

Figure 10. DSIAT is expressed in differentiated neurons.
Double staining of the embryonic and larval CNS with anti-HA (green) and anti-ELAV (red) antibodies to visualize DSIAT and ELAV (differentiated neuron marker) expression.
(A) Ventral view of stage 16 embryo. The peripheral DSIAT expressing neurons (arrows) lie most ventrally in the CNS. The middle rows of DSIAT expressing neurons (arrowhead) lie more dorsally relative to peripheral neurons.
(B) Magnified lateral section of the 16 stage embryonic CNS. Image was obtained using 40X objective magnification.
(C) Dorsal view of the third instar larval CNS. (D) Magnified portion of the lateral section of ventral ganglion of third instar larval brain.
(A) and (C) images were obtained using 20X objective magnification. (B) and (D) images were obtained using 40X objective magnification.
Scale bar corresponds to 20 μm.

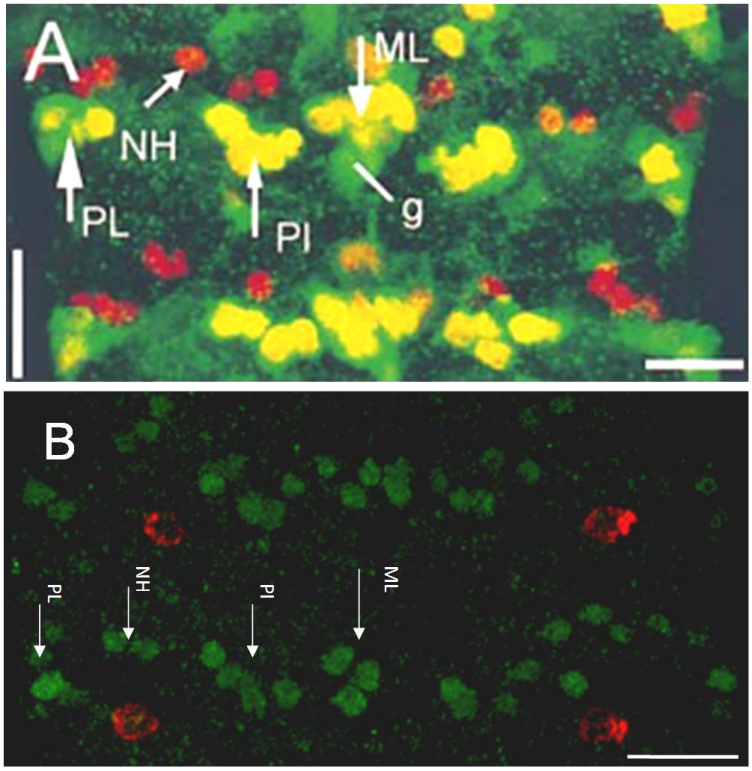

Figure 11. DSIAT is not expressed in engrailed-positive cells at embryonic stage.
Engrailed is expressed in subset of interneurons but not motor neurons of the CNS. Region shown in (A) and (B) comprises anteroposterior distance of two neuromeres with anterior at the top.
(A) At embryonic stage 16 *engrailed* expression (green) occurs in two bilateral groups of neurons at the posterior of each hemineuromere, PL and PI, in the midline (ML) neurons, NH neurons and some glia (g). (Modified from (Siegler and Jia, 1999)).
(B) DSIAT expressing cells (red) do not overlap with engrailed positive cells (green) at embryonic stage. ML neurons appear most ventrally in the nerve cord. PI neurons lie more dorsally to ML plane. Sialyltransferase expressing neurons lie in the same plane as PI engrailed positive neurons. Scale bar corresponds to 30 μm.

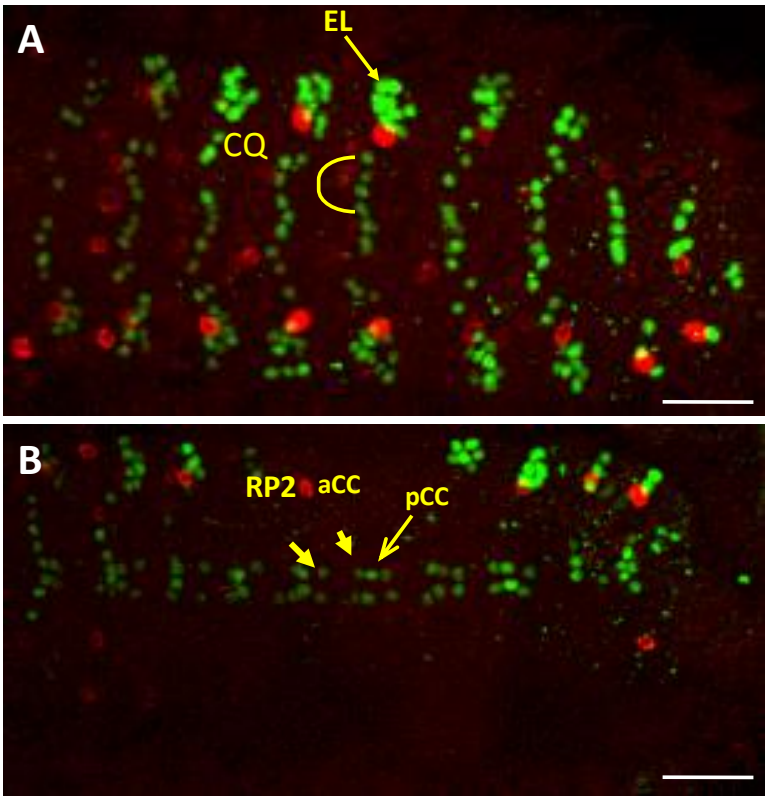

Figure 12. DSIAT is not expressed in even-skipped positive neurons at embryonic stage. Two different focal planes of the same 16 stage embryo stained with anti-Eve (green) and anti-HA (red) are shown in (A) and (B). (A) depicts ventral plane of the embryonic CNS. The distal rows of DSIAT expressing cells in (A) lie in the same focal plane as EL cluster of interneurons and CQ cluster of motor neurons.
(B) shows dorsal plane of the embryonic CNS with expression of pcc interneuron and two motor neurons RP2 and aCC that projects their axons to dorsal oblique muscle 2 and 1 correspondingly. Clearly, none of the DSIAT expressing cells express *even-skipped* at this stage. Anterior is to the left. Scale bar corresponds to 30 μm.

By double immunolabeling of the third instar larval brains we found that a large portion of DSIAT expressing neurons are cholinergic interneurons (Figure 13B). Some DSIAT expressing neurons are motor neurons (Figure 13A). None of the dopaminergic or serotonergic neurons expresses sialyltransferase (Figure 13C). Certain number of neurons expressing sialyltransferase still remains of unknown identity.

We found that DSIAT is expressed in the hemisegmentally-repeated pattern in a group of dorsomedial motor neurons in the third instar ventral ganglion. We performed an immunolabeling of glutamatergic motor neurons with anti-DVGLUT antibody and with anti-HA antibody to visualize DSIAT expressing neurons. The two dorsomedial motor neurons with most prominent DSIAT expression were identified as MN1-Ib and MN30-Ib (see (Choi et al., 2004), Figure 14 and Table 2). We also confirmed that the most posterior motor neuron in this cluster of 5 motor neurons is MN1-Ib motor neuron and has aCC embryonic identity by labeling it with RKK-eve-Gal4; UAS-CD8-GFP (Figure 14D1). The RKK even-skipped (eve)-GAL4 driver is expressed in 1–2 identified motor neurons per hemisegment, and specifically is expressed in the aCC motor neuron which innervates dorsal longitudinal muscle 1 via a type Ib NMJ (Featherstone et al., 2000; Renden et al., 2001). We couldn't detect DSIAT in the axonal processes or the NMJ synapses in KI48 line by anti-HA staining.

We also analyzed the expression of DSIAT at the pupal stage (This work was performed by Dr. Kate Koles – a postdoctoral researcher in the lab). We found that early pupal brain expresses DSIAT at even higher levels than third instar larval brains, but the expression is significantly diminished by the late pupae stage.

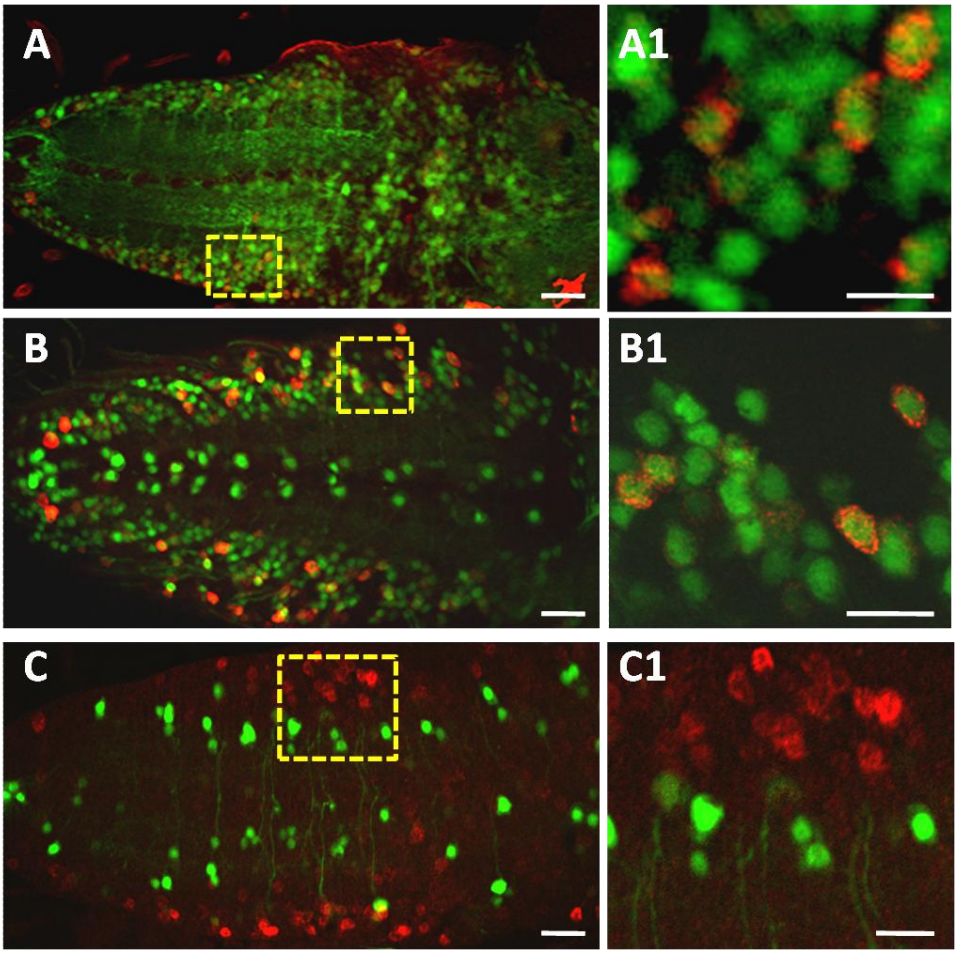

Figure 13. DSIAT is expressed in many cholinergic interneurons and motor neurons, but is absent from dopaminergic and serotonergic interneurons.
(A) OK6-Gal4 expresses in the CNS motor neurons (green) of the larval ventral nerve cord. Inset (A1) shows close up of (A) where some DSIAT expressing cells are co-localized with GFP-labeled motor neurons.
(B) Cha-Gal4 expresses in the CNS interneurons (green) of the larval ventral nerve cord. Inset (B1) shows close up of (B) with DSIAT expressing cells co-localized with GFP-labeled cholinergic interneurons.
(C) DDC-Gal4 expresses in serotonergic and dopaminergic interneurons (green) of the larval ventral nerve cord. Inset (C1) shows close up of (C) where DSIAT expressing cells do not co-localize with serotonin or dopamine expressing cells.
Anterior is to the right on all images. Images (A), (A1), (B) and (C) were taken at 20X magnification. Scale bar, 20 µm. (C1) was taken at 40X magnification. Image (B1) was taken at 63X magnification. Scale bar for (A1), (B1) and (C1), 20 µm.

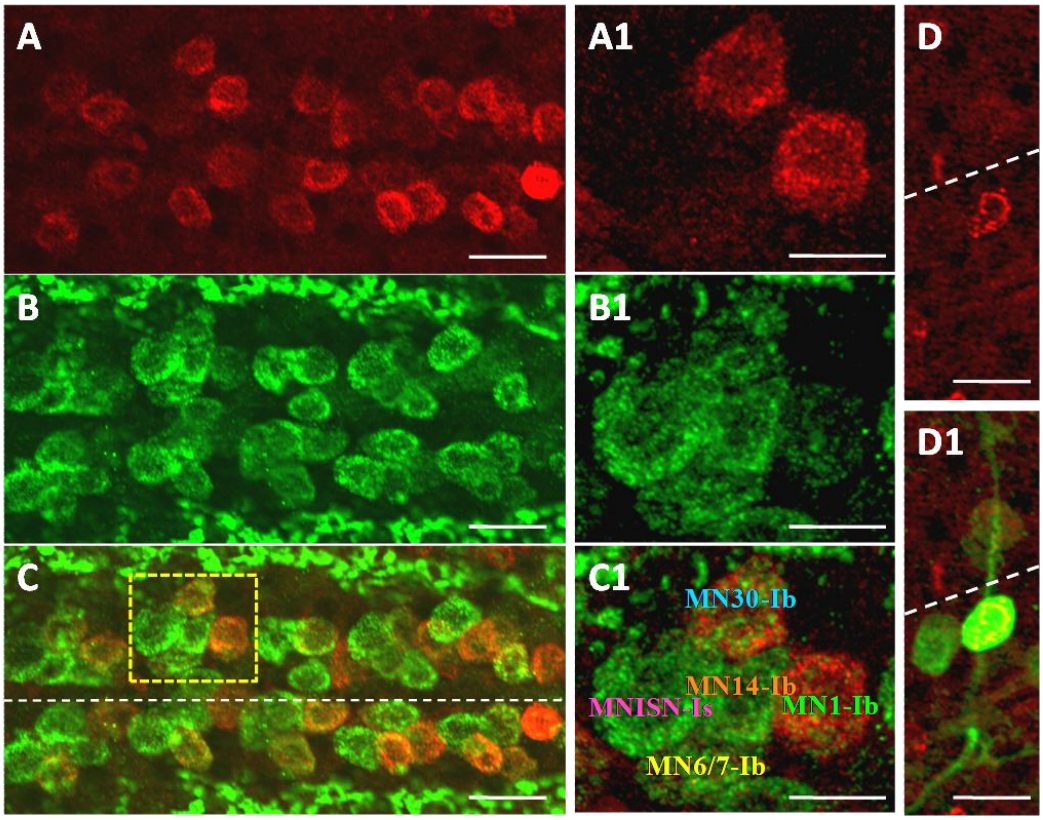

Figure 14. DSIAT is expressed in dorsomedial motor neurons in the larval ventral ganglion.
(A) and (B) show 40X view of abdominal segments of a 3rd instar ventral ganglion stained for DSIAT-HA (red) and DVGLUT (green). Motor neuron clusters are seen in every segment. (C) shows 40X overlay of red (A) and green (B) channels. The CNS midline is denoted by the dotted line. (A1) and (B1) show 63X single channel image close up of the 4th abdominal segmental cluster highlighted with yellow dotted line on (C).
(C1) shows close up of 63X overlay of (A1) and (B1) with labeled motor neuron identity according to (Choi et al., 2004). In 5-motor neuron cluster the most lateral DSIAT expressing neuron belongs to MN30-Ib motor neuron and the most posterior DSIAT expressing neuron belongs to MN1-Ib motor neuron.
(D) shows 40X image of DSIAT-expressing MN1-Ib motor neuron. (D1) shows 40X image of MN1-Ib motor neuron expressing mCD8-GFP under control of RKK-Gal4. Motor neuron MN1-Ib as visualized by GFP staining and dye injection (Choi et al., 2004) makes ipsilateral projection as well as dendritic arborizations concentrated around axonal tract. On all images, anterior is on the left. Scale bar on (A), (B), (C), (D) and (D1), 20 μm. Scale bar on (A1), (B1), (C1), 10 μm.

Table 2	Morphology of the third instar larval dorsomedial motor neurons (modified from Choi et al., 2004)				
Third instar motor neuron	Embryonic identity	Nerve	Target muscles	Projections	DSIAT positive
MN1-Ib	aCC	ISN	1	Ipsilateral axon, major contralateral process	yes
MNISN-Is	RP2	ISN	1,2,3,4,10, (18), 19, 20	Ipsilateral axon	no
MN14-Ib	RP1 or RP4	SN	14	Contralateral axon	no
MN6/7-Ib	RP3	SN	6/7	Contralateral axon, major ipsilateral process	no
MN30-Ib	RP1 or RP4?	SN	30	Contralateral axon	yes

Central brain and optic lobes still had detectable levels of DSIAT expression, while there were only a few DSIAT positive cells found in the thoracic ganglion. Staining of the dissected adult brains of one day old KI48 flies showed elevated DSIAT expression in the central brain and also lobula and medulla regions of the optic lobe. A few DSIAT expressing cells have been found in the adult thoracic ganglion (data not shown).

The DSIAT-HA staining reduced drastically with the age of the flies and disappeared almost completely by 6th day after fly eclosion.

DISCUSSION

Studies on *Drosophila* sialylation provide some evidence for the presence of a sialyltransferase enzyme that functions in a developmentally-regulated and tissue-specific manner (see INTRODUCTION). In this chapter we have attempted to answer some important questions: what is the pattern of DSIAT expression? What type of tissue and cell expresses DSIAT? Answers to these questions can give us an idea about biological function of *Drosophila* sialyltransferase that can shed light on the function of its human counterparts.

Analysis of the HA-tagged sialyltransferase protein expression pattern shows that it first appears in the brain and ventral nerve cord at late embryonic stage 15. The level of expression significantly increases in the hemispheres and ventral ganglion during the larval stages. By the adult stage pattern of DSIAT expression changes drastically. In the young, one day old adult fly, DSIAT expression is very high in the optic lobes and central brain, while only 4-8 cells in the thoracic ganglion continue to express DSIAT. The level of expression strongly decreases every day and is almost abolished in 5 day old fly brains. The CNS-specific expression of DSIAT during development suggests that the function of sialylation in *Drosophila* is mainly limited to the nervous system. We observed attenuation of level of DSIAT expression during adult stages and it complete disappearance by 6th day after fly eclosion. We think there could be two explanations for this. First, DSIAT might only be necessary for the early development of neurons or neuronal network and is not required for the matured ones, and therefore its expression decreases with fly age. Second, the expression of DSIAT is high during the first days of

fly development, when neuronal synaptic connections are constantly formed and neuronal network is built. With age, DSIAT expression diminishes and is maintained at low undetectable with antibodies level for the rest of fly life. We will discuss this issue in more details in Chapter V.

Three primary cells types in the Drosophila CNS include: motor neurons, interneurons and glia. Using antibodies anti-ELAV and anti-REPO for embryonic and larval CNS we found that DSIAT expressing cells are all differentiated neurons and not glial cells. By double immunolabeling for DSIAT and some known neuronal cell specific markers we found that the majority of DSIAT expressing cells are motor neurons and cholinergic interneurons.

Motor neurons in *Drosophila* are glutamatergic and they project their axons via segmental nerves outside CNS to the corresponding muscle. The release of glutamate neurotransmitters into the synaptic cleft causes muscle contractions and results into coordinated larval locomotion. Expression of sialyltransferase in the *Drosophila* motor neurons suggests that it might be required for motor neuron development and/or functioning.

We also determined the identity of two of the dorsal motor neurons where DSIAT is expressed. One of those motor neurons (MNI-Ib) innervates larval dorsal longitudinal muscle 1, the other one (MN30-Ib) innervates ventral longitudinal muscle 30 (Figure 14). This result will help us further answer the following questions: how elimination of DSIAT activity affects neuronal and synaptic transmission of this particular motor axon, and how the disruption of DSIAT expression changes the morphology of the axon

terminals. Using electrophysiological techniques and morphological analysis we will elaborate these questions in Chapter III.

It is worth mentioning that we observed HA-tagged version of the sialyltransferase expression solely in the Golgi compartment of the neuronal soma. Interestingly, flies containing knocked-in HA-tagged version of DSIAT exhibited locomotion problems and had temperature-sensitive paralysis similar to the *DSIAT* knockout flies (see Chapter III). We think that even though DSIAT-HA knocked-in fly CNS reflects endogenous pattern of DSIAT expression, HA-tag inserted into the stem region hinders DSIAT transport into axons from neuronal soma (data not shown). Indeed, overexpression of DSIAT with the HA-tag inserted at the catalytic domain not only gives the complete rescue of the mutant locomotion and paralytic phenotypes, but also shows DSIAT transport into the axons and to some sites of motor neuronal synaptic connections. However, this hypothesis needs to be further elaborated.

Temporal and spatial pattern of DSIAT expression at different developmental stages of *Drosophila* suggests that DSIAT plays a role in nervous system development and functioning. To support this statement I will further address the question: what fly phenotypes are caused by the loss of DSIAT activity? In Chapter III I will give the detailed characterization of the neurological phenotypes identified in DSIAT mutants.

MATERIALS AND METHODS

Drosophila *strains and maintenance*

Drosophila stocks were cultured in standard corn syrup supplemented with soy flour. All flies unless specified were kept at room temperature (23^0C). For DSIAT pattern characterization the following fly strains were obtained from Bloomington Stock center: Cha-Gal4, OK6-Gal4, C380-Gal4. DDC-Gal4 was provided by J. Hirsh (Li et al., 2000). RKK-eve-Gal4 was provided by David Featherstone (Featherstone et al., 2000).

Ends-in targeting crosses and statistics

Mobilization of the targeting construct on the second chromosome was accomplished according to (Rong and Golic, 2000). Males carrying constructs P[DSIAT-HA] on X and 3rd chromosome were crossed independently to virgin females w^-; +/+; hsFLP, I-SceI/ Tm6, Ser. 3 day-old progeny was heat-shocked at 38^0C for 1 hour. Virgin mosaic females from these crosses (+/2HA; +/+; hsFLP, ISceI/+ and w^-; +/+; hsFLP, I-SceI/2HA-DSIAT) were collected and crossed with w^-; FLP/FLP;+/+ males. Adults were discarded and the progeny was screened for red eye flies. Red eyed males were then selected and crossed individually to Bl/CyO to establish lines. Red-eyed non CyO progeny was screened for targeting.

Total number of screened flies was 31,209. Out of them 50 red eye flies have been selected and only 12 of them had targeted DSIAT-HA construct.

I-CreI reduction to single allele

Reduction was carried out using the following cross: virgin females $w^{-/-}$;KI-DSIAT-HA;+/+ were crossed to males $w^{-/-}$; +/CyO;70I-CreI, Sb;+. After 3 days, parents were removed and bottles were heat-shocked at 36^0C for 1 hour. 10 Individual white eye males from each cross $w^{-/-}$;KI-DSIAT-2HA/CyO; Sb/+ were crossed to $w^{-/-}$; Bl/CyO virgin females. Individual males with reduced DSIAT-2HA copy $w^{-/-}$; KI-DSIAT-2HA (reduced)/CyO were crossed again to virgin Bl/CyO females to establish fly lines. Non-CyO flies were crossed to each other for homozygous stocks.

Verification of targeting by Southern blot analysis

To identify fly strains that contains targeted constructs Southern blot analysis of fly genomic DNA was performed according to (Sambrook et al., 1989).

Immunohistochemistry

For embryonic staining, embryos were collected on apple juice plates. Embryos were then dechorinated by 50% bleach in distilled water for 2 minutes. Then after rinse with distilled water, embryos were fixed in 4% paraformaldehyde solution by vigorous shaking for 20 minutes followed by rinses in methanol. Embryos were stored in methanol at -20^0C overnight prior to rehydration in PBT (1X PBS, 0.1% Tween-20). After 1 hour washes in PBT, embryos were blocked for 1hr in 5% goat or donkey serum (which is chosen according to secondary antibody). Incubation with primary antibodies in 5 % goat/donkey serum was as follows: rat anti-HA (1:400) (Roche), mouse anti-HA (1:500)

(Covance), rat anti-ELAV (1:00) (Developmental Studies Hybridoma Bank University of Iowa, Iowa City, IA), mouse anti-REPO (1:5) (Developmental Studies Hybridoma Bank University of Iowa, Iowa City, IA), mouse anti-even skipped (1:20) (gift from N. Patel), mouse anti-engrailed (1:300) (Developmental Studies Hybridoma Bank University of Iowa, Iowa City, IA).

Immunostaining for DSIAT larval CNS was performed according to (Sullivan, 2008). Briefly, larval brains were dissected in Ringers solution and fixed in 4% paraformaldehyde followed by night incubation at -20^{0}C in methanol.

For larval brain staining the following primary antibodies were used: rat anti-HA (1:1000) (Roche), rabbit anti-DVGLUT (1:4000) (gift from A. DiAntonio), rat anti-Elav (1:100) (Developmental Studies Hybridoma Bank University of Iowa, Iowa City, IA), mouse anti-Repo (1:5) (Developmental Studies Hybridoma Bank University of Iowa, Iowa City, IA).

Both for embryonic and larval CNS, following incubation with primary antibodies, tissues were rinsed in PBT four times (15 minutes each wash), and incubated in 5% goat/donkey serum in PBT and corresponding secondary antibody for 1 hr 30 min. The following secondary antibodies were used: Alexa anti-mouse 488 (1:150), Alexa anti-rat 488 (1:150), anti-mouse Cy3 (1:250) (Jackson laboratories), anti-rat Cy3 (1:250) (Jackson laboratories). After staining embryos and larval CNS were mounted with VectaShield (Vector Laboratories) for visualization. Images were captured on a Zeiss Axioscope microscope using a 20X and 63X objectives.

CHAPTER III

CHARACTERIZATION OF *DROSOPHILA* SIALYLTRANSFERASE MUTANT PHENOTYPES

INTRODUCTION

Biological expression and role of sialyltransferases varies quite a lot. Studies in mammals have shown that mutations in sialyltransferases lead to developmental problems, tissue degeneration, malignancy transformation etc. For example, mutation in lactosylceramide α2,3 sialyltransferase, hST3GalV (GM3 synthase) involved in the nervous system lipid glycosylation in humans leads to seizures at early age and diffuse brain atrophy at older age (Jaeken and Matthijs, 2007). ST3GalV knockout mice showed increased sensitivity to insulin due to enhanced insulin receptor phosphorylation in the skeletal muscle (Yamashita et al., 2003). ST8SiaII and ST8SiaIV polysialyltransferases are involved in the biosynthesis of polysialic acids. ST8Sia II is involved in memory and behavioral processes formation. ST8SiaII knockout mice exhibit misguidance of infrapyramidal mossy fibers and formation of ectopic synapses in the hippocampus (Takashima, 2008). ST8SiaIV knockout mice show impaired synaptic plasticity, suggesting important role of this glycosyltransferase in this neuronal process (Eckhardt et al., 2000).

Overexpression of ST6Gal I has been reported to occur in several human malignancies; several clinical and experimental studies suggest a positive correlation between high levels ST6Gal I and invasive behavior of cancer cells (Chiricolo et al.,

2006). In addition, ST6Gal I knockout mice showed severe immunosuppression phenotypes, such as reduced serum IgM levels, impaired T and B cell function (Hennet et al., 1998).

Drosophila sialyltransferase is highly homologous to hST6Gal II sialyltransferase (hST6Gal family sialyltransferases). It has been noted earlier that hST6Gal II is highly expressed in fetal brain. No mutant phenotypes associated with mutation in *hST6Gal II* have been reported so far and biological functions of hST6Gal II have not been elucidated.

Obtaining and characterizing a gene's mutant phenotype is an important step towards understanding the protein function. However difficultly of genetic manipulation with mammalian organisms presents a real challenge in uncovering the gene function. The *Drosophila* model organism has been used for genetic studies for almost a century. It is a very powerful tool for elucidating functions of novel genes based on its advanced genetic approaches, complete genome sequence and well-characterized development. The possibility to apply RNA interference and 'reverse' genetics techniques to generate knockout flies makes *Drosophila* an even more attractive model organism to understand the function of DSIAT and its human counterpart, hST6Gal II.

We used 'reverse genetics' technique approach using engineered construct to mutate *DSIAT*. In contrast to traditional X-ray or EMS mutagenesis it allows obtaining mutants with the predicted lesion. Using gene targeting approach (Rong and Golic, 2000), two different DSIAT knockout fly lines were previously generated in our lab. The first DSIAT mutant allele (*s23*) was engineered by introducing two stop-codons within

DSIAT open reading frame using ‘ends-in’ gene targeting approach. The first stop codon should lead to premature translation termination after 17 amino acids of DSIAT protein. Alternative initiation of translation that may occur downstream of this stop codon should not produce an active protein because of lack of signal sequence. The downstream stop codon should result in sialyltransferase protein truncated in the middle of S-sialylmotif. But this protein should be inactive (Datta et al., 2001). Successful integration of donor construct into the *DSIAT* genomic region was confirmed by Southern blot analysis (data not shown).

The second DSIAT mutant allele (*L22*) was engineered by introducing deletion within DSIAT open reading frame using ‘ends-out’ gene targeting approach. This induced targeting event was also confirmed by Southern blot analysis.

In this Chapter I will present characterization of DSIAT adult mutant behavioral phenotypes. I will also focus on characterization of DSIAT mutant phenotypes at larval stage of *Drosophila* development. Because we found that DSIAT is expressed in motor neurons in the developing CNS, special attention will be given to characterization of electrophysiological characteristics of developing neuromuscular junctions in the *Drosophila* larvae.

RESULTS

Based on DSIAT expression pattern, we hypothesized that DSIAT has a specialized function in the nervous system. Interestingly enough, DSIAT mutant flies (line *s23* and *L22*) were homozygous viable and did not show any developmental and morphological abnormalities. However, we noticed that mutant larvae and adults appear more sluggish and uncoordinated than wild type flies.

We found that *DSIAT* mutant larvae crawl much more slowly than the wild type. To characterize this, we compared mutant and wild type larval crawling in locomotion test modified from (Roberts, 1998). In addition to slower crawling, we noticed that the pattern of crawling of DSIAT mutant larvae is somewhat different from the wild type larvae. We recorded the tracks of wild type and DSIAT mutant third instar larvae that were placed in the middle of the agar plate and allowed to crawl for 30 seconds.

Summarizing our observations, wild type larvae prefer to crawl straight from the middle to the edge of the Petri dish, occasionally making sharp turns and finally tries to escape the dish. DSIAT mutant larvae look uncoordinated, frequently pause, crawl back and make lots of turns. We manually digitized the tracks of twenty wild type and twenty DSIAT mutant larvae travelled from the center of the agar plate for 30 seconds (Figure 15). There was a striking difference in crawling patterns between the genotypes. We couldn't rescue this phenotype either with genomic copy of P-element inserted DSIAT, or with UAS-Gal4 system using neuronal specific drivers. The observed phenotype was confirmed for two different DSIAT mutant alleles placed into two independent genetic backgrounds.

Then we also measured mutant and wild-type larva speed, number of contractions and distance crawled in one contraction on a linear path. On an agar plate *DSIAT* mutants travel shorter distance per contraction (Figure 16C) and have less number of contractions per second than the wild type larva (Figure 16B). We completely rescued these phenotypes using genomic wild type copy of DSIAT on a separate chromosome.

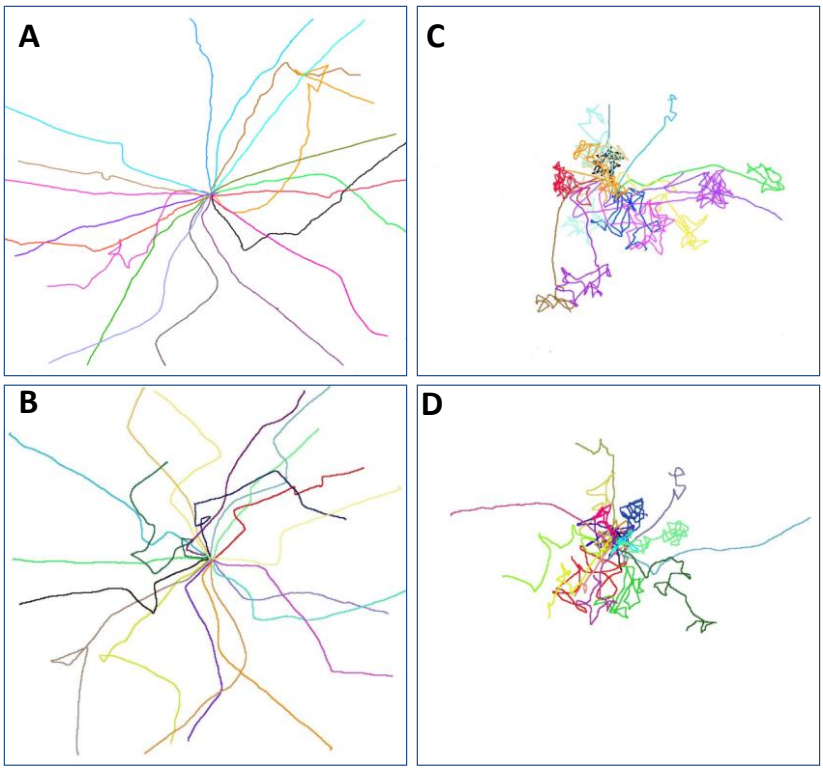

Figure 15. Representative crawling patterns of *Drosophila* wild type and *DSIAT* mutant larvae at wandering stage.
Figure (A)-(D) depict outlines of 20 different wild type larval tracks. Larval movement on agar plate was captured by 30 second video recording and manually digitized by tracing larvae head. The following genotypes were tested: (A) CS (wild type), (B) *s23* (DSIAT mutant in Canton S background), (C) *yw* (wild type) (D) *L22* (DSIAT mutant in *yw* background).

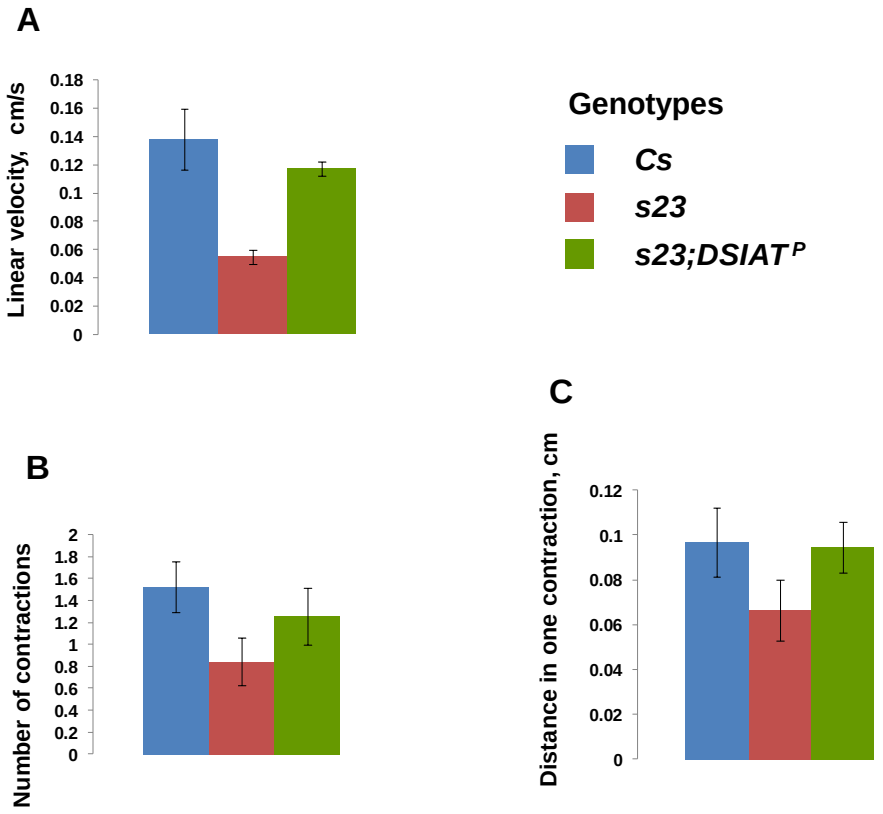

Figure 16. Analysis of DSIAT mutant and wild type larva locomotor defects. Larval movement on agar plate was captured by 30 second video recording for wild type (*Cs*), DSIAT mutant (*s23*) and rescue (*s23;DSIAT* ^{P}) larvae. (A)-(C) shows velocity, number of contractions and distance traveled in one contraction on a linear path, calculated for used genotypes. *DSIAT* mutant larvae exhibit 40 to 50% reduction in measured characteristics compared to wild type larvae. All assayed larvae had the same Canton S genetic background to minimize its potential influence on the behavioral characteristics. Values shown are mean ±standard deviation. The number of larvae assayed for each data point was 20. For wild type and mutant larvae the difference between compared phenotypes is statistically significant (t-test, $p < 0.001$). For wild type and rescue larvae the difference between compared phenotypes is statistically insignificant (t-test, $p > 0.05$).

We examined muscle morphology of DSIAT mutants for notable muscle defects. Overall the muscles 6, 7 (most widely studied in *Drosophila* larvae) and 1 (muscle, innervated by axon, which soma expresses DSIAT) morphologically looked normal in the mutants.

We performed thermotaxis and phototaxis assay on DSIAT mutant and wild type larvae and found that there is no difference in sensory efficacy to heat/cold or light stimuli (data not shown).

To elucidate the biological function of DSIAT, we also characterized the phenotypes of *DSIAT* mutants at adult stage. These flies were homozygous viable and fertile without noticeable morphological defects. However, they appeared less healthy than wild type genotypes, and they were rare in balanced heterozygous stocks, indicating that homozygous mutants have decreased viability during development. In addition, the adult mutants had significantly decreased longevity. In both individual and group assays, the longevity of mutants was decreased by ~40% as compared to controls, wild type and rescue genotypes (Figure 17).

Since DSIAT mutants, compared to wild type flies, were noticeably sluggish, we decided to characterize their locomotor abilities using behavioral assays. We compared performance of adult flies in climbing and righting assays. Geotaxis tests showed that *DSIAT* mutants take longer time to climb a 6 cm line in a vertical tube after brief mechanical agitation (Figure 18B). In a righting assay, *DSIAT* mutants were not able to right themselves as fast as wild-type flies (Figure 18A).

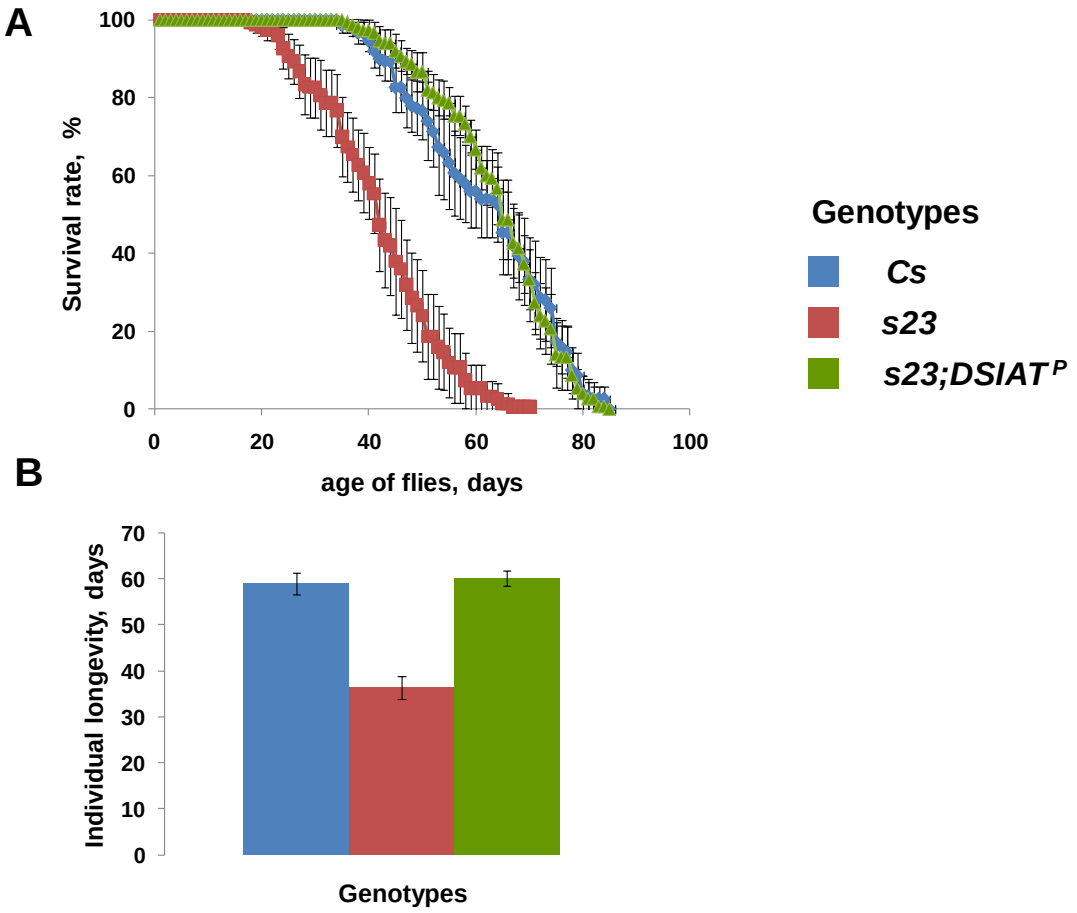

Figure 17. Male population and individual longevity.
(A) shows percent survivorship of population consisting of 10 male *Drosophila* in a vial. At least 20 vials were set up for each assay giving population size of 200.
(B) shows average lifespan for individual males of the assayed genotypes.
For both assays vials were stored horizontally and flies were transferred every three days. There is a drastic reduction of population and individual fly longevity in DSIAT mutants compared to wild type. The mean life span for the population assay reached 65 days for the wild type and only 43 days for the DSIAT mutant (95% confidence interval < 0.001). The average individual life span for DSIAT mutants is almost 40% shorter than for the wild type flies (t-test $p<0.001$).
Number of flies assayed for individual longevity was 20 for each genotype. Error bars for both graphs indicate SEM.

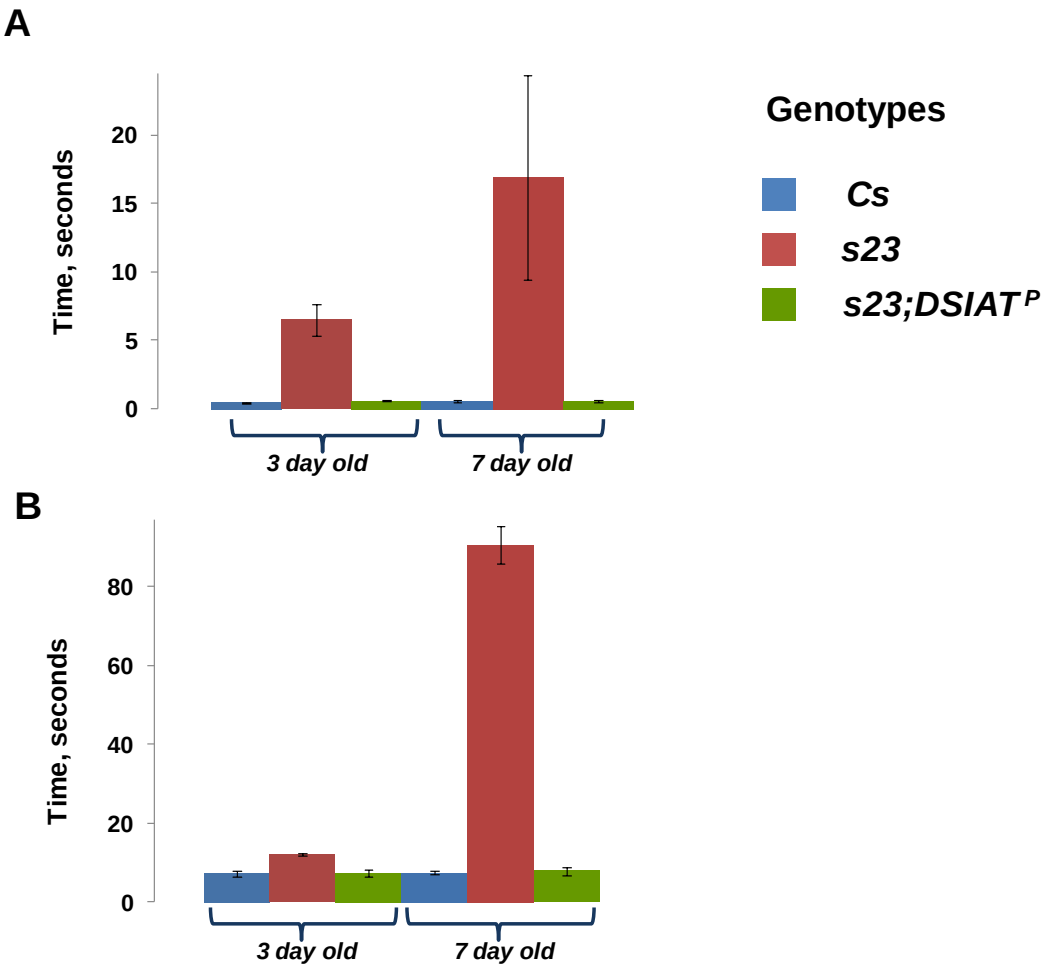

Figure 18. Behavioral phenotypes of DSIAT mutants.
(A) Mechanical agitation assay. Flies of each genotype were scored for their ability to right themselves after falling on their back in response to mechanical shock of the vial. Five trials were given to each fly. Difference in righting time between DSIAT mutant and wild type is statistically significant (t-test $p<0.001$). This mutant phenotype was rescued by P-element insertion of DSIAT genomic copy. Error bars indicate SEM.
(B) Climbing assay. Flies of each genotype were scored for their ability to climb 6 cm up in a vial. Time of crossing a 6 cm line was recorded. Difference in climbing time between DSIAT mutant and wild type is statistically significant (t-test $p<0.001$). Error bars indicate SEM.
For (A) and (B) number of flies assayed for each data point equals 20.

We also found that *DSIAT* mutant flies are paralyzed when temperature is shifted above 36 ^{0}C (Figure 19A, B). We empirically defined paralysis as the condition in which the animal lies on its back with little effective movement of the legs and wings (Sanyal et al., 2005). Time that is required for DSIAT mutant fly to be paralyzed significantly decreases by raising the temperature. For example, when the temperature of the water bath is increased to 38^{0}C, only 20% of flies in the vial are paralyzed within the three minute time interval. If the temperature of the water bath is increased to 39^{0}C, 60% of mutant flies are paralyzed within the three minute time interval.

We also characterized the paralysis phenotype in flies using *s23* mutant allele at different ages. Using fixed temperature 38^{0}C we looked at kinetics of fly paralysis (Figure 19B). At first day after eclosion approximately 13% of DSIAT mutants are paralyzed within five minute time interval. On the third day after eclosion almost 50% of DSIAT mutants are paralyzed within five minute time interval, while on the tenth day after eclosion all flies are paralyzed by the 5th minute of assay. Thus it is clear that the severity of the locomotor abnormalities in adult mutants notably increases with fly age.

Analysis of protein expression pattern (see Chapter II) showed that DSIAT is expressed in the Golgi compartment of the motor neuron soma. Each motor neuron projects an axon to the specific body wall muscle and forms neuromuscular junction.

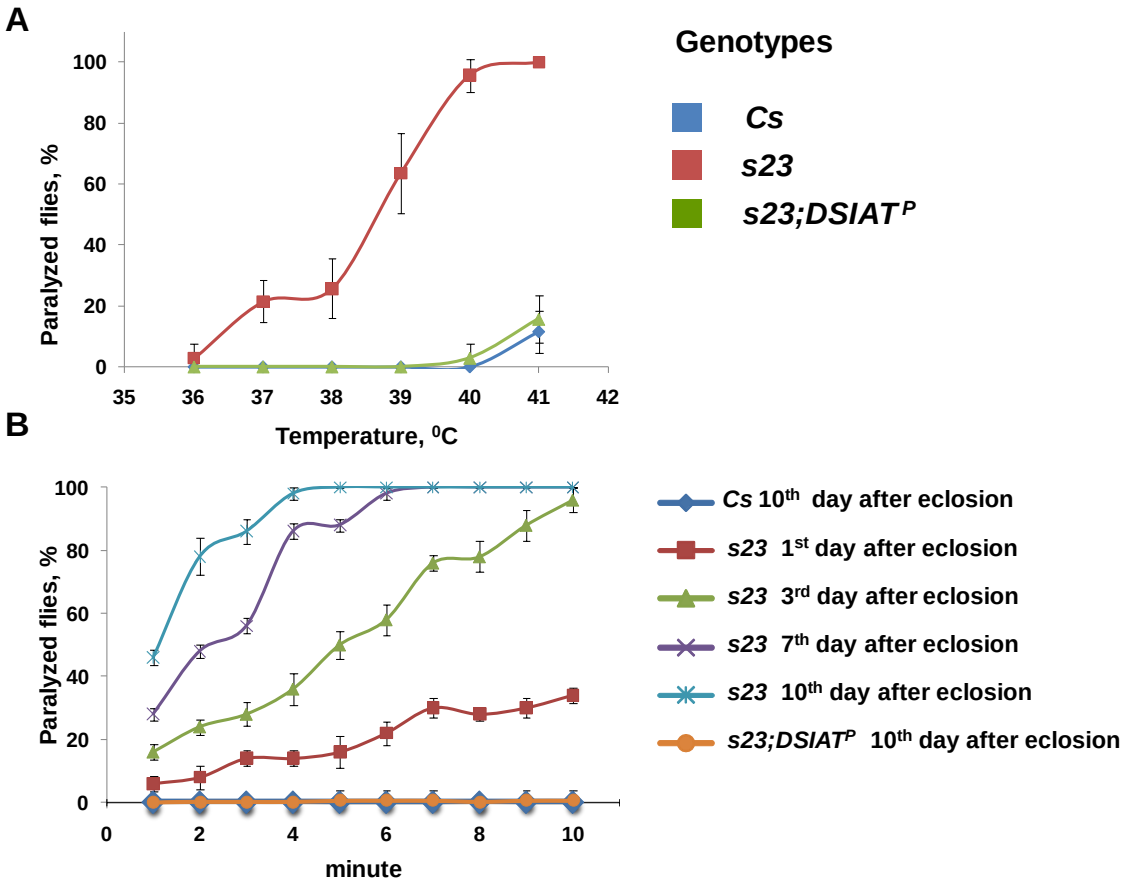

Figure 19. Kinetics of fly paralysis.
(A) Ten *DSIAT* mutants (*s23*), wild type (*Cs*) and rescue flies (*s23;DSIAT*^{P}) (3 day old) were placed into empty vials and submerged into water bath heated to corresponding temperature. Percentage of paralyzed flies within 3 minute time period was recorded.
(B) Ten *DSIAT* mutants (1, 3, 7 and 10 day old), wild type and rescue flies (10 day old) were placed in empty vials which were submerged into the water bath heated to 38^{0}C. Percentage of flies paralyzed every minute within 10 minute period was recorded.
For (A) and (B) total number of flies analyzed for each graph point is 100. Error bars indicate SEM. Difference in percentage of paralyzed flies between wild type and mutants is statistically significant (t-test, $p<0.001$)

Neuromuscular junction consisting of synapses transmits signal to the muscle. This subsequently drives muscle contraction. Some neurological mutations impinge on the function of neuromuscular junctions (NMJs) during development (Bate and Broadie, 1995). Examples include mutations in voltage-gated ion-channel genes such as voltage-gated K^+ channels (e.g. *Shaker* (Wu and Ganetzky, 1992)), Na^+ channels (e.g. *paralytic* (Loughney et al., 1989)), mutations in genes involved in axonal transport (e.g. *kinesin* (Saxton et al., 1991)), mutations in genes involved in synaptic transmission (e.g. *shibire* (Chen et al., 1991)). Thus, to reveal the cellular basis of locomotor abnormalities of DSIAT mutants, we decided to characterize NMJ morphology in the DSIAT mutant larvae.

In *Drosophila* larvae, the morphology of NMJs varies between different muscles but exhibit a stereotypic pattern for corresponding muscles in different animals of the same developmental stage. Characteristic features of NMJ architecture include its branching pattern (the number and length of branches) and the size of terminal connections (the number of boutons). We focused our analysis on the NMJ of the dorsal abdominal muscle 1, a muscle innervated by a DSIAT-expressing motor neuron (see Figure 20A).

The formation of neuromuscular junction contacts entails a complex sequence of events that can be broadly divided into three major categories: axonal pathfinding, target recognition and synapse development. We examined whether *DSIAT* mutants have motor neurons pathfinding or target recognition problems by looking at the third instar

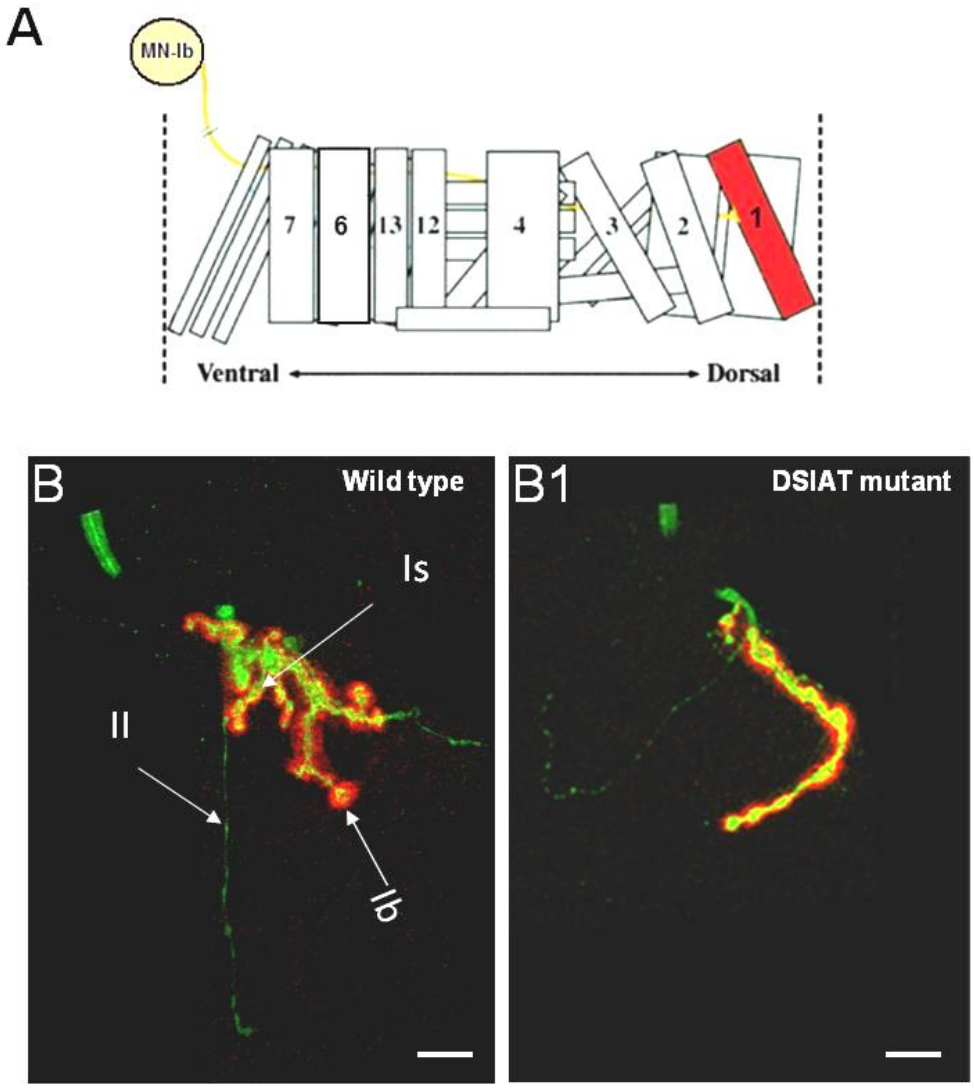

Figure 20. Morphology of DSIAT mutant and wild type NMJ innervating muscle 1.
(A) Diagram of *Drosophila* neuromusculuture. The ventral and dorsal midlines are represented by dashed lines. NMJ morphology was analyzed at muscle 1 (green). Modified from (Featherstone et al., 2000)
Fluorescent images of wild type NMJ (B) and DSIAT mutant NMJ (B1) innervating abdominal larval muscle 1. Antibodies specific to pre (HRP, red) and post (Dlg, green) synaptic markers have been used to visualize NMJ morphology. On (B) arrows point on bouton types Ib, Is and II.

larval NMJ innervating dorsal muscle 1. Motor axons contacted the appropriate target muscles as revealed by immunostaining with anti-HRP antibody that cross-reacts with a glycoprotein on neural membranes. Therefore we concluded that DSIAT does not likely affect motor neuron pathfinding or targeting.

Next we asked whether motor neuron synaptic morphology is altered. The stereotypic architecture of the NMJ is defined by its branching pattern, terminal size and nerve entry point (Prokop and Meinertzhagen, 2006). We compared NMJ morphology in mutants and wild type third instar larvae. It is known that larval population density affects synaptic morphology by regulating Fasciclin-II levels (Stewart and McLean, 2004). Therefore we grew *DSIAT* mutant and wild type larvae at the same density (20 larvae per vial) and then dissected them for NMJ analysis. Study of the fluorescently labeled NMJ in *DSIAT* mutants and wild type larvae showed that mutants have lower number of synaptic boutons and branches (Figure 21). The decreased number of boutons and branches was not rescued by a P-element construct carrying a genomic copy of *DSIAT* locus. To exclude the possibility that these NMJ phenotypes are associated with a genetic background influence, we analyzed NMJs of another *DSIAT* mutant allele in a different (*yw*) genetic background and found a similar decrease in the number of boutons and branches (Figure 21).

The Drosophila larval NMJ is particularly amenable to the investigation of synaptic transmission because of its electrophysiological accessibility (Koh et al., 2000). To elucidate a potential synaptic function of DSIAT we decided to directly examine the synaptic physiology of larval NMJs in DSIAT mutants by intracellular recording of

excitatory junctional potentials (EJPs) and miniature excitatory junctional potentials (miniEJPs) from dorsal oblique muscle 1. We didn't detect significant changes in resting membrane potential (*CS/2J* -59±0.84 mV; *DSIAT* -60.1±0.59 mV*; CS/2J;s23;P(III)* -60.8±1.6 mV; *yw* -60.1±0.24 mV; *yw;L22* -60.7±0.42 mV; *yw;L22;P(III)* -61±0.47 mV) at external 1mM Ca^{2+} concentration. Using intracellular recording from muscle 1 of segments A3 and A4 in the third larval instar, we analyzed miniEJPs that reflect a 'background' synaptic activity due to spontaneous fusion of synaptic vesicles and evoked excitatory junction potential (EJP, i.e. a response to electric stimulation of the nerve) in DSIAT mutants. Usually, synaptic vesicles fuse with the presynaptic membrane in response to action potentials, but spontaneous fusion resulting in the release of neurotransmitters (so called miniEJP) has been recorded in the absence of action potentials (Verstreken and Bellen, 2001). We detected no significant difference between control and mutants in the amplitude or frequency of miniEJPs (Figure 22), suggesting that spontaneous release of neurotransmitters from the presynaptic membrane is not affected in the mutants; synaptic vesicles dock and fuse normally with the presynaptic membrane (Bronk et al., 2001; Ly et al., 2008). We then evoked EJPs by stimulating the cut segmental nerve with 0.2ms, 6 mV step pulse at 1mM Ca^{2+} HL3 saline. The evoked EJP was reduced to approximately 68.7% of control at homozygous *DSIAT* NMJs in *CS* genetic background ($p<0.0001$, Figure 23) and to 64% in *yw* genetic background ($p<0.0001$). EJPs from homozygous DSIAT rescue larva were indistinguishable from controls in *CS* genetic background ($p=0.35$) suggesting that decreased EJP amplitude

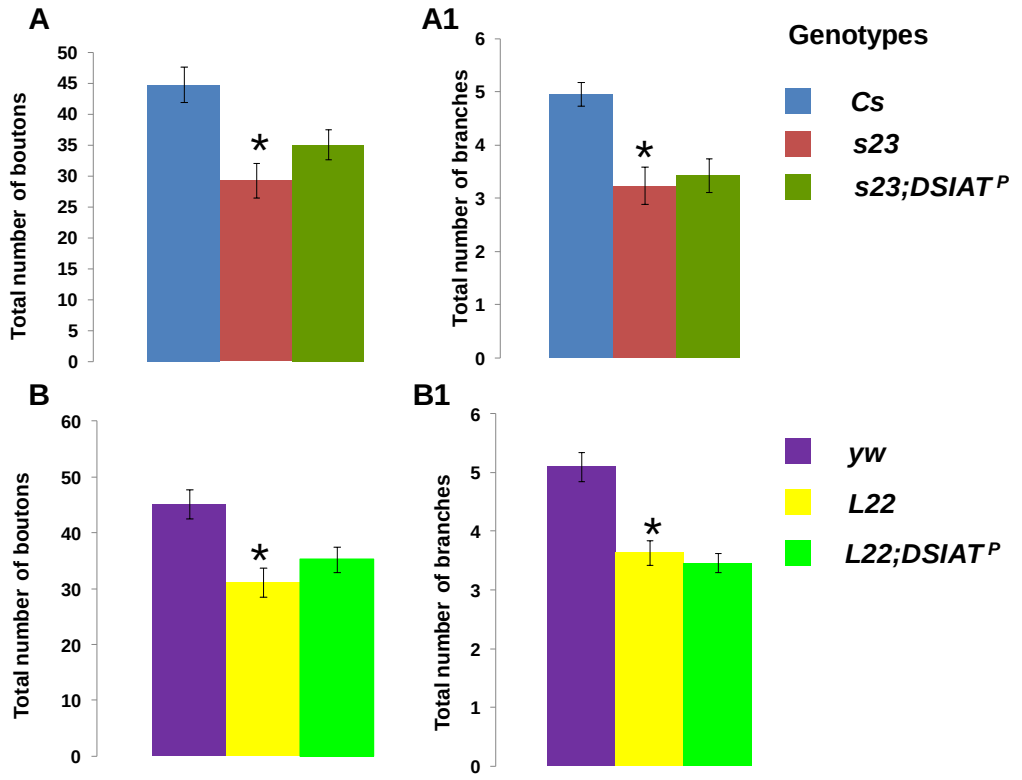

Figure 21. Quantification of bouton and branch number.
(A) and (B) shows total number of boutons at NMJ innervating muscle 1 for two different DSIAT mutant alleles: *s23* and *L22* in two different genetic backgrounds, *Canton S* (A), (A1) and *yw* (B), (B1).
(A1) and (B1) shows total number of branches at NMJ innervating muscle 1.
Number of boutons and branches is significantly reduced in DSIAT mutants for both alleles (t-test, *p<0.001). However, genomic wild copy of DSIAT does not rescue reduction of boutons or branches.
The number of assayed NMJs for each genotype >20. Error bars represent SEMs.

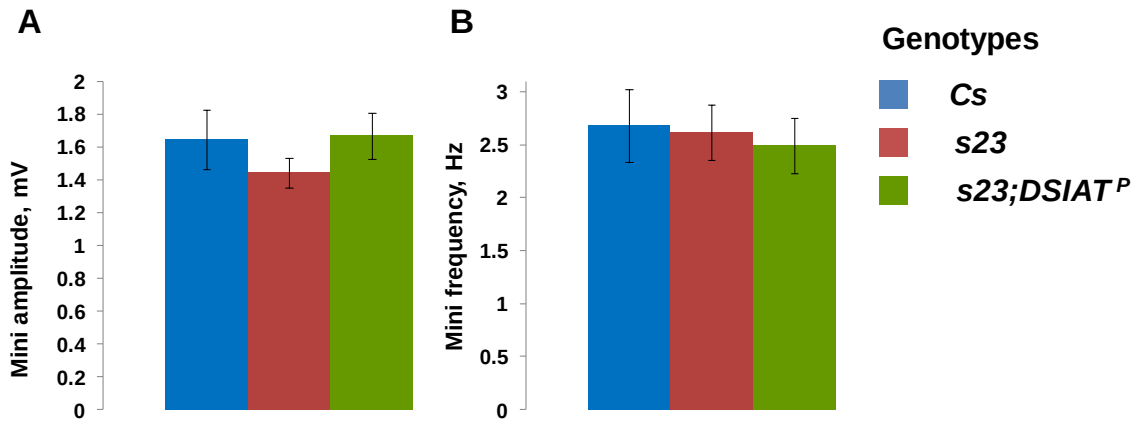

Figure 22. Current clamp electrophysiology reveals normal spontaneous exocytosis in DSIAT mutants.
(A) MiniEJP amplitude is not different for wild type, DSIAT mutant and rescue larvae.
(B) MiniEJP frequency is not different for wild type, DSIAT mutant and rescue larvae.
n=10 for all genotypes. No statistically significant difference was observed for assayed genotypes (t-test, $p>0.05$). MiniEJPs were recorded in 1mM Ca^{2+}.

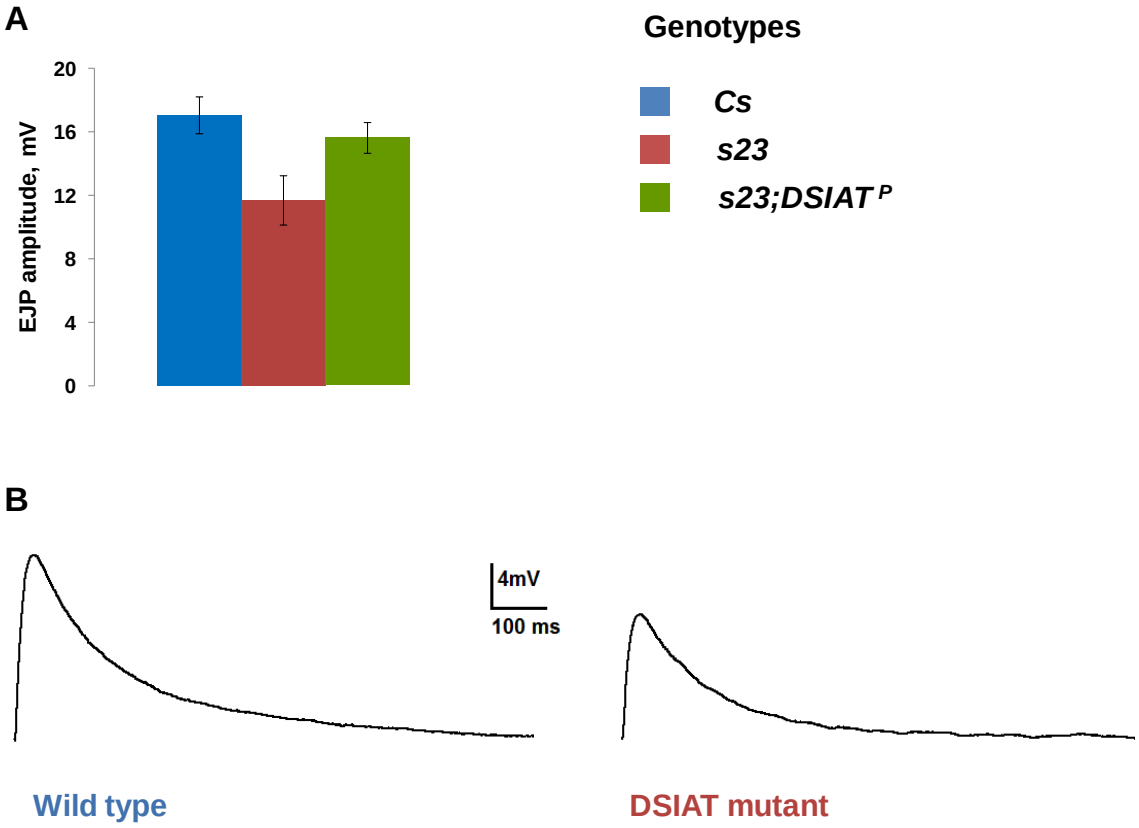

Figure 23. Current clamp electrophysiology of DSIAT mutants reveals reduced neuromuscular transmission.
(A) Quantification of EJP amplitude for relevant control (*Cs*), DSIAT mutant (*s23*) and rescue (*23;DSIAT*^{P}) larva recorded in 1 mM Ca^{2+}. EJP is almost 30% reduced in DSIAT mutants. This difference is statistically significant (t-test, $p<0.001$). EJP for rescue larvae is not statistically different from DSIAT mutant and the wild type ($p>0.03$). Number of larvae used for EJP recording for selected genotypes is 10. Error bars indicate SEM.
(B) Sample EJPs recorded in 1mM Ca^{2+}.

is due to the mutation in *DSIAT* locus. Reduction of the EJP amplitude in *DSIAT* mutants suggests that the excitability of axonal membrane or/and the mechanism that triggers the release of neurotransmitters in response to an action potential are compromised.

Decreased EJP amplitude could result from 1) altered action potentials arriving at the terminal 2) affected postsynaptic (muscle) membrane or 3) affected synaptic release from the presynaptic terminal.

Binomial model defines EJP as the product of quantal content (number of vesicles involved in evoked release event (i.e., synaptic response to single AP) and the membrane potential change induced by a single vesicle of neurotransmitters (i.e. mini amplitude) (DiAntonio et al., 2001; Zucker and Regehr, 2002). From analysis of miniEJPs we know that amplitudes and frequencies of single vesicle release in mutants are not different from wild type. Therefore, DSIAT mutants do not have obvious reductions in the ability for spontaneous exocytosis, altered amount of neurotransmitters per vesicle, or altered postsynaptic sensitivity to neurotransmitters. Then, decreased EJP amplitude in DSIAT mutants at 1mM Ca^{2+} concentration suggests that quantal content (in other words, the average number of vesicles released during a synaptic event in response to action potential, or ratio of the post-synaptic potential to potential produced by release of a single vesicle) is decreased in DSIAT mutants. By definition quantal content (m) is product of n (the number of vesicles released following an action potential) and p (the probability of any vesicle fusing and releasing its neurotransmitter content).

First, we decided to test if the pool of vesicles n or the process of vesicle recycling is altered in DSIAT mutants by measuring the response of mutant and wild type NMJs to

high-frequency (10 Hz) stimulation. High frequency stimulation affects transmission via a number of mechanisms. Those include but are not limited to modulation of actin cytoskeleton-dependent processes (Bader et al., 2004) and changes in vesicle recycling dynamics. Both mutant and control NMJs show similarly sustained EJPs at 1 mM Ca^{2+} during prolonged stimulation (Figure 24A). In addition both DSIAT mutant and the wild type show similar reduction from the highest amplitude and sustainability of EJP in presence of high external 4 mM Ca^{2+} concentrations (Figure 24B).

We also observed that at high 4mM external Ca^{2+}, when the probabilities of vesicle release in DSIAT mutant and the wild type synapse are similar, we completely rescued the defect in reduction of EJP amplitude observed at normal 1mM Ca^{2+} concentration (Figure 25). Together these last two experiments suggest that pool of vesicles *n* and the process of vesicle endocytosis are not affected in DSIAT mutants.

We also decided to test whether the efficacy of synaptic release is affected in DSIAT mutants by measuring paired-pulse (short-term) facilitation. Paired-pulse facilitation occurs in low calcium on a millisecond timescale and is characterized by increased EJP amplitude after the second stimulation in a closely spaced pair (Mattaliano et al., 2007). According to one of the explanations the increased amplitude of the second pulse is due to increased residual presynaptic calcium (Zucker and Regehr, 2002).

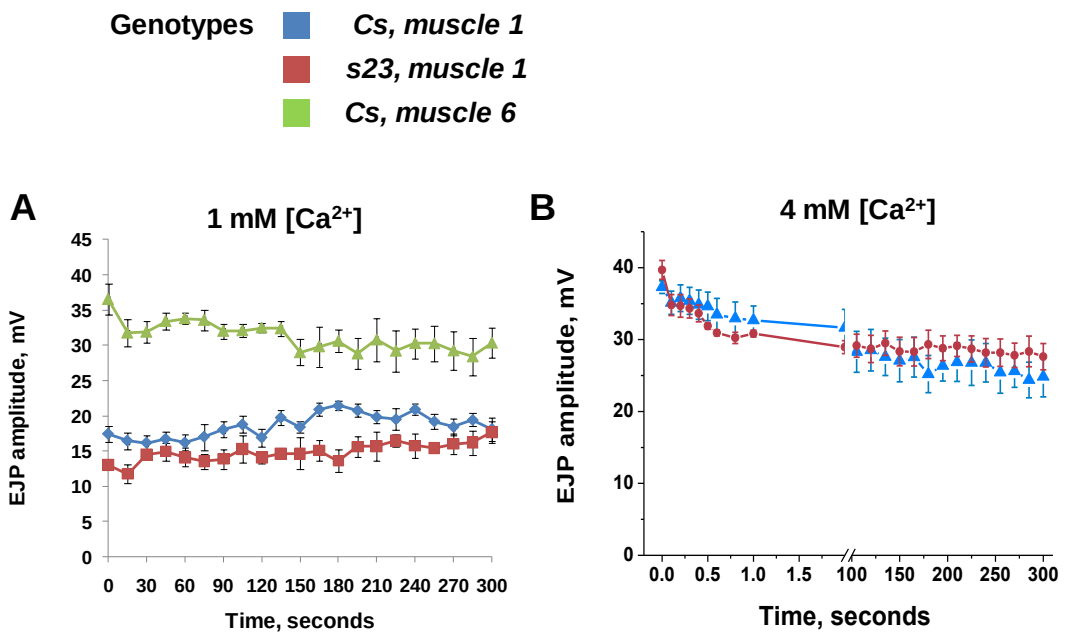

Figure 24. The density of vesicles in readily releasable pool is similar in wild type and DSIAT mutants.
(A) Dynamics of evoked release during repetitive stimulation (10Hz) measured at muscle 1 of control (*Cs*) and homozygous DSIAT mutants (*s23*) and at muscle 6 of control (*Cs*) flies at 1 mM Ca^{2+}. While EJP measured at muscle 6 in the presence of 1 mM Ca^{2+} drops from its maximum value by about 20% and is then sustained by endocytosis, EJP amplitude measured at muscle 1 doesn't change during the same simulation period. This data suggests that overall muscle 1 NMJ has different from muscle 6 response to high-frequency stimulation. In addition similar response in wild type and mutants suggests that the pool of readily releasable vesicles of muscle 1synapse is completely recovered between interpulses for the mutant and the wild type. This result is further confirmed at 4 mM Ca^{2+} concentration.
(B) Dynamics of evoked release during repetitive stimulation measured at muscle 1 of control (*Cs*) and DSIAT mutants (*s23*) at 4 mM Ca^{2+}. EJPs were stimulated at 10Hz for 5 min and recorded from muscle 1. No statistically significant difference in reduction of EJP from its maximal value in presence of high external Ca^{2+} concentration was observed ($p>0.3$).
For each indicated point mean amplitudes were obtained from at least 4 larvae. Error bars indicate SEM.

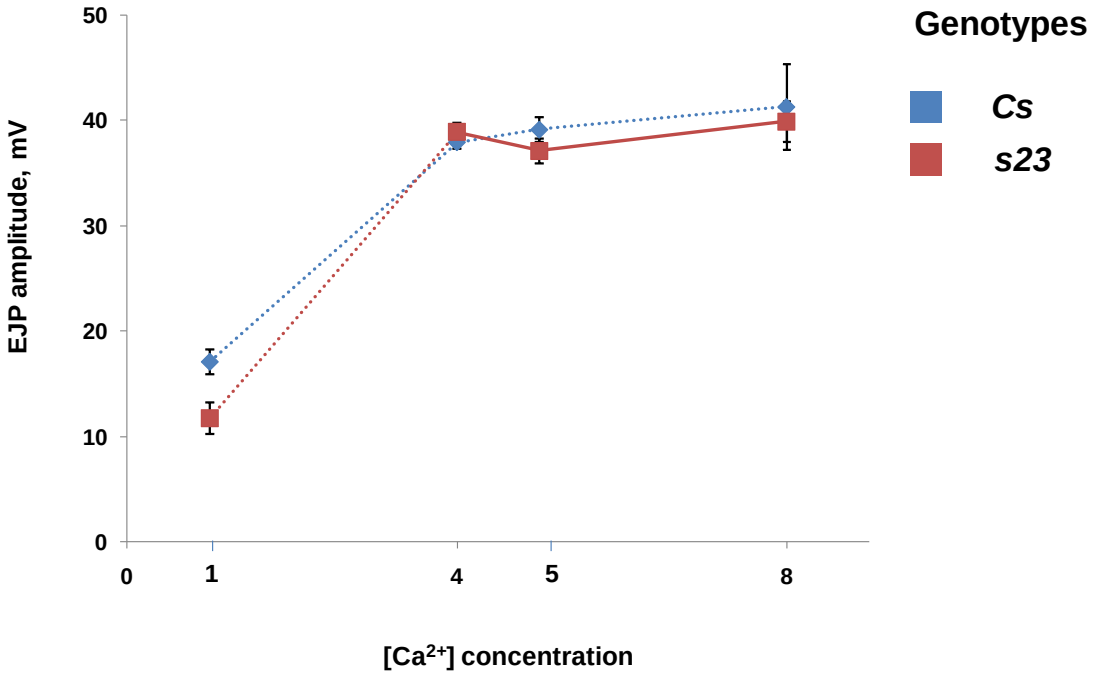

Figure 25. High calcium concentration rescues reduced EJP amplitude of DSIAT mutants.
For 1 and 4, 5 and 8 mM Ca^{2+} external concentrations average EJP amplitude value was obtained from at least 4 larvae. Statistically significant difference in EJP amplitude is observed in DSIAT mutant and the wild type at 1mM [Ca^{2+}] ($p<0.001$). However, no statistically significant difference in EJP amplitude is seen at 4, 5 and 8 mM [Ca^{2+}] between DSIAT mutant and wild type ($p>0.2$). Error bars indicate SEM.

We observed no significant difference in the ratio of the second to the first EJP amplitude measured at dorsal muscle 1 in DSIAT mutants comparing to the wild type (EJP_2/EJP_1 for 50 ms interpulse interval: *CS*, 1.37±0.06; *Cs;s23*, 1.24±0.05; P>0.1 for wild type and DSIAT mutant, Student's t-test) (Figure 26).

These experiments, together with the identical synaptic responses of mutants and wild type larvae to high frequency stimulation, suggest that the synapses of DSIAT mutant have similar secretion capabilities (i.e., readily releasable pools of vesicles, *n*, are similar), as quantal content is not different between groups when the probability of release is equivalent in high calcium (Figure 27).

Thus, since high calcium can rescues the DSIAT synaptic phenotype and spontaneous release of neurotransmitters is not different between groups, it is not likely that reductions in quantal content are inherent to the secretory mechanism itself, but rather to an impact of DSIAT on upstream excitability (i.e., the motor neuronal action potential).

To test further whether decreased EJP amplitude might be due to abnormal action potential arriving at the synaptic terminal we tried to measure the synaptic response by direct electrotonic stimulation with 10μM TTX which suppresses action potentials. Electrotonic stimulation of a nerve is a way of passive depolarization of the presynaptic terminal by generating strong electric field. It can be done by bringing the stimulation electrode close to the synapse and elevating the voltage. It is generally used to isolate the defects in presynaptic function from defects in upstream neuronal properties (e.g., action potential propagation).

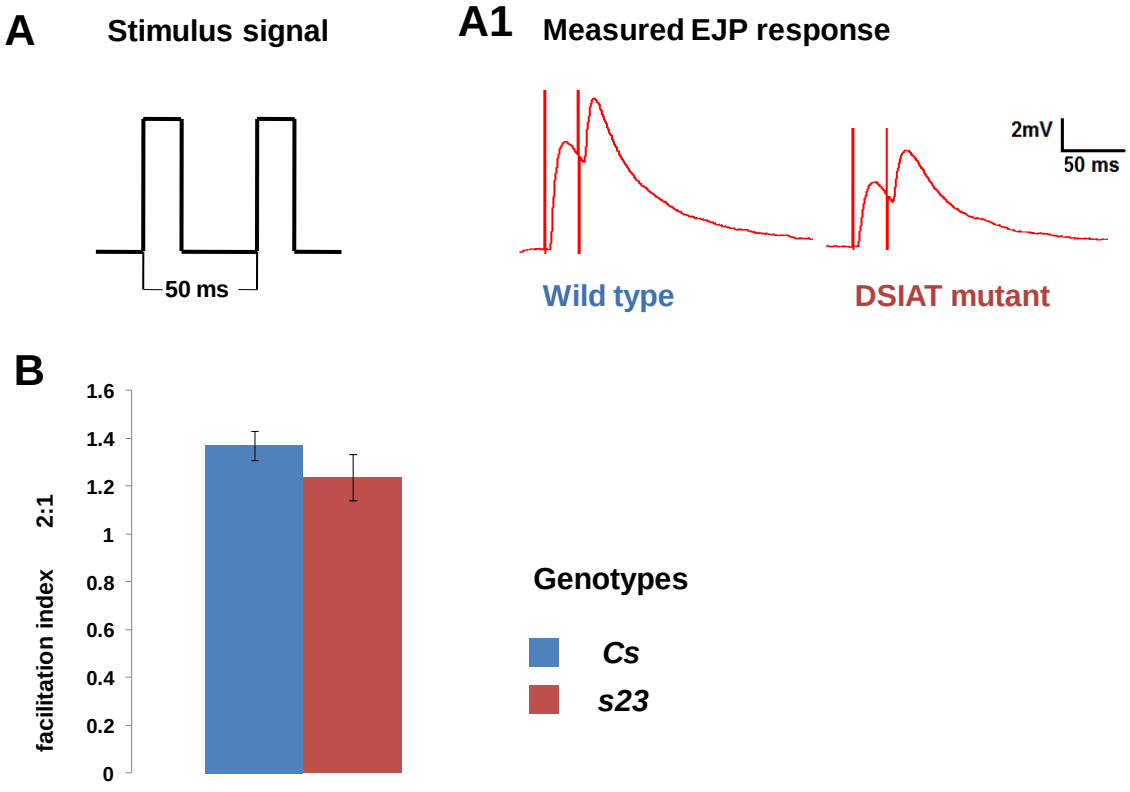

Figure 26. Mutation in *DSIAT* does not alter short-term plasticity at the larval neuromuscular junction.
Paired-pulse facilitation occurs in low calcium on a millisecond timescale and is characterized by increased EJP amplitude after the second stimulation in a closely spaced pair. The enhancement of the second EJP is believed to be a result of residual presynaptic calcium. (A) shows a pair of two closely spaced stimuli separated by 50 msec from the generator.
(A1) shows first and second EJP sweeps in response to train of stimuli in wild type (*Cs*) and DSIAT mutant (*Cs;s23*).
(B) No difference in the ratio of the second to first EJPs measured at muscle 1 in 0.75 mM external Ca^{2+} was seen between the wild type and DSIAT mutant (t-test, $p>0.05$). For each indicated time point mean amplitudes were obtained from 10 EJPs of 10 larvae. Error bars indicate SEM.

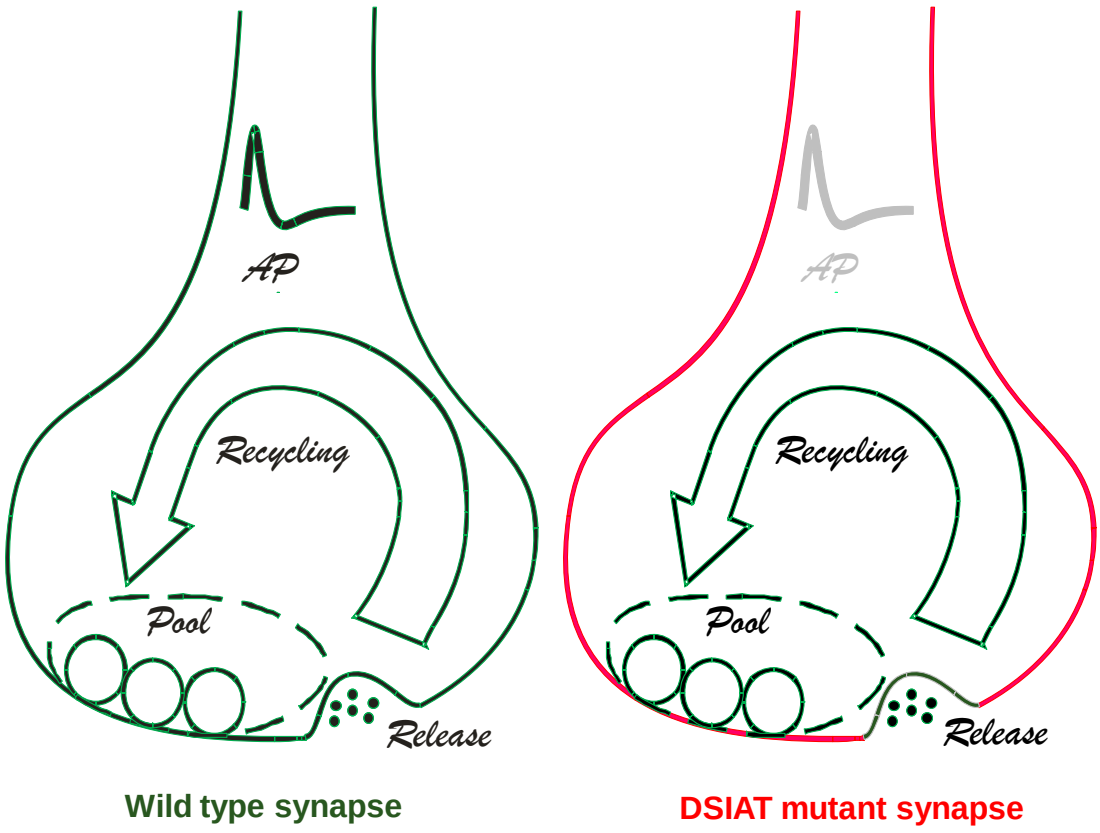

Figure 27. Schematics of wild type and DSIAT mutant synapse.
In picture showing wild type synapse, green color denotes normal action potential propagation along the axon and selected processes in the synapse that overall contribute to normal functioning of the bouton. In the picture showing DSIAT mutant synapse, red color denotes impaired neuronal functioning of the synaptic bouton reflected by reduced EJP at 1 mM Ca^{2+}. Electrophysiological experiments showed that high calcium concentrations rescue defective mutant EJP amplitude. In addition DSIAT mutant and wild type synapses show identical responses to high-frequency stimulation and paired-pulse facilitation. Also, mini analysis showed that amount of neurotransmitters per vesicle and postsynaptic sensitivity to neurotransmitters in not altered in DSIAT mutants. Therefore, DSIAT mutant have similar secretion capabilities (pool of vesicles, number of neurotransmitters, and vesicle recycling is not impaired and labeled as green). We hypothesize that reduced EJP can be due to decreased neuronal excitability (motor neuronal action potential) and labeled it with grey color.

If reduced EJP is due to action potential defect, then direct electrotonic stimulation should rescue the reduced EJP signal in the mutants and show normal wild type EJP response. In this experiment, larvae were dissected along the lateral midline in the standard saline and then the preparation was superfused with 1mM Ca^{2+} HL3 saline containing 10μM tetrodotoxin. This concentration was sufficient to completely eliminate EJPs which depend on action potential propagation. Then, the stimulating electrode with the segmental nerve was moved as close as possible to the synapse on muscle 1. Voltage was gradually increased 20-fold larger than conventional threshold of nerve stimulation used for inducing action-potential mediated EJPs. However, no EJPs were observed at muscle 1. In control experiments with muscle 6/7, potentials could be easily evoked by passive electrotonic depolarization of the nerve terminal by applying strong stimulus (approximately twice the normal threshold for induction of regular EJP).

This result can be explained by the fact that it is impossible to put the stimulus electrode in the nearest vicinity of the synapse at muscle 1 (the nerve bundle entering under muscle 7 and muscle 1 are spatially distant from each other) (see Figure 19A). Therefore the generated strength of electric field is insufficient to trigger vesicle release from presynaptic terminals.

We then decided to measure EJP response in DSIAT mutant and wild type larvae over time after addition of TTX into the recording saline. We first measured EJP response at muscle 1 of the wild type larvae and then added 10 μM TTX into the saline and traced EJP signal decay by measuring it every minute until it disappears. Comparison of the EJP amplitude decay in DSIAT mutant larvae after addition of the same amount of TTX

showed that DSIAT mutants are more resistant to TTX block (Figure 28). Tetrodotoxin binds to the pores of the voltage gated Na^+ channels in nerve cell membranes and completely blocks action potential propagation. Since DSIAT mutants showed lower sensitivity to TTX, therefore we think that DSIAT may influence properties of PARA and thus have an effect on action potential.

The conducted electrophysiological experiments showed that DSIAT mutant transmitter release can be rescued by elevating calcium in the saline. In addition DSIAT mutant and wild type have equal amplitudes and frequencies of miniEJPs, and DSIAT mutants are less sensitive to TTX effect. All these data suggest mutation in DSIAT affects action potential thus decreasing the probability of vesicle release.

DISCUSSION

In this study, we characterized phenotypes associated with a mutation in *Drosophila* sialyltransferase gene that codes for a functional protein which is structurally related to ST6Gal family of vertebrate sialyltransferases. In *Drosophila*, inability to sialylate targeted proteins leads to severe behavioral abnormalities both at larval and adult stages. In addition, it leads to structural abnormalities at the neuromuscular junction and disrupts proper neuronal signaling.

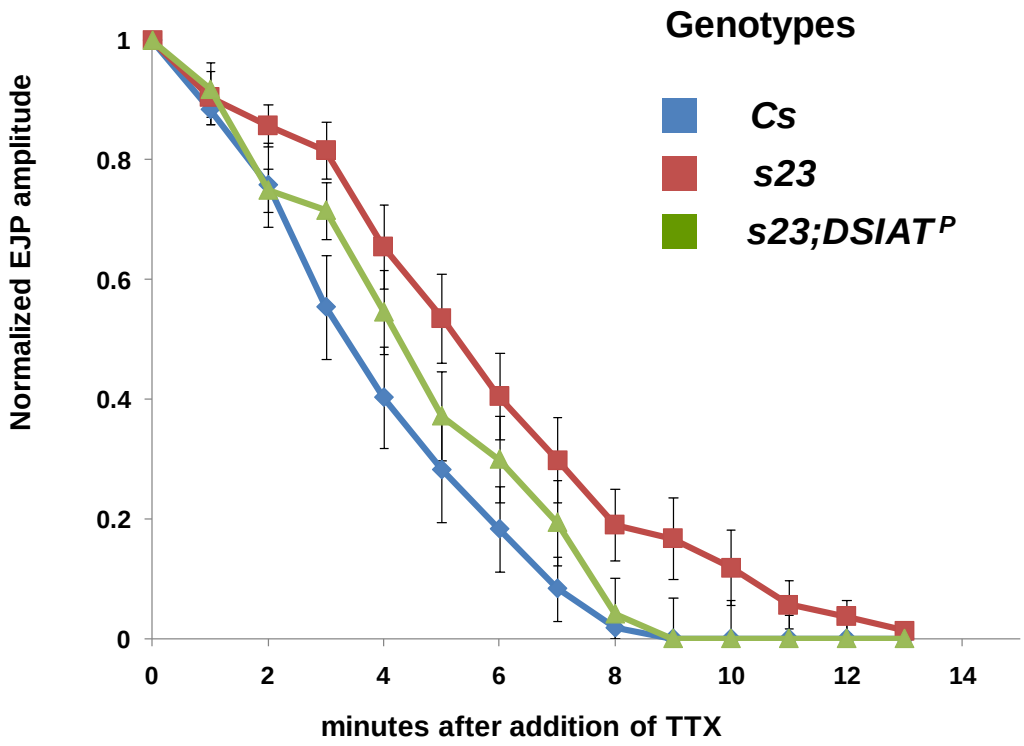

Figure 28. DSIAT mutants are less sensitive to tetrodotoxin.
Larva of the corresponding genotype was dissected in chilled 0mM Ca^{2+} saline. 0mM Ca^{2+} saline was then replaced with 1 mM Ca^{2+} and EJP amplitude was measured at muscle 1 upon axonal stimulation. Then, 10 µm of TTX was added into saline and EJPs were recorded precisely each minute until EJP amplitude becomes indistinguishable from noise. On the graph 0-minute corresponds to EJP amplitude measured prior to addition of TTX. 1-minute point corresponds to EJP amplitude measured after 1minute of addition of TTX and so on. All EJP amplitudes were normalized to EJP amplitude recorded prior to addition of TTX. Starting from the 3rd minute DSIAT mutant EJP response is statistically different from the wild type (t-test, $p<0.05$). Rescue of the observed DSIAT mutant resistivity is statistically different from the mutant starting from 8th minute (t-test $p<0.05$). For each indicated time point mean amplitudes were obtained from 5 EJPs of 16 larvae. Error bars indicate SEM.

Behavioral phenotypes

Wandering behavior in *Drosophila* larvae precedes metamorphosis. At the beginning of this stage, larvae crawl out of food on the walls of the vial searching for pupation site. We performed a videotaped analysis of wandering *DSIAT* mutant and wild type larvae to see the difference in larval locomotion behavior. We found that DSIAT mutant larvae frequently pause and turn and rarely crawl straight for a long distance. We marked out two different phenotypes. First, *DSIAT* mutant larvae were found to have significantly inferior crawling abilities, as judged by decreased crawling speed, frequency of body muscle contractions, and the distance traveled per contraction on the linear path. This data could be linked to impaired muscle performance in DSIAT mutants, which may result from muscle degeneration (postsynaptic side) or abnormalities in signal transmitted to muscle (presynaptic side). Because DSIAT protein is not expressed in muscles and morphologically muscles look normal in DSIAT mutants, we concluded that the abnormalities in mutant larvae behavior come from the presynaptic side. Defects in signal transmitted to the muscle may arise from defects in axonal part of the motor neuron or motor neuronal synapse.

Second, mutations in *DSIAT* resulted in stereotypic modifications in locomotion crawling pattern. It was previously reported that mutations in single ion channel subunits, like *para* (gene encodes for α-subunit of voltage-activated Na^+ channel (Pittendrigh et al., 1997)) or *Hk* (gene encodes for β-subunit of voltage-activated K^+ channel) led to stereotypic defects in larval locomotion (Wang et al., 1997) and (Figure 29). Comparison of larval crawling pattern suggests that *DSIAT* mutants look very similar

to *para* but not *Hk* mutants. Because $para^{ts1}$ mutation affect Na^{+} channels and presumably decrease their neuronal excitability, the abnormal crawling pattern seen in $para^{ts1}$ larvae can be attributed to hypoexcitablity of neuronal cells of the relevant circuits (Wang et al., 1997). Therefore we think that altered crawling pattern that includes 'decision-making' pausing stops and peristaltic locomotion in *DSIAT* mutants reflects significant problems in neural signaling, but not muscle performance.

Characterization of adult phenotypes showed that DSIAT mutants are uncoordinated, display locomotion problems and exhibit temperature-sensitive paralysis. This data suggests that DSIAT may affect both adult muscles and/or adult nervous system. However, immunostaining of the larval tissues for DSIAT-HA showed that DSIAT is expressed in the central brain and thoracic ganglion. No DSIAT-HA staining was detected in the muscles. Therefore described phenotypes of the mutants indicate that DSIAT functions in neuronal signaling, thus potentially affecting electric properties of neuronal membranes or synaptic connections. The described phenotypes of *DSIAT* mutants strongly progressed with age. This indicates that mutation in *DSIAT* gene may cause neurodegeneration. We performed electron microscopy on the mutant adult photoreceptors and didn't detect apoptotic neuronal death. This can be explained by the fact that DSIAT is not expressed in the lamina regions of the brain. However, we also performed paraffin sectioning on central brain, medulla and lobula regions of the adult brain and again didn't see any signs of neurodegeneration (data not shown). We think that a more thorough investigation is necessary to confirm this result. We also suspect that

neurodegeneration may occur at the neuromuscular junction sites, direct connectors of the neuron and muscle. This hypothesis needs to be tested as well.

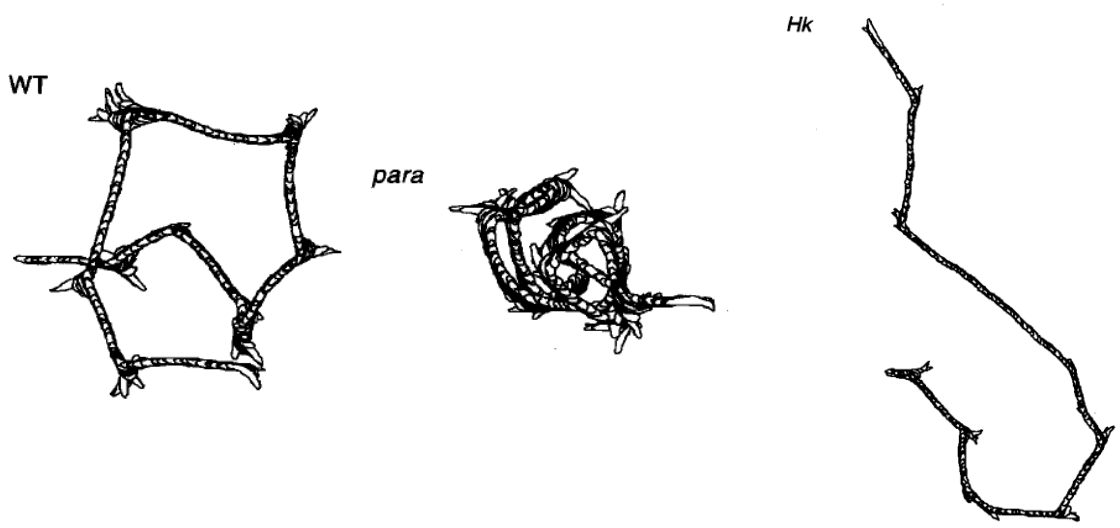

Figure 29. Representative crawling patterns of *Drosophila* wild type, *para* and *Hk* larvae at wandering stage.
Larval positions and outlines on leveled agar plates were captured by video recording and digitized for duration of 2 minutes. Modified from (Wang et al., 1997)

DSIAT is required for proper NMJ morphology and electrophysiology

Because DSIAT may regulate synaptic transmission at the third instar larval NMJ, it may have a role in determining the structure of the synapse. In fact, quantification of the number of boutons and branches at muscle 1 NMJ in segment A3 in the third instar larvae showed their significant decrease as compared to wild type.

Electrophysiological recording from muscle that is innervated by DSIAT expressing motor neuron showed severe impairment in the evoked release of neurotransmitters. We investigated the mechanism underlying this phenotype. First, we observed approximately 25% reduction in the evoked transmitter release in DSIAT mutants as compared to wild type measured in 1 mM Ca^{2+} HL3 saline. The decrease in evoked release indicates that the nerve is releasing fewer synaptic vesicles. Reduction of EJP amplitude in DSIAT mutants was rescued by a P-element construct carrying a genomic copy of DSIAT locus. Hence, the loss of DSIAT is responsible for the reduction of EJP amplitude is DSIAT mutants, and therefore DSIAT is important for normal neuronal signaling at the *Drosophila* NMJ.

EJP response measured at the muscle is a cumulative response from all synaptic boutons targeting this muscle. Therefore observed alteration of electrophysiological properties could be a result of failure to properly develop synaptic arborizations and synaptic boutons. In fact we observed approximately 25-30% reduction of number of boutons that can be a direct indication of decreased EJP signaling. However we think that even though absence of DSIAT affected number of synaptic boutons, nervous system switched on some compensatory mechanism and developed stronger individual synaptic boutons that overall made DSIAT mutant synapse similar to the wild type. In this case the total number of neurotransmitters or active zones per NMJ should be similar in the mutants and the wild type and therefore the observed reduction of evoked response can be due to abnormal neuronal activity that for example involves impaired action potential

propagation along the axon or synaptic transmission but not due to decreased number of neurotransmitters or active zones in the synapse.

High-frequency stimulation applied to axon lead to a decrease in synaptic strength of the NMJ. The most common mechanism is presynaptic decreases in the release of neurotransmitters that likely reflects a depletion of a release-ready pool of vesicles (Zucker and Regehr, 2002). To test whether DSIAT affects pool of vesicles in synaptic boutons or vesicle recycling, we performed high frequency stimulation. We didn't observe a difference in the reduction of EJP amplitude after prolonged stimulation in DSIAT mutants in comparison to wild type. The results of high-frequency stimulation suggest that DSIAT is not involved in vesicle recycling at that motor neuronal synapse. We also tested whether the pool of synaptic vesicles is affected in DSIAT mutants by elevating external Ca^{2+} concentration. We found that the pools of vesicles in mutants and wild type are similar. In addition, we couldn't detect any difference in the miniEJP amplitude of frequency, suggesting the docking mechanism of synaptic vesicles in DSIAT mutants is not affected as well.

The probability of evoked release of a single synaptic vesicle release depends on its successful docking and fusion at the presynaptic membrane, as well as the action potential arriving at the synapse. Analysis of spontaneously released vesicles (miniEJP analysis) suggests that vesicles fuse and dock normally in DSIAT mutants.

Therefore decreased EJP may be caused by defective shape and/or propagation of action potential. We tried to bypass action-potential stimulated release by direct electrotonic stimulation with addition of TTX, but failed. We then tested sensitivity of

Na^+ channels in DSIAT mutants by adding TTX into the recording medium. Results showed that EJPs in the mutants are sustained for longer period of time as compared to wild type. We think that sialic acids are located close to the pore of the voltage gated Na^+ channel substantially influencing its gating properties. Thus removal of sialic acids perturbs proper functioning of these channels and may affect the shape and/or the amplitude of the action potential (Figure 30).

Revealing evolutionary role of hST6Gal family human sialyltransferases

Multiple sequence alignment of protein sequences of human ST6Gal I, ST6Gal II and DSIAT shows high degree of conservation between their catalytic domains suggesting that enzymatic role of these sialyltransferases are highly conserved between vertebrates and protostomes (Figure 2). We predicted that DSIAT and its human homologs should have similar functions. To test this hypothesis, we tried to rescue DSIAT mutant phenotypes by neuronal or ubiquitous overexpression of its human counterparts. Analysis of transgenic flies showed neither human sialyltransferase does rescue of locomotor and paralytic behavior of *DSIAT* mutants (data not shown).

We think that this could be due to several reasons: human sialyltransferase is not properly folded or properly secreted when it is expressed in *Drosophila*; it may also use different from DSIAT pool of protein acceptors. Additionally, we constructed chimera protein consisting of *Drosophila N*-terminus, transmembrane domain, stem region and hST6Gal II catalytic domain to ensure proper sialyltransferase folding and subcellular localization.

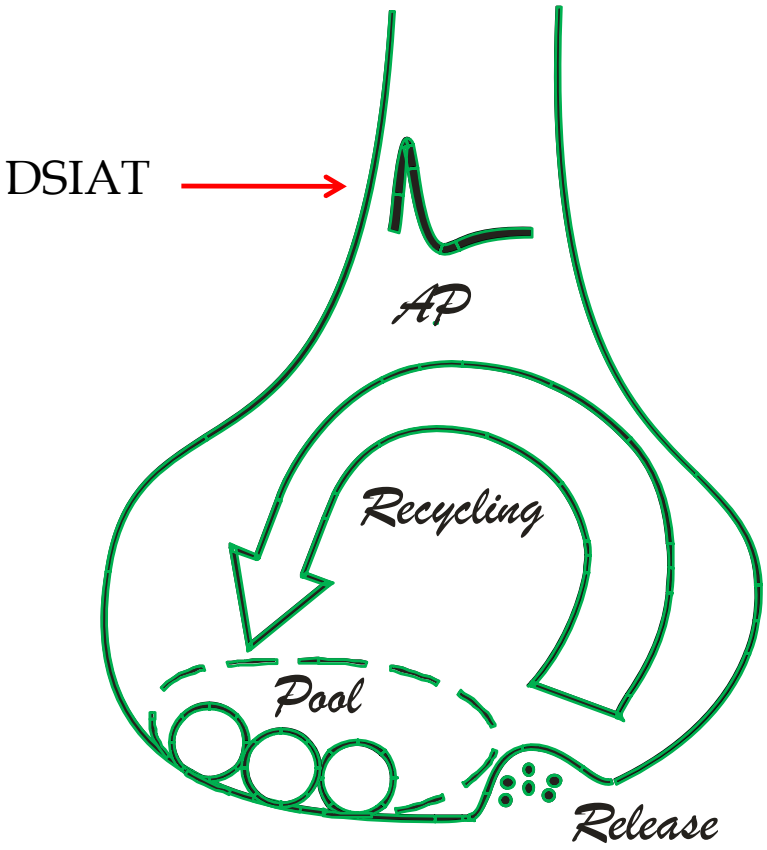

Figure 30. Excitability of motor neurons in DSIAT mutants is altered due to defective action potential.
Electrophysiological experiments suggest that the excitability of the motor neurons is altered in mutants, resulting in differences in synaptic function compared to wild type flies. The following electrophysiological results support this conclusion: 1) DSIAT mutants do not have obvious reductions in the ability for spontaneous exocytosis, altered amount of neurotransmitters per vesicle, or altered postsynaptic sensitivity to neurotransmitters based on mini analysis, 2) identical synaptic responses of mutants and wild type larvae to high frequency stimulation, rescue of EJP amplitude in DSIAT mutants in the presence of high Ca^{2+}, presence of paired-pulse facilitation in wild type and mutants which suggest that the synapses of DSIAT mutant work normally. Therefore it is not likely that reductions in quantal content are inherent to the secretion mechanism itself at 1mM Ca^{2+}, but rather to an impact of DSIAT on upstream excitability (i.e. the motor neuronal action potential).

Immunostaining for this chimera protein overexpression in transgenic flies showed that it is targeted to Golgi and ER compartments but is not transported to the axons. We couldn't rescue DSIAT mutant phenotypes with chimera protein as well. This result also correlates with our hypothesis that DSIAT needs to be transported to the axons for its normal functioning in flies (see Chapter II). This interesting observation calls for further investigation.

MATERIALS AND METHODS

Fly strains and maintenance

Prior to all behavioral assays flies were kept at 25^0C and 25% humidity day-night cycle controlled incubator.

Larval behavior

To analyze larval behavior adult flies were allowed to lay eggs on the grape juice plate within 3 hours. 24 hours later, 20 first instar larvae were transferred to the food vials until they reach mid third instar stage (between 80-84 hours after egg laying). Individual larva was rinsed briefly in 25% sucrose, then in distilled water and placed gently in the center of a100 mm 3% agar plate using a brush. Water has been soaked by a Kimwipe® paper around the larva. The larva has been given 3 minutes to adapt to the agar plate. After that the plate was placed on a 0.5X0.5 mm grid paper. 30 seconds movie was recorded from each larvae using Sony HD digital camera, secured on the tripod right above center of the plate. Speed and number of contractions per second of each larva that crawled on a straight path interval without stops were obtained from recorded movies.

Distance per contraction was calculated by dividing speed to contraction frequency of each larva. Crawling track of larva was digitized using active screen pen and Vitrite® and GIMP software packages.

Motility assays

Flies were collected at day 0 and aged until 3 and 7 days old in the temperature and humidity controlled incubator. Before the assay, each male was placed into the empty vial and allowed to rest for 10 minutes. For the climbing assay each fly was tested for the ability to cross 6 cm line in a vial after being gently tapped to the bottom of the vial. This assay was done in the red dim light twice for each fly with 10 minute recovery after each trial.

Mechanical agitation assay was done as described in (Haines and Stewart, 2007). Aged male was placed in the empty vial. The vial containing a single fly was banged onto soft pad 5 times. The time that it took the fly to right itself after falling on the back and number of falls was recorded. This assay was done in the room temperature conditions for each fly with 2 trials and 10 minute recovery after each trial.

Life span measurement

For individual longevity, males were collected within 24 hours following eclosion. Each male was placed into individual vial with fresh food and transferred to fresh food every 3 days. The day when the fly dies was recorded. 20 flies for each genotype were assayed.

For the group longevity, newly hatched males were collected in groups of 10, placed in the vials transferred to fresh food every 3 days and dead flies were counted until all died. 15 vials total per each genotype were assayed. All vials were kept horizontally in the incubator.

Analysis of the paralytic behavior

We defined a paralysis as a condition when the fly lies on its back with shaking movement of its legs and head and inability to walk. To analyze the kinetics of fly paralysis we isolated flies in groups of 10 within 24 hours after their eclosion and aged to 1, 3, 7 and 10 days. Kinetics of fly paralysis graphs were obtained by introducing a vial with 10 flies in the water bath heated to 38^0C and counting number of flies paralyzed at fixed intervals of time.

Temperature endurance of flies was determined by introducing a vial with 10 flies into the water bath heated to different temperatures and recording number of flies paralyzed within 3 minutes.

NMJ analysis

Adult flies were allowed to lay eggs on apple-agar media overnight. To control population density (Stewart and McLean, 2004) 20 first instar larvae were collected and transferred to plastic vials containing approximately 7 mL of the food. Larvae then grew at 25% humidity and 25^0 C incubator until wandering third instar stage. Third instar larvae were then dissected along the dorsal midline in PBT and fixed in 4%

paraformaldehyde for 10 minutes at room temperature. To visualize NMJs, the following primary antibodies were used: mouse anti-DLG 4F3 (Developmental Studies Hybridoma Bank, Iowa City, IA) 1:200 dilution; rabbit anti-HRP (Jackson ImmunoResearch Labs) 1:800 dilution. Secondary antibodies anti-mouse Cy3 at 1:250 dilution (Jackson ImmunoResearch Labs) was used to label DLG, and anti-rabbit 488 (Molecular Probes) at 1:140 dilution was used to label HRP. Fluorescent images of muscle 1 abdominal segments 4 were obtained using Zeiss Fluorescent microscope using Apotome z-stack sectioning. The stack was then projected onto a single plane using AxioVision 4.6 software for NMJ analysis. We quantified number of boutons and branches (with at least 3 boutons in each branch).

Electrophysiology

Current-clamp recordings were made at room temperature from abdominal dorsal muscle 1 (primarily abdominal segment A3) of climbing third instar larvae raised at 25°C as described earlier. Drosophila third instar larvae was dissected in cold Ca^{2+} free HL3 solution. For recordings, HL-3 solution was supplemented with $CaCl_2$ as indicated in the specific experiments. The composition for HL-3 saline is (in mM): NaCl, 70; KCl, 5; $MgCl_2$, 20; $NaHCO_3$, 10; Trehalose, 5; HEPES, 5; Sucrose, 115. Suction electrodes were beveled with a Sutter BV-10 Microelectrode Beveler (Sutter Instrument Co, Novato, California). Plots were made using Origin 4.0 (Microcal Software Inc., Northampton, Massachusetts) and Excel software. Muscles 1 and 6 were identified using Olympus microscope under the transmitted light. To elicit a postsynaptic response, the segmental

nerve was stimulated (0.2 ms pulse duration) at 2 times the stimulus amplitude required for a threshold response. Membrane potentials or synaptic currents were amplified through an Axoclamp 200B amplifier (Axon Instruments), filtered at 10 kHz, digitized and recorded on a Dell PC computer equipped with pClamp10 software (Axon Instruments). Microelectrodes had inner diameter of 8–10 μm (filled with 3 M KCl) with an input resistance of 10–15 MΩ were used for intracellular recording. mEJP analysis was done in a semi-automated manner using the event detection analysis in pCLAMPv10.0 (Molecular Devices, Sunnyvale, CA). Excitatory junctional potentials (EJPs) were analyzed using the cursor options of Clampfit 10.0.

EJP analysis was done by averaging ten events in each muscle and counting the average amplitude as one measurement for the genotype assayed. High-frequency stimulation was performed in 1mM and 4mM Ca^{2+}. 3000 pulses with frequency of 10Hz were delivered to the stimulated axon. Paired-pulse facilitation was performed in 0.75 mM Ca^{2+} HL3 saline and was quantified by recording a train of two pulse events separated by 50 ms time interval from a single muscle, then dividing the second EJP amplitude by the first EJP amplitude.

For electrotonic stimulation recordings were performed as described by (Song et al., 2002). For EJP tracing analysis 10 μM of TTX was added into the recording saline and EJP response was recorded every following minute until response is completely gone. For plotting EJPs during specific time points were normalize to EJP amplitude before addition of TTX.

CHAPTER IV

REVEALING MOLECULAR TARGETS AND GENETIC PARTNERS OF *DROSOPHILA* SIALYLTRANSFERASE

Studies of sialylation in vertebrate nervous system showed that this process plays important and dynamic role. Despite the fact that biosynthetic pathway of sialic acids is relatively well studied, it is still very difficult to reveal functionally relevant endogenous targets of sialylation. In vertebrate nervous system sialic acids are abundantly present on gangliosides, neural cell adhesion molecules, voltage gated ion channels. Invertebrates give a unique opportunity to identify some sialylated structures that are possibly conserved among species.

Drosophila represents an attractive model because of recently identified functional DSIAT enzyme and sialylated glycopeptides in its nervous system (Koles et al., 2007).

Little is known about the sialylated structures in protostome lineage of species (see INTRODUCTION). The presence of functional sialyltransferase and sialylated glycans in *Drosophila* indicate that DSIAT functions as a sialyltransferase in vivo. Previous attempts to capture α2-6 sialylated glycoproteins from membrane-associated protein extract from *Drosophila* heads using SNA lectin-affinity chromatography were not successful. Enrichment of *N*-glycosylated glycoproteins from membrane fraction of *Drosophila* heads using WGA (wheat germ agglutinin) lectin revealed many glycopeptides sequences that may contain sialylated glycans (Koles et al., 2007). Some

of the potential sialylated glycoproteins isolated from *Drosophila* nervous system include but are not limited to: voltage-gated potassium channel, voltage-gated calcium channel, ATP binding transporter, transmembrane receptor protein tyrosine kinase etc.

In addition to biochemical approach we embarked on genetic interaction studies that could potentially reveal DSIAT targets and elucidate sialylation pathway in *Drosophila*. Genetic interaction studies showing the effects of mutations on the phenotypes may shed light on biological functions regulated by a gene of interest. In addition, they are important for our understanding of the role of a particular gene or the biological process it mediates. Thus, we decided to test whether *DSIAT* genetically interacts with the some of the genes involved in neural functioning and development, as well as genes involved in the biosynthesis of sialic acids.

RESULTS AND DISCUSSION

Genetic interaction analysis

One of the most useful ways to uncover the biological role of a gene is through analyzing genetic interaction with other genes showing phenotypes similar to those observed with mutation in the gene of interest. Therefore we assembled a list of genes for genetic interaction studies based on the similarity of mutant paralytic phenotypes and possible sialylation of the genes' products (Table 3). In *Drosophila*, mutations in genes involved in neural transmission are commonly associated with temperature-sensitive (TS) paralysis phenotype (Siddiqi and Benzer, 1976). The group of so-called TS-paralytic mutants includes mutations in more than 20 genes affecting the synaptic vesicle cycle and

synaptic transmission or the properties of action potential and membrane excitability (Vijayakrishnan and Broadie, 2006). Our primary candidates for direct target of DSIAT activity are voltage-gated ion channels, since they were shown to be sialylated in vertebrates (Johnson et al., 2004; Shi and Trimmer, 1999). Others include genes involved in synaptic transmission (Table 3).

Table 3 Genes tested in genetic interaction experiments

Mutant	Gene product	Phenotype	Proposed function
Neuronal excitability			
para	α1 subunit of Na^+ channel	Impaired nerve conduction	Action potential propagation
cacophony	α1 subunit of Ca^+ channel	Decreased evoked release	Presynaptic Ca^{2+} influx
Sh	α subunit of K^+ channel	Behavioral response to ether	Membrane repolarization
Hk	β subunit of K^+ channel	Behavioral response to ether	
Endocytosis			
Shibire	Dynamin GTPase	Depletion of vesicles	Involved in endocytosis
Exocytosis			
comatose	NSF ATPase	Loss of evoked release	Disassembly of cis SNAREs after fusion

Heat shock-induced paralysis phenotype was tested for different homo- and heterozygous combinations of mutant alleles. Genetic interaction between *DSIAT* and *Sh, comatose*, cac^{ts2} alleles were inconclusive. We couldn't detect obvious genetic interaction in temperature-sensitive assays and sensitivity in exposure to ether (for *Sh*, *Hk* and

DSIAT). We found some genetic interactions between *DSIAT* and shi^{ts} alleles. However this data is preliminary and needs to be confirmed as well.

We found a strong genetic interaction between *DSIAT* and *para* gene that encodes for α-subunit of voltage-gated sodium channel. In temperature-sensitive assay, five day old *DSIAT* mutant females were paralyzed within 6-8 minutes at 38^0C heated water bath. At the same time recessive $para^{ts1}$ mutation (temperature-sensitive hypomorphic allele) causes five day old females to be paralyzed within approximately 8 seconds. Addition of single $para^{ts1}$ copy into *DSIAT* mutant background enhances *DSIAT* temperature-sensitive phenotype and decreases the temperature of a complete fly paralysis to 30 seconds. To prove that enhancement of this phenotype is because of the addition of *para* mutation in *DSIAT* mutant background, we added normal copy of *para* on the 4th chromosome to genotype carrying a single copy of $para^{ts1}$allele and two mutant copies of *DSIAT*. We observed partial rescue of this enhancement suggesting that phenotype enhancement was caused by the genetic combination of *para* and *DSIAT* alleles (Figure 31).

Para is also known to be affected by DDT (1,1,1-trichloro-2,2 *bis*(*p*-chlorophenyl) ethane) insecticide. In insects it was shown that DDT binds to voltage-gated sodium channel and locks it in the open state. Prolonged influx of sodium ions makes nerves to fire repeatedly and this causes death of the insect. Several mutations in *para* cause flies to confer resistance to DDT by either direct alteration of binding site or by delaying the normal voltage-dependent mechanism of its inactivation (Pittendrigh et al., 1997; Soderlund and Bloomquist, 1989).

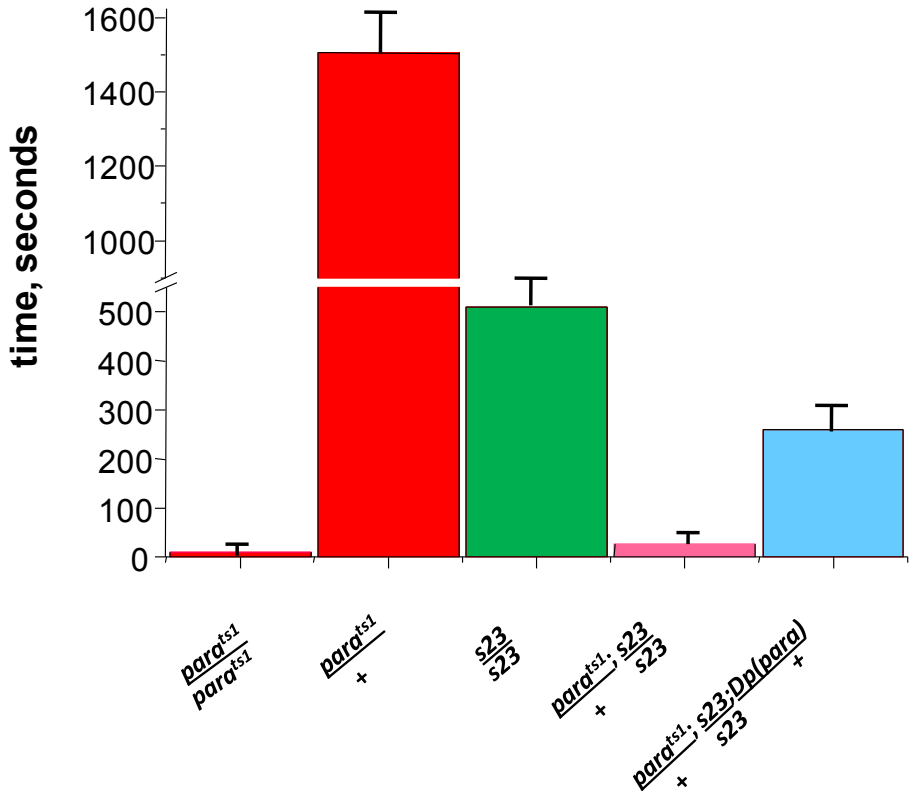

Figure 31. Single copy of recessive *para*^{ts} allele enhances *DSIAT* temperature-sensitive phenotype.
Bars show time that it takes for three day old females of the assayed genotypes to be paralyzed at 38 ^{0}C. Flies *para*^{ts1} are typically paralyzed within 8 seconds. Flies *para/+; s23/+* and *para/+* are not paralyzed at this temperature. *DSIAT* mutant females on average are paralyzed within 8-10 minutes. Flies *para/+; s23/s23* females are paralyzed 6 times faster than *DSIAT* mutants alone. This dramatic decrease in paralysis time can be reversed by bringing wild type *para* copy (*Dp(para)*) on the fourth chromosome in *para*/+;*s23/s23* mutant background.
Number of flies tested for each data point was 20.
Flies for this test have had similar genetic background. Error bars indicate SEM.

We decided to examine the sensitivity of DSIAT mutant and wild type flies to DDT. Briefly, 10 adults, 4 d old were exposed to insecticide-coated glass scintillation vial for 12 hours at 25^0C. After 12 hours mortality was scored as inability to move. Preliminary data suggests that DSIAT mutants are significantly less sensitive to DDT than wild type flies (Figure 32). This experiment gives another evidence of possible involvement of DSIAT in functional modification of voltage-gated sodium channels.

Voltage gated sodium channels (VGSC) are critical in action potential initiation and propagation in excitable cells. The typical VGSC consists of glycosylated subunits α, and one or more β. In vertebrates, 30% of mass of sodium channels belongs to carbohydrates, which are mostly comprised of *N*-acetylhexosamines and sialic acids, modulating properties of these channels (Cronin et al., 2005).

Drosophila α-subunit of VGSC protein, encoded by gene *para*, has approximate molecular mass of 250 kDa and consists of four highly homologous domains I-IV. Each of these domains is comprised of five hydrophobic transmembrane-spanning segments S1, S2, S3, S5, S6, one positively charged segment S4, a pore loop with linking regions between the domains, and extended aqueous N- and C-terminal domains (Loughney et al., 1989) (also see Figure 33).

S4 transmembrane segments in each homologous domain are voltage sensors, because of the positively charged amino acid (arginine or lysine) located within electric field at every third position of this segment separated by two hydrophobic residues. The inner lining of the pore of the channel is surrounded by the S5 and S6 transmembrane segments and SS1/SS2, short membrane-associated segments between them (also known

as P loops) form narrow ion-selective outer lining of the pore. Short intracellular loop connecting domains III and IV is an inactivation gate which may fold back and block the ion-conducting pore thus preventing ions to flow through it. Analysis of *Drosophila* PARA polypeptide sequence by NetNGlyc predicts 6 *N*-glycosylation sites in extracellular domains which may carry sialic acids (Figure 33A). Three of these appear to be between regions IS5 and IS6 (positions 313, 325 and 343). One sialylation site appears to be between IIS5 and IIS6 and two more between IIIS5 and IIIS6 (Figure 33B and (Loughney et al., 1989).

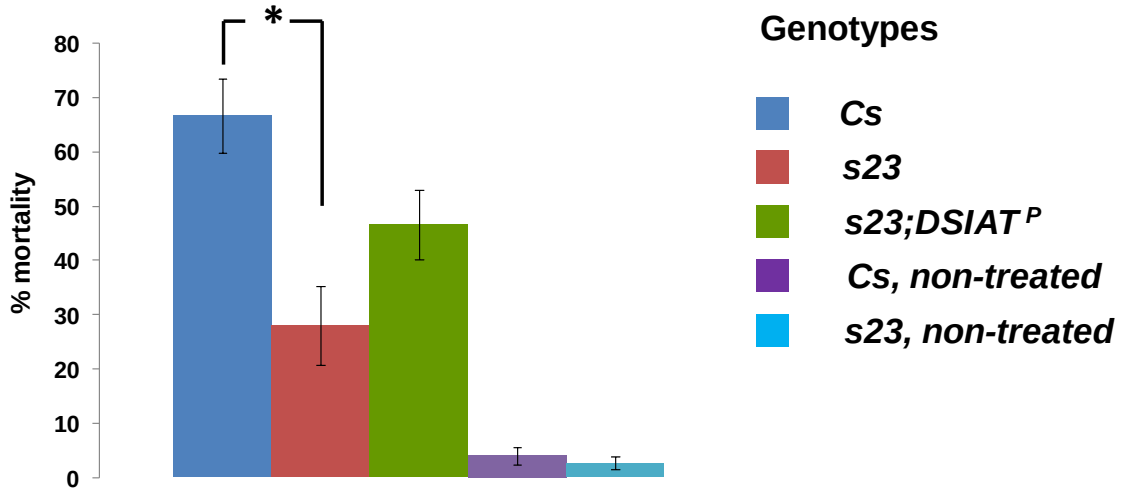

Figure 32. Mortality of flies exposed to DDT.
Comparison of mortality rates for wild type (*Cs*), DSIAT mutant (*s23*) and rescue flies exposed to 10µg of DDT per vial. DSIAT mutants show much higher resistivity to DDT estimated as percentage of dead flies per total number of flies in a vial (*p<0.001). No complete rescue using genomic copy of DSIAT was observed. Mortality rates for wild type and mutant flies non-treated with DDT are also presented in the graph. Total number of flies assayed per each genotype is 150. Error bars represent SEMs.

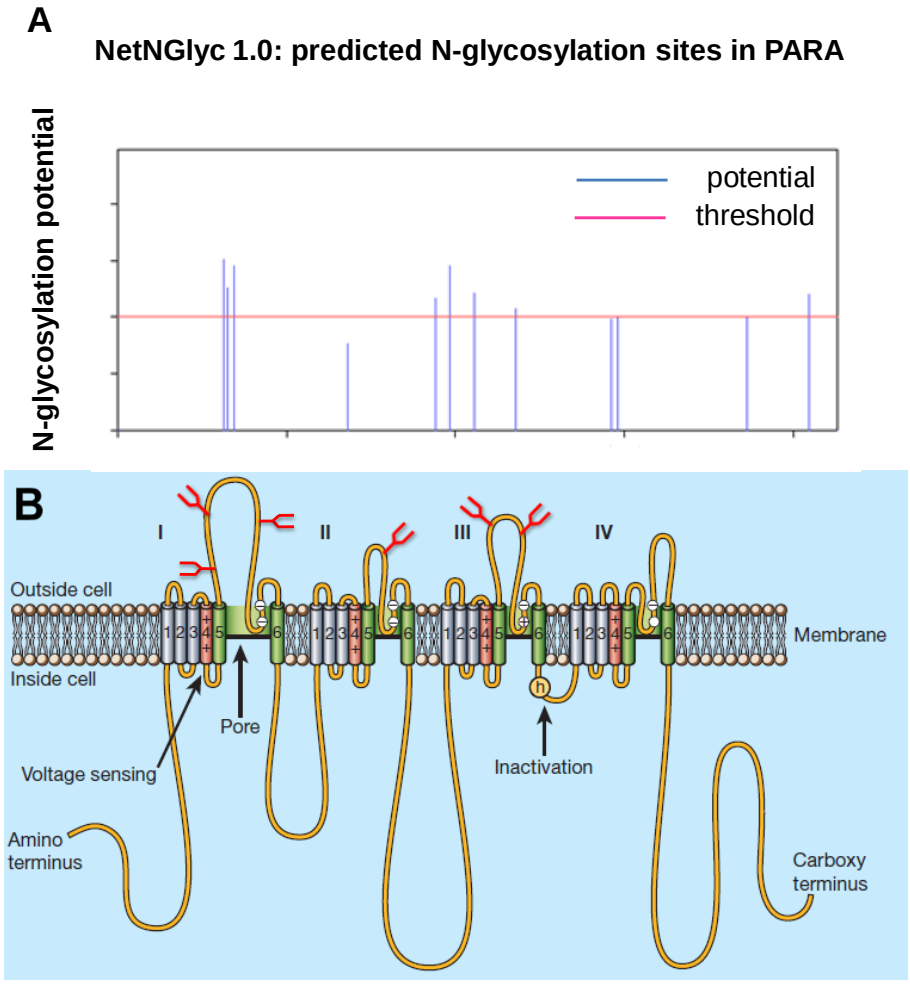

Figure 33. Prediction of N-glycosylation sites and molecular map *Drosophila* PARA with *N*-glycosylation sites carrying sialic acids.
(A) shows prediction of N-glycosylation sites in *Drosophila* PARA protein using NetNGlyc 1.0 Server.
(B) shows two-dimensional molecular structure of α-subunit of the voltage-gated sodium channel composed of four homologous domains (I to IV) each of which consists of six transmembrane segments. Potential N-glycosylation sites are shown in red on extracellular loops. Cylinders represent α-helical transmembrane segments. Red indicates the S4 transmembrane segment which serves as a voltage sensor. Green cylinders and shading between them between S5 and S6 segments form the walls of sodium-ion-conducting pore. Negatively charged amino acids between S5 and S6 transmembrane segment serve as TTX binding site. Modified from (Catterall, 2001)

The important question that awaits further investigation is whether PARA is sialylated by DSIAT?

We attempted to answer this question using genetic interaction analysis. Heat-shock paralytic assays showed that DSIAT strongly interacts with *paralytic* gene. Because of variety of *para* splice-forms (more than 23), cloning of correct *para* cDNA and consequent direct biochemical assay for sialylation presents a real obstacle. However, strong genetic interaction between *DSIAT* and $para^{ts1}$ allele suggest that α-subunit of voltage-gated sodium channels may be sialylated in flies.

This conclusion is also supported by pharmacological experiments that demonstrate an altered onset of neuromuscular paralysis (i.e., EJP failure) between mutants and wild type in the presence of TTX, a blocker of voltage-gated sodium channels. It is also supported by pharmacological effect of DDT insecticide that causes persistent activation of sodium channels in the wild type flies resulting in subsequent death and prolonged resistance to it in case of DSIAT mutant flies.

Based on studies in vertebrates, we predict that removal of sialic acids in *Drosophila* could reduce sodium channel opening probability thus affecting its conductance. Furthermore direct measurement of action potential in the mutant axon will help to understand how removal of sialic acids affected gating properties of the voltage-gated sodium channels.

Link to sialylation pathway

To find out if DSIAT is involved in sialylation process conserved among vertebrate species and to test if sialylation is the mechanism underlying DSIAT biological activity, we investigated genetic interactions between *DSIAT* and other genes predicted to be involved in sialylation pathway in *Drosophila* and tested if sialyltransferase activity of DSIAT is important for its function in vivo. In addition we investigated possibility to create a "sialic acid- negative" phenotype by sialidase overexpression.

The expression of *DmCSAS* that encodes for CMP-Sia synthetase is developmentally regulated and has restricted pattern of expression within the fly CNS (see INTRODUCTION). We first analyzed phenotypes caused by mutation in *CMP-Neu5Ac synthetase* (*DmCSAS*) gene. Interestingly mutation in *DmCSAS* phenocopies righting and paralytic behavior observed in *DSIAT* mutants (Figure 34). Rescue data suggests that observed phenotypes are specific to mutation in *DmCSAS* gene.

The expression of *DSIAT* and *DmCSAS* is similarly present in the developing CNS, which suggests that sialylation is possibly conserved between *Drosophila* and higher organisms. However their patterns of expression are slightly different. Future experiments on double fluorescent in situ hybridization may reveal if they have overlapping patterns. Although studies on *Drosophila sialylation* provide a strong evidence for the presence of sialylation pathway that functions in developmentally-regulated and tissue-specific manner, several questions await further investigation. What is the expression pattern of other genes (*DmSAS*, putative *Sia-P phosphatase*, *Sia*

transporter) involved in a biosynthetic pathway? What are the mutant phenotypes of these genes involved in sialylation and do they show any genetic interaction?

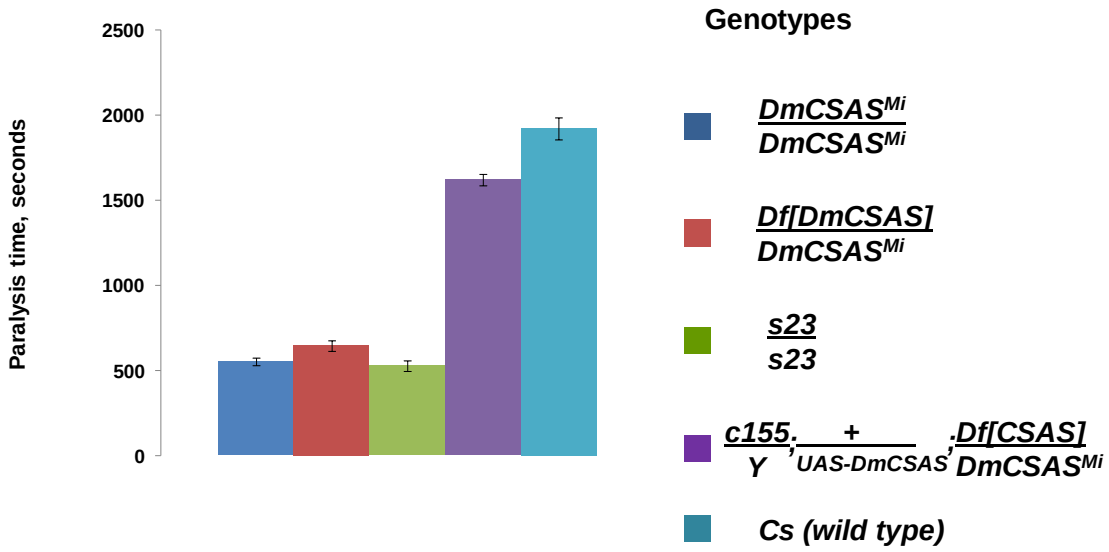

Figure 34. Mutations in *DmCSAS* phenocopy *DSIAT* paralytic behavior.
Figure shows time that is required for 3d old males to be paralyzed in the water bath at 38^0C. *DmCSAS* mutants (*DmCSAS*^{Mi} allele) exhibit similar paralytic behavior as *DSIAT* mutants (*s23* allele) with average times around 600 seconds (10 minutes). This *DmCSAS* mutant phenotype can be rescued by the expression of wild type copy of *DmCSAS* using UAS-Gal4 system under neuronal specific driver (t-test, $p<0.0001$).
All assayed flies were 3d old males. Number of assayed flies for each genotype is 20. Error bars indicate SEMs.

Next we wanted to discriminate between two possibilities for the requirement of DSIAT in neurons: first, whether the DSIAT protein exerts in vivo function via its enzymatic activity as a sialyltransferase, or, second, if DSIAT has some non-enzymatic role within the cell.

Considering that both *DSIAT* mutant alleles that we used in this study are predicted to produce a severely truncated protein (or no protein product at all), we could not discriminate between these scenarios based on their analysis. Thus we embarked on generating an enzymatically inactive form of DSIAT that would still be correctly folded and presumably preserve its other molecular features. Although there is no crystal structure available for vertebrate-type sialyltransferases, a conserved histidine residue within the VS motif was proposed to be involved in the catalytic activity of mammalian sialyltransferases (Kitazume-Kawaguchi et al., 2001). Mutations of this residue inactivated mammalian sialyltransferases, while presumably preserving their folding and substrate recognition (Jeanneau et al., 2004). We reconstructed this point mutation in the DSIAT protein by changing His^{406} to Lys ($DSIAT^{HK}$). Site directed mutagenesis as well as biochemical assay were performed by Dr. Kate Koles. The mutant protein had no detectable catalytic activity when expressed and purified from *Drosophila* cultured cells (Figure 35). At the same time, my experiments demonstrated that $DSIAT^{HK}$ protein was unable to rescue locomotor abnormalities and heat-induced paralysis when it was expressed in vivo in DSIAT mutants (Figure 36). This result indicated that the sialyltransferase catalytic activity of DSIAT is essential for its biological function.

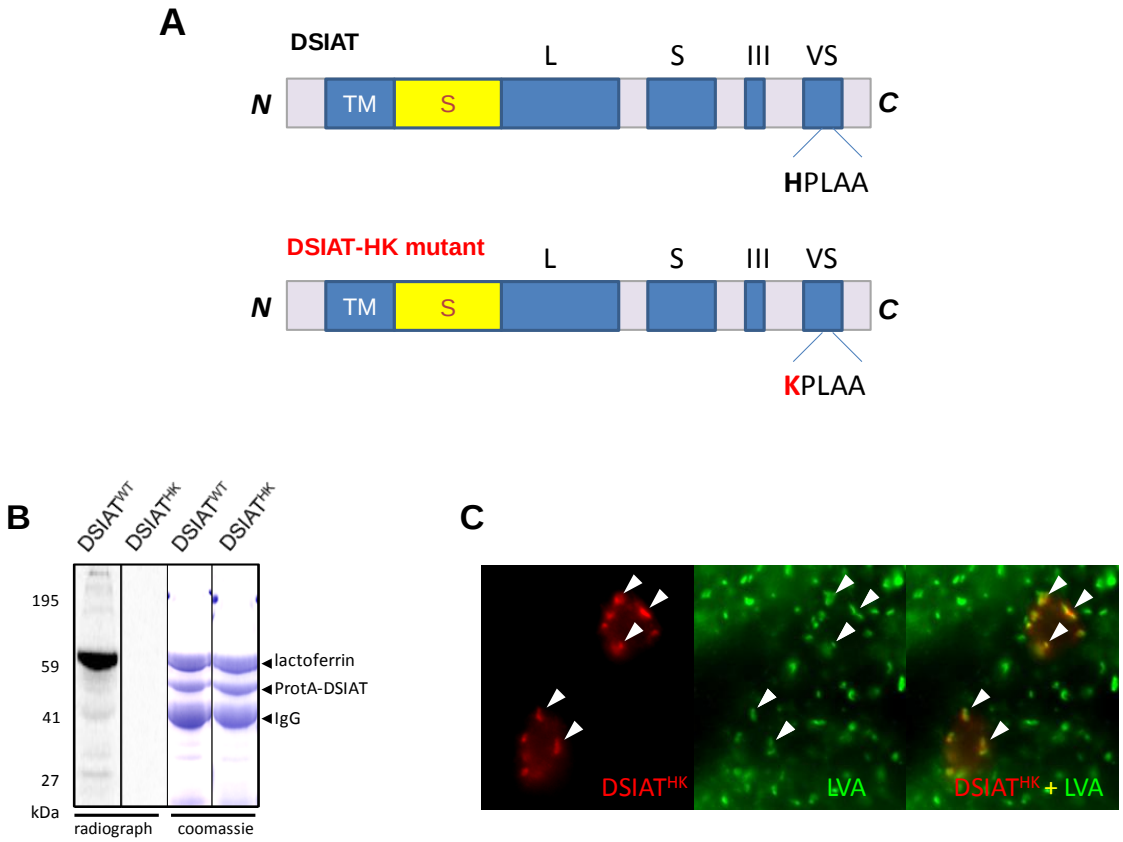

Figure 35. Subcellular localization and purification of DSIAT^{HK}.
(A) shows schematic of DSIAT protein with *N*-terminus (*N*), transmembrane domain (TM), stem region (S), catalytic domain that consists of 4 conserved sialylmotifs: Large (L), Short (S), III, Very Short (VS) and C-terminus (*C*). DSIAT-HK mutant has Histidine (H) to Lysine (K) substitution within sialylmotif VS.
(B) Autoradiograph of lactoferrin acceptor sialylated with [^{14}C]Neu5Ac by DSIAT (wild type) but not by DSIAT^{HK}. Coomassie staining of purified protein-A tagged DSIAT and DSIAT^{HK} shows that DSIAT^{HK} was produced at the same level as DSIAT^{WT}.
(C) Immunofluorescent staining of Drosophila brain cells expressing DSIAT^{HK} protein reveals its co-localization with Golgi resident protein (Lva): red(Cy3), DSIAT^{HK}; green(FITC), Lva. Right panel shows the overlay of red and green channels.

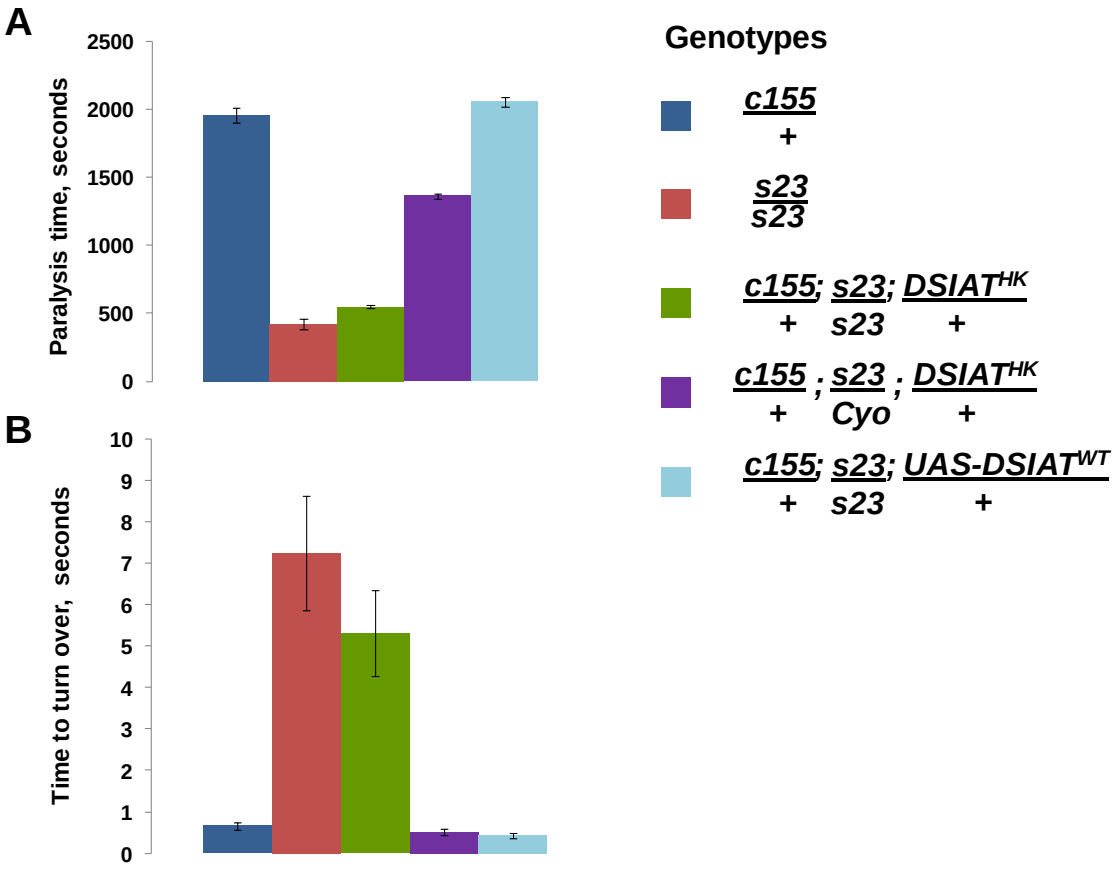

Figure 36. DSIAT protein possesses in vivo function via its enzymatic activity as a sialyltransferase and does not have a non-enzymatic role within the cell.
(A) shows average paralysis time of 5-d old females of tested genotypes in water bath heated to 38^0C.
(B) shows average time necessary for a 5-d old female to turn over after mechanical shock of the vial on a soft pad.
In the 'genotypes' legend: *c155* - pan-neuronal driver; *s23* – DSIAT mutant allele; $DSIAT^{HK}$ - point mutation in *DSIAT* changing His^{406} to Lys; $DSIAT^{WT}$ - wild type copy of *DSIAT* with *C*-terminal *HA*-tag.
In both assays, HK copy of DSIAT driven by c155 does not rescue paralytic and locomotor behavior of DSIAT mutant (compare third and second bar), while wild copy of DSIAT perfectly rescues the described behavioral phenotypes.
Number of flies assayed for each genotype was 20. Error bars indicate SEM.

'Sialic acid negative' phenotype

Finally we decided to test if overexpression of bacterial sialidase can phenocopy locomotor abnormalities and paralytic behavior observed in DSIAT mutants. We used neuronal specific driver to express sialidase from a non-pathogenic bacterium, *Arthrobacter ureafaciens*, which was reported to cleave terminal sialic acids linked via α,2-3, α,2-6 and α,2-8 in wild type background (Saito et al., 1979). We found only minor locomotor abnormalities in these flies (Figure 37). These flies did not have a temperature-sensitive paralysis phenotype. This data suggests that *Arthrobacter ureafaciens* sialidase activity is not enough to produce complete cleavage of all sialic acids in *Drosophila* CNS.

MATERIALS AND METHODS

Fly strains and maintenance

Prior to all behavioral assays flies were kept at 25^0C and 25% humidity day-night cycle controlled incubator. The following Drosophila mutant alleles, chromosomal aberrations, and transgenic insertions used in the study were obtained from the Bloomington Drosophila Stock Center, Indiana University: $para^{ts1}$, *tipE*, cac^{ts2}, shi^{ts1}, *Sh*, *Hk*, $comt^{6}$, *DmCSAS*. Stocks $Dp(1:4)r^{+}f^{+}$ -duplication of entire $para^{+}$ gene and $para^{lk5}$ were generously provided by Dr. B. Ganetzky (University of Washington) (Ganetzky, 1984).

For DDT resistance assay, wild type (*Cs*), DSIAT mutants (*s23* allele) and rescue ($s23;DSIAT^{P}$) flies were collected and aged to 4 days old. 10 µg of DDT was dissolved

in 100 µl of acetone. Scintillation vials were coated with this mix until acetone dries out. Control vials were treated with acetone alone. Flies were then left in a vial closed with cotton plug in humidity and light controlled incubator. Number of dead flies was scored after 12 hours.

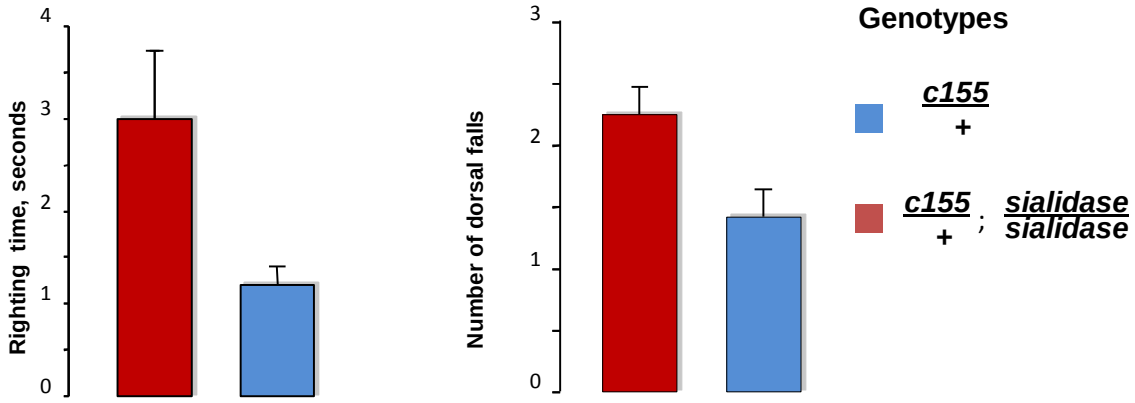

Figure 37. Ectopic expression of *A. ureafaciens* sialidase in *Drosophila* neurons causes impairment of locomotion.
Overexpression of two copies sialidase increases time that mutant fly spends on its back to turn over in comparison to wild type (t-test, $p<0.05$) and it also increases the number of dorsal falls (t-test, $p<0.05$). Number of tested flies for each assay was 20. Error bars indicate SEMs.

CHAPTER V

CONCLUSION

Sialylation has been shown to be present in different organisms, including deuterostomes (vertebrates, ascidians, and echinoderms), some bacteria, fungi and protozoa. In plants and the majority of protostomes, sialic acids appear to be absent or their presence is controversial. In higher animals, sialylation plays a fundamental role in the development of variety of tissues including nervous system. Sialylated glycoconjugates are highly expressed in vertebrate brain and spinal cord and undergo qualitative and quantitative changes in sialic acid content during nervous system development (Hildebrandt et al., 2007; Ngamukote et al., 2007). Regulated expression of sialyltransferases, enzymes involved in the biosynthesis of the sialylated glycoconjugates, is one of the mechanisms that control these changes. However, the complexity of these regulatory mechanisms and the sialylation pathway in humans presents a real obstacle for understanding the functions of sialylation.

Recently several genetic and biochemical studies suggested potential for sialylation in flies. First of all, a search of *Drosophila* genome revealed the presence of several genes that encode orthologues of genes involved in vertebrate sialylation pathway including *Neu5Ac phosphate synthetase, CMP-Neu5Ac synthetase, CMP-Neu5Ac/CMP antiporter* and *DSIAT.* Alignment of protein sequences of human and *Drosophila* sialyltransferases shows a significant degree of conservation between their catalytic domains suggesting that the enzymatic role of these sialyltransferases is highly conserved

between vertebrates and protostomes. In addition, sialic acids have been identified in *Drosophila* embryos and adult heads (Aoki et al., 2007; Koles et al., 2007). These findings reveal significant similarities between sialylation pathways of vertebrates and insects and provide evidence for their common evolutionary origin.

Drosophila represents an invertebrate model organism that is often used for analysis of gene functions and offers more advantages over some vertebrates such as zebrafish and mice. Because of its lower complexity and available arsenal of genetic techniques, *Drosophila* is powerful model system and it is more amenable to functional analysis than vertebrate models.

Still it is an open question if we can use *Drosophila* model organism to understand the function of sialylation in protostomes and further extend this knowledge to higher organisms.

In this dissertation work I tried to answer this question and reveal the role of sialylation in *Drosophila*.

Vertebrate sialyltransferases in the CNS

Great strides have been made in uncovering sialylated structures and understanding the exact biological impact of sialylation on properties of glycoproteins and glycolipids in the central nervous system (Troy, 1992). Yet our understanding of the basic biological roles of sialylation and sialyltransferases in the development and functioning of the CNS is rudimentary.

Vertebrates comprise the largest and most studied family of at least 20 different intracellular Golgi membrane bound sialyltransferases (Table 1 and Chapter I). In spite of their fundamental role in the synthesis of specific sialylated structures, there is still limited information available on their protein structure, mechanism of action and regulation of expression (Harduin-Lepers et al., 2005). Our knowledge of structure, enzymatic activity and expression of sialyltransferases working in the nervous systems is even more unclear. To date it is known that ST3Gal II and ST3Gal V (ST3Gal subfamily), ST6GalNAc V and VI (ST6GalNAc subfamily), ST8Sia I, III and V (ST8Sia subfamily) function in the nervous system and are implicated in ganglioside biosynthesis. However, for the majority of them, mutants are not available, and no extensive phenotypic characterization of gene knockouts has been done.

Polysialyltransferases ST8Sia II and ST8Sia IV (ST8Sia subfamily) also function in the nervous system of vertebrates and catalyze transfer of multiple sialic acids on neural cell adhesion molecule (NCAM). These are the only two sialyltransferases out of the whole family of mammalian sialyltransferases that are extensively studied; phenotypic characterization of their knockouts and vital role of NCAM polysialylation has been revealed to some extent (Angata et al., 2002; Close et al., 2001; Franceschini et al., 2001).

In the vertebrate developing nervous system, sialyltransferases so far have been implicated in the biosynthesis of a few glycoproteins. The best studied sialylated molecules include neural cell adhesion molecule (NCAM), voltage-gated sodium channels and gangliosides (sialylated glycosphingolipids) (Close and Colley, 1998;

Curreli et al., 2007; Finne, 1982; Muhlenhoff et al., 1996; Sato et al., 2000; Zuber et al., 1992).

Let's discuss how removal of sialic acids or ablation of sialyltransferases affects properties of these glycoproteins in different organisms. This will help us to gain insights in understanding biological role of this type of post-translational modification and determine if *Drosophila* can be used to study sialylation.

Voltage-gated sodium channels

The importance of voltage-gated sodium channel sialylation has been suggested by studies in vertebrate muscle and brain cells. These channels have significant content of sialic acid residues on the glycosylation sites of the extracellular region (Zuber et al., 1992). Sialic acids highly contribute to the total negative surface charge near voltage-gated channels. They are thought to be transferred by polysialyltransferases forming long chains, however no direct evidence of which polysialyltransferase accomplishes this has been shown. In addition, no functional characterization of sodium channel properties was performed in polysialyltransferase knockout vertebrates.

Nevertheless, studies have shown that reduction of sialylation level of eel electroplax or rat skeletal muscle sodium channel protein by neuraminidase treatment causes a large depolarizing shift of steady-state activation and inactivation (Bennett et al., 1997; Recio-Pinto et al., 1990). Expression of different voltage-gated sodium channel subunits of rat cardyomyocytes in cell culture showed that the level of sialylation of

sodium channels during postnatal development strongly influences voltage-dependent channel gating (Stocker and Bennett, 2006).

The functional role of sialylation of a central neuronal voltage-gated channel has been examined recently. In particular, it was found that neuraminidase treatment causes a large depolarizing shift of voltage-gated sodium channel activation/inactivation and action potential threshold in rat hippocampal CA3 pyramidal neurons (Isaev et al., 2007). Thus, sialylation appears to be a powerful mechanism to control neuronal and network excitability in vertebrates.

We decided to establish the role of *Drosophila* sialyltransferase in the CNS motor neurons by detailed characterization of electrophysiology of mutant neuromuscular junctions (NMJ) deficient for sialyltransferase. We identified the motor neuron which expresses DSIAT and measured its electrophysiological characteristics.

We used DSIAT sensitized genetic background to identify genes that function in the same pathway in *Drosophila* organism. We decided to use such sensitized genetic background based on the premise that if the activity of a key component of a pathway is reduced to a threshold level of function, other components of the pathway can be made susceptible to a reduction in gene dosage. In fact we obtained strong genetic evidence that DSIAT can regulate neuronal excitability by modulating function of PARA, an alpha subunit of voltage gated sodium channel. By genetic interaction experiments we showed that mutation in *para* gene strongly enhances DSIAT mutant phenotype.

Pharmacological experiments with tetrodotoxin, a blocker of sodium channel and action potential propagation, suggested that in the absence of DSIAT, desialylated mutant

channels are more resistant to binding this toxin. Additionally we showed that DSIAT mutant flies are less sensitive to DDT (a type I pyrethroid pesticide targeting voltage gated sodium channels (O'Reilly et al., 2006)).

We think that DSIAT mutation affects binding of either TTX or DDT by causing functional changes in voltage gated sodium channel that alleviate consequences of exposure to the agents. Such changes may alter voltage dependence or kinetics of activation or inactivation of voltage-gated sodium channels.

Thus all these data support the hypothesis that DSIAT is involved in regulating excitability of axonal membranes. It potentially affects the shape and/or the amplitude of the action potential propagating along the axon.

In fact, studies of gating kinetics of the sodium channels in vertebrates showed that desialylation of these channels causes increase in the action potential threshold. This means that shape of the rising phase of the action potential will be less rapid and the resulting amplitude of the action potential will be decreased. We expect to see less steep rise and lower amplitude action potential in DSIAT mutants. Additionally, channel gating is dependent directly on the membrane potential and sialic acids contribute to the surface charge of the membrane. Thus, desialylation of channels may alter membrane potential and subsequently affect gating. Taken together, these factors will lead to decreased power of action potential, thus decreased Ca^{2+} influx into the synapse and lower postsynaptic response. Analysis of the relationship among action potential amplitude, Ca^{2+} current and postsynaptic response in normal and desialylated fly synapses will help us to predict how amplitudes of action potential generated by sialylated and desialylated channels in

vertebrates affect the synaptic potential. This will help us to gain further understanding how sialylation affects neuronal signaling, muscle contraction and overall vertebrate physiology.

Our research is the first work on the in vivo involvement of an alpha 2,6 glycoprotein-specific sialyltransferase in the function of the CNS. And the results strongly suggest that this involvement is principally based on the sialylation of voltage-gated sodium channels. We think that sialylation of extracellular loops of PARA protein may influence their confirmation and subsequently functional properties of the voltage-gated sodium channel.

Voltage-gated sodium channels are charged. Negatively charged sialic acids highly contribute to channel charge, because they polarize the surrounding environment and establish an electrostatic potential. Based on our electrophysiological analysis performed on *Drosophila* NMJ, sialic acids seem to affect action potential. We think of three mechanisms how sialic acids can modulate voltage-gated Na^+ activity.

First, sialic acids may decrease the polarization of the axonal membrane and their absence will cause slight decrease in the Na^+ current. Decreased inward sodium current will then decrease the rising phase of the action potential and will act against K^+ outward current thus reducing the falling phase of the action potential. Because neuronal activity will occur at less depolarized potentials, this will impact neuronal excitability in a negative fashion. Potentially this will create a less easily excitable cell, and affect the integrity of spreading of the action potential. Additionally, depolarizing shift caused by absence of sialic acids will diminish the amplitude of the action potential leading to less

number of vesicles released from the synapse and reduced muscle response and EJP amplitude.

Second, sialic acids may increase the rate of recovery from fast inactivation. If functional sialic acids increase the recovery rate from inactivation, then they will decrease the effective time between successive depolarizing/repolarizing waves because of the redistribution of voltage-gated Na^+ channels among functional states toward a larger population of activated channels. Then in the absence of sialic acids a larger percentage of voltage-gated sodium channels remain inactivated for longer period of time which will subsequently limit further membrane depolarizations and may also result in decreased response of the muscle.

Third, we know that voltage-gated sodium channels themselves play essential role in establishing structural stability of axonal segments. Therefore, removal of sialic acids from VGSCs may cause decreased expression of the functional channels on neuronal surfaces. This will subsequently result in decrease of the action potential amplitude and further reduce the synaptic response.

The hypothesis about the influence of negatively charged sialic acids on the properties of α-subunit of voltage-gated sodium channel and action potential modulation in *Drosophila* is in a good agreement with data from vertebrate models.

Influence of voltage-gated channel properties by their functional modification with sialic acids may also explain observed age-dependent progression of locomotor abnormalities and higher sensitivity to increased temperature in flies deficient of sialyltransferase. In humans, it is known that nerve conduction velocities are high by age

of 3-5, then they slightly increase in the arms and decrease in legs during childhood up to 19 years. With aging though, nerve conduction velocities decline after 50 years of age and they are 10% reduced at 60 years of age. Aging also causes diminution of the amplitude of action potential which decline slowly after the age of 60 years sometimes dropping to 50% after age of 70 (Bradley, 2004).

We found that mutation in DSIAT causes significant progression of locomotor abnormalities with aging in comparison to wild type flies. We think of two mechanisms that may explain this observation.

First, impaired action potential caused by undersialylation of voltage-gated sodium channels from early development may erroneously sculpt the functional neuronal network through adulthood causing neurodegeneration of axonal and/or synaptic surfaces. Progressive degeneration will subsequently diminish synaptic response causing dramatic physiological response of flies with aging.

Second, desialylation of alpha subunit of VGSC may cause retention of other functional subunits in the secretory compartment and their further downregulation. If this is the case, then absence of sialyltransferase and further channel desialylation will prevent VGSC translocation to the cell surface and may subsequently decrease turnover of the functional channels. Desialylated channels that have been originally targeted to the cell membrane will deteriorate with time, may leak and each time generate reduced action potential. Thus, if they are not replaced by new functional channels overall performance of axonal membrane should drastically change with fly age causing decreased response of the muscle side.

Thus we think that our research provides a strong basis for further understanding how sialic acids affect surface charge and gating properties of voltage-gated channels in protostomes and for revealing evolutionary conservation of function of sialylation in higher organisms.

Undoubtedly direct biochemical assay and mass spectrometry analysis will tell us if the *para* gene product is indeed a sialylated glycoprotein in flies. This will not only identify the first sialylated protein in *Drosophila*, but will also confirm the evolutionary conserved sialylation of Na^+ channels across the animal kingdom.

Although our findings are preliminary, they can be useful for understanding the possibilities of epilepsy and seizure treatment in humans by reducing the negative surface charge contributed by sialic acids and thus decreasing neuronal excitability.

In fact, it is known that mutations in human voltage gated sodium channels can essentially lead to altered action potential and cause severe disorders. Examples include paramyotonia congenital and hyperkalemic periodic paralysis associated with mutations in the human skeletal muscle Na^+ channel alpha subunit (Bouhours et al., 2005; Brancati et al., 2003; Lehmann-Horn et al., 1987). Generalized epilepsy with febrile seizures, intractable childhood epilepsy with generalized tonic-clonic seizures, benign familial-infantile seizures and myoclonic epilepsy in infancy are associated with mutations in voltage-gated sodium channels in the central nervous system (Kanai et al., 2004; Nagao et al., 2005; Pineda-Trujillo et al., 2005; Yamakawa, 2005).

In addition, some disorders resulted from defects in synthesis of sialic acids and their attachments to other compounds have been reported in humans. They include

psychomotor retardation, seizures, hypotonia, etc. (for full review see (Jaeken and Matthijs, 2007).

Therefore we think undersialylation of voltage-gated sodium channels, a potential disorder that has not been identified yet, may directly affect cellular excitability and ultimately lead to similar phenotypes. Understanding how the level of sialylation regulates the surface charge and neuronal excitability in *Drosophila* is very promising. It will give us more complete understanding how by regulating level of sialylation we can modify excitability of the cells and ultimately suppress seizures in humans.

Neural cell adhesion molecule (NCAM)

Neural cell adhesion molecule is a homophilic binding glycoprotein expressed on the surface of neurons, glia, skeletal muscles and natural killer cells, which has been implicated in cell-cell adhesion, neurite outgrowth and synaptic plasticity. NCAM is polysialylated in vertebrates. Even though posttranslational modification of NCAM with polysialic acid is one of the most studied type of sialylation in the developing mammalian nervous system, our understanding of the functions of these terminal glycans is still limited.

Vertebrate polysialyltransferases ST8Sia II and ST8Sia IV are expressed in the nervous system of most vertebrates where they catalyze the transfer of hundreds of sialic acids mainly on *N*-glycans of NCAM affecting neuronal migration and plasticity (Chang et al., 2009; Nakayama et al., 1998). This conclusion is supported by multiple studies performed on cell lines and vertebrates. For example, in neuroblastoma cells, removal of

polysialic acids induced *cis*-dimerization of non-polysialylated NCAMs at cell-cell contact leading to their reduced proliferation and favoring neuronal differentiation (Seidenfaden et al., 2006).

Double knockout mice for ST8Sia II and ST8Sia IV polysialyltransferases displayed severe defects in anatomical organization of the forebrain and wiring defects, progressive hydrocephalus, postnatal growth retardation and early death (Hildebrandt et al., 2007). Removal of polysialic acids by endoneuraminidase *N* injection in chicken eggs caused severe defects in fasciculation, collateral branching, terminal arborizations of motor neurons and optic fibers (Maarouf et al., 2003; Rafuse and Landmesser, 2000). The likely explanation of morphogenetic defects induced in polysialic acid deficient chickens as well as polysialyltransferase mutant mice is increased adhesiveness of cell surfaces after polysialic acid removal. It also suggests that the major function of polysialic acid is to mask NCAM by steric hindrance and thus guarantee that NCAM contacts take place in a highly organized time- and site-specific manner (Hildebrandt et al., 2007).

Other models involving modification of NCAMs by polysialyltransferases have been also proposed recently. Binding of polysialic acid to heparin sulfate proteoglycans of the cell surface or the extracellular matrix may modulate synapse formation (Dityatev et al., 2004; Storms and Rutishauser, 1998).

In the nervous system of developing zebrafish, polysialic acid is transiently formed on NCAM, where it acts in guiding outgrowing axons (Marx et al., 2001). Defects in midline crossing of hindbrain commisures caused by endoneuraminidase *N*

treatment in zebrafish could be reproduced by injections of soluble polysialic acids indicating competitive inhibition of polysialic acid-specific binding functions.

In addition to its role in cell adhesion, modulation of NCAM properties by sialic acids has been also implicated in signal transduction. It was shown that NCAM may affect neurite outgrowth and matrix adhesion by assembling a fibroblast growth factor receptor signaling complex (Cavallaro et al., 2001).

Therefore removal of polysialic acids by enzymatic treatment or knockout of polysialyltransferases is crucial for modulating NCAM-activity and thus CNS development and functioning in vertebrates.

Fasciclin II glycoproteins which are most highly related to NCAM are abundantly present on *Drosophila* motor axons and in subset of interneuronal axons. However, polysialic acids have not been detected in flies so far (Koles et al., 2009). Additionally, analysis of sialyltransferase deficient motor axons in *Drosophila* didn't show any axonal pathfinding or target recognition defects. Despite this, we do not exclude the possibility that fly interneuronal surfaces may express sialylated fasciclins or similar adhesion molecules, which may affect axonal growth cone guidance, neuronal recognition or defects in cell positioning.

In the adult vertebrate brain polysialylated NCAM is expressed in olfactory bulb, hypothalamus and hyppocampus, where neural generation and neural plasticity persist (Rutishauser and Landmesser, 1996). Studies in mice revealed a crucial role of NCAM for chain migration of neuronal precursor cells, fasciculation and pathfinding of axons,

and synaptic plasticity, which are primarily mediated by polysialic acid (Cremer et al., 1998; Muller et al., 1996; Ono et al., 1994; Tomasiewicz et al., 1993).

We have detected high expression of DSIAT in interneurons of lobula and medulla regions and in the projections neurons of the central brain at adult stage of fly development (data not shown). Furthermore, we attempted to use MARCM method to perform clonal analysis of projection neurons and reveal any changes in their number or any axonal pathfinding defects, because dendritic targeting specificity of projection neurons is known to be controlled by axonal guidance molecules and cell adhesion molecules. This work was initiated by Kate Koles. Her studies suggested that projection neurons deficient of sialyltransferase may have targeting defects. However, this data is preliminary and needs to be carefully investigated.

We think that the enormous potential of *Drosophila* for cellular and molecular genetics studies, as well as the availability of sialyltransferase mutants may lead to greater insights into the mechanisms controlling such complex processes as neuronal substrate recognition and axonal fasciculation. The homology in adhesion and recognition molecules between vertebrates and invertebrates will help us to gain understanding how sialylation of fasciclins mediates recognition of specific axonal pathways in simple organisms like *Drosophila* and directly apply this knowledge to complex organisms.

Gangliosides

Gangliosides belong to family of sialic-acid containing glycosphingolipids present on the outer leaflet of the plasma membranes of most vertebrate neuronal cells. They play important roles in regulation of highly organized multi-cellular systems. Sialic acids present on gangliosides were shown to play important role in neurite outgrowth, cell adhesion, toxin-binding, signal transduction, membrane stability and apoptosis (Hashiramoto et al., 2006; Ledeen and Wu, 2006).

Biosynthesis of the common series of gangliosides involves sequential activities of sialyltransferases and glycosyltransferases. As the mammalian brain develops, there is an increase in the content of gangliosides and in their degree of sialylation. In mammals, sialyltransferases ST8Sia I (GM3 synthase) and ST8Sia V (GT3 synthase) catalyze transfer of α2-8 sialic acids on specific gangliosides. ST8SiaIII is also thought to be implicated in the biosynthesis of GT3 ganglioside. Other members of vertebrate sialyltransferase family: ST3Gal II, ST3Gal V, ST6GalNAc V and ST6GalNAc VI were also shown to transfer specific sialic acids on glycosphingolipids in the nervous system (proposed function is described in Table 1).

A complete understanding of the biological role of sialyltransferases in the synthesis of various gangliosides is still emerging. Nevertheless, attempts to reveal their function have already been made. For example, analysis of ST8SiaI sialyltransferase knockout mice behavior suggested a critical role of sialic acids in the development and maintenance of the sensory nervous system (Handa et al., 2005). Overexpression of the ST3Gal V transcript in the developing zebrafish led to neuronal cell death particularly

apparent in the forebrain, midbrain and mid/hindbrain boundary indicating that these regions are sensitive to GM3 overexpression (Sohn et al., 2006).

It was also observed that mice lacking GM3 synthase showed impaired electrophysiological responses at mutant NMJs suggesting involvement of sialic acids in motor neuronal function in vertebrates (Zitman et al., 2009).

Patients with a mutation in the GM3 synthase gene (hST3GalV) show disrupted biosynthesis of gangliosides derived from lactosylceramide and develop infantile-onset symptomatic epilepsy associated with developmental stagnation and blindness (Simpson et al., 2004).

Although neurobiological functions of gangliosides are not fully understood, they are essential for the nervous system development and maintenance. One important function that has been recently addressed is contribution of gangliosides to stability of myelinated junctions and ion channel clusters in the myelinated nerve fibers in vertebrates. To achieve a normal function in both motor and sensory neurons, a rapid and efficient propagation of action potential is required. Studies have recently shown that mutant mice lacking complex gangliosides have disrupted paranodal junctions along the axon, altered lipid raft composition, mislocalization and dysfunction of ion channels and slowing motor nerve conduction (Qiao et al., 2008; Susuki et al., 2007). These data also suggest that gangliosides play important role in conduction and excitability of axonal membrane.

Also, engineered mice or cells lacking most of the complex gangliosides have been produced. Surprisingly, the mice and the cells appeared to be largely normal in their

neuronal development (Takamiya et al., 1996). However, recent studies have revealed that mice lacking complex gangliosides exhibit axonal degeneration and myelination defects (Chiavegatto et al., 2000).

Gangliosides have not been identified in insect nervous system yet. In vitro analysis failed to show *Drosophila* sialyltransferase activity towards glycolipids (Koles et al., 2004). Therefore it is questionable if *Drosophila* can be used to study function of gangliosides and further research is needed. However, if similar structures are present in fly axons they may play a crucial role in maintaining and facilitating neuronal excitability in *Drosophila* unmyelinated nerve fibers via, for example, modulation of voltage-gated sodium channels. In addition, they may play important role for axonal stability and prevention of axonal degeneration.

Presence of sialylated structures in the fly nervous system similar to gangliosides may also explain deterioration of locomotor and temperature-sensitive phenotypes in the DSIAT mutant flies. For example, sialidases are the key enzymes that are involved in ganglioside degradation. Therefore removal of sialic acids on gangliosides is an important step for their general catabolism, modulation of cellular function and proper axonal functioning. Absence of sialic acids on gangliosides will lead to their accumulation on neuronal surfaces. This may subsequently accumulate intracellular toxic proteins and organelles and further affect axonal properties and cause its progressive dysfunction.

If neurodegeneration of axonal and synaptic membranes is caused by the absence of sialylated structures on fly neuronal surfaces, this may lead to progressive loss of

structure and function of neurons, their subsequent death and progressive deterioration of neuronal performance.

This hypothesis may also explain why the severity of the locomotor phenotypes progresses with a fly age in DSIAT mutants.

Evolutionary implications of sialylation in the CNS

The expression of sialic acids is originally thought to be limited to deuterostomes, some pathogenic bacteria infecting these animals and fungi. Structural studies of natural and recombinant glycoproteins of plants showed no evidence of sialic acids.

Deuterostomes express the highest structural diversity of sialic acids. To date twenty different sialyltransferases have been found and cloned in vertebrates with at least nine of them functioning in the nervous system. Each of them is highly specific for the linkage formed, but more flexible with the acceptor substrate.

Recent studies revealed presence of sialic acids in protostomes, specifically in *Drosophila melanogaster*. A functional α2,6 sialyltransferase which occurs in the CNS and stage-specific manner has been extensively characterized. We have tried to reveal its evolutionary relation to hST6Gal family of vertebrate sialyltransferases. Alignment of protein sequences showed high homology between catalytic domains of DSIAT and hST6Gal family members. DSIAT also seem to have similar in vitro acceptor preferences as hST6Gal II sialyltransferase (Koles et al., 2004). However, expression of hST6Gal sialyltransferases in DSIAT mutant background failed to rescue behavioral phenotypes identified in DSIAT mutants suggesting a different glycoprotein acceptor specificity of

hST6Gal sialyltransferases or different from DSIAT function within the CNS (for example, non-enzymatic activity).

It is quite interesting though that *C. elegans* shows no evidence for sialic acid expression. It can be speculated that the development of more complex organisms coincided with the evolution of more complex level of sialylation. In *C. elegans* this has been accomplished by fucosylation instead of sialylation since sialic acid and fucose compete for the same acceptors and lack of sialic acids in *C. elegans* coincides with expression of 18 different fucosyltransferase genes (Lehmann et al., 2004; Oriol et al., 1999). Absence of sialylated structures in *C.elegans* may also be explained by significantly reduced complexity of its CNS organization and functioning.

Thus, it is likely that divergence and subfunctionalization of vertebrate sialyltransferases that function in the CNS coincided with the appearance of increasingly more complex organism with new structures and functions.

Summary

The complexity of sialic acid biosynthesis and its biological role in vertebrates necessitates the use of a system with powerful genetics and reduced genomic redundancy. In this study we used the *Drosophila* model system to address several important questions.

First, can *Drosophila* be used to study the process of sialylation and functions of sialylated glycoproteins? To answer this question we analyzed the spatio-temporal expression pattern and the function of *Drosophila* sialyltransferase (DSIAT) implicated

in the biosynthesis of α2-6-sialylated glycoconjugates in fruit flies. We also revealed that another putative member of sialylation machinery, DmCSAS, has a similar phenotype and expression pattern.

Second, given the experimentally confirmed presence of Neu5Ac, how is the de novo pathway of sialylation initiated in insects? In other words, what is the source of ManNAc or ManNAc-6-P? Potential sources of ManNAc or ManNAc-6-P that may include a dietary source, symbiotic microorganisms (such as *Wolbachia*) and a yet unknown GlcNAc epimerase present in insect cells have been discussed earlier (Angata and Varki, 2002). However, considering the high degree of overall conservation between *Drosophila* and vertebrate sialylation pathways (Figure 1), each of these possibilities would represent somewhat exotic biological solutions from biochemical and evolutionary standpoints.

Even though UDP-GlcNAc 2-epimerase/ManNAc kinase and sialidase homologs are absent from the *Drosophila* genome, the possible presence of KDN, a different type of sialic acid, suggests an alternative scenario for sialylation in flies. However, KDN has not been detected in protostomes so far, and mass spectrometry analysis of N- and O-linked glycans failed to detect KDN in *Drosophila* brains (Koles et al., 2009). Thus presence of KDN in *Drosophila* and protostome lineage of species needs to be further investigated.

There are still important questions that need to be investigated. Earlier we mentioned that *Drosophila* sialyltransferase is most homologous to the hST6Gal subfamily of vertebrate sialyltransferases, consisting of hST6Gal I and hST6Gal II.

hST6Gal I is ubiquitously expressed and hST6Gal I-deficient mice were shown to be immunosuppressed, had low serum IgM levels and decreased proliferation of their B cells (Hennet et al., 1998). However, no neurological phenotypes have been described yet. For hST6Gal II sialyltransferase, which is highly homologous to *Drosophila* sialyltransferase, no data about phenotypes or possible mechanism of action has been described so far. Thus, can we infer that due to subfunctionalization, most CNS functions are performed by hST6Gal II in higher animals? And if yes, does hST6Gal II sialylates voltage-gated sodium channels in vertebrates?

Other important questions that need to be answered are the developmental regulation and expression of sialylation pathway genes in *Drosophila*: *DmCSAS*, putative *Sia-P phosphatase* and *Sia transporter*. What are the mutant phenotypes of these genes involved in sialylation, do mutations in their loci show genetic interactions and can they phenocopy each other? What are the in vivo targets of sialylation in *Drosophila*? What is the specific role of sialylation in the CNS glycoproteins in *Drosophila*? Can we further extend this knowledge to understand problems caused by mutations in sialyltransferases functioning in the CNS in humans?

In summary my results have demonstrated that sialylation is an evolutionary conserved mechanism important for *Drosophila* nervous system. This work establishes *Drosophila* as a useful model system to study sialylation which may shed light on related biological functions in higher organisms including humans.

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