Sialyltransferase Regulates Nervous System Function in *Drosophila*

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In vertebrates, sialylated glycans participate in a wide range of biological processes and affect the development and function of the nervous system. While the complexity of glycosylation and the functional redundancy among sialyltransferases provide obstacles for revealing biological roles of sialylation in mammals, *Drosophila* possesses a sole vertebrate-type sialyltransferase, *Drosophila* sialyltransferase (DSiaT), with significant homology to its mammalian counterparts, suggesting that *Drosophila* could be a suitable model to investigate the function of sialylation. To explore this possibility and investigate the role of sialylation in *Drosophila*, we inactivated DSiaT *in vivo* by gene targeting and analyzed phenotypes of *DSiaT* mutants using a combination of behavioral, immunolabeling, electrophysiological, and pharmacological approaches. Our experiments demonstrated that DSiaT expression is restricted to a subset of CNS neurons throughout development. We found that *DSiaT* mutations result in significantly decreased life span, locomotor abnormalities, temperature-sensitive paralysis, and defects of neuromuscular junctions. Our results indicate that DSiaT regulates neuronal excitability and affects the function of a voltage-gated sodium channel. Finally, we showed that sialyltransferase activity is required for DSiaT function *in vivo*, which suggests that *DSiaT* mutant phenotypes result from a defect in sialylation of N-glycans. This work provided the first evidence that sialylation has an important biological function in protostomes, while also revealing a novel, nervous system-specific function of α 2,6-sialylation. Thus, our data shed light on one of the most ancient functions of sialic acids in metazoan organisms and suggest a possibility that this function is evolutionarily conserved between flies and mammals.

Introduction

Sialic acids, a family of nine-carbon backbone acidic sugars, mainly occupy terminal positions of carbohydrate modification of glycoproteins and glycolipids (Angata and Varki, 2002). In vertebrates, sialylated glycans are abundantly present on cell surfaces and in the extracellular milieu; they are involved in cell adhesion and cell communication, participating in a variety of crucial biological processes (Varki, 2007). Abnormal sialylation has been implicated in a number of pathobiological conditions, including neurological diseases, immunodeficiency, and cancer (Varki, 2008). Glycoprotein sialylation has been extensively studied in mammals and some roles of this posttranslational modification have been determined. These roles often depend on the

nature of acceptors, as well as on the linkage of sialylation, both determined by properties of sialyltransferases, enzymes that attach sialic acid to specific acceptors (Harduin-Lepers et al., 2001). Phenotype analysis of mouse knock-outs for genes involved in sialylation has been an important approach in revealing biological functions of sialylated structures (Hennet et al., 1998; Eckhardt et al., 2000; Moody et al., 2001; Angata et al., 2004). Although the prominent role of polysialylation of the NCAM (neural cell adhesion molecule) in mammalian brain development and neurophysiology has been established (Rutishauser, 2008; Mühlenhoff et al., 2009), the function of other types of glycoprotein sialylation in the nervous system remains mostly unknown. The complexity of the mammalian nervous system, functional redundancy among sialyltransferases, and intricate regulation of sialylation pathway impede the analysis of neural functions of sialylation in mammals (Harduin-Lepers et al., 2001; Martin et al., 2002; Hildebrandt et al., 2007), suggesting that a suitable model organism could be a useful tool for this analysis. Yet, little is known about sialylation in animals besides vertebrates. Recently, *Drosophila* has been shown to possess functional homologues of vertebrate enzymes for several key steps in sialylation pathways, including sialic acid phosphate synthetase (Kim et al., 2002), CMP-sialic acid synthetase (Viswanathan et al., 2006), and a sialyltransferase, *Drosophila* sialyltransferase (DSiaT) (Koles et al., 2004). Functional characterization of DSiaT revealed its evolutionary relationship to mammalian ST6Gal sialyltransferases (Fig. 1), suggested that DSiaT functions in the nervous system, and predicted that N-linked glycans are putative

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Figure 1. Phylogenetic relationship between *Drosophila* and human sialyltransferases. DSiaT is closely related to ST6Gal family of mammalian sialyltransferases. Sialyltransferase subfamilies with distinct substrate/linkage specificities are indicated by different colors: DSiaT and ST6Gal enzymes, red; ST6GalNAc enzymes, magenta; ST3Gal enzymes, green; ST8Sia enzymes, blue. The phylogenetic tree was constructed by Clustal W2 program at EMBL-EBI site (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) (Larkin et al., 2007) using neighbor-joining method. The accession numbers of protein sequences are as follows: NP_523853 (DSiaT), NM_173216 (HST6Gal I), XM_038616 (HST6Gal II), NP_060884 (HST6GalNAc I), NP_006447 (HST6GalNAc II), NP_694541 (HST6GalNAc III), NP_778204 (HST6GalNAc IV), NP_112227 (HST6GalNAc V), Q969X2 (HST6GalNAc VI), NP_003024 (HST3Gal I), Q16842 (HST3Gal II), NP_777631 (HST3Gal III), NP_006269 (HST3Gal IV), NP_003887 (HST3Gal V), NP_006091 (HST3Gal VI), NP_003025 (HST8Sia I), NP_006002 (HST8Sia II), NP_056963 (HST8Sia III), NP_005659 (HST8Sia IV), NP_037437 (HST8Sia V), and NP_001004470 (HST8Sia VI).

targets of sialylation *in vivo* (Koles et al., 2004). The presence of predicted α 2,6-sialylated N-linked glycans has been recently confirmed in *Drosophila* by mass spectrometry (Aoki et al., 2007; Koles et al., 2007). However, until now the biological function of sialylation in *Drosophila* or any other protostome species (including arthropods, annelids, and mollusks) was unknown.

To shed light on this function and analyze its relationship to the role of sialylation in higher animals, we generated *Drosophila* sialyltransferase knock-out mutants and analyzed their phenotypes using behavioral, genetic, electrophysiological, and pharmacological approaches. We found that DSiaT plays a pivotal role in the nervous system, regulating excitability of neurons, affecting development of neuromuscular junctions (NMJs), and influencing behaviors. Our results demonstrate that sialyltransferase enzymatic activity is required for DSiaT *in vivo* function and suggest that DSiaT modulates the function of voltage-gated sodium channels. Together, our results reveal a novel, neuron-specific function of ST6Gal-type sialyltransferases and suggest a possibility that this function is evolutionarily conserved in animals.

Materials and Methods

Drosophila strains

KKKeve-GAL4 was obtained from David Featherstone (University of Illinois at Chicago, Chicago, IL) (Featherstone et al., 2000). Wild-type control *w¹¹¹⁸ Canton-S* was from Josh Dubnau (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (Dubnau et al., 2001); *para^{LK5}* (loss-of-function allele), *para^{ts1}* (hypomorph, conditional temperature-sensitive allele), and *para^{DP}* (aka *Dp(1;4)r⁺f⁺*, a duplication of *para* on the fourth chromosome) were from Barry Ganetzky (University of Wisconsin-Madison, Madison, WI) (Ganetzky, 1984); *tipE¹* (loss-of-function paralytic allele) was obtained from Linda Hall (University of California-Davis, Davis, CA) (Feng et al., 1995), and *cac^{ts2}* (temperature-sensitive paralytic mutant) was received from Richard Ordway (Penn State University, University Park, PA) (Brooks et al., 2003). *y¹w¹*, *C380-GAL4*, *Cha-GAL4*, *UAS-GFP* and *UAS-CD8-GFP* lines were from the Bloomington Stock Center (Indiana University). All *Drosophila* strains were reared in a controlled environment incubator (25°C, 35% humidity, 12 h light/darkness) on standard cornmeal–malt–yeast medium.

Generation of DSiaT alleles

Two loss-of-function alleles were created by homologous recombination. The S23 loss-of-function allele was generated by an ends-in gene-targeting approach using pTV2 vector-based donor construct (Rong and Golic, 2000) including 8 kb *DSiaT* genomic region. S23 includes two premature stop codons within the *DSiaT* coding region that are predicted to inactivate the gene. The upstream stop codon is expected to prematurely terminate translation after the first 17 aa of the DSiaT protein. The downstream stop codon is predicted to result in DSiaT truncated in the middle of its S-sialylmotif and missing 84 C-terminal amino acids. Both engineered stop codons also introduced additional restriction sites (BspHI and NheI), which facilitated the analysis of the mutant (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Another loss-of-function allele, L22, was generated with an ends-out targeting technique (Gong and Golic, 2003) by introducing a deletion that removes most of the *DSiaT* coding sequence. Targeting was performed essentially as previously described (Gong and Golic, 2004). Briefly, the donor construct was based on pW25 vector and included 8 kb genomic region of *DSiaT* with engineered 1.7 kb deletion of the 2 kb *DSiaT* open reading frame (ORF). The donor construct also included a 729 bp fragment encoding green fluorescent protein (GFP), so the resulting mutant ORF encoded a fusion of first 44 aa of DSiaT with GFP. The design was aimed at creating a mutant allele with GFP expression in endogenous DSiaT pattern; however, the GFP-coding sequence was damaged by a short insertion (~30 bp) during the targeting event, which abolished GFP expression. The *mini-white* marker associated with donor construct was removed from targeted locus by Cre recombinase (Gong and Golic, 2004). The *KI48* allele was generated using the same strategy that was applied for creating S23. *KI48* includes a short sequence encoding two tandem hemagglutinin (HA) tags (Niman et al., 1983) in frame with endogenous ORF of *DSiaT*, which results in the expression of the DSiaT protein with two HA tags within its stem region. All three alleles were confirmed by Southern blot hybridization and sequencing of *DSiaT* coding region plus adjacent 2 kb flanking genomic fragments (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

To minimize potential influence of genetic background, *DSiaT* mutant alleles were outcrossed at least 10 times to their matching “wild-type” control genotypes, *w¹¹¹⁸ Canton S* (designated as WT) and *y¹w¹* (designated as yw) for S23 and L22 alleles, respectively. In all functional assays, S23 and L22 alleles were indistinguishable from each other, and they were used in this work interchangeably. The *P[DSiaT⁺]* transgenic insertion on the third chromosome was generated by P-element-mediated transformation. The construct contained 8 kb genomic locus of *DSiaT* including ~3 kb upstream and downstream regions of the gene and no other predicted ORFs.

Behavioral assays

Larvae. For larval locomotion assays, we used larvae from controlled-density populations (Stewart and McLean, 2004). To this end, adult parent flies were allowed to lay eggs on a grape juice plate for 3 h. Twenty-four

hours later, first-instar larvae were transferred from the plate to vials with ~7 ml of food, 20 larvae per vial, and allowed to develop until mid-third instar (80–84 h after egg laying). Before analyses, individual larvae were briefly rinsed in 25% sucrose and then in distilled water. Using a brush, larvae were placed gently in the center of a 100 mm, 3% agar plate. Each larva was given 2 min to adapt to the plate. The plate was placed onto a 0.5 × 0.5 mm grid paper, and movies were recorded for 30 s for each larva using Sony HD digital camera. Speed and number of waves of body muscle contractions per second were obtained from straight-path crawling intervals. Distance traveled per one wave of body muscle contraction ("distance per muscle contraction") was calculated as larva speed divided by contraction frequency. For crawling pattern assay, the motion of individual third-instar larvae on agar plates was recorded during 30 s, and digitized tracks were obtained from recorded movies by tracing larval head movements.

Adult flies. For individual longevity, adult male flies were collected within 24 h after eclosion. Each fly was placed into individual vial with food and transferred to new vial with fresh food every 3 d. Group longevity was assayed with groups of 10 flies (males)/vial. Flies were transferred to fresh food every 3 d, and dead flies were counted every day. For locomotion assays, individual flies were collected on the day of enclosure and aged for 3, 5, and 7 d, as indicated. Before the assay, each fly was placed in an empty vial and allowed to adapt for 10 min. Locomotion assays were performed essentially as described previously (Haines and Stewart, 2007). Briefly, the vial with a single fly was banged five times onto a soft rubber pad. The time that it took the fly to right itself after falling on its back was recorded. For each fly, the assay was performed with two trials and 10 min recovery in between. For the temperature-sensitive (TS)-paralysis assay, flies were transferred to empty vials and temperature was shifted to 38°C by submerging vials in a controlled-temperature water bath. We defined paralysis as a condition when a fly lies on the bottom of a vial and is unable to stand and walk. To analyze the kinetics of paralysis, flies were collected on the day of enclosure and aged as indicated. Paralysis was assayed in groups of 10 flies in empty plastic vials while counting the number of paralyzed flies in 1 min intervals. For genetic interactions, flies were assayed individually, and the time of paralysis onset was recorded for every fly.

NMJ analysis

We used third-instar larvae collected from controlled-density populations. Larvae were dissected along the ventral midline in ice-cold PBS, fixed in 4% paraformaldehyde, 50 mM NaCl, 0.1 M PIPES, pH 7.2, for 10 min at room temperature, and then their NMJs were analyzed by immunostaining. Fluorescent z-stack optical sections of muscle 1 from third abdominal segments were used to analyze the number of boutons, branches, and active zones. Since identified DSiaT-expressing motoneuron (MN1-Ib) produces type Ib boutons, we did not include type II boutons in our analyses. However, we counted type Ib and type Is boutons together because morphological difference between them is sometimes ambiguous. For active zone analysis, muscle 1 NMJs were double stained with rabbit anti-HRP and mouse nc82 (anti-Bruchpilot) primary antibodies and anti-rabbit Alexa 488 and anti-mouse Cy3-conjugated secondary antibodies. Distinct nc82 puncta were counted in optical sections and verified with three-dimensional reconstructed images of individual boutons using AxioVision software. For nc82 puncta analysis, five Ib boutons (3–6 μm in size) were randomly selected from each analyzed muscle 1 NMJ in abdominal segment 3 of late third-instar larvae.

Immunofluorescent staining and microscopy

Immunostaining was performed essentially as described previously (Lyalin et al., 2006). The following primary antibodies and corresponding dilutions were used: rabbit anti-Lva (a gift from John Sisson, University of Texas, Austin, TX), 1:2000, was used to label the Golgi compartment (Sisson et al., 2000); rabbit anti-DVGlut (a gift from Aaron DiAntonio, Washington University, St. Louis, MO), 1:4000, was used to label motor neurons (Daniels et al., 2008); rat anti-HA (Roche), 1:800, was used to reveal the pattern of DSiaT expression in *K148* allele; rabbit anti-HRP (Jackson ImmunoResearch Laboratories), 1:800, and mouse anti-Dlg (Developmental Studies Hybridoma Bank), 1:200, were used to visualize

presynaptic membrane (Jan and Jan, 1982) and subsynaptic reticulum (Budnik et al., 1996), respectively. We used mouse anti-Repo antibody (1:5) to label glial cells (Alfonso and Jones, 2002). Mouse nc82 antibody from Developmental Studies Hybridoma Bank was used at 1:50 dilution to visualize adult brain neuropil and to label active zones in synaptic boutons of larval NMJs. We used the following fluorescent secondary antibodies (with corresponding dilutions): anti-mouse-Cy3 (1:250), anti-rabbit-FITC (1:150), anti-mouse-Cy5 (1:250) (all from donkey; Jackson ImmunoResearch Laboratories), and goat anti-rabbit Alexa-488 (1:140) (Invitrogen). Fluorescent images were acquired using an Olympus FV1000 confocal microscope or a Zeiss Axioplan 2 microscope with ApoTome module for optical sectioning. Z-projections were generated using Zeiss AxioVision and ImageJ software (Abramoff et al., 2004).

Electrophysiology

Current-clamp intracellular recordings were performed from muscles of dissected third-instar larvae essentially as previously described (Stewart et al., 1994). Briefly, *Drosophila* third-instar larvae were dissected in ice-cold Ca²⁺-free HL3 solution, and recordings were performed at room temperature. The recording solution was supplemented with CaCl₂ as indicated for the specific experiments. We used microelectrodes with an input resistance of 8–16 MΩ prepared from 1.2 mm borosilicate glass capillaries and filled with 3 M KCl. Membrane potentials were amplified by the Axoclamp 200B amplifier (Molecular Devices), filtered at 10 kHz, digitized, and recorded on a Dell PC computer equipped with pClamp10 software (Molecular Devices). For spontaneous miniature excitatory junction potential (mini-EJP) recordings, the extracellular Ca²⁺ was 1 mM. Mini-EJP was analyzed using a semiautomated protocol with the event detection mode in pCLAMP and a template obtained from mini-EJP recording of wild-type control genotype based on >200 individual events. To elicit postsynaptic response, segmental nerves were stimulated with 0.2 ms pulses at 2 times the stimulus amplitude required for a threshold response. Excitatory junction potentials (EJPs) were analyzed using the cursor options of Clampfit 10.0 (Molecular Devices). EJP analysis was performed by averaging 10 events for each muscle and considering the average amplitude as one measurement for the genotype assayed. Paired-pulse facilitation was performed in 0.75 mM Ca²⁺ HL3 saline essentially as described previously (Wairkar et al., 2008), with 50 ms period between stimulating pulses. Facilitation index was calculated as the ratio between EJP amplitudes evoked by the second and the first pulses. High-frequency stimulation was performed in 4 mM Ca²⁺ HL3-saline as described previously (Song et al., 2002; Verstreken et al., 2002) with 3000 pulses delivered to stimulated nerves at a frequency of 10 Hz. For tetrodotoxin (TTX) sensitivity assays, 10 μM TTX was added to the recording saline (1 mM Ca²⁺ HL3), and then evoked EJP was recorded from segment A3 muscle 1 with 1 min intervals until the response disappeared. EJP amplitudes were normalized to the amplitude before TTX addition.

Dichloro-diphenyl-trichloroethane sensitivity assay

Flies were tested for sensitivity to dichloro-diphenyl-trichloroethane (DDT) essentially as previously described (Pittendrigh et al., 1997) with some modifications. Briefly, groups of 10 4-d-old flies were exposed to the insecticide in DDT-coated empty vials at 25°C for 12 h. The vials were prepared by adding 10 μg of DDT dissolved in acetone and drying the solvent completely. In control experiments, vials were treated with acetone alone.

DSiaT^{HK} mutant

Construct encoding DSiaT with H406K substitution (DSiaT^{HK}) was generated by changing the corresponding CAT codon to AAG in the sequence encoding C-terminally HA-tagged wild-type DSiaT (DSiaT^{WT}) (Koles et al., 2004). Site-specific mutagenesis was performed by PCR-based method (Panin et al., 2002). Full-length DSiaT^{WT} and DSiaT^{HK} were subcloned into pUAST for *in vivo* expression. For cell culture experiments, we generated *ProtA-DSiaT^{HK}* construct in pMK33 vector applying the strategy previously used to obtain protein A-tagged wild-type DSiaT (Koles et al., 2004).

Figure 2. Behavioral phenotypes of *DSiaT* mutants. **A**, *DSiaT* mutants have significantly reduced longevity. For each genotype, 150 flies were assayed for group longevity; error bars represent 95% confidence intervals. **B**, **C**, Mutant larvae have crawling defects as revealed by decreased distance per muscle contraction (**B**) and reduced speed of muscle contractions (**C**). Twenty larvae were assayed for each genotype. **D**, Mutant larvae have a crawling pattern abnormality, making many more turns. Graphs show 20 superimposed larval tracks with the initial positions overlaid in the middle. **E**, Mutant flies have a defect in the ability of righting themselves after falling on the back. Twenty flies were assayed for each genotype. **F**, Mutant flies have TS-paralysis phenotype. Flies were assayed at 38°C, 50 flies per genotype. Note that the severity of locomotor defects (**E**) and the sensitivity of mutants to elevated temperature (**F**) significantly increased with age. In all experiments, the rescue genotype was *w¹¹¹⁸; S23; P[DSiaT⁺]*, where *P[DSiaT⁺]* is a transgenic insertion of wild-type *DSiaT* gene. Error bars represent SD in **F**, and SEM in **B**, **C**, and **E**. The asterisks indicate statistically significant differences (*t* test, *p* < 0.01). Similar behavioral phenotypes were observed with the *L22* allele (supplemental Fig. S3B, available at www.jneurosci.org as supplemental material).

Protein purification and sialyltransferase assay

Cell culture expression, purification, and *in vitro* sialyltransferase assays of *DSiaT*^{WT} and *DSiaT*^{HK} were performed using previously described protocols (Koles et al., 2004).

Results

Drosophila sialyltransferase mutants have decreased longevity, locomotor abnormality, and temperature-sensitive paralysis

With only one sialyltransferase gene present in the genome (Koles et al., 2004), *Drosophila* represents an attractive system to explore the biological function of sialylation using reverse genetics. We generated two independent loss-of-function *DSiaT* alleles by introducing premature stop codons or a large deletion within the gene ORF (alleles S23 and L22, respectively) via gene targeting (Maggert et al., 2008) (see Materials and Methods) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Both mutants were homozygous viable and fertile, and did not have noticeable morphological defects. However, homozygous mutant flies were rare in balanced heterozygous stocks, which indicated their decreased viability. In addition, we found that these mutants have significantly decreased longevity (Fig. 2A; supplemental Fig. S2A, available at www.jneurosci.org as supplemental material) and pronounced locomotor defects, as revealed by behavioral assays in larvae and adults. Mutant larvae crawl significantly slower than wild-type and rescued mutant controls, as measured by the frequency of body muscle contractions and the distance traveled per contraction (Fig. 2B,C). In addition, mutant larvae make many more turns compared with wild-type controls when subjected to an open field crawling assay

(Fig. 2D; supplemental Fig. S2B, available at www.jneurosci.org as supplemental material). Mutant adults have impaired coordination, and they are unable to promptly right themselves after being knocked down to the bottom of a vial by a gentle agitation (Fig. 2E), with the severity of this defect rapidly increasing with age. Since neurological mutations in *Drosophila* often exhibit TS paralysis (Suzuki et al., 1971), we tested the behavior of *DSiaT* mutants at elevated temperature and found that they become paralyzed at 38°C within several minutes. Paralysis is reversible as flies fully recover within 10–15 min once temperature is shifted back to 25°C. Similar to the locomotion phenotype, the sensitivity to elevated temperature notably increases with age (Fig. 2F), suggesting that the mutant nervous system undergoes a progressive age-dependent deterioration.

DSiaT expression is restricted to a subset of CNS neurons throughout development

To elucidate the mechanism underlying behavioral phenotypes of *DSiaT* mutants, we investigated the expression of *DSiaT* at different developmental stages. To this end, we introduced a short sequence encoding two tandem HA epitopes into the endogenous *DSiaT* locus using gene targeting. We found that the pattern of endogenously expressed HA-tagged *DSiaT* closely reflects the distribution of *DSiaT* mRNA detected by *in situ* hybridization (Koles et al., 2004, 2009). The embryonic expression of *DSiaT* is initiated at stage late 15 in 10–14 cells within the ventral ganglion, with the number of prominently expressing cells gradually increasing during development, until it reaches ~400 in the late third-instar larval brain (Fig. 3A,B). Dual labeling for glial

cells with anti-Repo antibody revealed that DSiaT is not expressed in glia, whereas staining with the pan-neuronal marker Elav confirmed that all DSiaT-expressing cells are differentiated neurons (Fig. 3C–F). Although no expression was detected in dopamine and serotonin neurons labeled by dopa decarboxylase DDC-GAL4 driver (data not shown) (Li et al., 2000), labeling with interneuron and motor neuron-specific drivers revealed that DSiaT is present in subsets of interneurons and motor neurons throughout the CNS (supplemental Fig. S2C, available at www.jneurosci.org as supplemental material). In addition, we found that DSiaT is expressed in the hemisegmentally repeated pattern in a previously described group of dorsomedial motor neurons in the third-instar ventral ganglion (Fig. 3G–I). The two motor neurons with prominent DSiaT expression were identified as MN1-Ib and MN30-Ib (Choi et al., 2004). The expression of DSiaT in MN1-Ib was also confirmed by labeling with RKKve-Gal4 driver (Featherstone et al., 2000) (supplemental Fig. S2D, available at www.jneurosci.org as supplemental material). In the young adult brain, DSiaT is expressed in a distinct pattern, including medulla and lobula plate regions, ocelli, the region of olfactory projection neurons, as well as some other neurons (Fig. 3J). We did not detect the expression of DSiaT outside of the CNS at any developmental stage. Thus, these results suggest that DSiaT function is limited to subsets of neurons in the CNS throughout all developmental stages, which is consistent with the neurological phenotypes of *DSiaT* mutants.

***DSiaT* mutants have defects in the development and physiology of neuromuscular junctions**

Many neurological mutations in *Drosophila* are known to impinge on the function and development of NMJs (Collins and DiAntonio, 2007). Thus, we characterized the neuroanatomy of NMJs in *DSiaT* mutant larvae. We focused our analyses on the dorsal abdominal muscle 1 innervated by a DSiaT-expressing motor neuron MN1-Ib. We found no targeting defects as all examined muscle 1 NMJs were innervated properly in *DSiaT* mutants; however, the number of branches and synaptic boutons was significantly reduced (Fig. 4A,B; supplemental Fig. S2E,F, available at www.jneurosci.org as supplemental material), indicating a role for DSiaT function in NMJ development. The NMJ morphology defect was rescued by introducing a transgenic *DSiaT* genomic construct. However, the rescue was partial, which is likely attributable to the fact that the construct does not fully recapitulate the endogenous expression of *DSiaT*.

Since the mutant behavioral phenotypes suggested dysfunctional neural signaling, we examined neuromuscular synaptic function in *DSiaT* mutants using electrophysiological ap-

Figure 3. DSiaT expression is restricted to the CNS neurons at different developmental stages. **A**, DSiaT is expressed in the ventral ganglion (arrows) and the brain region (arrowheads) in the stage late 16 embryo CNS. **B**, The third-instar larval brain has numerous cells expressing DSiaT in the ventral ganglion (arrows) and hemispheres (arrowheads). **C**, DSiaT is not expressed in glial cells. Glial cells were stained with glial marker Repo (green), and DSiaT is red. **D–F**, DSiaT is expressed in a subset of neurons in third-instar larval brain. Immunofluorescent double staining for DSiaT (green) and pan-neuronal marker Elav (red). Medial focal plane of the posterior region of ventral ganglion is shown. **F** is the overlay of **D** and **E**. **G–I**, DSiaT expression in dorsomedial motor neurons in the third-instar ventral ganglion. Double staining for DSiaT (red) and DVGluT (green) reveals prominent DSiaT expression in two motor neurons, MN1-Ib (arrowheads) and MN30-Ib (arrows). The dotted line indicates the approximate position of midline. **I** is the overlay of **G** and **H**. Four abdominal segments (3–6, from left to right) of the ventral ganglion are shown. **J**, Expression of DSiaT (green) in the pharate adult brain. The overall morphology of the brain is visualized by neuropil staining with nc82 antibody (magenta). The expression of DSiaT is present in the optic lobes (MC, medulla cortex; LP, lobula plate), the region of projection neurons (PN) and ocelli (OC). **A–I**, Anterior is to the left; **J**, frontal view, dorsal is up. Scale bars: **A**, **C–F**, **J**, 20 μ m; **B**, 40 μ m; **G–I**, 10 μ m.

proaches. We did not detect any significant difference in the resting membrane potential of muscle 1 between mutants and control genotypes [*S23* mutants, -60.1 ± 0.6 mV; *WT* control, -59.0 ± 0.8 mV; *L22* mutants, -60.7 ± 0.4 mV; *yw* control, -60.1 ± 0.3 mV (for each genotype, errors are SEM; sample size, $N = 10$)], which suggested that muscle functions are not affected in *DSiaT* mutants. Using intracellular recordings, we analyzed the EJP evoked in muscles in response to extracellular nerve stimulation. The evoked EJP amplitude was significantly decreased at mutant NMJs (Fig. 4C,D), indicating that the excitability of axonal membrane and/or the mechanism of NMJ synaptic transmission are compromised. Notably, evoked EJP was normal at muscle 6/7 NMJ innervated by MN6/7-Ib, a motor neuron without detectable DSiaT expression (supplemental Fig. S3A,B, available at www.jneurosci.org as supplemental material), which suggested that DSiaT affects EJP cell-autonomously. To further investigate the mechanism underlying the defect in evoked EJP,

Figure 4. Morphological and electrophysiological phenotypes of *DSiaT* mutants. **A, B**, *DSiaT* mutants have decreased number of muscle synaptic branches (**A**) and boutons (**B**). **C**, Representative traces of evoked EJPs (top) and mini-EJPs (bottom). **D**, Evoked EJP amplitude is decreased in *DSiaT* mutants. Recordings were performed at 1 mM Ca^{2+} concentration. **E**, *DSiaT* mutant MN1-Ib boutons have normal nc82 staining that visualizes active zones. Anti-HRP antibody (green) was used as a presynaptic membrane marker. Images show Z-projections obtained from optical sections of boutons. Scale bar, 5 μm . **F**, The defect in evoked EJP amplitude is rescued by increased Ca^{2+} concentration. In all panels, analyses were performed in third-instar larvae for muscle 1 NMJ in abdominal segment 3. Error bars are SEM. The asterisks indicate statistically significant differences (t test, $*p < 0.05$ and $**p < 0.01$). Rescue genotype was $w^{1118}; S23; P[DSiaT^+]$. Number of larvae analyzed were as follows: **A, B**, WT, 22; S23, 32; Rescue, 23; yw, 29; yw; L22, 36; **D**, WT, 10; S23, 10; Rescue, 10; **F**, WT, 10; S23, 10.

we first examined mini-EJP activity as a measure of spontaneous synaptic transmission in the absence of nerve stimulation (Fig. 4C). We detected no significant difference between control and mutants in either the amplitude or frequency of mini-EJP: amplitude was 1.56 ± 0.16 and 1.52 ± 0.09 mV, and frequency was 2.43 ± 0.34 and 2.55 ± 0.25 Hz for wild-type and S23 mutants, respectively (errors are SEM; t test, $p > 0.7$; 10 larvae were analyzed for each genotype). These results suggested that the stimulus-independent synaptic vesicle exocytosis, the amount of neurotransmitter per vesicle, and the postsynaptic sensitivity to neurotransmitter are not affected in mutants. Thus, we concluded that the decreased evoked EJP is likely associated with a defect of excitation (action potential function) at the synapse on nerve stimulation, or with abnormal synaptic functions downstream of the action potential. In general, the synaptic response depends on the number of functional active zones, the sites of neurotransmitter release, and the probability of release of synaptic vesicles at these sites (Zucker and Regehr, 2002). To test the possibility that the evoked EJP defect in *DSiaT* mutants might be associated with a decreased number of active zones, we labeled them with nc82 antibody [anti-Bruchpilot (Kittel et al., 2006; Wagh et al., 2006)] and estimated the density of nc82 puncta in MN1-Ib synaptic boutons. No decrease in active zone density or the intensity of nc82 staining was found in mutants (Fig. 4E). Moreover, the density of nc82 puncta was slightly increased in mutant boutons (0.56 ± 0.04 puncta/ μm^2 in S23 mutants vs 0.46 ± 0.04 puncta/ μm^2 in wild-type control; errors are SEM; t test, $p < 0.0005$; 11 larvae were analyzed for each genotype), suggesting a possible compensatory mechanism that tends to maintain the overall strength of synaptic connection at mutant synapses with decreased NMJ size. To further shed light on synaptic processes, we measured evoked EJP amplitude in the presence of high external Ca^{2+} concentrations. This condition is

expected to increase the probability of synaptic vesicle fusion and transmitter release to nearly saturated level and has been shown to rescue defective EJPs with a diminished probability of release when the number of functional active zones is not effected (Waikar et al., 2008). However, elevated Ca^{2+} concentration would unlikely rescue the phenotype associated with decreased number of active zones. We found that EJP amplitude reached saturation at ≥ 4 mM extracellular calcium concentration and that this elevated level of external Ca^{2+} fully rescued the EJP defect (Fig. 4F), further suggesting that the function of active zones is not affected in *DSiaT* mutants. Moreover, this result suggests that Ca^{2+} -triggered exocytosis is unaffected in *DSiaT* mutants, since increased Ca^{2+} concentration cannot rescue decreased neural transmission in SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) mutants (Stewart et al., 2000), as well as in several other mutants with impaired Ca^{2+} -triggered exocytosis (Aravamudan et al., 1999; Bao et al., 2005; Long et al., 2008). To further elucidate the physiology of Ca^{2+} -dependent excitation–secretion coupling, we analyzed short-term plasticity that is known to depend on spatiotemporal regulation of action potential-triggered Ca^{2+} influx (Atwood, 1967; Zucker and Regehr, 2002). We applied a paired-pulse facilitation assay that is commonly used to reveal abnormalities of synaptic transmission. In this assay, facilitation results from residual Ca^{2+} enhancing release probability at a closely spaced second stimulation (Katz and Miledi, 1968). The absence of facilitation could be explained by a depletion of readily releasable pool of vesicles (von Gersdorff et al., 1997). On the other hand, abnormally enhanced facilitation is indicative of a decreased probability of release at the initial pulse that results in a significant potentiation of vesicle release by accumulated intracellular Ca^{2+} in response to the following stimulus [as, for instance, observed in mutants with defects in synaptic vesicle release, such as *bruchpilot*, *rotated abdomen*, *synaptotagmin 1*, and *lap* (Bao et al., 2005; Kittel et al., 2006; Saraswati et al., 2007; Waikar et al., 2008)]. We detected no difference in short-term facilitation between *DSiaT* mutants and wild-type controls: facilitation index was 1.48 ± 0.07 and 1.46 ± 0.05 for wild-type and *DSiaT* mutants, respectively (errors are SEM; t test, $p > 0.3$; 19 larvae were analyzed for each genotype) (supplemental Fig. S3C, available at www.jneurosci.org as supplemental material). This result suggested that probability of release is not affected by *DSiaT*. Finally, we tested the cycling of synaptic vesicles by applying high-frequency prolonged stimulation (10 Hz, 5 min), a protocol that results in dramatic synaptic depression in mutants with defective vesicle cycling and compromised pool of synaptic vesicles (Verstreken et al., 2002, 2003; Koh et al., 2007). We did not detect any difference between wild-type control and mutants in this assay (supplemental Fig. S3D, available at www.jneurosci.org as supplemental material), indicating that the synaptic vesicle cycling is not significantly affected by *DSiaT*. Together, our electrophysiological results are most consistent with the conclusion that synaptic secretory mechanisms are not

significantly affected in *DSiaT* mutants, while suggesting that the evoked EJP defect is a by-product of abnormal neuronal excitability because of an impaired action potential.

DSiaT mutations affect the function of voltage-gated sodium channel

To investigate the possibility that a neuronal excitability defect (i.e., compromised action potential) may underlie the physiological and behavioral phenotypes of *DSiaT* mutants, we have conducted additional genetic and pharmacological analyses. Action potential properties depend on the function of several families of voltage-gated ion channels (Bean, 2007), with a voltage-gated Na^+ channel encoded by the *para* gene playing a major role in action potential initiation and propagation in most fly neurons (Loughney et al., 1989). Interestingly, mutations in *para* also result in TS paralysis (Suzuki et al., 1971), whereas a hypomorphic *para* mutant has a larval crawling pattern phenotype similar to that of *DSiaT* mutants (Fig. 2D) (Wang et al., 1997). We analyzed genetic interactions between *para* and *DSiaT* using the TS-paralysis assay and found that a copy of a temperature-sensitive conditional (*ts1*) or a loss-of-function (*LK5*) allele of *para* could significantly enhance the TS-paralysis phenotype of *DSiaT* mutants, indicating strong synergistic interactions between these genes (Fig. 5A). No significant interaction was detected by this assay for two other TS paralytic mutants, *cac^{ts2}* [conditional TS allele of voltage-gated Ca^{2+} channel $\alpha 1$ subunit (Kawasaki et al., 2002)] and *tipE¹* [loss-of-function allele of auxiliary subunit of voltage-gated Na^+ channel (Feng et al., 1995)] (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). These results further supported the specificity of genetic interactions between *para* and *DSiaT*, suggesting that the function of Para is affected in *DSiaT* mutants. To examine this effect more directly, we used a pharmacological strategy based on the specific inhibition of Para by TTX, a neurotoxin that blocks the channel by binding its pore loops with high affinity (Catterall, 2000). We hypothesized that if Para properties are changed in *DSiaT* mutants, it may result in an altered TTX-mediated inhibition of Para. Indeed, we found that the kinetics of inhibition measured by the gradual disappearance of the stimulus-evoked EJP in muscle 1 after TTX addition was significantly slower in *DSiaT* mutants than in wild-type controls (Fig. 5B, C). Yet another evidence suggesting that Para function is affected in *DSiaT* mutants came from the decreased sensitivity of the mutants to DDT (Fig. 5D; supplemental Fig. S3E, available at www.jneurosci.org as supplemental material), an insecticide that specifically interacts with voltage-gated sodium channels and alters their gating (Davies et al., 2007). The DDT sensitivity phenotype was rescued by the *DSiaT* transgene (the rescue was incomplete, probably because the transgene did not fully restore the endogenous level of *DSiaT* activity). Together, these results indicate that *DSiaT* regulates the functional properties of Para, strongly suggesting that decreased neural transmission in *DSiaT*

Figure 5. The function of voltage gated Na^+ channel is affected in *DSiaT* mutants. **A**, *DSiaT* and *para* have synergistic genetic interactions. Although heterozygous *para* mutations do not cause TS paralysis, they dramatically enhance the phenotype of *S23* mutants. This synergistic phenotype is significantly rescued by a *para* genetic duplication (*para^{ts1}*). Twenty 3-d-old flies were assayed for each genotype. **B**, **C**, Sensitivity to TTX is decreased in *DSiaT* mutants (**B**), whereas a copy of wild-type *DSiaT* gene rescues this phenotype (**C**). Sixteen larvae were analyzed for each genotype. **D**, *DSiaT* mutants are more resistant to DDT than wild-type flies. "DDT −," Mortality in mock-treated vials. Twenty groups of 10 flies each were analyzed for each genotype. In all panels, error bars are SEM. The asterisks indicate significant differences (*t* test, **p* < 0.05 and ***p* < 0.01). Rescue genotype was *w¹¹¹⁸; S23; P[DSiaT⁺]*.

mutant neurons is associated with altered neuronal excitability because of an action potential defect.

Sialyltransferase activity is required for *DSiaT* function *in vivo*

We tested whether the sialyltransferase activity of the *DSiaT* protein is important for its function *in vivo*. To this end, we embarked on generating an enzymatically inactive form of *DSiaT* that would still be correctly folded, retaining an overall normal structure. A conserved histidine residue within the VS sialylmotif 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 preserving their folding and substrate binding (Kitazume-Kawaguchi et al., 2001; Jeanneau et al., 2004). Thus, we generated an expression construct encoding *DSiaT^{HK}*, a point mutant with the conserved His⁴⁰⁶ in the VS motif changed to Lys (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). This mutant was expressed as a protein A-tagged secreted construct in *Drosophila* S2 cells, purified, and assayed for enzymatic activity *in vitro*. We found that the mutant protein has no detectable sialyltransferase activity (Fig. 6A). At the same time, when the full-length *DSiaT^{HK}* protein was expressed *in vivo* using UAS-GAL4 system, the level of expression and subcellular localization of *DSiaT^{HK}* were indistinguishable from those of the matching wild-type control, *DSiaT^{WT}* (Fig. 6B), suggesting that the H406K mutation indeed does not affect the folding and proper localization of the sialyltransferase protein within the cell.

Figure 6. Sialyltransferase activity is required for *in vivo* DSiaT functioning. **A**, DSiaT^{HK} mutant protein has no detectable activity *in vitro*. DSiaT^{HK} and DSiaT^{WT} expressed in *Drosophila* S2 cells as protein A-fusion constructs were assayed for sialyltransferase activity using CMP-[¹⁴C]Sia as a donor and lactoferrin as an acceptor. The left two lanes are the autoradiograph of products of the assays separated on SDS gel; the right two lanes show the Coomassie staining of the same gel as a control for protein amount. IgG, Ig leached from beads used in purification (Koles et al., 2004). **B**, DSiaT^{HK} is expressed and localized normally in brain neurons. DSiaT^{HK} and DSiaT^{WT} were expressed *in vivo* as C-terminally HA-tagged full-length constructs using C155-Gal4 driver. There is no difference in the level of expression or subcellular localization of these proteins, as revealed by double staining with a Golgi marker, Lva (green) (Sisson et al., 2000). **C**, The transgenic expression of DSiaT wild-type construct with a neuronal driver fully rescues the TS-paralysis phenotype of *DSiaT* mutants; however, the expression of enzymatically inactive DSiaT protein, DSiaT^{HK}, is unable to rescue the mutant phenotype. **D**, Sialyltransferase activity is required for rescuing locomotor defect of *DSiaT* mutants. Whereas transgenic DSiaT^{WT} fully rescues locomotor abnormalities, DSiaT^{HK} is unable to rescue the mutant phenotype. Genotypes are as follows: WT, Wild-type control (*w¹¹¹⁸* Canton S); Driver, C155-GAL4/+; S23 + Driver::DSiaT^{WT}, C155-GAL4/+;S23/S23;UAS-DSiaT^{WT}/+; S23 + Driver::DSiaT^{HK}, C155-GAL4/+;S23/S23;UAS-DSiaT^{HK}/+. Twenty 5-d-old flies were assayed for each genotype. Error bars are SEM. The asterisks indicate the results with significant differences from wild-type phenotypes (*t* test, ***p* < 0.01) (but these results are not significantly different from each other).

Yet the expression of DSiaT^{HK} was unable to rescue the TS paralysis and locomotor abnormalities of *DSiaT* mutants (Fig. 6C,D). Thus, these results demonstrated that the sialyltransferase activity of DSiaT is essential for its biological function.

Discussion

Our results revealed that *Drosophila* sialyltransferase functions specifically in the CNS throughout development, modulating excitability of individual neurons, influencing NMJ development, and affecting behaviors and longevity at the organismal level. This is the first demonstration, to our knowledge, of a specific biological role of an ST6Gal sialyltransferase in the nervous system. Mammals have two ST6Gal enzymes, with one of them, ST6Gal I, being implicated in immune system function and cancer (Hennet et al., 1998; Hedlund et al., 2008). No mutants have been yet characterized for the second enzyme, ST6Gal II, whose function remains unknown. Whereas ST6Gal I is expressed ubiquitously, ST6Gal II has a tissue-specific expression, with elevated level being detected in the fetal and adult brain (Takashima et al., 2002; Krzewinski-Recchi et al., 2003). Based on its structural and enzymatic properties (Takashima et al., 2002; Krzewinski-Recchi et al., 2003; Rohfritsch et al., 2006), ST6Gal II represents the closest mammalian counterpart of DSiaT (Fig. 1) (Koles et al.,

2004). Thus, our results suggest an intriguing possibility that ST6Gal II, similar to its *Drosophila* homolog, may be involved in the regulation of nervous system function, which will require additional investigation.

DSiaT has a substantial impact on CNS function at the organismal level. Although DSiaT activity is required for properly coordinated locomotion and longevity under normal conditions, its effect on neural functions is compounded at elevated temperature. Together, these results implicate DSiaT-mediated sialylation in essential neuronal network regulation. Moreover, the severity of behavioral phenotypes in *DSiaT* mutants rapidly increases with age, which suggests a progressive deterioration of neural functions and potential neurodegeneration. This is consistent with significantly decreased life span of *DSiaT* mutants, and with the fact that the mutants exhibiting TS paralysis and defects in membrane excitability commonly have neurodegeneration (Palladino et al., 2002; Fergestad et al., 2006, 2008). Additional studies will reveal the cellular mechanisms underlying the age-dependent exacerbation of behavioral phenotypes in *DSiaT* mutants.

Our analysis of larval NMJ morphology revealed a decreased number of synaptic boutons and branches in *DSiaT* mutants, which suggests that DSiaT activity promotes the growth of syn-

aptic connections. It is interesting that, despite the decreased size of the neuromuscular synapses, the synaptic activity appears to be properly maintained in *DSiaT* mutants at the whole-muscle level, as suggested by increased density of active zones, normal mini-EJP, unchanged paired-pulsed facilitation, and proper synaptic vesicle dynamics, as well as by the rescue of evoked EJP phenotype by increased Ca^{2+} concentration. This conclusion is consistent with the fact that transgenic *DSiaT* construct can fully rescue the EJP defect, albeit it does not completely restore NMJ morphology (likely because its expression does not fully recapitulate the endogenous *DSiaT* expression during NMJ development), which indicates that the decreased EJP and abnormal NMJ are two separable phenotypes. The effect of *DSiaT* on synaptic morphology may be explained by a decrease in neural excitability during the growth and development of larval NMJ synapses. Neural excitability was shown to positively regulate the postembryonic development of motor terminals, whereas reduced impulse activity results in smaller NMJs (Budnik et al., 1990; Sigrist et al., 2003; Zhong and Wu, 2004; Guan et al., 2005). Moreover, genetic manipulations with *para* activity revealed that the increased level of *para* results in NMJ synapse overgrowth, while reducing *para* activity (e.g., using *nap* mutations), has an opposite effect, leading to a decreased number of branches and fully suppressing the NMJ overgrowth induced by elevated temperature or by the mutations resulting in membrane hyperexcitability, such as *eag* and *Sh* (Budnik et al., 1990; Zhong and Wu, 2004). These data are consistent with our results indicating that *DSiaT* affects Para function.

We demonstrated that sialyltransferase activity is required for *DSiaT* *in vivo* function, which indicates that *DSiaT* mutant phenotypes result from a deficiency of sialylation. To our knowledge, this is the first evidence that sialylation has an important biological function in protostomes. Previous research on sialylation in insect cells has been controversial, with some experiments suggesting the presence of sialylated structures or biochemical potential for sialylation, and others unable to detect sialic acids (Marchal et al., 2001; Angata and Varki, 2002; Koles et al., 2009). The absence of reliable evidence of sialylation or its function in protostomes has led to the long-standing paradigm that sialic acid biosynthesis is a late evolutionary invention of deuterostomes (vertebrates, ascidians, and echinoderms), whereas protostomes, including *Drosophila*, are beings devoid of sialic acids or any important role of sialylation (Angata and Varki, 2002). Although more recently sialylated structures have been unambiguously detected in *Drosophila* (Aoki et al., 2007; Koles et al., 2007), their biological role remained elusive. Our results shed light on the function of sialylation in *Drosophila* and suggest that sialylation is tightly regulated by spatially and temporally restricted expression of *DSiaT* throughout development. These results also indicate that sialylated glycans may be confined in *Drosophila* to a subset of CNS neurons.

Using behavioral, genetic, electrophysiological, and pharmacological approaches, we present four different lines of evidence that *DSiaT* activity impinges on the function of Para, a voltage-gated sodium channel. A possible scenario involves a direct mechanism of sialylation of putative N-linked glycans located on Para pore loops (Loughney et al., 1989). The potential sialylation of pore loops may influence Para interactions with TTX and DDT, or/and it may modify channel gating, thus affecting the efficacy of their action. This possibility is consistent with the fact that single amino acid substitutions within the pore loops can confer resistance to TTX and DDT (Pittendrigh et al., 1997; Catterall, 2000). In vertebrates, voltage-gated Na^+ channels are known to be heavily sialylated (Miller et al., 1983; James and

Agnew, 1987; Schmidt and Catterall, 1987), with sialic acids being responsible for changes in gating properties of the channels *in vitro* (Recio-Pinto et al., 1990) and in cell culture in an isoform-specific manner (Bennett, 2002). In rat models, the channel glycosylation was found to be developmentally regulated, and treatments with glycosidases that remove sialylated glycans suggested that the membrane sialylation modulates voltage-gated Na^+ channels in dorsal root ganglion neurons (Tyrrell et al., 2001) and in the hippocampus, while influencing network excitability and seizure threshold in kindling epilepsy models (Isaev et al., 2007). Our experiments demonstrated that sialylation plays an important role in *Drosophila* CNS, regulating excitability of neuronal membranes and modulating the function of a voltage-gated Na^+ channel. These findings not only reveal the evolutionary conservation of the biological function of sialylation between *Drosophila* and mammals but also present the first *in vivo* evidence that N-linked sialylation plays a specific role in the control of neuronal transmission, while demonstrating the significance of such a role at the organismal level. Importantly, our results suggest a novel function of sialylation in the regulation of voltage-gated Na^+ channel since *DSiaT*-generated structures are predicted to be monosialylated biantennary N-glycans (Koles et al., 2004, 2007; Aoki et al., 2007), as opposed to the heavily sialylated carbohydrates modifications present on vertebrate voltage-gated Na^+ channels (Miller et al., 1983; Roberts and Barchi, 1987). So far, the effect of sialylation on the function of voltage-gated Na^+ channels in neurons has been attributed to a large negative charge caused by a significant number of sialic acid residues attached to the channel [estimated as >100 residues per channel (Miller et al., 1983; Bennett, 2002)], including polymers of sialic acid (James and Agnew, 1987; Zuber et al., 1992). Our data, however, imply that sialylation can have a more specific effect on ion channel function, not necessarily associated with a significant charge accumulated in the vicinity of channel pore, and suggest that sialylated N-glycans may have a novel role in regulating neural excitability. Such a specific function of sialylation in modulating the voltage-gated sodium channel is consistent with the fact that Endo-N treatment that specifically removes polysialic acids has no effect on evoked synaptic transmission and intracellularly recorded action potentials in rat hippocampus (Muller et al., 1996). This function may be also involved in the regulation of cardiac sodium channel, which is significantly less sialylated than its neuronal counterparts and whose properties appear to be affected by certain “functional” sialic acids (Stocker and Bennett, 2006) rather than by a total surface charge accumulated because of sialylation (Ufret-Vincenty et al., 2001; Bennett, 2002; Fozzard and Kyle, 2002). Molecular mechanisms underlying the role of sialylated N-glycans in the function of Para, including direct analysis of its potential sialylation, will require additional investigation, while taking into account that a large number of Para variants can be produced by alternative splicing (Olson et al., 2008) and RNA editing (Palladino et al., 2000), and that these variants may be differently affected by *DSiaT*. Together, our results suggest that *Drosophila* can be a useful and experimentally amenable model for elucidating the function of sialylation in the nervous system.

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