Cell Type-Specific Requirements for Heparan Sulfate Biosynthesis at the *Drosophila* Neuromuscular Junction: Effects on Synapse Function, Membrane Trafficking, and Mitochondrial Localization

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Heparan sulfate proteoglycans (HSPGs) are concentrated at neuromuscular synapses in many species, including *Drosophila*. We have established the physiological and patterning functions of HSPGs at the *Drosophila* neuromuscular junction by using mutations that block heparan sulfate synthesis or sulfation to compromise HSPG function. The mutant animals showed defects in synaptic physiology and morphology suggesting that HSPGs function both presynaptically and postsynaptically; these defects could be rescued by appropriate transgene expression. Of particular interest were selective disruptions of mitochondrial localization, abnormal distributions of Golgi and endoplasmic reticulum markers in the muscle, and a markedly increased level of stimulus-dependent endocytosis in the motoneuron. Our data support the emerging view that HSPG functions are not limited to the cell surface and matrix environments, but also affect a diverse set of cellular processes including membrane trafficking and organelle distributions.

Introduction

The function of heparan sulfate proteoglycans (HSPGs) has been the subject of intense investigation in the last decade, and it is now clear they play essential roles in development, controlling both responses of cells to secreted growth factors, as well as the distribution of growth factors in the matrix (Lin, 2004; Bülow and Hobert, 2006; Kirkpatrick and Selleck, 2007). HSPGs are also critical for nervous system development, affecting such diverse events as axon guidance and the localization of synaptic components (Hoch et al., 1994; Yamaguchi, 2002; Lee et al., 2004; Rontundo et al., 2005; Jenniskens et al., 2006; Lindwall et al., 2007). A number of growth factors that have a high affinity for heparan sulfate (HS), including neuregulins and hepatocyte growth factor, affect neuromuscular junction (NMJ) development or function (Loeb, 2003; Madhavan and Peng, 2006). The HSPG syndecan-2 participates in ephrin-mediated dendritic spine development (Irie and Yamaguchi, 2004). Thus, HSPGs can potentially affect synapse development in a number of ways, but their contributions to synapse formation and function have not been

examined in a genetically accessible system in which heparan sulfate synthesis or modification can be readily manipulated.

In *Drosophila*, two heparan sulfate-modified core proteins affect the assembly of the neuromuscular junction (Johnson et al., 2006). Syndecan (Sdc), a transmembrane proteoglycan (PG), influences presynaptic terminal growth, whereas Dally-like protein (Dlp), a glycosylphosphatidylinositol (GPI)-linked HSPG, participates in active zone assembly. These two proteoglycans are proposed to play antagonistic roles in regulating the function of the receptor tyrosine phosphatase Dlar.

The analysis of HSPGs to date has mostly focused on their activities at the cell surface and in the extracellular matrix. More recent work has highlighted their cellular functions, demonstrating that HSPGs are not simply growth factor coreceptors, but control growth factor distributions by regulating endocytic trafficking (Marois et al., 2006; Gallet et al., 2008). There are also compelling data suggesting that one or more HSPGs serve to internalize triglyceride-rich lipoprotein particles (MacArthur et al., 2007). Studies of mice bearing mutations in *Ndst2*, a HS-modifying enzyme critical for mast cell function, suggest that normal HS biosynthesis is required to properly package the components of secretory vesicles, indicating HS has important biosynthetic functions (Forsberg et al., 1999; Humphries et al., 1999).

We are interested in understanding the function of HSPGs in cell–cell communication and chose the *Drosophila* neuromuscular junction as a model system to explore both their role in the assembly of a defined synapse, as well as their contribution to the well characterized architecture and cellular processes required for normal NMJ activity. We report here a detailed functional inves-

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Figure 1. HSPGs are localized at the *Drosophila* NMJ. **A–L**, NMJs at muscles 6 and 7 were stained with 3G10 antibody (green) to display the localization of HSPGs and anti-HRP (red) to label motoneuron processes. Scale bars, 10 μ m. **A–C**, In wild-type animals, HS is highly enriched at the NMJ, including both type Ib and Is boutons, but it is also enriched on trachea (**A**, asterisk). At muscle 13 (**C**, inset), HS is found at type Ib boutons (large arrow) and Is boutons (small arrow), but not at type II boutons (arrowhead). **D–I**, 3G10 anti-HS staining is nearly eliminated in *botv*⁵¹⁰/*botv*⁴²³ and *ttv*^{00681b} mutant animals. **J–L**, *sfl*^{9B4}/*sfl*⁰³⁸⁴⁴ mutants show normal levels of 3G10 antibody staining. **M–R**, NMJs at muscles 6 and 7 stained with anti-Dlp antibody (green) and anti-HRP (red). Scale bars, 10 μ m. **M–O**, Anti-Dlp staining is enriched at the NMJ and has a somewhat punctate appearance. It overlaps with anti-HRP staining, but has a broader distribution. The inset in **O** shows a type Ib bouton (**O**, arrow) in which anti-Dlp staining mostly surrounds anti-HRP staining. **P–R**, There is no anti-Dlp staining in *dlp*¹/*dlp*² mutants, demonstrating specificity of the anti-Dlp antibody.

tigation using mutants that affect HS chain initiation, polymerization, or sulfation. We find that HS is concentrated at the NMJ, as in vertebrates, and that mutations compromising HS synthesis deplete HS from this cell–cell junction. Loss of HS affects both NMJ structure and function, and causes surprising defects in membrane trafficking and organelle localization in the neuron and in the muscle cell.

Materials and Methods

Drosophila strains. All flies were cultured at 25°C in vials or bottles containing standard cornmeal, agar, sugar, and yeast medium. Oregon-R (+/+) flies were used as wild-type controls. *sfl*^{9B4} *FRT2A/TM3 Ser GFP* and *sfl*⁰³⁸⁴⁴ *P[w⁺, FRT2A/TM3 Ser GFP]* were crossed to generate *sfl* transheterozygous null mutants (Lin and Perrimon, 1999) and sibling

controls. *yw; FRT[42D] botv*⁵¹⁰/*CyO GFP* and *yw; FRT[42D] botv*⁴²³/*CyO GFP* were crossed to generate *botv* mutants (Takei et al., 2004) and sibling controls. *FRTG13 ttv*^{00681b}/*CyO ubq::GFP* was used as a source of homozygous *ttv* null mutants (Bellaïche et al., 1998). Heterozygous controls carried *TM3 Ser GFP* or *CyO ubq::GFP* balancer chromosomes with wild-type *sfl* or *ttv* alleles, except for FM1-43 labeling (see Fig. 7) in which mutant stocks were outcrossed to Oregon-R to generate *sfl*^{+/+} or *ttv*^{+/+} heterozygotes. *dlp*¹ *st ry/TM6B, Tb* and *ru h dlp*² *st ry e/TM6B, Tb* or *Df(3L)jz.D21* were crossed to generate *dlp* mutants. *sdc* mutants were created by crossing *sdc*¹⁰⁶⁰⁸/*CyO ubq::GFP* with *Df(2R)48/CyO ubq::GFP*. For rescue of *ttv* mutants, strains bearing a *Gal4* driver (*elav-Gal4*, *G14-Gal4*, or *da-Gal4*), *ttv*^{00681b}, and *CyO ubq::GFP* were crossed to *FRTG13 ttv*^{00681b}/*CyO ubq::GFP*; *UAS-ttv-myc*. The genotypes of progeny expressing *UAS-ttv-myc* with a *Gal4* driver are shown as *elav>ttv-myc*,

Figure 2. Functional analysis of *sfl* and *ttv* mutant synapses. **A**, Representative traces of evoked neurotransmission at abdominal muscle 6 of wild type (+/+), *sfl*^{9B4}/*sfl*⁰³⁸⁴⁴ mutants, *ttv*^{00681b} mutants, and *ttv*^{00681b} mutant larvae expressing a UAS-*ttv-myc* transgene driven by *elav-Gal4*, *G14-Gal4*, or *da-Gal4*. **B**, Average EJP amplitudes evoked in *sfl* or *ttv* heterozygous and mutant animals, and in *ttv* mutant animals expressing UAS-*ttv-myc* as in **A**. In all graphs, error bars represent SEM. ***p* < 0.001 relative to +/+ and the relevant heterozygous control. Sample numbers (*n*) shown below the graph also apply to **D**. Both *sfl* and *ttv* mutants display increased EJP amplitudes compared with heterozygous controls. Expression of *Ttv-myc* with *elav-Gal4* or *G14-Gal4*, but not *da-Gal4*, rescues the EJP amplitude of *ttv* mutants. **C**, Representative traces of spontaneous neurotransmission in abdominal muscle 6 of animals with the same genotypes as in **A**. mEJP amplitudes are increased in *sfl* and *ttv* mutants, and rescued by neuronal (*elav-Gal4*) or, to some extent, muscle (*G14-Gal4*) expression of *Ttv-myc*. **D**, Average mEJP frequency for the indicated genotypes (as in **B**). *sfl* and *ttv* mutants have decreased mEJP frequencies relative to +/+ and heterozygous controls; this phenotype is rescued by UAS-*ttv-myc* expression with *G14-Gal4* or *da-Gal4*, but not *elav-Gal4* (Table 1).

G14>*ttv-myc*, or *da*>*ttv-myc*. Similar crosses were performed to express UAS-*mitoGFP* or UAS-*CD8:GFP*. UAS-*ttv-myc* flies were a generous gift from I. The (Utrecht University, Department of Developmental Biology, Utrecht, The Netherlands); UAS-*mitoGFP* was obtained from M. O'Connor (Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN). Other fly strains used are described in Flybase and are available from the Bloomington Stock Center.

Antibodies and immunohistochemistry. Third-instar larvae were dissected in ice-cold Ca²⁺-free HL-3 saline (70 mM NaCl, 5 mM KCl, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 100 mM sucrose, 5 mM HEPES, pH 7.2) (Rawson et al., 2003). Larvae were fixed with Bouin's fixative (70% picric acid, 25% 37% formaldehyde, 5% glacial acetic acid) for staining of NMJs with anti-Dally-like (30 min), anti-cytochrome *c* (5 min), and anti-glutamate receptor IIA (5 min). For staining with other antibodies, larvae were fixed with 4% formaldehyde in PBS for 30 min. Primary antibodies used were mouse anti-cysteine string protein (anti-CSP) (kind gift from K. Zinsmaier, University of Arizona, Arizona Research Laboratories Division of Neurology, Tucson, AZ) at 1:1000, mouse anti-myc (Roche) at 1:100, mouse anti-binding Ig protein (BIP) (Nventa Biopharmaceuticals) at 1:100, mouse anti-p120 (Calbiochem) at 1:200, mouse anti-Dally-like (Developmental Studies Hybridoma Bank) at 1:4, mouse anti-cytochrome *c* (BD Biosciences Pharmingen) at 1:50, and mouse anti-glutamate receptor IIA (anti-GluRIIA) (Developmental Studies Hybridoma Bank) at 1:100. Staining was visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen). For heparan sulfate staining, larvae were dissected, fixed with Bouin's fixative for 60 min, and treated with 20 mU of heparitinase I (Seikagaku) in heparitinase I buffer (100 mM sodium acetate, 10 mM calcium acetate, pH 7.0) for 2 h at 37°C. After washing, samples were stained with 3G10 antibody

(Seikagaku) at 1:100. Anti-Dally-like, anti-myc, and anti-heparan sulfate signals were amplified with Tyramide Signal Amplification Fluorescence Systems (PerkinElmer). Larvae were stained with fluorescein (FITC)-conjugated goat anti-horseradish peroxidase (anti-HRP) (Jackson ImmunoResearch Laboratories) at 1:50 to show axons and synaptic boutons (Bodmer et al., 1987). Images were captured with a Nikon C1 confocal microscope and analyzed with ImageJ 1.34 s and Adobe Photoshop. Identical confocal settings were used when imaging mutant, rescued, and control samples to allow direct comparisons.

Electrophysiology. Intracellular recordings were performed as described previously (Rawson et al., 2003) with minor modifications. Thin-walled glass intracellular electrodes were filled with 3 M KCl and had resistances of 10–20 MΩ. Preparations were immersed in room temperature HL-3 saline containing 1.2 mM Ca²⁺. Miniature excitatory junction potentials (mEJPs) at muscle 6 of the A2 or A3 hemisegment were collected for 1 min to derive an average frequency and strength of spontaneous release. Excitatory junction potentials (EJPs) were recorded by stimulating the nerve with 15 ms pulses at a frequency of 1 Hz and at a strength 1.5 times that necessary to evoke the compound response. Only recordings with resting membrane potentials of at least −65 mV were used. Recordings were acquired with an Axonclamp 2B amplifier (Molecular Devices). Mini Analysis program (Synaptosoft) was used to analyze the recordings. The quantal content for a given EJP was calculated by dividing the evoked EJP amplitude by the mEJP amplitude, and then adjusted for nonlinear summation of EJPs (Martin, 1955).

Behavioral assay: muscle contraction assay.

Third-instar larvae were placed on a fresh agar plate. For larvae undergoing continuous forward movements, the numbers of body wall contractions during 1 min were counted.

Electron microscopy. Third-instar larvae were dissected in Ca²⁺-free HL-3 saline and processed as described previously (Marqués et al., 2002). Synaptic boutons between muscles 6 and 7 were examined using the JEOL 1200-EXII transmission electron microscope and analyzed with ImageJ 1.34 s. Three *sfl*⁰³⁸⁴⁴ mutants and three Oregon-R control animals were analyzed. Between 5 and 45 different sections were used to quantify each phenotype.

Assay for vesicle recycling. Third-instar larvae were dissected in Ca²⁺-free normal saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 36 mM sucrose, 5 mM HEPES, pH 7.2). Boutons were stained in 1 μM FM1-43 in high K⁺ saline (75 mM NaCl, 60 mM KCl, 2 mM MgCl₂, 36 mM sucrose, 1.5 mM CaCl₂, 5 mM HEPES, pH 7.2) to depolarize the nerve (Ramswami et al., 1994). Preparations were washed thoroughly in Ca²⁺-free normal saline to remove noninternalized dye. Images of synaptic boutons at muscle 4 in segment A3 were captured using a Nikon Eclipse E800 fluorescence microscope. Only synaptic boutons in focus were analyzed with Adobe Photoshop and ImageJ 1.34 s.

Statistics. Numerical data are presented as mean ± SEM. Student's *t* test was used to compare means between mutant and control groups; significance is indicated as follows: **p* < 0.01 and ***p* < 0.001 compared with +/+ (Oregon-R) and the relevant heterozygous control.

Results

HS is localized at the *Drosophila* NMJ

A variety of HSPGs are concentrated at the synapse of the vertebrate NMJ (Jenniskens et al., 2006). These include agrin, which is

critical for clustering acetylcholine receptors in the postsynaptic membrane (Hoch et al., 1994), and perlecan, a protein that binds the collagen-tailed form of acetylcholinesterase (Arikawa-Hirasawa et al., 2002). Two HSPGs have been localized to the NMJ in *Drosophila*, Sdc and Dlp, a transmembrane and a GPI-linked PG, respectively (Johnson et al., 2006). To evaluate the distribution of HS at the *Drosophila* NMJ, we treated larval preparations with heparitinase, which digests HS chains leaving desaturated uronate epitopes that are recognized by the monoclonal antibody 3G10 (David et al., 1992). At body wall muscles 6 and 7 of third-instar larvae, substantial 3G10 antibody staining is found on axons and synaptic boutons of the NMJ (also labeled with anti-HRP) (Fig. 1A–C) as well as on tracheal branches, the respiratory conducting elements (Fig. 1A, asterisk). Muscles 6 and 7 are innervated by two motoneurons that both use glutamate as the neurotransmitter (Johansen et al., 1989). One motoneuron forms larger type Ib boutons on muscles 6 and 7, whereas the other makes smaller type Is boutons on these and several adjacent muscles (Hoang and Chiba, 2001). Both the type Ib and type Is-derived boutons are stained by 3G10 antibody. We examined 3G10 staining at muscle 13 to evaluate HS localization at type II boutons, which arise from a third type of motoneuron and use multiple neurotransmitters (Hoang and Chiba, 2001). HS is concentrated at type Ib and Is boutons of muscle 13 (Fig. 1C, inset, arrows), but there is little or no staining of type II boutons (Fig. 1C, inset, arrowhead), demonstrating that HS preferentially localizes to specific bouton types.

We used 3G10 antibody staining to evaluate the level of HS remaining at the NMJ in third-instar larvae bearing mutations in HS biosynthetic genes. These animals survive until the larval or pupal stage by relying on maternally provided gene products. The enzyme encoded by *brother of tout velu* (*botv*) initiates an HS chain by adding *N*-acetylglucosamine (GlcNAc) to a tetrasaccharide linker at specific sites on HSPG core proteins (Izumikawa et al., 2006). The HS chain is extended by a copolymerase produced from the *tout velu* (*ttv*) and *sister of tout velu* (*sotv*) genes (Izumikawa et al., 2006). Next, *N*-deacetylase/*N*-sulfotransferase (encoded by *sfl*) modifies the chain by deacetylating GlcNAc and introducing *N*-sulfate groups (Toyoda et al., 2000). We found no 3G10 staining at the NMJ in *botv* or *ttv* mutants (Fig. 1D–I), as expected for animals that cannot initiate or extend an HS chain. In contrast, *sfl* mutants exhibit normal 3G10 staining (Fig. 1J–L), showing that HS remains localized at these synapses even though sulfation is dramatically reduced in these animals (Toyoda et al., 2000).

We examined the localization of a particular HSPG, the glypican Dlp, at the NMJ. Anti-Dlp antibody staining showed that Dlp is concentrated in the region of the NMJ (Fig. 1M–O). Anti-Dlp staining appears to surround the synaptic boutons detected by anti-HRP staining, suggesting that Dlp may be present at higher levels on the surface of the muscle cell at the NMJ. This staining is lost in *dlp* mutant larvae (Fig. 1P–R).

The postsynaptic localization of Dlp protein contrasts with the mostly presynaptic staining seen with 3G10 antibody, suggesting that other presynaptic HSPGs are present at the NMJ. The other HSPG known to localize to the NMJ, Sdc, is also reported to show mostly postsynaptic staining (Johnson et al., 2006), again begging the question of which PG is present at significant levels presynaptically. To evaluate this issue, we used two approaches. First, we used an antibody staining protocol that provided greater penetration of the 3G10 antibody (0.3% Triton X-100) to improve sensitivity, and second, we examined 3G10 staining in both *dlp* and *sdc* mutant larvae. With this change in protocol, addi-

Figure 3. Locomotion defects in HS biosynthetic mutants. Locomotion was quantified as the number of muscular body contractions per minute during larval crawling. Error bars represent SEM. ** $p < 0.001$ relative to +/+ and the relevant heterozygotes. (***) $p < 0.001$ compared with *ttv* heterozygotes. Sample numbers (*n*) are shown below the graph. Both *sfl* and *ttv* mutants show a crawling defect. This defect is rescued in *ttv* mutants by *Ttv-myc* expression with *elav-Gal4* or *G14-Gal4*, but is not significantly rescued with *da-Gal4*.

tional 3G10 staining is observed surrounding the synaptic boutons in wild-type NMJs (supplemental Fig. S1A–F, available at www.jneurosci.org as supplemental material). A significant portion of this signal is lost in *dlp* mutants (supplemental Fig. S1G–L, available at www.jneurosci.org as supplemental material), although substantial presynaptic staining remains. In addition, *dlp* mutants have almost no staining on the rest of the muscle cell surface, suggesting that a low level of Dlp is normally present there. In *sdc* mutants (supplemental Fig. S1M–R, available at www.jneurosci.org as supplemental material), 3G10 staining at and around the synaptic boutons was reduced, but not eliminated; staining elsewhere in the muscle was unaffected. These results demonstrate that Dlp and Sdc are both significant contributors to the HS found at the NMJ, but suggest the possibility that other presynaptic HSPGs may also be present. Alternatively, Dlp and Sdc may have significant presynaptic localization in addition to their described levels postsynaptically.

HS biosynthetic mutants show defects in NMJ physiology

To assess the role of HS biosynthesis in synapse function, we analyzed spontaneous and evoked neurotransmission in third-instar larvae with mutations in HS biosynthetic genes. In response to nerve stimulation, both *ttv* and *sfl* mutants exhibited significantly increased EJPs compared with wild-type or heterozygous control larvae (20–25% increase over controls) (Fig. 2A,B). Spontaneous release events (mEJPs) were also affected in these mutants (Fig. 2C,D): mEJP frequency was substantially reduced in *sfl* and *ttv* mutants compared with heterozygous or wild-type controls, suggesting that loss of HS biosynthesis affected the spontaneous vesicle release probability in the motoneuron. We

Table 1. Summary of *sfl* mutant, *ttv* mutant, and *ttv* rescue phenotypes

	description	viability	EJP amplitude	mEJP frequency	locomotion	HS level (3G10 staining)	bouton number/muscle area	FM1-43 uptake (presynaptic endocytosis)	cyt c (mitochondrial marker) in muscle	BIP (ER marker) in muscle	p120 (Golgi marker) in muscle
<i>sfl</i> ^{9B4} / <i>sfl</i> ⁰³⁸⁴⁴	<i>sfl</i> mutant	pupal lethal	high	low	reduced	normal	normal	increased	reduced	ND	ND
<i>ttv</i> ^{00681b}	<i>ttv</i> mutant	pupal lethal	high	low	reduced	severely reduced	reduced	increased	reduced	defect	defect
<i>ttv</i> ^{00681b} , <i>UAS-ttv-myc</i>	<i>ttv</i> mutant with <i>ttv</i> transgene, no driver*	pupal lethal (like <i>ttv</i> ^{00681b})	high	low	reduced	ND	ND	ND	ND	defect	defect
<i>ttv</i> ^{00681b} , <i>elav>ttv-myc</i>	<i>ttv</i> mutant with pan-neuronal expression of <i>ttv</i> transgene	pupal lethal, but longer survival than <i>ttv</i> ^{00681b}	rescue	low	substantial rescue	restored at synapse	rescue	rescue	reduced	rescue	partial rescue
<i>ttv</i> ^{00681b} , <i>G14>ttv-myc</i>	<i>ttv</i> mutant with <i>ttv</i> transgene expressed in muscles	some adult escapers, mostly pupal lethal	rescue	rescue	rescue	partial rescue	rescue	substantial rescue	partial rescue	rescue, more than +/+	rescue
<i>ttv</i> ^{00681b} , <i>da>ttv-myc</i>	<i>ttv</i> mutant with ubiquitous high-level <i>ttv</i> transgene expression	pupal lethal (like <i>ttv</i> ^{00681b})	high	rescue	reduced	rescue	reduced further than <i>ttv</i> ^{00681b}	partial rescue	ND	partial rescue	defect

Mutant phenotypes are shown in red; fully or partially rescued phenotypes are shown in blue. ND, Not determined.

*With no *Gal4* driver present, there should be no expression of the *UAS-ttv-myc* transgene.

also found evidence for changes in postsynaptic responses in *sfl* and *ttv* mutants (Fig. 2C). mEJP amplitudes were modestly increased in *sfl* mutants relative to heterozygous and wild-type controls (*sfl* mutants, 1.18, compared with *sfl*/*TM3* heterozygotes, 0.79; $p < 0.001$; compared with +/+, 0.98; $p < 0.05$; *TM3* is a balancer chromosome bearing a wild-type *sfl* allele). mEJP amplitudes in *ttv* mutants were significantly elevated compared with *ttv*/*CyO* controls (*ttv* mutants, 1.05; *ttv*/*CyO* heterozygotes, 0.82; $p < 0.001$; *CyO* is a balancer chromosome bearing a wild-type *ttv* allele); this phenotype was rescued by neuronal or muscle-directed expression of a wild-type transgene ($p < 0.001$ and 0.01, respectively) (data not shown). Together, these electrophysiological phenotypes indicate that HSPGs affect both presynaptic and postsynaptic function.

The phenotypes of *ttv* and *sfl* mutants differ from those of *dlp* mutants (Johnson et al., 2006) in that spontaneous neurotransmitter release is affected as well as evoked responses. Thus, loss of HS biosynthesis has broader effects on both presynaptic and postsynaptic function at the NMJ than loss of a single HSPG. Loss of *sfl* function had more severe effects on mEJP frequency and amplitude than mutation of *ttv*, suggesting that lack of proper HS modification can be more deleterious than loss of HS chains (see below).

HS biosynthetic mutants have larval locomotion deficits

In addition to their electrophysiological phenotypes, we found that *ttv* and *sfl* mutant larvae have defects in crawling behavior. Larval crawling consists of repeated “strides” in which muscular contractions propagate from one end of the body to the other. To quantify the locomotion deficit, we counted the number of these peristaltic contractions while larvae crawled on a flat surface. Both *sfl* and *ttv* mutants had significantly reduced numbers of contractions per minute in this assay (Fig. 3). As with some of the

physiological measures, crawling behavior was more severely affected in *sfl* than *ttv* mutants.

Rescue of NMJ phenotypes by *ttv* transgene expression

To determine what cells require HS biosynthesis for normal NMJ function, we sought to rescue *ttv* mutants with a *ttv* transgene. We characterized the expression patterns of the *Gal4* drivers for these experiments using a membrane-tethered green fluorescent protein (GFP) reporter (*UAS-CD8::GFP*) (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). *elav-Gal4*, a common neuronal driver, drove GFP expression in the CNS and in the motoneuron at the NMJ (supplemental Fig. S2C,D, available at www.jneurosci.org as supplemental material). In addition, *elav-Gal4* directed a low level of GFP in muscle tissue (supplemental Fig. S2D, inset, available at www.jneurosci.org as supplemental material). *G14-Gal4*, which is described as a muscle driver, drove strong expression of GFP at the NMJ and throughout muscles 6 and 7 (supplemental Fig. S2F, available at www.jneurosci.org as supplemental material). However, *G14-Gal4* is not solely muscle-specific, because we also observed high levels of neuronal GFP in the mushroom bodies and lower levels in other neurons in the brains of these animals (supplemental Fig. S2E, available at www.jneurosci.org as supplemental material). *da-Gal4* is a strong ubiquitous driver, and gave very high levels of GFP in all tissues examined (supplemental Fig. S2G,H, available at www.jneurosci.org as supplemental material).

Expression of a myc-tagged form of Ttv with *G14-Gal4* was able to rescue *ttv* mutant animals to adulthood or to a later pupal stage (Table 1). Expression of *UAS-ttv-myc* with *elav-Gal4* also improved viability, but expression with *da-Gal4* did not. We evaluated HS biosynthesis in these animals by staining heparitinase-treated NMJ preparations with 3G10 antibody. All three drivers increased 3G10 staining, suggesting that HS synthesis was at least

partially restored (Fig. 4A–O). In *elav-Gal4>UAS-ttv-myc* animals, HS appeared to be localized primarily to the synapse. *G14-Gal4* and *da-Gal4* gave similar or even more substantial 3G10 staining at the synapse, as well as additional staining in the muscles and tracheal system. *da-Gal4>UAS-ttv-myc* animals expressed very high levels of Ttv-myc protein relative to those with the *G14-Gal4* driver, as judged by anti-myc antibody staining (Fig. 4P–R). The failure to rescue viability suggests that the HS chains in *da-Gal4>UAS-ttv-myc* animals might be defective in some way or that this level of Ttv protein is somehow deleterious.

Expression of *UAS-ttv-myc* with these same drivers rescued some of the electrophysiological and behavioral phenotypes of *ttv* mutants (Figs. 2, 3; Table 1), but sometimes with surprising cell type requirements for HS synthesis. For example, the reduced mEJP frequency in *ttv* mutants suggests a defect in presynaptic function, but this phenotype was rescued by expression of *UAS-ttv-myc* using the mostly muscle-directed *G14-Gal4* driver (Fig. 2D). Pan-neuronal expression with *elav-Gal4>UAS-ttv-myc* did not increase the mEJP frequency so *G14-Gal4*-driven expression in neurons cannot be responsible for this rescue. Therefore, we conclude that HS biosynthesis is required specifically in muscles for this motoneuron physiological attribute. The elevated EJP amplitude of *ttv* mutants was substantially rescued with either *G14-Gal4* or *elav-Gal4>UAS-ttv-myc* (Fig. 2B), suggesting that neuronal expression of Ttv-myc may be sufficient to restore normal muscle responses to motoneuron stimulation. However, since *elav-Gal4* gave a low level of *UAS-CD8::GFP* expression in muscle tissue (supplemental Fig. S1D, available at www.jneurosci.org as supplemental material), we cannot be completely certain about the critical site(s) of HSPG action for these phenotypes. Expression of Ttv-myc using *G14-Gal4* also restored normal crawling behavior, whereas *elav-Gal4* gave substantial rescue (Fig. 3). The rescue of these multiple electrophysiological and behavioral defects by *UAS-ttv-myc* expression shows conclusively that these phenotypes result from loss of *ttv* activity.

Expression of *UAS-ttv-myc* with the strong ubiquitous *da-Gal4* driver did not ameliorate *ttv* mutant phenotypes to the same extent as the other drivers. *da-Gal4*-directed expression rescued the mEJP frequency (Fig. 2D), but did not reduce the abnormally elevated EJP amplitude (Fig. 2B), nor did it rescue crawling behavior (Fig. 3). Clearly rescue of *ttv* mutants cannot be achieved simply by a high level of ubiquitous Ttv expression but depends on careful control of the sites and level of *ttv* product.

Figure 4. Rescue of HS biosynthesis by Ttv-myc expression. **A–O**, Anti-HRP (green) and 3G10 anti-HS (red) staining of NMJs at muscles 6 and 7 from wild type, *ttv* mutants, or *ttv* mutants expressing Ttv-myc driven by *elav-Gal4*, *G14-Gal4*, or *da-Gal4*. The penetration of the 3G10 antibody was increased by staining in buffer containing 0.3% rather than 0.1% Triton X-100, so the tracheal staining in wild type (**A**, **C**) is more apparent than in Figure 1. The insets in **G–O** show larger images of boutons. Scale bar, 10 μ m. **D–F**, 3G10 staining is lost in *ttv00681b* mutants. **G–I**, 3G10 staining is restored to synapses when Ttv-myc is expressed with *elav-Gal4*. **J–L**, *G14-Gal4* expression of Ttv-myc increases 3G10 staining primarily at synapses, and to a limited extent in the muscles and trachea. Note that 3G10 staining appears to surround the synaptic boutons (insets). **M–O**, *da-Gal4* expression of Ttv-myc almost fully rescues 3G10 staining. **P–R**, Expression level of Ttv-myc as shown by anti-myc antibody staining (red) of muscle 6 and 7 NMJs from *ttv* mutants expressing Ttv-myc with *elav-Gal4*, *G14-Gal4*, or *da-Gal4*. Anti-HRP staining is shown in green. *da-Gal4* gives extremely high levels of Ttv-myc expression relative to *elav-Gal4* or *G14-Gal4*. Neuronal Ttv-myc (in *elav-Gal4>UAS-ttv-myc* animals) should localize to the cell bodies, which are not visible in these images.

Morphological changes at the synapse of HS biosynthetic mutants

To evaluate the importance of HS biosynthesis for synapse development, we examined the morphology of the NMJ in *sfl* and *ttv* mutant larvae (Fig. 5). *ttv* mutant synapses are reduced in size and have significantly fewer boutons per unit muscle area compared with controls (Fig. 5C,G), whereas *sfl* synapses are smaller than normal but with no reduction in number of boutons relative to muscle area (Fig. 5B,G). We also noted alterations in bouton morphology: both *sfl* and *ttv* motoneuron boutons are bigger than normal, with a reduced number of “buds” (Fig. 5A–C, in-

Figure 5. Synapse morphology in HS biosynthetic mutants. **A–F**, Anti-HRP staining of muscle 4 NMJs from wild type, *sfl* or *ttv* mutants, and *ttv* mutants expressing *UAS-ttv-myc* driven by *elav-Gal4*, *G14-Gal4*, or *da-Gal4*. Scale bar, 10 μm. The insets show larger views of terminal type Ib boutons. *ttv* mutants have fewer, larger boutons than controls, with fewer buds. *sfl* mutants also have larger boutons with fewer buds. *G14-Gal4*>*UAS-ttv-myc* completely rescued *ttv* synapse and bouton morphology. *elav-Gal4*>*UAS-ttv-myc* rescued bouton number and size, but caused increased bouton budding. *da-Gal4*>*UAS-ttv-myc* enhanced the *ttv* mutant phenotypes. **G**, Average numbers of boutons per unit muscle area for the indicated genotypes. Error bars represent SEM. ***p* < 0.001 compared with +/+ and the relevant heterozygote control. Sample numbers (*n*) are shown below the graph. *ttv* mutant synapses have fewer boutons than controls. This phenotype is rescued by *elav-Gal4* or *G14-Gal4* driving *Ttv-myc* expression, and exacerbated by expression of *Ttv-myc* with *da-Gal4* (*p* < 10^{−4} compared with *ttv*^{00681b} mutants).

sets). During larval growth, new boutons can be added to the extending nerve terminals, with the result that larger boutons sometimes display small buds known as satellites. In contrast to normal synapses, we found that *sfl* and *ttv* synapses consisted primarily of large boutons without any buds; a similar increase in *ttv* bouton size was observed previously (Kraut et al., 2001). Our results suggest that these mutants have some defect in initiating new bouton formation, and are consistent with a role for HSPGs in normal synapse growth.

We rescued the growth defect of *ttv* mutant synapses by presynaptic and postsynaptic expression of *UAS-ttv-myc*. Expression with either *elav-Gal4* or *G14-Gal4* increased the number of boutons per unit muscle area to normal levels (Fig. 5G). Expression with *G14-Gal4* also restored a normal level of bouton budding (Fig. 5E). In contrast, expression of *UAS-ttv-myc* with *elav-Gal4* resulted in elevated bouton budding (Fig. 5D, inset), suggesting that presynaptic overexpression of *Ttv-myc* causes too many new buds to initiate. Ubiquitous overexpression of *UAS-ttv-myc* with *da-Gal4* had the opposite effect on synapse development: the number of boutons relative to muscle area was significantly reduced compared with *ttv* mutants and we observed no bouton budding (Fig. 5F). Overexpression of *UAS-ttv-myc* with *da-Gal4* in a wild-type animal also reduced bouton number significantly ($5.01 \times 10^{-4}/\mu\text{m}^2$ compared with $7.54 \times 10^{-4}/\mu\text{m}^2$ for +/+; *p* < 0.001); this effect was not seen with *elav-Gal4* (8.44×10^{-4} boutons/ μm^2 ; *p* = 0.12). Rather than rescuing the morphology, the HS synthesized in *da-Gal4*>*UAS-ttv-myc* animals seems to inhibit synapse growth, showing that appropriate *Ttv* expression is critical for synapse development.

Abnormal synaptic ultrastructure in *sfl* mutants

To assess the morphology of HS-deficient synapses in greater detail, we examined *sfl* mutants by transmission electron microscopy (TEM). The most striking phenotype we observed was a change in the distribution of mitochondria in *sfl* mutant muscles. In a wild-type synapse, mitochondria are densely packed beneath

the postsynaptic membrane near the boutons, but the number of these mitochondria is grossly reduced in *sfl* mutant NMJs (Fig. 6A,B) ($1.11/\mu\text{m}^2$ compared with $1.82/\mu\text{m}^2$ for +/+; *p* < 0.01). The numbers of mitochondria in other parts of the muscle cell, such as between the contractile fibers, were unaffected (Fig. 6C,D) ($0.265/\mu\text{m}^2$ compared with $0.325/\mu\text{m}^2$ for +/+; *p* = 0.32), and mitochondrial numbers in motoneurons were unchanged, based on counts of presynaptic mitochondria in TEM sections (data not shown). Thus, the defect may represent a failure to properly localize or retain mitochondria in a specific region of the muscle cell.

sfl mutant synapses appeared to be normal in many respects, with well aligned presynaptic and postsynaptic membranes. We did not detect any changes in the number or morphology of motoneuron active zones (Fig. 6E–J). This finding was confirmed by anti-NC82 staining, a marker for synaptic active zones in *Drosophila* (our unpublished data). Boutons were surrounded by a normal amount of subsynaptic reticulum, but *sfl* mutant muscles had gaps in the membranous structures around ~40% (17 of 44) of the boutons (Fig. 6, compare E, F, with G, H). These enlarged postsynaptic “pockets” are similar to those seen in synapses that developed under partial loss of Wingless (Packard et al., 2002).

We also noted a change in the vesicle population of the presynaptic cells. Wild-type and *sfl* boutons have similar numbers and distributions of synaptic vesicles (35 nm in diameter), but *sfl* mutants have reduced numbers of larger 70 nm vesicles, or cisternae (Fig. 6E,I, arrows; compare with G, J). TEM characterization of NMJs in animals with endocytosis defects has demonstrated increased numbers of 70 nm cisternae; these intracellular vesicular structures accumulate when endocytosis is compromised and are presumed to be intermediates in synaptic vesicle recycling (Wucherpfennig et al., 2003; Zhang, 2003). We observed a greater than twofold reduction in the number of cisternae in *sfl* mutant terminals ($0.46/\mu\text{m}^2$ compared with $1.29/\mu\text{m}^2$

for $+/+$; $p < 0.001$), suggesting that *sfl* mutants have a vesicle trafficking defect.

sfl and *ttv* mutants have endocytosis abnormalities

To directly assess membrane trafficking at the *Drosophila* NMJ, we observed the internalization of a lipophilic dye, FM1-43, after repetitive motoneuron stimulation. Repeated low-frequency depolarization mediated by incubation in high K^+ saline results in selective uptake of this fluorescent dye into the recycling pool (also known as the exo/endo pool) of synaptic vesicles (Kidokoro et al., 2004; Rizzoli and Betz, 2005). Using this assay, we found a dramatic increase in FM1-43 fluorescence in the motoneuron terminals of both *ttv* and *sfl* mutants compared with wild-type and heterozygous controls (Fig. 7*A–C,G*). This increase depends on nerve stimulation, because it did not occur when preparations were treated with FM1-43 without high levels of K^+ (our unpublished observation). Mutant neurons showed no more fluorescence than controls in the absence of stimulation, demonstrating that mutation of *sfl* or *ttv* does not affect membrane affinity of FM1-43 or fluorescence of the dye in the membrane (our unpublished observation). Therefore, we conclude that loss of HS biosynthesis or modification produces an increase in activity-dependent endocytosis at the NMJ. This increase in endocytosis documented by FM1-43 uptake is consistent with the decrease in the number of cisternae observed by TEM: both of these phenotypes are opposite to those observed in endocytic mutants (Wucherpfennig et al., 2003; Zhang, 2003). We did not see a change in the overall number of synaptic vesicles in *sfl* mutants by TEM; however, the recycling pool labeled by our FM1-43 protocol is reported to represent a modest proportion (14–19%) of all synaptic vesicles (Delgado et al., 2000; Rizzoli and Betz, 2005) so an increase in this portion of the total vesicle pool might not be apparent.

To confirm that the stimulus-dependent increase in FM1-43 uptake is mediated by loss of HS biosynthesis, we demonstrated that the elevated level of FM1-43 internalization found in *ttv* mutants is restored to normal by *elav-Gal4*-directed expression of *UAS-ttv-myc* (Fig. 7*D,G*). FM1-43 uptake is rescued to somewhat lesser degrees by muscle-directed (*G14-Gal4*) or ubiquitous expression (*da-Gal4*) (Fig. 7*E–G*). These rescue experiments support the conclusion that HSPGs serve to limit synaptic vesicle recycling at the NMJ.

Morphological changes in muscle cells of HS biosynthetic mutants

The TEM analysis of *sfl* mutants showed that the muscles of these animals have a number of morphological abnormalities, in particular a maldistribution of mitochondria beneath the cell surface in the vicinity of synaptic boutons. To further explore this phe-

Figure 6. Ultrastructure of *sfl* mutant synapses. *A, B*, Electron micrographs showing synaptic boutons (large arrowheads) and the underlying muscle in wild-type and *sfl*⁰³⁸⁴⁴ mutant NMJs. Wild-type NMJs have an abundance of mitochondria (arrow) between the boutons and the contractile fibers of the muscle, but *sfl* mutants have significantly fewer. Scale bar, 1 μ m. *C, D*, Electron micrographs showing the contractile muscle fibers from wild-type and *sfl* mutant animals (same scale as in *A* and *B*). Similar numbers of mitochondria (arrows) are found within the contractile apparatus of *sfl* mutant muscles as in wild type. *E–H*, Higher-magnification views of boutons from wild-type and *sfl* mutant animals. Scale bars: *E, G*, 0.5 μ m; *F, H*, 0.1 μ m. The arrows in *E* point to 70 nm cisternae, which are more numerous in wild type than in *sfl* mutants. The arrows in *G* point to enlarged pockets between the postsynaptic membrane and the subsynaptic reticulum in *sfl* mutants. *I, J*, Electron micrographs showing synaptic vesicles near active zones in wild type and *sfl* mutant NMJs. Scale bar, 0.1 μ m. Numerous 35 nm vesicles are seen in both genotypes, but *sfl* mutants have fewer 70 nm cisternae (arrows) than controls.

notype, we examined both *sfl* and *ttv* mutant NMJs using a mitochondria-specific antibody (anti-cytochrome *c*) and a mitochondrial-directed GFP marker (*mito-GFP*). Anti-cytochrome *c* staining of wild-type NMJs revealed the concentration of mitochondria surrounding the motoneuron boutons, as well as some enrichment around nuclei (Fig. 8*A–C*). Confirming our TEM analysis, neither *sfl* nor *ttv* mutants show a concentration of anti-cytochrome *c* staining surrounding type Ib synaptic boutons (Fig. 8*D–G,J*). Muscle-directed (*G14-Gal4*) expression of *UAS-ttv-myc* in *ttv* mutants partially restored the staining around boutons and nuclei, whereas *elav-Gal4*>*UAS-ttv-myc* had little effect (Fig. 8*G–L*). Mitochondrial number in the pre-synaptic terminals of *sfl* mutants is unaltered as measured by the levels of Mito-GFP (Fig. 8*M,N*), a result confirmed by direct counts of mitochondria in TEM sections of *sfl* nerve terminals (our unpublished data). Thus, HS biosynthetic mutations specifically affect mitochondrial localization in the postsynaptic cell near the motoneuron boutons.

The abnormalities in mitochondrial distributions in the mus-

Figure 7. Endocytosis abnormalities in HS biosynthetic mutants. **A–F**, Images of FM1-43 dye uptake in synapses at muscle 4 of wild type, *sfl* or *ttv* mutants, and *ttv* mutants expressing *UAS-ttv-myc* with *elav-Gal4*, *G14-Gal4*, or *da-Gal4*. Scale bar, 10 μ m. **G**, Average FM1-43 uptake for the indicated genotypes. Error bars represent SEM. **/**p* < 0.001 relative to +/+ and < 0.01 relative to *sfl*/+; ***p* < 0.001 compared with +/+ and *ttv*^{00681b}/+. Sample numbers (*n*) are shown below. *sfl* and *ttv* mutant synapses show substantial increases in vesicle recycling as measured by FM1-43 uptake. This phenotype is rescued by expression of *Ttv-myc* with *elav-Gal4*, *G14-Gal4*, and, to a lesser extent, *da-Gal4*.

cles of *sfl* and *ttv* mutants prompted us to examine other membrane compartments in muscle cells. We used anti-BIP antibodies to visualize endoplasmic reticulum (ER) and anti-p120 as a Golgi marker (Sanyal et al., 2005; Tsruya et al., 2007). *ttv* mutants show a marked reduction in the level of anti-BIP immunostaining (Fig. 9*A,B*); BIP levels in the muscle are rescued by expression of a *UAS-ttv-myc* transgene in neurons with *elav-Gal4*, and increased above normal levels by muscle (*G14-Gal4*) expression (Fig. 9*C,D*). Golgi organization is also altered in *ttv* mutant muscles, with dramatically reduced levels of p120 that can be rescued with *G14-Gal4*-directed expression of *UAS-ttv-myc*, or partially rescued with *elav-Gal4* (Fig. 9*E–H*). It should be emphasized that these changes in intracellular membrane organization are not a consequence of compromising cell viability: the muscle cells of

HS biosynthetic mutants show a normal membrane potential and an increased EJP in response to motoneuron stimulation. These findings demonstrate that the loss of heparan sulfate affects intracellular membrane organization. They suggest that HSPGs participate in intracellular compartment or membrane trafficking, either directly, or indirectly by affecting signaling between the muscle and the motoneuron.

Discussion

Our analyses of *Drosophila* HS biosynthetic mutants demonstrated that proper HS synthesis is important for many facets of NMJ structure and function. Defects in HS biosynthesis affected synapse growth and the formation of new boutons, disturbed both spontaneous and evoked electrophysiological properties, and produced locomotion defects in mutant animals. Notably, loss of HS also increased synaptic vesicle endocytosis and had profound consequences for intracellular organization in the muscle cell, suggesting that HSPGs normally regulate membrane trafficking and organelle localization both in neurons and muscles.

Mutations affecting different aspects of HSPG biosynthesis cause distinct phenotypes

The consequences of losing HS biosynthetic functions were broader and more substantial than the previously reported defects caused by loss of the individual HSPGs Sdc or Dlp. Johnson et al. (2006) found that both Sdc and Dlp contribute to synaptic development, but Sdc specifically promotes synapse growth, whereas Dlp influences active zone morphology. *dlp* mutants show defects in synapse physiology as well: muscle responses to nerve stimulation are substantially increased, although spontaneous neurotransmitter release is normal. Although HS biosynthetic mutants had features resembling both *sdc* and *dlp* mutants (for example, *ttv* mutants have a similar reduction in synapse size as *sdc* mutants, and increased EJP amplitudes like *dlp* mutants), *sfl* and *ttv* also affect spontaneous neurotransmission, unlike *dlp*.

Also, there were no apparent changes in active zone morphology in *sfl* mutants like those caused by loss of *dlp*. These differences indicate that *sfl* and *ttv* mutants have some defects distinct from those caused by loss of individual HSPGs.

Our analysis of mutants affecting either HS chain synthesis or sulfation shows that their phenotypes are not precisely the same; in fact, loss of sulfation has more severe consequences for spontaneous neurotransmission and locomotion than simple loss of HS chains. Although we cannot know precisely the level of gene function that remains in these animals, either from maternal contributions or some residual gene activity of the mutant alleles [both described as genetic nulls (Bellaiche et al., 1998; Lin and Perrimon, 1999)], we have conducted biochemical and structural

analysis of the HS chains in the third-instar larvae of these mutants and showed that the levels of HS were reduced at least 15-fold in *ttv* and sulfated HS was undetectable in the *sfl* alleles used (Toyoda et al., 2000). These observations suggest that the unmodified HS chains in *sfl* mutants have more deleterious biological activities than the few or shortened HS chains that remain in *ttv* mutants. Proteoglycans carrying unsulfated chains may localize correctly in cells (as reflected in the normal 3G10 staining pattern in *sfl* mutants), but may fail to bind critical partner proteins or interfere with normal HSPG functions.

Appropriate HS biosynthesis is required for normal NMJ structure and function

We determined the cell type requirement for HS biosynthesis by rescue experiments with *ttv* mutants. One surprising finding was that widespread high-level expression of *ttv*⁺ did not provide full rescue. Indeed, *ttv*; *da-Gal4*>*UAS-ttv-myc* animals showed significantly less rescue than mutants with only neuronal or muscle expression of *UAS-ttv-myc*, and synapse size was reduced even further in these animals than in *ttv* mutants. Since overexpression of Ttv-myc with *da-Gal4* in an otherwise wild-type animal also reduced synapse size, we conclude that high levels of Ttv protein are detrimental in some way. Ttv is part of a copolymerase with Sotv (Han et al., 2004; Izumikawa et al., 2006); excessive levels of Ttv may enhance HS polymerization (Busse et al., 2007) or interfere with the activity of this protein complex. In addition to potential changes in HS chain length produced by overexpression of Ttv, these polymers may have aberrant levels or patterns of sulfation (Presto et al., 2008).

Our rescue analysis showed some unexpected tissue requirements for HS synthesis. Most strikingly, the reduced mEJP frequency of *ttv* mutants, a presynaptic defect, was rescued by muscle rather than neuronal expression of *ttv*⁺, demonstrating that HS biosynthesis in muscle is critical for a motoneuron property. In addition, *G14-Gal4*-driven expression rescued other neuronal phenotypes such as synapse growth and activity-dependent endocytosis, which were also ameliorated with *elav-Gal4*. Conversely, neuronal expression of *UAS-ttv-myc* restored normal BIP staining and rescued other muscular defects. Although we cannot rule out rescue by the very low level of *elav-Gal4*-driven expression in muscle cells, neuronal HS synthesis appears to be critical for muscle integrity. These results may reflect the need for HS molecules on the surface of one synaptic partner modulating activities in the other cell (Marqués, 2005).

The organization of intracellular organelles is altered in HS biosynthetic mutants

The localization of mitochondria beneath the postsynaptic membrane was abnormal in HS biosynthetic mutants, as shown both

Figure 8. Abnormal mitochondrial localization in muscles of HS biosynthetic mutants. **A–L**, Muscle 6 and 7 NMJs stained with anti-cytochrome *c* (red) to visualize mitochondrial localization and anti-HRP (green) to label motoneuron processes. The images in **C** and **F** are single confocal images from the superficial region of the NMJ near the boutons; other images are composites showing staining from the surface of the boutons down into the contractile apparatus of the muscle. Scale bar, 10 μ m. **A–C**, In wild-type NMJs, mitochondria are enriched in the vicinity of the boutons and around nuclei (arrows), but are also distributed throughout the muscle. **D–G**, *sfl* and *ttv* mutants do not show the normal enrichment of mitochondria around type Ib boutons and nuclei. **H**, **I**, **K**, **L**, Expression of Ttv-myc with *G14-Gal4*, but not *elav-Gal4*, restores significant cytochrome *c* staining. **M**, **N**, NMJs from muscles 6 and 7 of *sfl* heterozygous or mutant animals expressing a mitochondrial GFP fusion protein (green) driven by *elav-Gal4*. Anti-CSP staining (red) labels boutons. Scale bar, 10 μ m. Mitochondrial localization is unaffected in *sfl* mutant neurons.

by TEM and anti-cytochrome *c* staining. The defect was specific to the vicinity of the boutons, because mitochondrial distributions within the muscle contractile fibers were normal, as were levels of mitochondria in the presynaptic terminal. This phenotype was substantially rescued in *ttv* mutants by expression of a wild-type transgene in muscle, suggesting there is a cell-autonomous requirement for HS production to maintain normal mitochondrial organization in the postsynaptic cell. Presynaptic targeting of mitochondria to axon terminals has been well documented (Stowers et al., 2002; Guo et al., 2005; Glater et al., 2006); anterograde transport depends on kinesin motors and microtubules, whereas anchoring in response to nerve growth factor and phosphoinositide 3-kinase signaling appears to require F-actin (Chada and Hollenbeck, 2004; Hollenbeck and Saxton, 2005; Frederick and Shaw, 2007). Less is known about the importance of postsynaptic mitochondrial localization, although the devel-

Figure 9. Disruptions of muscle morphology in *ttv* mutants. **A–D**, Anti-BIP antibody staining (red) labels the endoplasmic reticulum in NMJs from wild-type, *ttv* mutant, and *ttv* mutant animals expressing Ttv-myc with *elav-Gal4* or *G14-Gal4*. Anti-HRP staining is shown in green. Scale bar, 10 μ m. *ttv* mutants have decreased levels of anti-BIP staining in muscles. Expression of Ttv-myc with *elav-Gal4* restores normal anti-BIP staining, whereas expression with *G14-Gal4* substantially increases anti-BIP staining above the normal level. **E–H**, Anti-p120 antibody staining (red) labels the Golgi in NMJs of the same genotypes. *ttv* mutant muscles show reduced levels of anti-p120 staining. Comparatively normal staining is restored by expression of Ttv-myc with *G14-Gal4*, whereas *elav-Gal4* gives partial rescue.

opment and plasticity of dendritic spines depends on mitochondrial distribution (Li et al., 2004). HSPGs could influence mitochondrial organization at the NMJ by affecting extracellular signaling events, such as that described in axon terminals in which nerve growth factor signaling recruits axonal mitochondria to specific sites (Chada and Hollenbeck, 2004; Hollenbeck and Saxton, 2005; Frederick and Shaw, 2007). Alternatively, HSPGs could affect mitochondrial transport within cells, or in some way regulate the fission and fusion events that are a critical feature of mitochondrial biology (Cervený et al., 2007).

We found that levels of the ER-resident protein BIP and the Golgi protein p120 were reduced in the muscle cells of HS biosynthetic mutants. Expression of a wild-type transgene in either neuron or muscle restored the levels of these ER and Golgi markers, demonstrating that the loss of HS caused these cellular phenotypes. Given that HS-modified proteins are primarily known as molecules of the cell surface and the extracellular matrix, these changes in cellular organization and mitochondrial localization were, on the face of it, surprising. However, there is growing evidence that the activity of HSPGs is not limited to events in the extracellular space or at the plasma membrane. For example, HSPGs, synthesized in the Golgi, may affect the biosynthesis of other protein components destined for secretion or delivery to the cell surface, perhaps serving as a scaffolding molecule within the Golgi. The cellular phenotypes of *Ndst2* mutant mice support this model. *Ndst2* encodes a sulfotransferase critical for synthesis of heparin, the highly sulfated form of heparan sulfate that is a component of mast cell secretory vesicles. In addition to lacking heparin, mast cells from *Ndst2* mice lack a number of proteases that are normally found within secretory granules; this protease defect is posttranslational, suggesting that proper biosynthesis of secretory granule components depends on the ability to make heparin (Forsberg et al., 1999; Humphries et al., 1999). If HSPGs serve as molecular scaffolds for a variety of membrane-associated proteins, loss of HS biosynthesis could affect other cellular compartments as well as secretory granules.

HSPGs may also affect intracellular organization via effects on membrane trafficking, particularly endocytosis. We found that the rate of synaptic vesicle recovery measured by FM1-43 dye internalization was more than doubled in HS biosynthetic mu-

tants compared with controls. This increase in endocytosis was entirely corrected by expression of the wild-type transgene in neurons, and mostly corrected by muscle-driven expression. The number of 70 nm cisternae, presynaptic vesicles known to be affected by rates of endocytosis (Wucherpfennig et al., 2003; Zhang, 2003), found in *sfl* mutants also support the conclusion that HSPGs limit the recovery of synaptic vesicles. A few previous studies have implicated HSPGs in other endocytic and trafficking processes. For example, Syndecan-2 participates in trafficking of membrane proteins such as FGF receptor and β 1-integrin in cultured cells (Zimmermann et al., 2005). Dlp promotes internalization of the lipid-modified morphogens Hedgehog and Wingless (Gallet et al., 2008), perhaps by association with lipoprotein particles (Eugster et al., 2007). HSPGs also play important roles in lipoprotein uptake by the liver: selective loss of *Ndst1* in hepatocytes greatly reduces uptake of triglyceride-rich lipoprotein particles, leading to elevated serum triglyceride levels (MacArthur et al., 2007). Clearly, additional studies will be required to elucidate the mechanisms of these complex effects of HSPGs on trafficking and cellular organization.

References

- Arikawa-Hirasawa E, Rossi SG, Rotundo RL, Yamada Y (2002) Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. *Nat Neurosci* 5:119–123.
- Bellaiche Y, The I, Perrimon N (1998) Tout-velu is a *Drosophila* homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature* 394:85–88.
- Bodmer R, Barbel S, Sheperd S, Jack JW, Jan LY, Jan YN (1987) Transformation of sensory organs by mutations of the cut locus of *D. melanogaster*. *Cell* 51:293–307.
- Bülow HE, Hobert O (2006) The molecular diversity of glycosaminoglycans shapes animal development. *Annu Rev Cell Dev Biol* 22:375–407.
- Busse M, Feta A, Presto J, Wilén M, Grönning M, Kjellén L, Kusche-Gullberg M (2007) Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation. *J Biol Chem* 282:32802–32810.
- Cervený KL, Tamura Y, Zhang Z, Jensen RE, Sesaki H (2007) Regulation of mitochondrial fusion and division. *Trends Cell Biol* 17:563–569.
- Chada SR, Hollenbeck PJ (2004) Nerve growth factor signaling regulates motility and docking of axonal mitochondria. *Curr Biol* 14:1272–1276.
- David G, Bai XM, Van der Schueren B, Cassiman JJ, Van den Berghe H (1992) Developmental changes in heparan sulfate expression: in situ detection with mAbs. *J Cell Biol* 119:961–975.

- Delgado R, Maureira C, Oliva C, Kidokoro Y, Labarca P (2000) Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a *Drosophila* mutant, shibire. *Neuron* 28:941–953.
- Eugster C, Panáková D, Mahmoud A, Eaton S (2007) Lipoprotein-heparan sulfate interactions in the Hh pathway. *Dev Cell* 13:57–71.
- Forsberg E, Pejler G, Ringvall M, Lunderius C, Tomasini-Johansson B, Kusche-Gullberg M, Eriksson I, Ledin J, Hellman L, Kjellén L (1999) Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature* 400:773–776.
- Frederick RL, Shaw JM (2007) Moving mitochondria: establishing distribution of an essential organelle. *Traffic* 8:1668–1675.
- Gallet A, Staccini-Lavanant L, Théron PP (2008) Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis. *Dev Cell* 14:712–725.
- Glater EE, Megeath LJ, Stowers RS, Schwarz TL (2006) Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent. *J Cell Biol* 173:545–557.
- Guo X, Macleod GT, Wellington A, Hu F, Panchumarthi S, Schoenfeld M, Marin L, Charlton MP, Atwood HL, Zinsmaier KE (2005) The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses. *Neuron* 47:379–393.
- Han C, Belenkaya TY, Khodoun M, Tauchi M, Lin X, Lin X (2004) Distinct and collaborative roles of *Drosophila* EXT family proteins in morphogen signalling and gradient formation. *Development* 131:1563–1575.
- Hoang B, Chiba A (2001) Single-cell analysis of *Drosophila* larval neuromuscular synapses. *Dev Biol* 229:55–70.
- Hoch W, Campanelli JT, Scheller RH (1994) Agrin-induced clustering of acetylcholine receptors: a cytoskeletal link. *J Cell Biol* 126:1–4.
- Hollenbeck PJ, Saxton WM (2005) The axonal transport of mitochondria. *J Cell Sci* 118:5411–5419.
- Humphries DE, Wong GW, Friend DS, Gurish MF, Qiu WT, Huang C, Sharpe AH, Hsueh GL (1999) Heparin is essential for the storage of specific granule proteases in mast cells. *Nature* 400:769–772.
- Irie F, Yamaguchi Y (2004) EPHB receptor signaling in dendritic spine development. *Front Biosci* 9:1365–1373.
- Izumikawa T, Egusa N, Taniguchi F, Sugahara K, Kitagawa H (2006) Heparan sulfate polymerization in *Drosophila*. *J Biol Chem* 281:1929–1934.
- Jenniskens GJ, Veerkamp JH, van Kuppevelt TH (2006) Heparan sulfates in skeletal muscle development and physiology. *J Cell Physiol* 206:283–294.
- Johansen J, Halpern ME, Keshishian H (1989) Axonal guidance and the development of muscle fiber-specific innervation in *Drosophila* embryos. *J Neurosci* 9:4318–4332.
- Johnson KG, Tenney AP, Ghose A, Duckworth AM, Higashi ME, Parfitt K, Marcu O, Heslip TR, Marsh JL, Schwarz TL, Flanagan JG, Van Vactor D (2006) The HSPGs Syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. *Neuron* 49:517–531.
- Kidokoro Y, Kuromi H, Delgado R, Maureira C, Oliva C, Labarca P (2004) Synaptic vesicle pools and plasticity of synaptic transmission at the *Drosophila* synapse. *Brain Res Brain Res Rev* 47:18–32.
- Kirkpatrick CA, Selleck SB (2007) Heparan sulfate proteoglycans at a glance. *J Cell Sci* 120:1829–1832.
- Kraut R, Menon K, Zinn K (2001) A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in *Drosophila*. *Curr Biol* 11:417–430.
- Lee JS, von der Hardt S, Rusch MA, Stringer SE, Stickney HL, Talbot WS, Geisler R, Nüsslein-Volhard C, Selleck SB, Chien CB, Roehl H (2004) Axon sorting in the optic tract requires HSPG synthesis by ext2 (dackel) and extl3 (boxer). *Neuron* 44:947–960.
- Li Z, Okamoto K, Hayashi Y, Sheng M (2004) The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* 119:873–887.
- Lin X (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. *Development* 131:6009–6021.
- Lin X, Perrimon N (1999) Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling. *Nature* 400:281–284.
- Lindwall C, Fothergill T, Richards LJ (2007) Commissure formation in the mammalian forebrain. *Curr Opin Neurobiol* 17:3–14.
- Loeb JA (2003) Neuregulin: an activity-dependent synaptic modulator at the neuromuscular junction. *J Neurocytol* 32:649–664.
- MacArthur JM, Bishop JR, Stanford KI, Wang L, Bensadoun A, Witztum JL, Esko JD (2007) Liver heparan sulfate proteoglycans mediate clearance of triglyceride-rich lipoproteins independently of LDL receptor family members. *J Clin Invest* 117:153–164.
- Madhavan R, Peng HB (2006) HGF induction of postsynaptic specializations at the neuromuscular junction. *J Neurobiol* 66:134–147.
- Marois E, Mahmoud A, Eaton S (2006) The endocytic pathway and formation of the Wingless morphogen gradient. *Development* 133:307–317.
- Marqués G (2005) Morphogens and synaptogenesis in *Drosophila*. *J Neurobiol* 64:417–434.
- Marqués G, Bao H, Haerry TE, Shimell MJ, Duchek P, Zhang B, O'Connor MB (2002) The *Drosophila* BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. *Neuron* 33:529–543.
- Martin AR (1955) A further study of the statistical composition of the end-plate potential. *J Physiol* 130:114–122.
- Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V (2002) The *Drosophila* Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell* 111:319–330.
- Presto J, Thuvesson M, Carlsson P, Busse M, Wilén M, Eriksson I, Kusche-Gullberg M, Kjellén L (2008) Heparan sulfate biosynthesis enzymes EXT1 and EXT2 affect NDST1 expression and heparan sulfate sulfation. *Proc Natl Acad Sci U S A* 105:4751–4756.
- Ramaswami M, Krishnan KS, Kelly RB (1994) Intermediates in synaptic vesicle recycling revealed by optical imaging of *Drosophila* neuromuscular junctions. *Neuron* 13:363–375.
- Rawson JM, Lee M, Kennedy EL, Selleck SB (2003) *Drosophila* neuromuscular synapse assembly and function require the TGF-beta type I receptor saxophone and the transcription factor Mad. *J Neurobiol* 55:134–150.
- Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. *Nat Rev Neurosci* 6:57–69.
- Rotundo RL, Rossi SG, Kimbell LM, Ruiz C, Marrero E (2005) Targeting acetylcholinesterase to the neuromuscular synapse. *Chem Biol Interact* 157–158:15–21.
- Sanyal S, Consoulas C, Kuromi H, Basole A, Mukai L, Kidokoro Y, Krishnan KS, Ramaswami M (2005) Analysis of conditional paralytic mutants in *Drosophila* sarco-endoplasmic reticulum calcium ATPase reveals novel mechanisms for regulating membrane excitability. *Genetics* 169:737–750.
- Stowers RS, Megeath LJ, Górski-Andrzejak J, Meinertzhagen IA, Schwarz TL (2002) Axonal transport of mitochondria to synapses depends on milton, a novel *Drosophila* protein. *Neuron* 36:1063–1077.
- Takei Y, Ozawa Y, Sato M, Watanabe A, Tabata T (2004) Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development* 131:73–82.
- Toyoda H, Kinoshita-Toyoda A, Fox B, Selleck SB (2000) Structural analysis of glycosaminoglycans in animals bearing mutations in sugarless, sulfateless, and tout-velu *Drosophila* homologues of vertebrate genes encoding glycosaminoglycan biosynthetic enzymes. *J Biol Chem* 275:21856–21861.
- Tsruya R, Wojtalla A, Carmon S, Yogev S, Reich A, Bibi E, Merdes G, Schejter E, Shilo BZ (2007) Rhomboid cleaves Star to regulate the levels of secreted Spitz. *EMBO J* 26:1211–1220.
- Wucherpennig T, Wilsch-Bräuninger M, González-Gaitán M (2003) Role of *Drosophila* Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *J Cell Biol* 161:609–624.
- Yamaguchi Y (2002) Glycobiology of the synapse: the role of glycans in the formation, maturation, and modulation of synapses. *Biochim Biophys Acta* 1573:369–376.
- Zhang B (2003) Genetic and molecular analysis of synaptic vesicle recycling in *Drosophila*. *J Neurocytol* 32:567–589.
- Zimmermann P, Zhang Z, Degeest G, Mortier E, Leenaerts I, Coomans C, Schulz J, N'Kuli F, Courttoy PJ, David G (2005) Syndecan recycling [corrected] is controlled by syntenin-PIP2 interaction and Arf6. *Dev Cell* 9:377–388.