Identification of dietary alanine toxicity and trafficking dysfunction in a *Drosophila* model of hereditary sensory and autonomic neuropathy type 1

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**Abstract**

Hereditary sensory and autonomic neuropathy type 1 (HSAN1) is characterized by a loss of distal peripheral sensory and motoneuronal function, neuropathic pain and tissue necrosis. The most common cause of HSAN1 is due to dominant mutations in serine palmitoyl-transferase subunit 1 (*SPT1*). *SPT* catalyses the condensation of serine with palmitoyl-CoA, the initial step in sphingolipid biogenesis. Identified mutations in *SPT1* are known to both reduce sphingolipid synthesis and generate catalytic promiscuity, incorporating alanine or glycine into the precursor sphingolipid to generate a deoxysphingoid base (DSB). Why either loss of function in *SPT1*, or generation of DSBs should generate deficits in distal sensory function remains unclear. To address these questions, we generated a *Drosophila* model of HSAN1. Expression of *dSpt1* bearing a disease-related mutation induced morphological deficits in synapse growth at the larval neuromuscular junction consistent with a dominant-negative action. Expression of mutant *dSpt1* globally was found to be mildly toxic, but was completely toxic when the diet was supplemented with alanine, when DSBs were observed in abundance. Expression of mutant *dSpt1* in sensory neurons generated developmental deficits in dendritic arborization with concomitant sensory deficits. A membrane trafficking defect was observed in soma of sensory neurons expressing mutant *dSpt1*, consistent with endoplasmic reticulum (ER) to Golgi block. We found that we could rescue sensory function in neurons expressing mutant *dSpt1* by co-expressing an effector of ER–Golgi function, Rab1 suggesting compromised ER function in HSAN1 affected dendritic neurons. Our *Drosophila* model identifies a novel strategy to explore the pathological mechanisms of HSAN1.

**Introduction**

Hereditary sensory and autonomic neuropathy type 1 (HSAN1) is a rare disorder pathologically characterized by distal sensory loss and peripheral ulceration, predominantly in lower limbs (1–5). Degeneration of motoneurons is also known to occur in addition to distal limb weakness and muscle atrophy (4). The condition is caused in the majority of cases by inheritable dominant mutations in the genes encoding subunits of the enzyme serine palmitoyl-transferase (*SPT*). *SPT* function is essential for catalysis of the first step in *de novo* sphingolipid synthesis at the ER (6,7). *SPT* exists as a heterodimer of homologous subunits, *SPT* long chain base subunit 1 (*SPTLC1/SPT1*) and *SPT* long chain base subunit 1 (*SPTLC1/SPT1*) and *SPT* long chain base subunit...
subunit 2 (SPTLC2/SPT2), that catalyse the pyridoxal phosphate-dependent condensation of l-serine and palmitoyl-coenzyme-A to generate 3-ketosphinganine (8). A third subunit present in mammals, SPTLC3 contributes to the generation of short C16 sphingoid bases (9). At present, the manner in which the dominant mutations in SPT1 affect neuronal function, particularly in peripheral nerves, is unclear. 

A number of HSAN1-related missense mutations (C133W, C133Y and V144D) have been identified in SPT1 from 24 affected families (6,7) of which C133W appears to be the most common (7). More recently, mutations in SPT2 that cause a form of HSAN1 have been identified (10,11). A structural determination of the homologous prokaryotic enzyme, which exists as a homodimer, has given some insight into how disease mutations may affect the activity of the enzyme (8,12). Initially, reports suggested that disease-related mutations in SPT1 generated a gain-of-function mechanism in SPT resulting in increased cellular glucosylceramide levels (7). Others suggested a dominant-negative mechanism, albeit a mechanism of selective or reduced sphingolipid synthesis while overall sphingolipid levels were maintained (13,14). Recent data, however, have suggested that the SPT1 disease causing mutations may be neomorphic and cause a shift in substrate specificity from serine to alanine or glycine incorporation (15,16). This in turn generates atypical (un)modifiable deoxysphingolipid bases (DSBs) which, based on in vitro activity, are proposed to be neurotoxic (15,16). Whether the mutations act in a predominantly neomorphic (production of toxic DSBs) or dominant-negative (reduction of sphingolipid synthesis) manner, or if the two mechanisms overlap to generate pathology, remain unclear. Why peripheral neurons are predominantly affected by mutations in this enzyme similarly remains unexplained.

To understand the pathological function of HSAN1 mutations in SPT1, and the possible toxic effect of dietary alanine in vivo, we have employed a transgenic approach using the genetically tractable Drosophila larval peripheral nervous system as a model system. Using a novel nociception assay (17), selective enrichment in SPT1, and the possible toxic effect of dietary alanine has given some insight into how disease mutations may affect the activity of the enzyme (8,12). Initially, reports suggested that disease-related mutations in SPT1 generated a gain-of-function mechanism in SPT resulting in increased cellular glucosylceramide levels (7). Others suggested a dominant-negative mechanism, albeit a mechanism of selective or reduced sphingolipid synthesis while overall sphingolipid levels were maintained (13,14). Recent data, however, have suggested that the SPT1 disease causing mutations may be neomorphic and cause a shift in substrate specificity from serine to alanine or glycine incorporation (15,16). This in turn generates atypical (un)modifiable deoxysphingolipid bases (DSBs) which, based on in vitro activity, are proposed to be neurotoxic (15,16). Whether the mutations act in a predominantly neomorphic (production of toxic DSBs) or dominant-negative (reduction of sphingolipid synthesis) manner, or if the two mechanisms overlap to generate pathology, remain unclear. Why peripheral neurons are predominantly affected by mutations in this enzyme similarly remains unexplained.

To understand the pathological function of HSAN1 mutations in SPT1, and the possible toxic effect of dietary alanine in vivo, we have employed a transgenic approach using the genetically tractable Drosophila larval peripheral nervous system as a model system. Using a novel nociception assay (17), selective enrichment of amino acids in the diet and examination of membrane traffic in sensory neurons, we determined a direct relationship between the disease mutations, dietary alanine, the presence of DSBs in sensory neurons, we determined a direct relationship between the disease mutations, dietary alanine, the presence of DSBs and sensory function. Additionally we identify a potential pathological mechanism of disrupted ER to Golgi traffic in these neurons. Our findings suggest a novel mechanism for sensory dysfunction that is consistent with deficits in distal peripheral neurons and further highlight a critical functional role for the secretory system in peripheral dendritic processes.

**Results**

**dSpt1** is an essential gene in Drosophila

HSAN1 patients have peripheral- and motoneuronal functional deficits (4). To try to dissect the mechanism driving SPT1C133W dysfunction in motor neurons, we examined the effects of expression of a mutated Drosophila SPT1 protein at the larval neuromuscular synapse.

We obtained the cDNA of the Drosophila SPT1 (dSpt1), introduced a C to W substitution at amino acid position 129 (analogous to the human 133, Fig. 1A) by site-directed-mutagenesis and subcloned both the wild-type and mutant subunit into the pUASt vector (22). We generated multiple independent UAS-dSpt1 and UAS-dSpt1C129W transgenes by microinjection, allowing us to express both forms in a tissue-specific manner under the control of the GAL4 transcriptional activator. When expressed with ppk-GAL4, all UAS-dSpt1-C129W transgenes generated a defective nociception response (see Fig. 2), while expression of UAS-dSpt1 gave responses no different to wild-type animals.

To ascertain the effect of dSpt1C129W expression on neuronal growth, we expressed the dSpt1C129W protein in the nervous system of the developing Drosophila larva using the pan-neuronal Elav-GAL4 driver and examined the effect on third instar neuromuscular junction (NMJ) morphology (Fig. 1B and C). The Drosophila larval NMJ is a genetically tractable and accessible model glutamatergic synapse that is well characterized for growth and morphology (23,24). Using homozygotes of a previously isolated hypomorphic allele of dSpt2, dSpt2lace (21) with a sphingolipid content known to be 2.58% of wild-type levels (25) we determined that a loss of SPT activity generated a reduction in synaptic bouton number, reducing bouton number by 50%.

Pan-neuronal (Elav-GAL4) expression of UAS-dSpt1C129W resulted in no change in synaptic bouton number at the larval muscle 6/7, hemisegment A3 NMJ (Fig. 1B and C). Introduction of heterozygous alleles of dSpt1, or dSpt2/lace in the presence of globally expressed dSpt1C129W–induced lethality at pupal stage. Introduction of heterozygous alleles of dSpt1 or dSpt2/lace in...
the presence of the neuronally expressed \textit{dSpt1C129W} induced a reduction of bouton number of 50%, phenocopying the homozygous loss-of-function allele of \textit{dSpt2/lace} \(^6\) (Fig. 1B and C). This genetic interaction between \textit{dSpt1C129W} expression and heterozygous mutations of \textit{dSpt1} and \textit{dSpt2/lace} \(^6\) strongly suggests a dominant-negative function for the \textit{dSpt1C129W} mutation when examined in the context of lethality and synaptic growth.

\textit{dSpt1C129W} expression in sensory neurons of \textit{Drosophila} larvae induces a sensory deficit

HSAN1 manifests as distal sensory loss, allowing recurrent and progressive tissue damage due to injury. We probed our \textit{Drosophila} model of HSAN1 for any effect in the response to noxious heat stimuli. It has been shown previously that in response to noxious...
heat thresholds, third instar larvae perform a rapid and characteristic barrel-rolling escape behaviour. This behavioural response was previously identified to promote pain sensitivity (29). We recently developed a novel thermosensation/nociception assay based on this rolling behaviour elicited by excessive heat in a droplet of water and used it to ask whether third instar larvae expressing in the class IV da neurons, and global hypomorphic conditions of dSpt1 (32). Light activation of the channelrhodopsin elicited a response to heat, and that dSpt1C129W protein could be partially alleviated by co-feeding 100 mM serine. Serine feeding had no effect on animals expressing either the wild-type dSpt1 or dSpt1C129W in the presence of alanine or glycine in the diet of larvae expressing the dSpt1C129W protein to be toxic.

We raised larvae globally expressing either wild-type dSpt1 or dSpt1C129W on diets containing 10 mM or 100 mM alanine. We found enriched alanine to be 100% toxic to larvae expressing dSpt1C129W, but not animals expressing wild-type dSpt1, control wild-type animals or siblings bearing a balancer chromosome (and therefore lacking the tubulin-GAL4 element to drive expression of dSpt1C129W) (Fig. 3A and B). Toxicity of alanine for animals expressing dSpt1C129W protein could be partially alleviated by co-feeding 100 mM serine. Serine feeding had no effect on animals expressing either the wild-type dSpt1 or dSpt1C129W protein in the absence of alanine supplementation. On normal lab yeast/agar food, survival for larvae expressing dSpt1C129W is unaffected (data not shown). Raising larvae on ‘instant’ laboratory food reduces survival for dSpt1C129W expressing larvae suggesting a high content of alanine or glycine (Fig. 3B). Supplementation ‘instant’ food with 100 mM serine brings survival of dSpt1C129W expressing larvae to wild-type levels. Our data support the findings that alanine is toxic in the presence of an HSAN1 mutation in SPT1.

Global expression of dSpt1C129W generates a reduction of normal sphingolipids, and upon feeding of alanine reveals the presence of DSBs

We interrogated our model of HSAN1 by globally expressing dSpt1C129W and assessed the abundance of sphingolipids. As can be seen in the graph (Fig. 3C and D), total sphingoid base abundance is reduced in animals globally expressing dSpt1C129W compared with controls, while the content of DSBs is only marginally elevated in the dSpt1C129W animals grown on normal lab food (with no DSBs found in the wild-type animals), suggesting a dominant-negative effect of this mutation on sphingolipid biosynthesis. However, following feeding with 100 mM alanine, total sphingoid base content in the dSpt1C129W expressing animals is restored back to untreated control levels but is coupled to an ∼8-fold elevation in the free DSB content compared with the animals grown on normal lab food, confirming a neomorphic effect following the change in diet. Confirmation of the identity of each sphingoid base analysed by HPLC is shown in Fig. 3D. Alanine fed animals globally expressing dSpt1C129W were used to confirm the identity of the DSB peaks and homozygous dSpt2/lace4 hypo-morphs were used as positive controls to identify endogenous sphingoid bases in the wild-type traces based on their absence in dSpt2/lace4 samples (previously shown to have a sphingolipid content of 2.58% (25)). Interestingly, feeding of serine can rescue
Figure 3. Feeding alanine to Spt1C129W expressing flies induces lethality and the appearance of DSBs. (A) Schematic representation of crossing scheme used to produce Spt1C129W expressing flies and control sibling flies lacking the Spt1C129W transgene for feeding experiments. (B) Feeding of alanine and serine to flies expressing Spt1C129W or wild-type Spt1. White boxes represent percentage experimental flies surviving, black boxes sibling controls surviving. (C) Quantification of total free endogenous sphingoid base (C14, C16 and C18 sphingosine and sphinganine) and free DSB (C14 deoxysphingosine and deoxysphinganine) content of Drosophila by HPLC analysis. ***p ≤ 0.001, two-way ANOVA with Tukey’s multiple comparison test. (D) Representative HPLC traces illustrating identification of each peak in the unknown samples based on known standards (green trace), confirmation of peaks representing endogenous sphingoid bases using the sphingoid base null lace mutants (black trace) or confirmation of the presence of DSBs via the alanine fed spt1C129W expressing animals (purple trace). Numbers correspond to endogenous free sphingoid bases, 1 and 2 = C14 chain length sphingosine and sphinganine respectively; 3 and 4 = C16 sphingosine and sphinganine, respectively; 5 and 6 = C18 sphingosine and sphinganine, respectively. A and B correspond to deoxysphingosine and deoxysphinganine respectively. For all experimental conditions n = 5–8, apart from lace where n = 2. ns = non-significant compared with control. (E, F). Disruption of larval synaptic growth parameters at the 6/7 muscle, hemisegment A3 in animals of the genotype Elav-GAL4/UAS-Spt1C129W, laceP can be rescued by the feeding of serine. Scale bar = 10 µm, ANOVA: F (d.f. 5) = 4.4, P < 0.01, Dunnett’s post hoc comparison to WT (**P < 0.01) and Bonferroni comparison between groups (**P < 0.01).
the synaptic development phenotype in an Elav-GAL4, UAS-dSpt1-C129W, lace/y+ animal (Fig. 3E and F). These data confirm that dSpt1-C129W over-expression in Drosophila acts in a manner similar to that identified in the mammalian system (15,16).

**Secretory and membrane dysfunction is observed in sensory neurons expressing dSpt1C129W**

During class IV da morphology analysis, accumulations of mCD8-EGFP fluorescence were observed in neurons expressing dSpt1-C129W that were largely absent in controls (Fig. 4A). It was considered therefore that, under conditions of altered sphingolipid expression, there is reduced trafficking of mCD8-EGFP out of the soma into the plasma membrane of the arbor to alter membrane function. In order to test this theory, fluorescence recovery after photobleaching (FRAP) was performed upon dendrite branches. Figures 4B and C show that FRAP is significantly reduced in class IV da neurons expressing dSpt1-C129W resulting in a t½ (time to half maximal recovery) of 31 s in comparison to 20 s in controls (P < 0.01, Student’s t-test). Co-expression of an ER marker, Lyz-KDEL-EGFP (33), with Spt1C129W reveals excessive accumulations of GFP compared with no Spt1-C129W expression (Fig. 4D). These data therefore support the theory that correct sphingolipid function is required for the correct trafficking of mCD8-EGFP to the plasma membrane and induces membrane trafficking defects. (Fig. 5A and B) (27). This subtle alteration in dendritic morphology, at least for the dSpt1-C129W expressing neurons may be caused by changes in dendritic morphology.

**dSpt1C129W or dSpt1 expression in Drosophila larval sensory neurons generates a subtle arborization defect**

With respect to the predominant sensory pathology observed in HSAN1, we then expressed dSpt1 and dSpt1-C129W in the class IV dendritic arborization (da) neurons using the class IV-specific GAL4 driver line pickpocket-GAL4 (ppk-GAL4) (26). Dendritic arbors were visualized using the co-expression of the plasma membrane marker mCD8-EGFP. Figure 5 shows the dendritic arbor of the v’ada neuron, in hemisegment 3 proximal to the location of the muscle 6/7 NMJ. The expression of either dSpt1 or dSpt1-C129W produces a subtle, yet statistically significant reduction in dendrite arborization, as shown by Sholl analysis (Fig. 5A and B) (27). This subtle alteration in dendritic morphology, at least for the dSpt1-C129W expressing neurons may be due in part to the trafficking deficit of the mCD8-EGFP marker observed in Fig. 4. Together with our data that expression of dSpt1 causes no nociception defect (Fig. 2), we suggest that the presence of the dSpt1-C129W mutation is sufficient to generate deficits in nociception and that such reductions in function are unlikely to be caused by changes in dendritic morphology.

**Enhancing ER to Golgi traffic rescues dSpt1C129W expression-induced nociceptive defects**

In a genetic screen for mutations affecting sensory dendritic arbor growth, a critical requirement for ER to Golgi trafficking function was identified (34). Identification of mutations in sec23, Sar1 and Rab1 suggests that ER to Golgi trafficking function is limiting to dendritic growth (34), a finding confirmed in mammalian neurons (35–37). To examine the suggestion that dSpt1-C129W expression may perturb ER to Golgi trafficking affecting sensory function, we introduced a known effector of ER to Golgi membrane trafficking, Rab1. Figure 5C demonstrates that the co-expression of the dominantly active form of Rab1Q70L completely restores the nociceptive defect observed with dSpt1-C129W expression while expression of the constitutively inactive Rab1L625N

![Figure 4. Spt1C129W expression in class IV da neurons reduces membrane trafficking to the plasma membrane and induces membrane trafficking defects.](https://academic.oup.com/hmg/article-abstract/24/24/6899/2384468)
alone phenocopies dSpt1C129W expression. Co-expression of Rab1S25N with dSpt1C129W in Class IV da neurons causes latency in the nociceptive response that is no more severe than either construct alone. Expression of Rab1Q70L generates a dendritic arbour indistinguishable from wild-type. These data together suggest that sphingolipid depletion, or the presence of DSBs, does indeed induce a trafficking bottle-neck at the ER to Golgi interface and that this can be circumvented via the expression of ER to Golgi trafficking effectors, rescuing nociceptor function.

**Discussion**

HSAN1 is characterized by a deficit in peripheral sensory function with ulceration and autonomic disturbances (3–5,38). It is unclear at present why mutations in the globally expressed SPT enzyme should lead to the specific peripheral phenotypes of HSAN1. An initial study suggested the mutation conferred a gain-of-function to the SPT enzyme (7), but later studies in yeast and mammalian cells (13,14) determined an inhibitory function, albeit with normal cellular levels of sphingolipids. A more recently proposed pathological mechanism, the presence of DSBs caused by alanine or glycine incorporation, rather than serine during sphingolipid synthesis suggests a neomorphic mode of action for the SPT mutations inducing HSAN1 (15,16) if DSBs are indeed toxic (39,40).

These proposed mechanisms are not mutually exclusive, and the data that we present with our Drosophila model of HSAN1 supports both.

For confirmation of the dominant loss-of-function mode of action of the dSpt1C129W mutant SPT subunit, we assayed the development of the larval NMJ. The lace locus in Drosophila encodes the dSpt2 enzyme (21). The laceP mutant is a hypomorphic transposon insertion in the dSpt2 locus that as a homozygote dies early in the pupal stage of development (21) and has been shown to express 2.58% of wild-type sphingolipid levels (25). Figure 1C and D shows that in the lace mutant, muscle 6/7 NMJ is severely underdeveloped in terms of bouton number, the observed boutons being larger and fewer than wild-type animals. This is the first description of a sphingolipid deficient synapse and suggests that these complex lipids are essential for regulation of synaptic structure. Similarly, expressing dSpt1C129W under the control of Elav-GAL4 neuronal promoter produces an aberrant developmental phenotype though not as severe. Introduction of dSpt1 or dSpt2/laceP heterozygous loss-of-function alleles in the presence of globally or neuronally expressed dSpt1C129W causes, respectively, early lethality and a synaptic phenotype identical to the dSpt2/laceP loss-of-function mutant. This phenocopy supports our sphingolipid quantification data shown in Fig. 3, suggesting that dSpt1C129W expression reduces sphingolipid

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**Figure 5.** Increased ER–Golgi membrane trafficking rescues Spt1C129W-induced nociception defects. (A) Exhaustive Z-stack confocal images of the v’ada class IV da sensory neurons in larval hemisegment A3 of larvae expressing mCD8-EGFP under control of the ppk-GAL4 driver in combination with UAS-Spt1, UAS-Spt1C129W, UAS-Rab1S25N or UAS-Rab1Q70L. Scale bar = 10 µm. (B) Sholl analysis performed on dendrite traces reveals that Spt1 or Spt1C129W expression mildly, yet significantly, reduces arborization. Kolmogorov–Smirnov test, **P < 0.05, z = 1.436. (C) Co-expression of dominant-active Rab1Q70L significantly rescues Spt1C129W expression-induced defective nociception. Expression of Rab1Q70L increases larval sensitivity to noxious heat, whereas expression of dominant-negative Rab1S25N causes defective nociception equivalent to that induced by Spt1C129W expression. Co-expression of UAS-Spt1C129W and UAS-Rab1Q70L does not significantly worsen the nociceptive defect. ***P ≤ 0.001, ****P ≤ 0.0001, ANOVA, ns = non-significant compared with control.
biogenesis consistent with the early reports of a dominant-negative mutant function (13,14). Reduction of wild-type copy number of dSpt1 or dSpt2/lace subunits of dSpt in the presence of the dSpt1C129W mutant induces a severe phenotype identical to a strong dSpt2 hypomorphic mutation with a very low sphingolipid content. These genetic combinations confirm the potential dominant-negative mode of action for the disease mutation in a peripheral neuron in our system.

Expression of dSpt1C129W in the class IV md sensory neurons responsible for thermo- and mechanosensory function in Drosophila larvae (28,41,42) induces a mild morphological reduction in dendrite terminals consistent with loss of epidermal innervation in SPT1C133W/− carrying HSAN1 patients (5,43). These larvae also display a deficit in thermosensation, showing a latency to respond to rising temperature consistent with a lack of function in the class IV md neurons. Interestingly, optogenetic stimulation of the class IV md neurons in the presence of the dSpt1C129W mutant can still elicit a nocifensive response (Supplementary Material, Movie S1) indicating some synaptic function remains in these compromised neurons. Synaptic structure of Class IV da neurons in the ventral nerve cord is preserved when dSpt1C129W is expressed (Supplementary Material, Fig. S2). These data suggest that the functional deficit may not centre on synaptic function in dSpt1C129W compromised sensory neurons but may lie in the dendritic arbour.

On examination of larval sensory neurons expressing dSpt1C129W, we observed a membrane trafficking deficit in cell soma. The data presented here, an expression of constitutively active Rab1 rescue of a dSptC129W-induced sensory deficit, suggests that dSpt1C129W expression induces an ER to Golgi trafficking restriction and that this disrupts nociception. Recent reports suggest a novel activity for the SPT enzyme bearing the mutated subunit. The C133W mutation is proposed to confer a preferred activity for alanine over serine in the condensation reaction generating dSptC129W expressing animal. In this scheme, it is proposed that the DSBs generate stress in the ER (16) and defects in neurite outgrowth and retraction (15). Membrane biogenesis is critically dependent on the orchestration between lipid and protein synthesis at the ER. Export of protein complexes from the ER can depend on the availability of ceramide (44), while the export of GPI-anchored proteins are critically dependent on the presence of sphingolipids and sterols (45,46). Disruption of the ORM1/2 proteins, important regulators of SPT function, leads to severe defects in ER function with a slowed ER-to-Golgi transport (47). Dendrites are particularly rich in components of the secretory apparatus with ER export sites and Golgi outposts assembled regularly throughout the dendritic tree (48–50). A genetic screen in Drosophila larvae, using the growth of the class IV md neurons as a model identified factors of ER-to-Golgi traffic as essential for proper dendritic growth (34). Studies in mammalian growing neurites confirm this requirement (35,36), suggesting a critical reliance in dendrites for secretory function. ER export sites are essential for dendrite outgrowth, but they are also observed to recruit neurotransmitter receptors upon metabotropic activation and neuronal activity reflecting a potential role in regulating dendritic function (35,48). Recent identification of dominant mutations in Atlastin1 and 3, proteins required for homotypic membrane fusion in the ER, in HSAN1 patients (51–54) similarly point to ER function as a ‘pinch-point’ in distal peripheral sensory function.

Oral supplementation of l-serine to the diet of mSPT1C133W expressing mice decreased DBS levels in plasma, paralleling similar observations in SPT1C133W carrying HSAN1 patients (55). We observe a similar effect in our Drosophila model system with alanine feeding to dSpt1C129W expressing animals inducing lethality that can be partially rescued by supplementation of l-serine in the food source. We also find that we can rescue synaptic structure in an Elav-GAL4/UAS-dSpt1C129W, lace− animal by feeding serine in the diet suggesting that the potential dominant-negative action of dSpt1C129W may be due to incorporation of alanine in nascent sphingolipids.

We have demonstrated that the causative mutation of HSAN1, SPT1C133W when expressed in Drosophila (as dSpt1C129W) acts dominantly to reduce sphingolipid synthesis, generate synthesis of DSBs and perturb the development of dendrites, neuromuscular synapses and nociceptive function of the class IV da sensory neurons. This reveals an essential function of sphingolipids in the development of these neurons in vivo. We also demonstrate that the expression of dSpt1C129W causes a significant reduction in the sensitivity of third instar larvae to noxious heat stimuli, producing a phenotype consistent with that observed in HSAN1 patients. Our data reveal that this sensory dysfunction may be due to an aberrant trafficking bottleneck in sensory dendrites, consistent with dysfunction in the ER. Thus we present a novel mechanism for the pathology observed in HSAN1 due to mutations in SPT1 in a simple model system amenable that recapitulates many aspects of the human disease.

Materials and Methods
Drosophila stocks and husbandry
Drosophila maintenance and crosses were performed using standard yeast-agar media at 25°C. Stocks used in this study: UAS-dSpt1C129W, UAS-Spt1 (this study), UAS-ChR2 (56) (Gero Miesenböck, Oxford), UAS-LYZ-GFP-KDEL (33) (Mary Lilly, NIH, Bethesda), UAS-Tetanus-toxin (29), pain1 (28) (Dan Tracey, Durham, NC), lace− (21) (John Roote, Cambridge), SPT1+/+HSAN1(26) (19) (Stephen Hou, NIH, Bethesda), pickpocket-GAL4 (26) (Wes Grueber, Columbia). The following stocks were obtained from the Bloomington Stock Centre, Indiana: w1118 (used throughout as wild-type), Df(2R)Vg-8, Elav-GAL4 tubulin-GAL4, UAS-mCD8-GFP, UAS-YFP-Rab1Q70L, UAS-YFP-Rab1S25N (57), i(2)49Fb1, i(2)49Fb2 (20).

Generation of UAS-dSpt1 and UAS-dSpt1C129W transgenes
The C129W mutation was introduced into the dSpt1 cDNA (R56823) by site-directed-mutagenesis using the following forward (CCGAGTTTGATCTTGGAGACCTCGG) and reverse (CCGAGTTCCAGATCCCAAACCTCGG) primers. Each construct was then sequence-verified and cloned into pUAST (23). Microinjection into w− embryos was then carried out using standard procedures.

NMJ analysis
Third instar wandering larvae were dissected in PBS, fixed in 3.7% formaldehyde/PBS for 7 min and stained using the appropriate primary antibodies, Cγ3 conjugated goat anti-HRP (1:200, Stratech scientific); rabbit anti-synaptotagmin (1:2000) (58). Synaptic bouton numbers at muscles 6/7, hemisegment A3, were determined by counting each distinct, spherical, anti-synaptotagmin positive varicosity contacting the muscle. As synaptic bouton number has been shown to increase proportionally with muscle surface area (MSA) synaptic bouton numbers were normalized against MSA by dividing the bouton number by the MSA and...
multiplying by mean wild-type MSA as described by (59). Muscles and synapses were imaged using an AxioCam HRC camera on a Axiovert 200e inverted fluorescence microscope using Plan-Neofluar 10×/0.3 and 40×/0.75 lenses, with Axiovision Rel. 4.8 software. Measurements were made from images using ImageJ.

**Dendritic arbour morphology**

Third instar larvae were dissected, and mounted. Dendritic field imaging was performed using a CarLiZeiss Axiovert 200M inverted confocal microscope with a 20× objective lens. High power excitation wavelength (488 nm) light and exhaustive Z-stack conditions were used to image the v’ ada class IV da neuron. Neurons were traced using NeuronJ imageJ plugin (60). Sholl analysis (27) was then performed upon these tracings using the Sholl analysis ImageJ plugin (62). Cumulative branch distribution data were interrogated using the Kolmogorov–Smirnov test, n > 5.

**Sphingolipid quantification**

Larvae were rapidly dissected in cold PBS to remove the fat bodies and subsequently frozen. After homogenization 1 mg was taken and used for each extraction. Total sphingolipids were extracted overnight in chloroform:methanol (1:1), and sample cleanup, labelling with o-phthaldialdehyde and analysis by HPLC were as described previously (61). Standards of C14 and C16 sphingoid bases were from Matreya, C18 sphingoid bases were from Sigma and an internal control C20 sphingosine was from Avanti Polar Lipids.

**Larval nociception analysis**

Third instar nociception assay based upon nocifensive characteristic escape behaviours to heat (17,28). Wandering third instar larvae were placed into a 30-μl water droplet on a petri dish. The petri dish was then placed onto a standard heater block set to 70°C. The time taken to perform escape behaviour was recorded, with respect to the first violent head-swing. Droplet temperature calibration was measured using a thermocouple with a digital read-out (Tenma 72-2060, Tenma Test Equipment Ohio, USA), held into the water droplet (n > 20). Nociception data interrogated using one-way ANOVA (n > 15). Nociception movies were recorded using a standard WebCam suspended above the heater block.

**Secondary dendrite fluorescence resonance analysis after photobleaching imaging**

Live larval dissection was performed as described for NMJ analysis above with the exception that experiments were performed in physiological saline. Specifically, wandering third-instar larvae were selected and were recorded in HL3 saline (70 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 115 mM sucrose, 5 mM trehalose and 5 mM HEPES) with 0.4 mM Ca²⁺. Class IV da neurons were located using CarlZeiss Axiovert 200M inverted confocal microscope with a 63× objective lens. The 100% laser power (488 nm, 20 mW) for 200 iterations was used to bleach region of interest 1 (ROI1) followed by 200 s post-bleach imaging to observe recovery. Acquisitional bleaching was controlled for using a second ROI and the following equation ((ROI1/ROI2) t/ROI1(1/t) = ROI2) * 100 = % recovery (t = 0, pre-bleach fluorescence) (n > 25). High magnification images of class IV da neuron cell bodies and muscle 6/7 NMJs were made using a CarlZeiss Axiovert 200M inverted confocal microscope with a 63× objective lens.

**Alanine and serine feeding**

Drosophila instant food (Carolina Biological Supply) was reconstituted using distilled water supplemented with l-serine or l-alanine. Drosophila crosses were added directly to this food source and allowed to lay for 3 days. Resultant offspring were counted and noted for genetic markers to assess survival. Crosses used were: (1) wild-type – w¹¹¹B; (2) tubulin-GAL4/TM6b, Hu, Tbx UAS-dSpt1¹²⁹W and (3) tubulin-GAL4/TM6b, Hu, Tb x UAS-dSpt1.

**Supplementary Material**

Supplementary Material is available at HMG online.

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