Lipid metabolic perturbation is an early-onset phenotype in adult spinster mutants: a Drosophila model for lysosomal storage disorders

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ABSTRACT Intracellular accumulation of lipids and swollen dysfunctional lysosomes are linked to several neurodegenerative diseases, including lysosomal storage disorders (LSD). Detailed characterization of lipid metabolic changes in relation to the onset and progression of neurodegeneration is currently missing. We systematically analyzed lipid perturbations in spinster (spin) mutants, a Drosophila model of LSD-like neurodegeneration. Our results highlight an imbalance in brain ceramide and sphingosine in the early stages of neurodegeneration, preceding the accumulation of endomembranous structures, manifestation of altered behavior, and buildup of lipofuscin. Manipulating levels of ceramidase and altering these lipids in spin mutants allowed us to conclude that ceramide homeostasis is the driving force in disease progression and is integral to spin function in the adult nervous system. We identified 29 novel physical interaction partners of Spin and focused on the lipid carrier protein, Lipophorin (Lpp). A subset of Lpp and Spin colocalize in the brain and within organs specialized for lipid metabolism (fat bodies and oenocytes). Reduced Lpp protein was observed in spin mutant tissues. Finally, increased levels of lipid metabolites produced by oenocytes in spin mutants allude to a functional interaction between Spin and Lpp, underscoring the systemic nature of lipid perturbation in LSD.

INTRODUCTION Abnormal lipid metabolism and lipid accumulation are hallmarks of neurodevelopmental and neurodegenerative disorders. Through detailed mass spectrometry studies, perturbations in lipid metabolism have been reported (Cutler et al., 2004; Han et al., 2011; Cheng et al., 2013; Panchal et al., 2014). But a systematic analysis through disease onset and progression has not yet been possible. With the help of recently established high-throughput lipidomics platforms (Shevchenko and Simons, 2010; Schwudke et al., 2011), rapid and...
quantitative analyses of lipids are now feasible for complex and large studies. This has been especially useful for the characterization of liposomes of routinely used biological model systems such as human cell lines (Sampaio et al., 2011), yeast (Ejsing et al., 2009), and Drosophila melanogaster. In the context of Drosophila, an extensively used model system, detailed lipidomics resources are now available with a focus on development and nutrition (Carvalho et al., 2010, 2012; Guan et al., 2013). The fly lipidome is distinct from its mammalian counterpart in many aspects, including that the major sterol is not cholesterol but ergosterol. In flies, the main membrane phospholipid is phosphatidylethanolamine (PE) and not phosphatidylcholine (PC), and the most abundant sphingolipid is ceramide phosphotidylethanolamine (CerPE), which substitutes an ethanolamine group for the choline group found in mammalian sphingomyelins (Carvalho et al., 2012). The sphingoid base chain length of Drosophila sphingolipids is shorter (predominantly C14) than in the mammalian system (C18). Typically, there are no polyunsaturated fatty acids (such as 20:4/20:5/22:5/22:6) present in Drosophila. Glycosphingolipids in flies are constituted by a core of mannose-glucosyl ceramide as opposed to galactose-glucosyl ceramide in mammals. Interestingly, none of the reported glycosphingolipids are sialylated as in mammals but instead have an N-acetyl-glucosyl head group (Seppo et al., 2000). Although gangliosides have not been described in Drosophila, orthologs of genes involved in ganglioside (GM2 and GM3) metabolism occur in the fly genome (Stern et al., 2000; Starostina et al., 2009). Despite these differences, many mammalian genes for lipid metabolism and transport are functionally conserved in Drosophila, suggesting common principles in their metabolism and transport (Hortsch et al., 2010). Furthermore, many relevant human disease associated genes connected to lipid metabolism and transport are functionally conserved; these include Taz, involved in mitochondrial lipid remodeling (Barth syndrome; Xu et al., 2006); Cerk, regulating ceramide metabolism (retinitis pigmentosa (Dasgupta et al., 2009); as well as dnp1c and dnp2c, critical for lipid transport and dSap-r, the pro-saposin ortholog for sphingolipid metabolism (Hindle et al., 2017). In addition to many genes involved in lysosomal storage disorders; reviewed in Hindle et al. (2011).

Using the advancements in lipidomics, we aim to understand the nature of lipid perturbations in disease onset and progression. Contrary to other disease models or patient samples, systematic and kinetic studies on lipids are relatively easily carried out in Drosophila, which is a well-accepted animal model for human disease (Bilen and Bonini, 2005). We focused on fly spinster (spin) mutants, which exhibit neurodegeneration with lysosomal storage disorders (LSD)-like features (Nakano et al., 2001; Sweeney and Davis, 2002; Dermaut et al., 2005; Hindle et al., 2011). Lipid metabolic changes in spin mutants have not been characterized despite their established link to neurodegeneration and their use as genetic tool to perturb sphingolipid levels (Osborne et al., 2008; Pantoja et al., 2013; Sasamura et al., 2013).

We have systematically analyzed and quantified lipids in adult spin brains at specific stages of disease onset and progression using lipidomics approaches while simultaneously describing the overall integrity of the nervous system using imaging, behavior, and lifespan analyses. Our experiments have helped establish a timeline of events that place loss of spin function before the ceramide metabolic imbalance in the progression of disease. Using proteomics approaches coupled with pull-down assays, we have compiled a resource list of Spinster (Spin) interactor proteins. For one of these, Lipophorin (Lpp), we present an interesting possibility that loss of Spin also causes a more general effect with changes in other lipid metabolites in tissues that regulate lipid metabolism (senocyttes).

**RESULTS**

Spin encodes a transmembrane protein that localizes to the late endolysosomal compartments. Early phenotypes associated with spin mutants include reduced viability and morphological abnormalities at the larval neuromuscular synapses and enlarged lysosomes (Sweeney and Davis, 2002). Typically, LSD are among the most common childhood neurodegenerative diseases (Hindle et al., 2011), and this is reflected in the shortened life span and reduced mobility of young spin mutant flies (Nakano et al., 2001; Dermaut et al., 2005). A hallmark of spin mutant brains is their characteristic autofluorescence, which is attributed to lipofuscin or the ageing pigment (Nakano et al., 2001). It is a mixture of accumulated/unmetabolized lipids and proteins and is distinguished by autofluorescence in aging and degenerating tissues (Gray and Woulfe, 2005). Related to this, adult spin mutant brains, retinas, and the larval neuromuscular synapse exhibit an accumulation of electron dense material and membrane whorls (Nakano et al., 2001; Dermaut et al., 2005).

**Increased ceramide levels and lipofuscin in spin mutant brains**

We imaged dissected adult brains for lipofuscin (recognized by its autofluorescence properties) and subsequently performed lipidomics on the imaged brains. To define the time course of lipofuscin accumulation and lipid profile alterations, we dissected brains at 1, 2, 4 and 8 d after eclosion. Our image analyses confirmed that spin mutant brains exhibit increased autofluorescence (Figure 1Ai), which is an indication of lipofuscin (Nakano et al., 2001; Venkatachalam et al., 2008). In contrast, age-matched controls showed no autofluorescence whatsoever (Figure 1Ai). The heteroallelic combination spin*/spin was used; it displays strong spin-associated phenotypes for viability and synapse morphology (Sweeney and Davis, 2002) and only a limited number of escapers are viable until adulthood. These escaper adults have a reduced lifespan (Supplemental Figure 1).

Using phalloidin as a general marker of brain volume, the autofluorescence in the green channel is used as an indicator of lipofuscin. In a maximum intensity projection of the mutant brain, lipofuscin-rich regions are visible in the entire brain (green channel; Figure 1Aii), revealing that lipofuscin (Nakano et al., 2001; Venkatachalam et al., 2008), and this is reflected in the shortened life span and reduced mobility of spin mutant brain. Lipofuscin accumulation in spin mutant brains was also verified with Sudan-Black-B (SBB; previously reported to label lipofuscin [Georgakopoulou et al., 2013]). A general enhancement of SBB staining was observed in the brain of mutant flies (Supplemental Figure 2). It should be noted that SBB, even in control fly tissues, appears to additionally label lipid droplets in the fat bodies. For the remainder of this study, we have used autofluorescence in spin brain as an indicator of lipofuscin.

Lipofuscin is evident in brains as early as adult emergence (1 d) and becomes progressively more pronounced by 8 d of age. Thus, there is progressive accumulation of lipofuscin in spin mutants as compared with age-matched control brains. Our lipidomics analysis of single adult brains comprising ~100,000 cells (Ito et al., 2013) enabled us to determine whether perturbations in major membrane lipids of the brain occur in conjunction with lipofuscin accumulation. In the course of this experiment, 41 membrane lipids belonging to six classes were quantified. PE, PC, and PE-ethers...
FIGURE 1: Lipofuscin accumulation and sphingolipid imbalance in spin mutant brains. (Ai) Box plot represents level of lipofuscin, indicated by autofluorescence, in dissected adult brains of spin\(^{4}/\)spin\(^{5}\) (green), genetic controls (spin heterozygotes, gray), and wild-type (+/+, black). Adult age in days is denoted after the genotype on the x-axis. The box ranges between 25th and 75th percentiles, whereas the whiskers denote the minimum and maximum values in the data set. Autofluorescence levels are variable, but it is evident that spin\(^{4}/\)spin\(^{5}\) (green) brains have much higher levels of autofluorescence than age-matched genetic controls (gray and light gray) or wild-type (black) brains. (Aii) Representative images of freshly dissected/nonpermeabilized adult spin\(^{4}/\)spin\(^{5}\) brains at 4 d of age stained with Alexa 555-phalloidin (magenta). Lipofuscin is visualized with autofluorescence (green). The top panel is a Z-stack visualized as a maximum intensity projection with the two channels separated out, and the bottom image is one optical slice with the two channels merged. The cerebrum (in the top panel) is demarcated with a white dashed line, and optic lobes (OL) are specified. Arrows (top panel) point to autofluorescent puncta, whereas the dotted white region (bottom image) denotes the CBR with autofluorescent puncta. Scale bar = 50 µm. (B) Values are mean ± SEM for lipid levels (quantified as picomoles/brain) for major phospholipid and sphingolipid classes. (C) Major Ceramides-1d values are shown with mean ± SEM for spin\(^{4}/\)spin\(^{5}\) mutants and wild-type controls.
Ceramides were the most prominently and consistently altered lipid class; an increase of more than 800% on day 1 in spin mutant brains (Figure 1B) as compared with controls was observed. This remarkable elevation in ceramide levels in spin brains was driven by increase in the major molecular species of ceramides, 32:1 and 34:1 (Figure 1C). It should be noted, however, that the magnitude of increase in ceramides in spin mutant brains is lowered on day 2 (more than 200%), day 4 (more than 350%), and day 8 (more than 590%) as compared with the initial surge on day 1. No change in ceramide levels was observed in spin mutant larval brains (Supplemental Figure 3). Because of partial lethality in the pupal stages (Nakano et al., 2001; Sweeney and Davis, 2002; Dermaut et al., 2005; Hindle et al., 2011), it is difficult to pinpoint the exact onset of the ceramide imbalance. From our results, ceramide levels are increased at the onset of adulthood but could have been initiated during adult brain development in metamorphosis.

The other lipid classes, such as phosphatidylinositol (PI), PE, and CerPE, show significant increases but the difference is neither sustained nor indicative of any consistent trend on the subsequent days (Figure 1B). Because of the significant and overall increase in ceramide levels in spin brains at all the time points, the remainder of our analyses has focused on sphingolipids and the early onset change on day 1.

Occurrence of lipofuscin correlates with phenotypic severity of spin mutants

The occurrences of lamellated whorls and endomembranous structures are characteristic ultrastructural hallmarks of several degenerative diseases including LSD. Indeed, such structures have been previously reported in spin mutant retinas and in other fly models of LSD (Nakano et al., 2001; Dermaut et al., 2005; Hickey et al., 2006). We therefore asked whether ultrastructural abnormalities in spin mutant brains coincide with altered lipid levels. Three categories of abnormal structures were observed in spin mutant brains that were not observed in age-matched control brains (Figure 2A); these include lamellated whorls/multi-lamellar whorls, prominent electron dense lysosomal structures, and a combination of dense bodies with PE-O are the most abundant phospholipids and CerPE is the most prominent sphingolipid class in adult brains, which is similar to the larval brain lipidome (Carvalho et al., 2010, 2012; Palm et al., 2012; Ghosh et al., 2013).
observed in another allelic combination, and ageing cells. Lamellated whorls and prominent lysosomes were observed at a higher frequency, and lipofuscin-like structures become increasingly abundant (Figure 2C). These structures are also consistently observed in another allelic combination, spin<sup>4</sup>/spin<sup>5</sup>, that exhibit lamellated whorls and membrane-bound structures (Figure 2B, left to right). The last category is reminiscent of lipofuscin—an outcome of increased oxidative stress and intracellular debris in degenerating and ageing cells. Lamellated whorls and prominent lysosomes were observed at a higher frequency at the onset (1–2 d of adulthood) as compared with 8 d, when lipofuscin-like structures become increasingly abundant (Figure 2C). These structures are also consistently observed in another allelic combination, spin<sup>4</sup>/spin<sup>58</sup>, that exhibit a milder phenotype with regard to adult life span (Supplemental Figure 1). When compared to several alleles, the more severe spin<sup>4</sup>/spin<sup>5</sup> versus the milder spin<sup>4</sup>/spin<sup>58</sup>, we recognized that the stronger phenotype with reduced life span (Supplemental Figure 1) correlated with an earlier occurrence and a higher frequency of lipofuscin (Figure 2C). It should be noted that these abnormal structures were not observed in age-matched control brains at these early stages of the adult lifespan, that is, up to 8 d after eclosion. We conclude that because lamellar whorls precede the formation of lipofuscin, they constitute an earlier phenotype than lipofuscin in the disease progression.

**Degree of ceramide and sphingosine elevation in adult spin brains correlates with the severity of neurodegeneration**

The increase in ceramide levels, observed with single brains (Figure 1), was further confirmed when we measured ceramide and its low-abundance breakdown metabolite, sphingosine (Sph), using larger amounts of brain tissue. Consistent with single brain measurements, we observed a dramatic increase in ceramides on day 1 of adulthood (Figure 3A) in spin<sup>4</sup>/spin<sup>5</sup> mutants. For sphingosine, we observed a tremendous increase in abundance for spin mutants (Figure 3). We also note a smaller but significant increase in sphingosine and ceramide levels in the second heteroallelic combination spin<sup>4</sup>/spin<sup>58</sup> but to a lower extent (Figure 3). Here, statistically significant changes were only detected in ceramide dienes. Overall, the changes for spin<sup>4</sup>/spin<sup>58</sup> are less pronounced when compared with spin<sup>4</sup>/spin<sup>5</sup> brains but are consistent with the milder survival and ultrastructural phenotypes.

The enhanced viability of spin<sup>4</sup>/spin<sup>58</sup> heteroallelic combination over spin<sup>4</sup>/spin<sup>5</sup> enabled us to investigate the chronological coincidence of ultrastructural abnormalities, lipid alterations, and behavioral impairment. Here we used changes in the climbing ability of adult flies as a general indicator of behavioral alteration/impairment. Decreased climbing ability is displayed only after 2 wk of adult age (Figure 3B; see the Supplemental Movies). Our experiments suggest that ceramide and sphingosine metabolic perturbations constitute an early-onset event in these mutants, after which other signs of neurodegeneration such as ultrastructural abnormalities, lipofuscin accumulation, and behavioral impairment become pronounced (time line summarized in Figure 3C).

**Ceramide imbalance is central to the role of spin in maintenance of adult neurons**

To show that spin function is causally connected to perturbed ceramide/sphingosine levels, we carried out a genetic rescue
Lipid imbalance in degeneration

FIGURE 4: Rescue of spin5/spin4 phenotypes with spin overexpression and modification of spin4/spin2 phenotypes with slab (Ceramidase) manipulation. (A) Bar graphs indicate percentage change in levels of sphingosine (blue bars) and total ceramides (black bars) measured on day 1 of eclosion in adult brains for mutant, genetic controls, rescue, and overexpression genotypes (also indicated along the x-axis). Lipid levels are quantified as picomoles of lipid/brain and percentage change with respect to genetic control (spin5+/+; spinGal4/+). Values are mean ± SEM, and **p < 0.000001 as calculated by post-hoc test following ANOVA. (B) Representative TEM images of cell bodies in the cortex of the adult brain at 4 d of spin5/spin4 (mutant), spin5/spin5; spin-Gal4-UAS-spin (genetic rescue) and spin5/spin5; slab5+/+ (mutant + decreased Cdase). In spin mutants alone and mutant + decreased Cdase combinations, abnormal structures (arrows) were observed. Insets show these abnormal structures at a higher magnification. In the case of the mutant alone, there are prominent lamellar whorls (orange) and a few lipofuscin-like structures (blue). These abnormal structures are strikingly absent on genetic rescue, but in the case of mutant + decreased Cdase combinations, there is an abundance of lipofuscin-like structures (blue). Nuclei of the cell bodies are indicated as n. Scale bar = 1.8 μm and 0.8 μm for insets. (C) Line profiles represent the mean ± SEM for normalized level of lipofuscin (indicated by autofluorescence) at 1 and 4 d of age in dissected adult brains of spin5/spin5 (green), wild-type (+/+; black), and on manipulation of slab (Cdase) in the background of spin mutants- spin5/spin5; slab5+/+ (blue dotted) and spin5/spin5; spin-Gal4-UAS slab (blue). The levels are normalized to that of spin mutants on day 1 of eclosion. Lipofuscin levels steadily increase in spin mutants between day 1 and day 4. Introduction of slab5+/+ in the spin background results in an even greater accumulation of lipofuscin whereas this trend is reversed with an overexpression of slab in the spin mutant background. (D) Graph represents climbing behavior at 20 d of age in adults for the genotypes indicated. Bars are mean ± SEM of percentage climbers. Mutants include spin5/spin5

experiment wherein full-length wild-type spin (isoform III) is overexpressed in the background of the mutant, spin5/spin5, specifically in cells where the endogenous spin-promoter is active (Sweeney and Davis, 2002). Lipid metabolic alterations are rescued by the transgene in this background. The abnormal high levels of brain ceramide and sphingosine in spin5/spin5 were reduced in rescued genotypes to levels almost comparable to the controls (Figure 4A). In the rescued genotypes, relative to the genetic controls, ceramide increase is only 74% as compared with 437% in the mutant, whereas sphingosine increase is only 61% as compared with 631% in mutants. The altered ceramide/sphingosine levels in the rescue genotypes also correlate with the life span of these animals; life span is longer in rescued animals than the mutant background but lower than in the controls (Supplemental Figure 1). Finally, the ultrastructural hallmark of spin mutant brains, which is the abundance of lamellar whorls, is significantly diminished on genetic rescue with spin overexpression (Figure 4B). Thus sphingolipid imbalance and the associated neurodegenerative attributes (reduced survival, progressively shortened life span, and ultrastructural abnormalities) described for spin mutants are all rescued by an overexpression of spin. These rescue experiments therefore underline that spin isoform III, as opposed to the other four isoforms (Nakano et al., 2001), is minimally sufficient to rescue most of the adult onset degeneration and related attributes in spin mutant flies. The residual effects on lifespan in these rescued genotypes could be explained due to the lack of the other spin isoforms.

To test whether ceramide/sphingosine imbalance is causal to the degenerative attributes in spin brains, the slab (ceramidase/Cdase) (Rohrbough et al., 2004) gene was manipulated in the background of spin mutants. Mutants and overexpression lines of slab exhibit altered levels of ceramides as characterized by MS measurements (Acharya et al., 2003, 2008). slab heterozygotes and overexpression of Cdase specifically in neurons showed an increase and decrease of brain ceramides by more than 200 and 30%, respectively (Hebbar et al., 2015).

(teal colored bar) and the same heteroallelic combination with spin-Gal4 in the background spin5/spin5, spin-Gal4/+ (teal and grey colored bar). Overexpression of slab in this background resulted in a rescue of the climbing behavior (blue bar for spin5/spin5, spin-Gal4-UAS slab). *p < 0.05 for each pair when compared with Student’s t test.
Therefore, these genetic tools were combined in the background of spin mutants. Ultrastructural defects as well as lipofuscin accumulation were used to score the extent of the degenerative phenotype against spin mutant alone. Reducing slab levels (with slab2 heterozygosity; slab2+/+) in the background of spin mutant, and consequently further pushing ceramide levels higher (documented for slab2/+ in Hebbbar et al., 2015), correlated with an intensification of these degenerative phenotypes. This effect is most clearly evidenced (at day 4 of adulthood) by the increased presence of ultrastructural defects (Figure 4B) and enhanced autofluorescence by 34% (Figure 4C) in spin2/spin2; slab2+/+. The abundance of lipofuscin-like structures as opposed to lamellar whorls in age-matched spin2/spin2; slab2+/+ neurons (Figure 4B) highlights the severity and progression of degeneration caused by a reduction of slab function (and increase in ceramides) in spin2/spin2 brains. In contrast, overexpression of slab, and consequently reducing ceramide levels (documented in Hebbbar et al., 2015), halted the formation and accumulation of lipofuscin with age (Figure 4C). A 42% reduction in lipofuscin levels was seen at the onset (day 1) and a 39% reduction was observed at a later stage (day 4) in spin2/spin2; spin-Gal4/+; UAS slab as compared with spin2/spin2. Further this interaction between spin and slab also extends to the phenotype of behavioral decline observed in spin mutants. Using a different allelic combination (spin5/spin5) that permits quantification of climbing behavior, we compared climbing behavior in spin mutants (spin2/spin5) alone and with overexpression of slab (spin2/spin5; spin-Gal4/+; UAS slab). On overexpression of slab in the spin mutant background, we observed that the reduced ability of spin mutants to climb is rescued (Supplemental Figure 4). By 20 d of adulthood, whereas spin mutants have ~30% reduced climbing ability, the overexpression of slab in spin mutants improved climbing ability to that seen in controls (Figure 4D).

This epistatic relationship between ceramide manipulation and spin mutant abnormalities suggests that lowered ceramide levels in spin brains correspond to lowered accumulation of lipofuscin and behavioral decline. However, it should be noted that slab manipulation did not correlate with developmental lethality/viability of spin2/spin2; we observed that slab overexpression or slab2+/+ in the spin2/spin2 background caused increases in spin2/spin2 viability (of 7 and 20%, respectively) to adulthood. This finding probably indicates that the genetic interaction of slab and spin is not critical during development but becomes important during adulthood.

The spin rescue experiments and the genetic interaction with slab help delineate that the removal of spin function in Drosophila causes an early-onset imbalance in sphingolipid metabolism during adulthood. Elevated levels of ceramides and sphingosine are responsible for the progression of the disease leading to a loss of neuronal health and to changes in behavior. It remains unclear if these lipids are specifically enriched in the observed ultrastructural abnormalities.

Interactors for Spin have roles in carbohydrate and lipid metabolism

In an effort to identify intracellular processes impaired by loss of function of spin, we sought to determine its direct protein interactors by coimmunoprecipitation and proteomics (see Supplemental Figure 5 for details). In this way, 29 interactors for Spin were detected and are summarized in Table 1. Using the PANTHER Classification System (Thomas et al., 2003; Mi et al., 2005), these proteins were classified according to their listed cellular component ontology. Thirteen proteins localize to the mitochondrion and are involved in glycolysis. Glycolytic enzymes are recruited to the synapses during energy demand (Jang et al., 2016). Since Spin is actively trafficked along axons (Kumarasamy et al., 2008; Lim and Kraut, 2009) via an energy demanding process, these proteins constitute a potentially intriguing cluster. Further, one protein (guanine nucleotide-binding protein subunit beta-like protein/Rack1) is assigned to the endosome, whereas the lipoprotein (Apolipopophin; ApoLpp) is assigned to the autophagosome; both of these subcellular locations are relevant given the previously published information on Spin localization to the late endosome/lysosome (Sweeney and Davis, 2002; Lim and Kraut, 2009; Rong et al., 2011). In the context of a multi-organ and systemic lipid metabolic imbalance in LSD, we focused on the relevance of Lpp (the functional lipoprotein produced from ApoLpp) as a direct interactor of Spin. Lpp constitutes the lipid transport protein in Drosophila that originates in the fat bodies and facilitates interorgan lipid movement, including the delivery of lipids (Brankatsch and Eaton, 2010; Palm et al., 2012). To confirm the interaction between Lpp and Spin, we investigated Lpp and Spin localization in tissues throughout the body. In the adult brain, Spin-GFP driven by spin-Gal4 is seen in the neuropil and cortex. In the cortical areas, punctate staining for Spin is evident and a subset of these structures are positive for Lpp immunoreactivity (Figure 5, A and A′). We further observed colocalization in fat bodies, where both Spin-GFP and Lpp (Brankatsch and Eaton, 2010) are also abundantly present (Figure 5B). Consistent with neuronal staining, a subset of Spin and Lpp are colocalized in compartments that are in close proximity to the membrane (Figure 5B).

In spin mutant adult brains, we observed a change in Lpp staining as compared with the rescue genotypes (Figure 6A). To better identify the nature of this change, 10-µm sections were imaged (Figure 6B) instead of the whole brain (Figure 6A). Focusing on the CBR, with neuronal nuclei marked with an antibody to elav, Lpp puncta are observed in the brains (Figure 6, B and B′). However, fewer Lpp-containing punctate structures are observed in mutant brains (Figure 6B′). Lowered Lpp staining in mutants was also consistently observed in other tissues; one such example includes the oenocytes. These cells are labeled very strongly when Spin-GFP is driven using spin-Gal4 indicative of spin expression (Figure 5C). Adult oenocytes are a band of cells present dorsally and ventrally along the abdominal cuticle (Figure 6, B and C, and Supplemental Figure 6; Billeter et al., 2009). Oenocytes regulate lipid metabolism and energy storage (Gutierrez et al., 2007), making them an ideal tissue to gain further functional insight into the Spin–Lpp connection. Adult oenocytes act as centers for the production of cuticular hydrocarbons, several of which function as pheromones (Billeter et al., 2009; Yew and Chung, 2015). Since cuticular hydrocarbons (CHCs) are synthesized by the extension and modification of fatty acid precursors (Yew and Chung, 2015), we hypothesize that Spin–LPP will influence CHC synthesis in the oenocytes. Support for this hypothesis comes from two observations. First, Lpp immunostaining in spin mutant oenocytes (Figure 6D) is altered, with a reduced intensity (as compared with rescue conditions [Figure 6C′] and wild-type conditions [Figure 6D]; see more examples in Supplemental Figure 6). Second, total amounts of CHCs are significantly increased in spin mutant females (Figure 6F) while there is no change in specific CHC content. Taken together, these observations point to an intriguing possibility that Spin–Lpp interaction plays a role in regulating CHC synthesis. Whether it is the delivery of the fatty acid precursors to the oenocytes, the actual biosynthesis, or the subsequent transport of the CHC from the oenocytes remains to be addressed. Moreover, whether there are general deficits in lipid metabolism, interorgan lipid transport, or Lpp production/transport are interesting questions for future research.
ages of 1-d-old brains (Figure 2). That spin function is causally connected to ceramide and sphingosine is further validated by our rescue experiments. The slab/Cdase and spin interaction experiments clearly establish loss of spin function upstream of the ceramide imbalance. The altered sphingolipid level is responsible for behavioral decline and lipofuscin accumulation. Given the chronology of events (summarized in Figure 3C) and the outcome of our genetic interaction/rescue experiments, it is evident that accelerated lipofuscin accumulation, as a result of perturbed ceramide levels, correlates with the loss of nervous system integrity in spin mutants.

How does the loss of Spin result in a perturbation of sphingolipids? There is no evidence from our study, or elsewhere, to suggest a direct role for spin in ceramide or sphingosine biosynthesis. However, Spin is assumed to function as a sphingosine-1-phosphate transporter (Osborne et al., 2008; Kawahara et al., 2009). Since our measurements are limited snapshots of the brain lipidome, we cannot make any speculations on the dynamics of ceramide/sphingosine and S1P transport in relation to spin function.

DISCUSSION
We identified alterations in brain ceramide and sphingosine levels in adults following loss of spin function. Perturbations in lipid quantities occur prior to the onset of endomembrane accumulation and before the functional and morphological attributes of neurodegeneration are evident (summarized in Figure 3C). These observations are comparable with reported elevation in sphingosine in cell lines on drug induction of LSD (Lloyd-Evans et al., 2008). Recent studies have linked oxidative stress, lipid droplet formation, neutral lipid storage, and neuronal health in Drosophila and mice tissues (Liu et al., 2015). Our approach has enabled us to define the nature and dynamics of these lipid perturbations and confirm the suspected links of lipid imbalance and pathological attributes of degeneration in a temporal and tissue-specific manner.

We argue that this loss of ceramide/sphingosine homeostasis is not due to apoptosis but is directly linked to loss of spin function. An indication that enhanced apoptosis is not the underlying cause is evident from the absence of any notable apoptotic-like features (vacuolization) in the transmission electron microscopy (TEM) im-

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TABLE 1: Identified Spin interactors.
It is possible that the loss of Spin causes an altered partitioning of sphingosine in the late endosomal/lysosomal compartments, which leads to aberrant signaling. It has been recently shown that sphingosine in cultured cells has the potential to release calcium from acidic compartments, which in turn can influence autophagy, relevant for the turnover of proteins and lipids (Hoglinger et al., 2015). A second likely possibility stems from observations that loss of Spin results in the incomplete progression of autolysosomal reformation and the accumulation of autolysosomes (Rong et al., 2011). We hypothesize that altered spin function in adult neurons accelerates or enhances the formation of autophagic structures such as autolysosomes. This disruption leads to a failure to completely recycle components, including lipids such as ceramides and sphingosine. Since the process is most likely stalled, and further exacerbated by the elevated levels of these signaling lipids, there is an eventual accumulation of intracellular debris in the form of lipofuscin. In motorneuron degeneration of adult spin mutants, a late endosomal protein, perturbations in autophagy could underlie the age-associated degeneration of adult spin mutants. Since all these three processes, namely a lipid metabolic imbalance, neurodegeneration, and autophagy, are implicated in LSD (Hoglinger et al., 2015), this work extends our understanding of the sequence of events in this disease model. Some relevant aspects of the above model are, however, unresolved. First, the relative contribution of the elevated ceramides and sphingosine in maintaining integrity in the brain is unknown. This is difficult to resolve because their metabolic pathways are interconnected. However, it will be relevant to elucidate the nature of ceramide/sphingosine downstream signaling that causes the aberrant progression of autophagy and leads to the formation of lipofuscin. Second, it will be important to also address if an imbalance of ceramides/sphingosine is only limited to signaling within, or from, lysosomal/autophagic structures. Indeed, our experiments with slab and spin suggest that changing overall levels, presumably in all membranes, certainly has an impact. Recently photoactivatable “caged” sphingosine was used to create elevated intracellular levels and its role in calcium signaling from the lysosome was demonstrated in cultured cells (Hoglinger et al., 2015). It will be important in the future to employ such approaches to enable manipulation of these lipids in specific subcellular compartments and monitor the consequences on disease progression.

Interestingly in the mutants, although ceramide levels are consistently higher than the controls, the initial dramatic surge is lowered by 8 d of age. In the context of a storage disease, it will be interesting to evaluate the general metabolic outcome of imbalances in ceramide and sphingosine in spin mutants. Given the relevance of glycosphingolipids in the pathology of LSDs (te Vruchte et al., 2004), it is crucial to ask whether the increased ceramide/sphingosine levels in spin mutants lead to the storage of complex glycolipids. Further, do the increased ceramides and sphingosine overlap with the increased oxidative stress? Indeed, spin mutant adult brains at 5 d posteclosion (Milton et al., 2011) exhibit increased oxidative stress signaling. We propose that elevated ceramide levels in spin brains could potentially influence pathways that lead to the generation of reactive oxygen species (ROS), as demonstrated in a mouse model for perturbed ceramide synthesis (Zigdon et al., 2013).

We have also identified potential metabolic and cellular interaction partners of Spin, including an interaction with Lpp and an impact of decreased spin function on Lpp levels in neurons and oenocytes. Our observations point to the likelihood that Spin and Lpp interact in the brain and also in adult oenocytic function, specifically in the overall production/transport of cumulation of lipofuscin in spin mutants. Thus, in a manner similar to bchs larval motorneuron degeneration, an overlap between perturbations in ceramide/sphingosine signaling and spin function in autophagy could underlie the age-associated degeneration of adult spin mutants. Since all these three processes, namely a lipid metabolic imbalance, neurodegeneration, and autophagy, are implicated in LSD (Hoglinger et al., 2015), this work extends our understanding of the sequence of events in this disease model. Some relevant aspects of the above model are, however, unresolved. First, the relative contribution of the elevated ceramides and sphingosine in maintaining integrity in the brain is unknown. This is difficult to resolve because their metabolic pathways are interconnected. However, it will be relevant to elucidate the nature of ceramide/sphingosine downstream signaling that causes the aberrant progression of autophagy and leads to the formation of lipofuscin. Second, it will be important to also address if an imbalance of ceramides/sphingosine is only limited to signaling within, or from, lysosomal/autophagic structures. Indeed, our experiments with slab and spin suggest that changing overall levels, presumably in all membranes, certainly has an impact. Recently photoactivatable "caged" sphingosine was used to create elevated intracellular levels and its role in calcium signaling from the lysosome was demonstrated in cultured cells (Hoglinger et al., 2015). It will be important in the future to employ such approaches to enable manipulation of these lipids in specific subcellular compartments and monitor the consequences on disease progression.

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**FIGURE 5:** Spin–Lpp interaction. A–B′: Representative images of (A–A′) adult brain and (B–B′) adult abdomen of spin-Gal4:Spin-GFP animals labeled with GFP (green) and Lpp (red) antibodies. Scale bar = 50 µm. In the abdominal fillet preparation, ventral midline is indicated with a dashed white line and fat bodies (gray box) and oenocytes (dashed outline) display LPP staining. Boxed regions are imaged at high magnification and shown in A′, A″, and B′. Arrowheads indicate subset of Spin-GFP-positive puncta that also display LPP immunoreactivity in neuronal cortex (above) and in fat bodies (below). Scale bar: 10 µm.
FIGURE 6: Decreased Lpp staining in spin mutants. (A, B) Labeling of Lpp in spin mutant and rescue neurons. (A) An optical section from a confocal Z-stack of dissected adult brain of spin rescue genotype (A, A′; spin+/spin5; spin-Gal4-UAS-spin) and mutant (A′; spin+/spin5). Tissues are immunostained with anti-GFP (Spin-GFP; green) and anti-Lpp (red). In the rescued genotype, the Spin-GFP (green) label is clearly visible in with the optic lobe (OL) and the cell body rind (CBR) of the adjacent neuropil in the cerebrum. Anti-Lpp immunostaining is shown separately in Spin-GFP (green) label is clearly visible in with the optic lobe (OL) and the cell body rind (CBR). Scale bar = 25 μm. (B) An optical section from a confocal Z-stack of a 10-μm adult head section of spin rescue genotype (B, B′; spin+/spin5; spin-Gal4-UAS-spin) and mutant (B′; spin+/spin5) immunostained with anti-GFP (Spin-GFP; green), anti-Lpp (red), and anti-elav (neuronal nuclei; cyan). The OL and associated CBR is visible in B. A higher magnification view of the CBR with neuronal nuclei (cyan) and anti-LPP (red) is shown in rescue (spin+/spin5; spin-Gal4-UAS-spin) and mutant (spin+/spin5). Lpp (red; indicated by white arrows) is decreased in the mutant. Scale bar = 25 μm (B) and 5 μm (B′, B′′). (C–E) Labeling of Lpp in spin mutant and rescue oenocytes. Female adult abdominal fillet labeled with GFP and Lpp antibodies in spin+/spin5; spin-Gal4-UAS-spin) rescued genotype. Rescue construct (Spin-GFP; green in C) is clearly evident in dorsal and ventral oenocytes (arrows in C); Ventral midline is indicated by white dashed line. Scale bar = 50 μm. Lpp distribution in ventral oenocytes (red outline) and adjoining fat bodies (orange arrow) is shown at a higher magnification (C′–E) in rescue (C′; spin+/spin5; spin-Gal4-UAS-spin), wild-type (D; +/+ ) and in mutant (E; spin+/spin5) genotypes. Lpp is visible at the membrane of oenocytic cells (white arrows), but the intensity of Lpp staining is reduced in spin mutants (E); there are some clusters of cells (white asterisk) wherein the membrane staining of Lpp appears diminished. Scale bar = 50 μm. (F) CHC quantification: Bars represent mean ± SEM for total cuticular hydrocarbon (CHC) levels in male and female flies from mutant (spin+/spin5) and (spin+/spin5; spin-Gal4-UAS-spin) rescued genotypes. Mutant females display significantly (p < 0.05) higher levels of CHCs as compared with rescued genotypes; n = 3 replicates of six to eight flies each.

Lipofuscin imaging and analyses

Adult brains were dissected in phosphate-buffered saline (PBS) on ice. They were placed in Alexa568-labeled phalloidin (1:40; Life Technologies, India) for 20 min on ice before transferring onto PBS on glass slides for imaging. Brains were imaged using Fluoview 1000 (Olympus Corporation, Japan) with an Argon 488 (autofluorescence) and HeNe (568 phalloidin) laser. Optical sections were taken and merged using ImageJ (Schneider et al., 2012) to create an image stack. Image stack was analyzed to give particle count to indicate number of fluorescent particles.

Materials and methods

Flies

All fly lines and genotypes used are listed in the Supplemental Material; see Supplemental Table 1. Flies were raised at 25°C on a regular yeast-sugar-cornmeal medium supplemented with fungicial mixture of phosphoric acid and propionic acid (http://flystocks.bio.indiana.edu/Fly_Work/media -recipes/caltechfood.htm).

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Immunostaining

Dissected adult brains and abdominal fillets were immunostained following Hebbar et al. (2006). The primary antibodies used were anti-GFP (A-11122, 1:200; Life Technologies, India), anti-elav (7E8A10, DSHB, University of Iowa, USA), and anti-Lpp (guinea pig, gift from Marko Brankatschk [Brankatschk and Eaton, 2010]). Fluor-tagged secondaries were obtained from Life Technologies, India.

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Transmission electron microscopy
Adult heads were dissected, placed in fixative (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4), and vacuum treated to removed adherent air. Heads were incubated in fresh fixative overnight at 4°C. All incubations were on a rotating wheel, unless otherwise stated. Heads were washed in 0.1 M sodium phosphate buffer (3 × 10 min), postfixed in 1% OsO4 for 1 h, followed by washes in 0.1 M sodium phosphate buffer (3 × 10 min), and dH2O (3 × 10 min). Heads were dehydrated in an acetone series (30%, 50%, 70%, 90%, 3 × 100%; 20 min each) and then incubated in increasing concentrations of Spurr’s resin:acetone (25%, 50%, 75%, 95%, 2 × 100% [at 37°C]; 45 min each) followed by incubation in 100% resin overnight at 4°C without rotation. Heads were embedded in Spurr’s resin for 24 h at 70°C.

After reaching the desired depth by semi-thick sectioning, ultra-thin sections (60–70 nm) were collected coated grids, treated with uranyl acetate in 50% ethanol (10 min), and submerged in dH2O to wash. Sections were stained with lead citrate (10 min) in the presence of sodium hydroxide pellets, followed by washing in dH2O. Images were acquired using analysis software on a TECNAI G (Version 2.18) transmission electron microscope (120 kV).

SBB staining
Fly heads were dissected and fixed in 4% formaldehyde, embedded in tissue freezing medium, and cryosectioned at 10-µm thickness. SBB staining was performed as described in Georgakopoulou in tissue freezing medium, and cryosectioned at 10-µm thickness. SBB staining Images were acquired using analysis software on a TECNAI G (Version 2.18) transmission electron microscope (120 kV).

Lipid extraction and lipidomics
Lipid extraction. Female adults of the required genotypes were collected on emergence and aged appropriately. Depending on the experiment, single brains or groups of three brains were dissected in PBS and flash frozen in liquid nitrogen and stored in the freezer at −80°C. For the mass spectrometric measurements, the samples were dissolved in 100 µl methanol containing 0.1% ammonium acetate.

Lipidomics of single brains. To study the correlation between lipfuscin accumulation and lipid metabolism (Figure 1), single brains were homogenized directly after imaging and extracted with the MTBE based extraction protocol. LC-MS was performed on a Agilent 1200 microLC system (Agilent Technologies, USA) coupled with a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using a split flow setup to perform nano-ESI with the Nanomate Triversa (Advion Biosciences Ltd, USA). Liquid chromatography was performed at a flow rate of 20 µl/min using a Zorbax SB-C18 column (0.5 mm ID, 5 µm, 150 mm), and 5-µl samples were injected. Solvent A was methanol containing 0.1% ammonium acetate and solvent B MTBE. The gradient was as follows: 0–3 min, B = 0.5%; 18–21 min, B = 50%; and 22–37, min B = 0.5%. Extracted ion chromatograms for designated lipids were integrated using accurate masses (±5 ppm) with Xcalibur software (Thermo Fisher Scientific, Germany)

MS² analysis of pooled brain lipid extracts. Lipid extracts were analyzed with a flow-injection system using a flow rate of 1 µl/min and 5 µl sample injection. Negative and positive ion mode spectra were acquired with a LTQ-Orbitrap XL equipped with a 1200 microLC system and a Nanomate Triversa utilizing 5-µm ID ESI-chips. In the negative mode, Pt, PE, PE-O, lysophosphatidylethanolamine (LPE), PC, CerPE, phosphatidylserine (PS), and phosphatidylglycerol (PG) were identified according to their accurate mass as described earlier (Schwendke et al., 2011). For PS, the specific neutral loss of 87 Da was monitored in the linear ion trap and used for quantification. In the positive ion mode Sph 14:1, ceramides and hex-ceramides were monitored with MS² in the linear ion trap using the long chain base-related fragment ions. All MS³ for quantifying sphingolipids were analyzed using Xcalibur software while all other analyses were performed using LipidXplorer (Herzog et al., 2011).

Annotation of lipids. Phospholipids were indicated as (lipidclass) (no. of carbons in all fatty acids):(no. of double bonds in all fatty acids). For the annotation of phosphatidyl ethanolamine vinyl ether and alkyl ether, the abbreviation PE-O was used along with the number of double bonds of the fatty acid and fatty alcohol moiety. Sphingolipids were annotated as (lipid class) (no. of carbons in the long-chain base and fatty acid moieties):(no. of double bonds in the long-chain base and fatty acid moieties). Lipid class abbreviations were utilized and defined throughout the article.

Coimmunoprecipitation and proteomics analyses
Spin-GFP protein was pulled down, and interaction partners identified using a proteomics-MS approach; see the Supplemental Material for schematic on workflow. Briefly, using the Gal4-UAS system, we overexpressed Spin-GFP under the control of the endogenous spin promoter (spin-Gal4; Sweeney and Davis, 2002). As a control, we also used flies that had GFP being overexpressed with spin-Gal4. Using lysates from whole organisms, we immunoprecipitated GFP using anti-GFP as the bait (GFP-Trap_A kit; Chromotek, Germany). The coimmunoprecipitated fractions were run on SDS–PAGE and each lane was cut into 10 slices, followed by in-gel digestion (Shevchenko et al., 2006). For each lane, slices were analyzed using 1200 nano-LC coupled to LTQ Orbitrap Discovery (Thermo Fisher Scientific, Germany) using Nanomate Triversa as ionsource. Peptide identification and protein assignments were performed using Protein Discoverer 1.3 (Thermo Fisher Scientific, Germany) and Mascot as a search engine. Only proteins with a minimum of two unique peptides and false discovery rate better than 0.01 were accepted as positive hits. Proteins specific to Spin-GFP co-IP fraction and not found in the control-GFP co-IP fraction were compiled from three independent experiments.

Climbing assays
Standard fly climbing assays were conducted to assay the climbing behavior in spin and control flies. For this, male and female flies were collected within 6 h of eclosion and separately maintained in groups of 10–20 flies with transfers into fresh food every 2–3 d. At 3 d of age, these flies were subjected to a climbing assay and subsequently assayed for the next 30 d. The climbing assay was conducted in a double-blind procedure (for the entire procedure until the data analysis was completed) in a room devoid of any obvious olfactory and visual...
cues and temperature was monitored and maintained at 25°C. Briefly, flies were transferred to a glass vial with an “8 cm” mark on it. After acclimatization, the assay was video recorded starting with the tapping of the vial. Climbers were defined as flies that reached a height of 8 cm within 18 s. The assay was repeated three times and averages for percentage climbers was calculated from video recordings from a set of three independent vials/repeats with 10–20 flies each.

Life-span analyses

Adult males and females of relevant genotypes were separately collected within 6 h of emergence and kept in groups of 15–20. Flies were transferred into fresh food vials every 2 d, and the total number of surviving flies was recorded. These observations were used to calculate life-span curves and 50% survivorship (T-50), which is the length in days at which 50% of the flies remained alive (Fergestad et al., 2008).

CHC extraction and analyses

Samples for were prepared by incubating five to eight of each genotype at room temperature for 20 min with 120 µL of hexane containing 10 µg/ml hexacosane as an internal standard. The extract (100 µL) was transferred into a fresh glass vial and allowed to evaporate at room temperature. Samples were stored at –20°C. Three replicates were prepared for each genotype.

Analysis by gas chromatography mass spectrometry (GCMS) was performed on a QP2010 system (Shimadzu, Kyoto, Japan) equipped with a DB-5 column (5%-phenyl-methylpolysiloxane column; 30 m length, 0.25 mm ID, 0.25 µm film thickness; Agilent). Ionization was achieved by electron ionization (EI) at 70 eV. One microliter of the sample was injected using a splitless injector. The helium flow was set at 1.9 ml/min. The column temperature program began at 50°C, increased to 210°C at a rate of 35°C/min, and then increased to 280°C at a rate of 3°C/min. A mass spectrometer was set to unit mass resolution and three scans/s from m/z 37 to 700. Chromatograms and mass spectra were analyzed using GCMSsolution software (Shimadzu). For total CHC levels, the area under each of the peaks for the spiked hexacosane standard. Statistical analysis was performed using a one-way ANOVA with post-hoc Holm-Sidak test.

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