

# Oxidative stress induces overgrowth of the *Drosophila* neuromuscular junction

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Synaptic terminals are known to expand and contract throughout an animal's life. The physiological constraints and demands that regulate appropriate synaptic growth and connectivity are currently poorly understood. In previous work, we identified a *Drosophila* model of lysosomal storage disease (LSD), *spinster* (*spin*), with larval neuromuscular synapse overgrowth. Here we identify a reactive oxygen species (ROS) burden in *spin* that may be attributable to previously identified lipofuscin deposition and lysosomal dysfunction, a cellular hallmark of LSD. Reducing ROS in *spin* mutants rescues synaptic overgrowth and electrophysiological deficits. Synapse overgrowth was also observed in mutants defective for protection from ROS and animals subjected to excessive ROS. ROS are known to stimulate JNK and fos signaling. Furthermore, JNK and fos in turn are known potent activators of synapse growth and function. Inhibiting JNK and fos activity in *spin* rescues synapse overgrowth and electrophysiological deficits. Similarly, inhibiting JNK, fos, and jun activity in animals with excessive oxidative stress rescues the overgrowth phenotype. These data suggest that ROS, via activation of the JNK signaling pathway, are a major regulator of synapse overgrowth. In LSD, increased autophagy contributes to lysosomal storage and, presumably, elevated levels of oxidative stress. In support of this suggestion, we report here that impaired autophagy function reverses synaptic overgrowth in *spin*. Our data describe a previously unexplored link between oxidative stress and synapse overgrowth via the JNK signaling pathway.

Lysosomal dysfunction leads to poor digestion of damaged macromolecules and organelles, resulting in the accumulation of biological waste. During aging, this "waste" manifests as lipofuscin, a nondegradable, autofluorescent intralysosomal polymeric agglomeration of lipids and proteins (1). Lipofuscin is viewed as a hallmark of aging cells and is notable in long-lived cells, such as neurons. Lysosomal storage disorders (LSD) are characterized in part by lipofuscin accumulation, reduced lysosomal function, and neurodegeneration. Ectopic synaptogenesis and dendritogenesis has also been demonstrated in models of LSDs (2, 3), although the mechanisms causing this growth are unclear. Lipofuscin deposition is proposed to hamper autophagic degradation in general and particularly organelle turnover, promoting the accumulation of senescent mitochondria and lysosomal iron, producing increasing amounts of reactive oxygen species (ROS) (4). A similar mechanism may occur in LSD, where lysosomal dysfunction can lead to an accumulation of autophagosomes, either because of a defect in autophagosomal clearance in the lysosome or an induction of autophagy (5, 6). Oxidative stress (OS), potentially activated by lysosomal accumulation, is an increasingly recognized feature of LSDs (see for example ref. 7) and may in turn induce a further increase in autophagy. The pathways by which OS/ROS go on to contribute to neuronal dysfunction in aging or diseased tissue currently remain obscure (8).

We have previously identified in *Drosophila* loss-of-function mutations in *spinster* (*spin*), an endosomal protein, that give rise to shortened lifespan, accumulation of lipofuscin, swollen lysosomes, neurodegeneration, and synaptic overgrowth (9–12), all

hallmarks of LSD. Neuromuscular synaptic overgrowth in *spin* was shown to require a permissive TGF- $\beta$  signal (9), an important developmental pathway regulating the growth of this synapse (9, 13–16). Synapse growth and function is also regulated by the jun N-terminal kinase (JNK)/activator protein-1 (AP-1) pathway (17–19). AP-1 is a transcriptional regulator composed of the DNA binding proteins fos and jun. In *hiw* mutants, loss of an E3-ubiquitin ligase and the consequent failure to degrade wallenda, a JNK kinase kinase (JNKKK) generates synaptic overgrowth by the activation of JNK/AP-1 (19, 20). In other cellular contexts the JNK/AP-1 pathway is well known to be activated by OS (21), particularly via the activation of apoptosis-signal-regulating kinase (ASK), a JNKKK stimulated by oxidation of thioredoxin (22) and the regulation of JNK itself by interaction with GST (23). OS has been shown to be a major activator of autophagy and antioxidative responses via JNK/AP-1 activity (24–26). Other than *hiw* and *wallenda*, factors signaling upstream of JNK regulating synaptic development are relatively unknown. The known activation and regulation of the JNK signaling pathway by ROS suggests that this stimulus may play a potent role in the activation of synapse growth and function.

We examined and found in our *Drosophila* LSD model *spin* evidence for an OS burden. We hypothesized and found that decreasing OS reduces synaptic overgrowth and alleviates physiological dysfunction. We further tested if larvae with OS generated by independent genetic and pharmacological manipulations have similarly overgrown synapses. Finally, we examined the role of JNK/AP-1 in regulating synaptic growth in response to OS. JNK/AP-1 are well known mediators of the cellular response to various stressors including OS. We demonstrate here that OS is a major activator of synapse growth via activation of the JNK/AP-1 pathway.

## Results

### Mutations in the Late Endosomal Protein *spin* Lead to an OS Burden.

The presence of lipofuscin and swollen late endosome/lysosomes in the *spin* mutant has previously been documented (9–11). Lipofuscin consists of undegraded material known to generate OS through Fenton reactions in the disrupted lysosome (1, 27). To test if neuromuscular junction (NMJ) overgrowth is caused by OS, we verified the presence of OS in *spin* using a lipid peroxidation assay (Fig. 1A) and a reporter transgene for OS (Fig. 1B). Lipid peroxidation is the degradation of polyunsaturated lipids to malondialdehyde (MDA) and 4-hydroxyalkenals by the action of free radicals (28) and adducts generated by MDA may give

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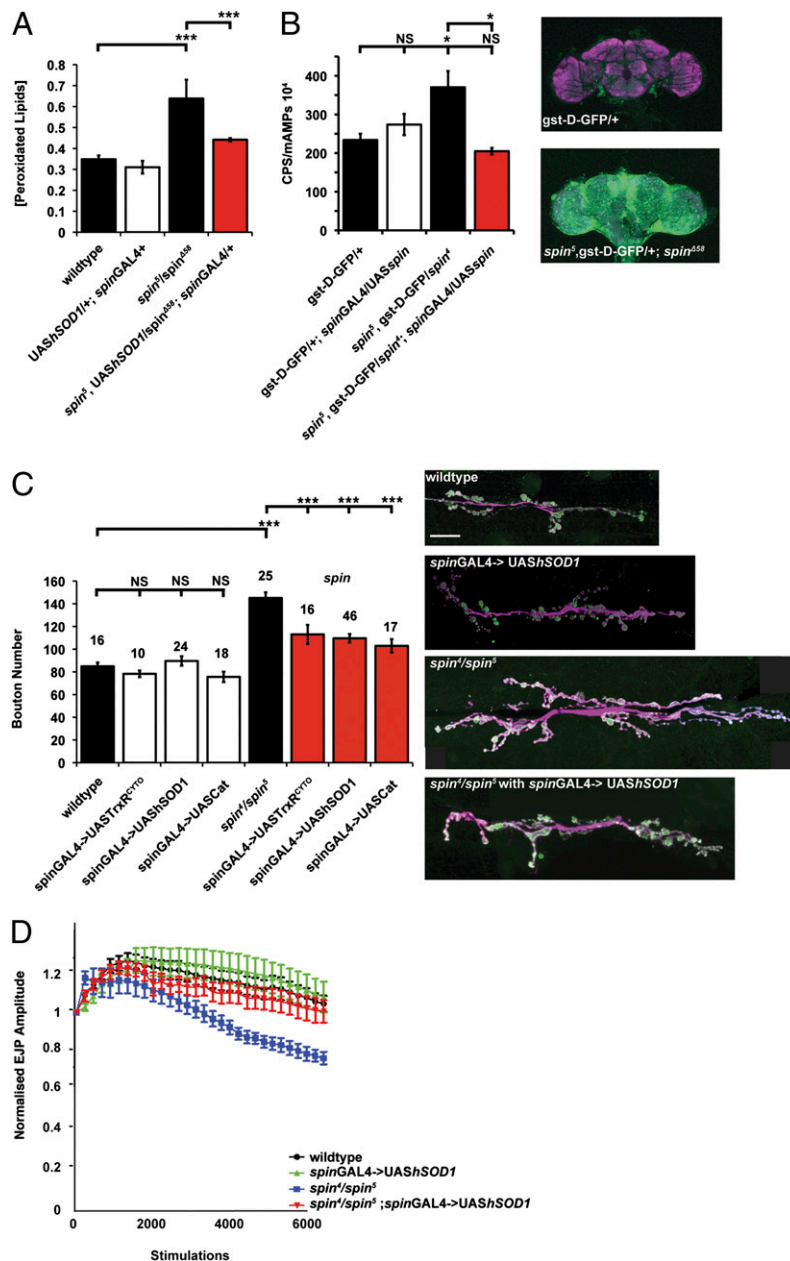
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**Fig. 1.** *spin* mutants bear a ROS burden: Relieving OS provides a partial rescue of overgrowth at the NMJ and alleviates electrophysiological deficits. (A) *spin* mutants have significantly increased levels of peroxidated lipids ( $***P < 0.001$ , Student *t* test), rescued by expression of *hSOD1* under the control of *spinGAL4*. Error bars show SEM ( $n = 3$ ). (B) *spin* mutants have increased levels of *gst-D-GFP* expression ( $*P < 0.05$ , ANOVA), indicating activation of the NRF2 OS response. Increased GFP is shown by fluorimetry, bars and show emission levels at the peak amplitude of GFP fluorescence; error bars show SEM ( $n = 3$ ). Increased GFP can also be seen in z-stacks of adult heads (neuropil, in magenta). Expression of *spin* under the control of *spinGAL4* rescues this phenotype. (C) Overexpression of antioxidant transgenes simultaneously expressed pre- and postsynaptically using *spinGAL4* significantly reduces overgrowth at the NMJ. Furthermore, *spin<sup>4</sup>/spin<sup>5</sup>* has a 96% increase in bouton number compared with wild-type, from  $85 \pm 3.3$  ( $n = 16$ ) to  $151 \pm 8.8$  ( $n = 32$ ) ( $***P < 0.001$ , ANOVA). Expression of UAS-*trxR1<sup>CYT0</sup>*, UAS-*hSOD1* or UAS-*cat* under control of *spinGAL4* does not change bouton number. UAS-*trxR1<sup>CYT0</sup>*; *spinGAL4<sup>4/+</sup>*, UAS-*hSOD1<sup>1/+</sup>*; *spinGAL4<sup>4/+</sup>*, and UAS-*cat<sup>1/+</sup>*; *spinGAL4<sup>4/+</sup>* have bouton numbers of  $78 \pm 2.7$  ( $n = 10$ ),  $90 \pm 3.3$  ( $n = 24$ ), and  $76 \pm 8.7$  ( $n = 18$ ), respectively. Expressing antioxidant transgenes in *spin* mutants significantly rescues bouton number to  $113 \pm 8.3$  ( $n = 10$ ),  $110 \pm 4.5$  ( $n = 46$ ), and  $103 \pm 4.5$  ( $n = 17$ ), respectively ( $***P < 0.001$ , ANOVA different from *spin* and  $*P > 0.05$  compared with wild-type). Error bars show SEM; numbers above each bar are the *n* values. Images of the NMJ at muscle 6/7 in segment A3, with nerves shown in magenta (HRP) and boutons in green (syt). (Scale bar, 20  $\mu$ m.) (D) EJP amplitudes decrease in *spin* with repeated stimulation. EJP amplitudes return to control levels when *hSOD1* is expressed. Expressing this transgene in a wild-type background does not affect responses to repeated stimulation.

rise to fluorescent lipofuscin (29). Elevated levels of aldehydes are commonly used as a marker of OS. We found that MDA levels in *spin* are increased by 80% in *spin* mutants, an increase rescued by the expression of a superoxide dismutase 1 (*hSOD1*) transgene, a well-characterized antioxidant enzyme (30) (Fig.

1A). This finding suggests pathological levels of ROS in *spin*. High levels of ROS in *spin* are also indicated using a GST (*gst-D-GFP*) reporter transgene where GFP expression is controlled by a *gst-D* promoter, an element recognized and activated by the NF-E2-related factor-2 (Nrf2) transcription factor under con-

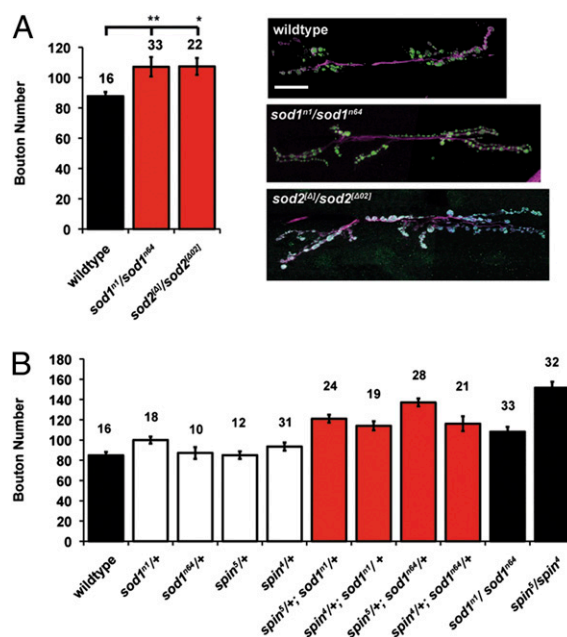
ditions of OS (31). Increased levels of GFP in *spin* are shown here both by fluorimetry and confocal microscopy (Fig. 1B), confirming activation of the cellular OS response pathway.

**Reducing ROS in *spin* Mutants Rescues NMJ Overgrowth and Electrophysiological Deficits.** Mutations in *spin* cause significant overgrowth of the NMJ, with an 80% increase in bouton number (9). Bouton number is a common measure of synaptic development at the *Drosophila* larval NMJ and is the quantification of synaptic growth that we use throughout our study as an assay for synaptic growth (32). The *spin* mutant muscles do not significantly differ in size from wild-type controls (Table S1). We therefore do not need to normalize bouton number to muscle area to allow for the presumed coupling during development between muscle size and synaptic growth (9, 33, 34). We asked if synapse overgrowth in *spin* mutants is generated by increased levels of ROS. Through expression of antioxidant transgenes we reduced OS in *spin*. To rescue cells affected by the loss of *spin*, UAS-*hSOD1*, UAS-*catalase* (*cat*), and UAS-*thioredoxin-reductase* (*TrxR1*) were expressed pre- and postsynaptically under the control of a *spin* promoter (*spin*GAL4, expressed both in muscle and nerve). In a wild-type background, expression of *hSOD1*, *cat*, and *TrxR1* does not affect bouton number. Overexpression of *hSOD1*, *cat*, or *TrxR1* in *spin* mutant larvae significantly rescue synapse overgrowth by 60% (Fig. 1C). In another synaptic overgrowth mutant, *hiw*, a modest but significant reduction in overgrowth can be seen when we express *hSOD1*. This finding suggests that OS contributes to the overgrowth seen in this mutant (Fig. S1). These data suggest increased levels of ROS are present in *spin* and contribute to synaptic overgrowth in *spin* and other synaptic overgrowth mutants.

Electrophysiological stimulation of control and *spin* mutant larval NMJs at a high frequency (10 Hz) resulted in an initial increase in excitatory junction potential (EJP) amplitudes over approximately the first 1,000 stimulations followed by a rundown in EJP amplitude. Similar responses have been reported in this preparation previously (35). Mutant *spin* larvae showed an increased rate and extent of rundown of (EJP) amplitudes to about 80% of their initial value, significantly different ( $P < 0.05$ ) from control larvae with EJP amplitudes of about 100% of initial values after 6,000 stimuli (Fig. 1D). This increased rate of rundown in *spin* mutants is similar to previous reports (12). Expression of *hSOD1* in a wild-type background had no effect on EJP amplitudes during high-frequency stimulation. Expression of *hSOD1* restored the ability of *spin* mutant synapses to maintain synaptic transmission during high-frequency stimulation. EJP amplitudes in rescued larvae were not statistically different from wild-type.

**OS Induces Synaptic Growth.** We examined further the role OS may have in generating synapse overgrowth independent of *spin*-induced lysosomal dysfunction. We determined synapse size in two different mutants known to have defective protection from ROS. The enzymes SOD1 and SOD2 are vital components of the cellular antioxidant defense system; SOD converts superoxide anions to hydrogen peroxide (30). SOD1 is predominantly localized to the cytosol and mitochondrial periplasm (36), and SOD2 is mitochondrially localized (37). Both *sod1* and *sod2* muscle fibers are undergrown; therefore, by not normalizing for synaptic growth coupling we underestimate the size of the synapse in these animals (Fig. S2 D–F and Table S1). On examination, larvae mutant for either *sod1* or *sod2* have a significant increase in bouton number, indicating a synaptic overgrowth (Fig. 2A). We observe a reduction in synaptic branching in *sod2* mutants (Fig. S3 and Table S2) and reduced bouton size in *sod1* mutants (Fig. S4 and Table S3); nonetheless, bouton number is increased compared with wild-type in all conditions.

Feeding paraquat to *Drosophila* has previously been used to induce OS. Paraquat is known to impair mitochondrial function by disturbing complex I and complex III of the mitochondrial



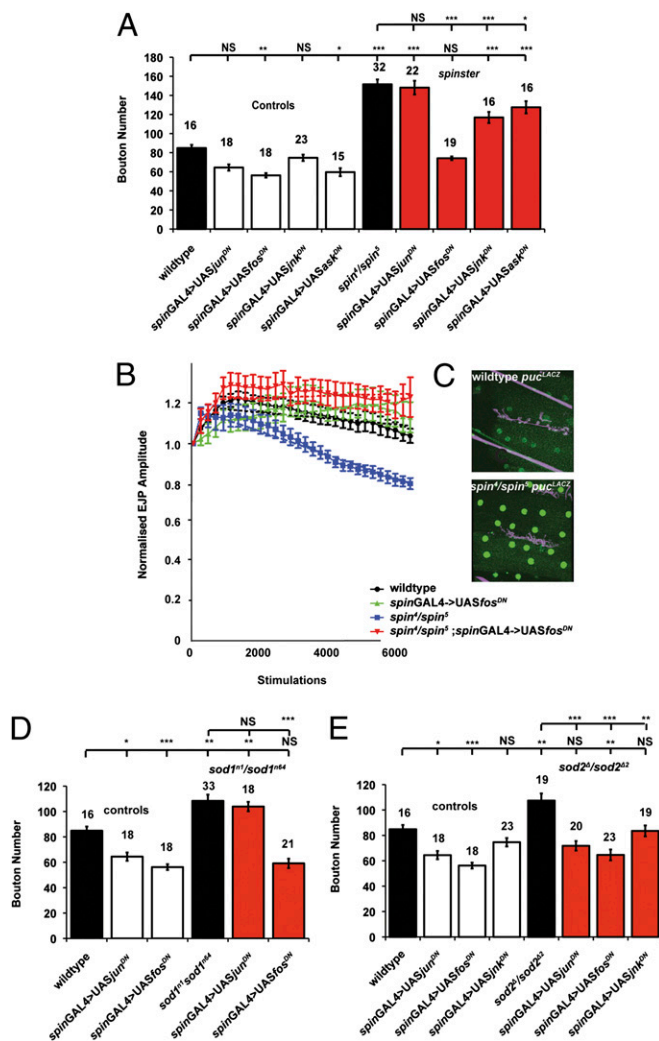
**Fig. 2.** OS induces growth of the NMJ. (A) Mutations in the OS protection system cause significantly overgrown NMJs. Wild-type larvae have a mean bouton number of  $85 \pm 3.3$  ( $n = 16$ ), which is increased by mutations in *sod1* to  $107 \pm 4.9$  ( $n = 21$ ,  $**P < 0.01$ , ANOVA) and *sod2* to  $107 \pm 5.9$  ( $n = 22$ ,  $*P < 0.05$ , ANOVA). Error bars show SEM; numbers above each bar are the  $n$  values. Images of the NMJ at muscle 6/7 in segment A3, with nerves shown in magenta (HRP) and boutons in green (syt). (Scale bar, 20  $\mu\text{m}$ .) (B) Larvae carrying heterozygous mutations for both *sod1* and *spin* are significantly overgrown compared with heterozygous mutations alone. Wild-type, *sod1*<sup>N1/+</sup>, *sod1*<sup>N64/+</sup>, *spin*<sup>A/+</sup>, and *spin*<sup>S/+</sup> are not significantly different ( $P > 0.05$ ). In all combinations, mutants of *spin* and *sod1* are overgrown compared with wild-type or the constituent heterozygotes alone ( $P < 0.001$ , ANOVA).

respiratory chain (38), increasing generation of ROS (39). *Drosophila* larvae were reared on food containing 10-mM paraquat from the first-instar stage. Although paraquat feeding resulted in smaller sized animals, expression of UAS-*hSOD1* in nerve and muscle reduced synapse growth by 30% compared with paraquat-fed controls (wild-type larvae with 10-mM paraquat bouton number,  $80 \pm 6.64$ ; *spin*GAL4/UAS-*hSOD1* with 10-mM paraquat,  $57 \pm 2.64$ ) (Fig. S24). Collectively, the observations that known mutations and a toxin, which increase OS, all induce synaptic growth suggest that exposure to OS during development can result in NMJ overgrowth.

**OS and *spin* Overgrowth Share the Same Genetic Pathway; JNK/AP-1 Signaling Is Required for ROS-Induced Synapse Overgrowth.** Heterozygotes of two *spin* mutants, *spin*<sup>A/+</sup> and *spin*<sup>S/+</sup> have bouton numbers similar to wild-type. Similarly, *sod1* mutant heterozygotes (*sod1*<sup>N1/+</sup> and *sod1*<sup>N64/+</sup>) do not lead to significant overgrowth ( $P > 0.05$ , ANOVA). Combining these mutations in a single animal by removing one functional copy of *spin* and one copy of *sod1* generates a significant increase in bouton number ( $P < 0.001$ , ANOVA). Animals carrying heterozygous mutations in both *sod1* and *spin* have a synaptic overgrowth that resembles the *spin* or *sod1* full-mutant phenotypes (Fig. 2B), suggesting a shared genetic pathway revealed by the synergy between the *spin* and *sod1* heterozygous mutants.

It is well documented that JNK and fos are activated in response to OS (21, 40, 41). Activation of the AP-1 pathway is known to directly transcriptionally activate *puckered*, a phosphatase inhibitor of JNK (42). We examined muscles in *spin* for the activation of the AP-1 pathway using the *puc*<sup>E69</sup> enhancer trap, examining tissue for the induction of the  $\beta$ -gal reporter trans-





**Fig. 3.** *spin*-, *sod1*-, and *sod2*-induced synaptic overgrowth is dependent on JNK/AP-1 signaling. (A) Expression of fos and JNK dominant-negative transgenes rescue synaptic overgrowth in *spin*. Depleting fos signaling rescues *spin* synaptic overgrowth, reducing bouton number from  $166 \pm 8.8$  ( $n = 25$ ) to  $74 \pm 1.7$  ( $n = 19$ ), generating a bouton number that is not significantly different from wild-type ( $P > 0.05$ , ANOVA) and significantly different from *spin* ( $***P < 0.001$ , ANOVA). Depleting jun did not significantly rescue synapse size, with a bouton number of  $148 \pm 3.6$  ( $n = 22$ ). Expression of a JNK dominant-negative transgene reduced bouton number to  $117 \pm 5.8$  ( $n = 16$ ), but expression of an ASK dominant-negative transgene did not rescue NMJ overgrowth with a bouton number of  $142 \pm 9.5$  ( $n = 16$ ). (B) EJP amplitudes decrease in *spin* with repeated stimulation. This rundown in EJP amplitudes is rescued to control levels when fos signaling is reduced by expression of *fos*<sup>DN</sup>. Expressing this transgene in a wild-type background does not affect synaptic responses to repeated stimulation. Expression of *fos*<sup>DN</sup> fully rescues the rundown of EJP amplitudes in *spin* mutants to the same level as wild-type EJPs. (C) Expression of *pucLacZ*, an enhancer trap-reporter of JNK/AP-1 activation, is increased in the muscle in *spin* compared with wild-type; nerves shown in magenta, LacZ in green. (D) In a wild-type background, *spinGAL4 > UASjun*<sup>DN</sup> and *spinGAL4 > UASfos*<sup>DN</sup> cause a significant reduction in bouton number, with mean bouton numbers of  $64 \pm 3.24$  ( $n = 18$ ) ( $*P < 0.05$ , ANOVA) and  $56 \pm 2.29$  ( $n = 18$ ) ( $***P < 0.001$ , ANOVA), respectively. Expression of *jun*<sup>DN</sup> in a *sod1* background does not rescue bouton number  $104 \pm 3.69$  ( $n = 18$ ), ( $P > 0.05$  compared with *sod1*;  $**P < 0.01$  compared with wild-type, ANOVA), whereas *fos*<sup>DN</sup> expression significantly reduces *sod1* overgrowth to  $59 \pm 3.7$  ( $n = 21$ ), back to wild-type levels ( $P < 0.001$  compared with *sod1*;  $P > 0.05$  compared with wild-type). (E) In a wild-type background *spinGAL4 > UAS-JNK*<sup>DN</sup> causes no change in bouton number,  $75 \pm 3.31$  ( $n = 23$ ) ( $P > 0.05$ , ANOVA). Expression of transgenes, *jun*<sup>DN</sup>, *fos*<sup>DN</sup>, or *JNK*<sup>DN</sup> in a *sod2* mutant background significantly reduces overgrowth to  $72 \pm 3.77$  ( $n = 20$ ) ( $***P < 0.001$ , ANOVA),  $65 \pm 4.25$  ( $n = 23$ ) ( $***P < 0.001$ , ANOVA), and  $84 \pm 4.28$  ( $n = 19$ ), respectively ( $**P < 0.01$ , ANOVA).

gene. Increased expression of *β-gal* compared with wild-type was observed by immunofluorescence, indicating an activation of the AP-1 pathway in muscles in *spin* (Fig. 3C). We then asked if this pathway is essential to generate OS-induced synaptic overgrowth. Dominant-negative transgenes (and RNAi) (Fig. S5) for components of JNK/AP-1 signaling were expressed simultaneously pre- and postsynaptically (using *spinGAL4*). In *spin*, synaptic overgrowth is significantly reduced by depleting fos and JNK signaling (Fig. 3A), but reducing jun and ASK activity did not reduce overgrowth. Similar results were observed using RNAi transgenes targeted to fos and JNK in a *spin* mutant background (Fig. S5).

As the *fos*<sup>DN</sup> transgene gave the greatest rescue of bouton number, we asked if depleting fos signaling could alleviate the electrophysiological deficit previously described in *spin* loss-of-function mutants. Expression of *fos*<sup>DN</sup> prevented the fatigue phenotype seen during repeated stimulation in *spin* (Fig. 3B). EJP amplitudes in the rescued larvae were not statistically different from controls, suggesting fos activation contributes to synapse dysfunction in *spin*.

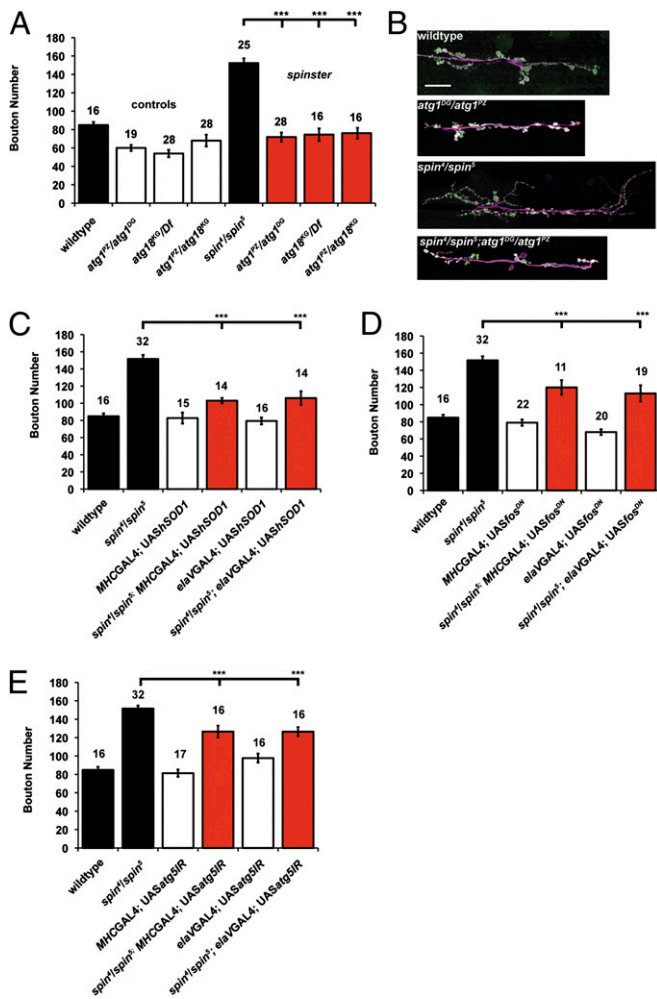
In *sod1* and *sod2* mutant animals, expression of the *fos*<sup>DN</sup> transgene using *spinGAL4* rescued synaptic overgrowth to wild-type levels (Fig. 3D and E), but a *jun*<sup>DN</sup> transgene rescued synaptic overgrowth in *sod2* animals only (Fig. 3E). A similar reduction in OS-induced synaptic growth using *fos*<sup>DN</sup> and *jun*<sup>DN</sup> transgenes is achieved in paraquat-fed animals (Fig. S6), although overall animal size is altered in paraquat-treated animals. These data suggest that fos is involved in all of the overgrowth phenotypes studied (*spin*, *sod1*, *sod2*, paraquat treatment), but we suggest that jun may be involved in synaptic growth where mitochondrial dysfunction may be the source for the generation of OS (*sod2* and paraquat treatment).

#### Autophagy Genes Are Required for OS-Induced Overgrowth in *spin*.

Autophagy is potentially activated in response to OS and JNK activation (24), and acts to protect the cell by degradation of damaged proteins, lipids, and organelles (43, 44). Activation of autophagy produces an added burden to lysosomal function, particularly in the context of LSD, where lysosomal clearance is blocked, resulting in added lysosomal storage. We reasoned that activation of autophagy function would be critical to the process of synaptic overgrowth induced by OS in *spin* and that blocking autophagy function would reduce accumulation in the lysosome. When introduced into a *spin* mutant background, mutations in *atg1* or *atg18* rescue synapse overgrowth, resulting in a significant reduction in bouton number (Fig. 4A and B). Heterozygous combinations of *atg1* and *atg18* similarly restrict synaptic growth in *spin* mutants. Although we observe a general decrease in branching in the *atg* and *atg*:*spin* mutant combinations, we suggest that the data demonstrate that an *atg* mutation can restrain *spin* synapse overgrowth by reducing the storage burden in the lysosomes.

#### Signaling from Pre- and Postsynaptic Compartments Contributes to Synaptic Overgrowth.

We expressed the *hSOD1* transgene in either muscles only or nerves only in a *spin* mutant background. Under either condition, we partially rescued synaptic overgrowth (Fig. 4C). When we expressed the *fos*<sup>DN</sup> transgene in either nerves or muscles only in a *spin* mutant, we partially rescued synaptic overgrowth, each time by ~50% (Fig. 4D). This finding suggests that both the pre- and postsynaptic compartment can contribute to synaptic overgrowth under conditions of OS via activation of the JNK/AP-1 pathway. In support of this observation, we note the elevated expression of the *puc*<sup>E69</sup> β-gal reporter in muscles of *spin* mutants (Fig. 3C). Similarly, depletion of *atg5* in either the nerve or muscle using transgenic RNAi significantly reduced synaptic overgrowth in *spin* (Fig. 4E). We propose that this result suggests that autophagy in muscle can contribute to synaptic overgrowth in *spin* by increasing the storage burden on lysosomes and potentiating the subsequent OS.



**Fig. 4.** Autophagy is required for *spin* induced overgrowth. (A) Autophagy is required for *spin*-induced synapse overgrowth. Mutations in the autophagy genes *Atg1* (*atg1<sup>PZ</sup>/atg1<sup>DS</sup>*), *Atg18* (*atg18<sup>KG</sup>/Df*), and hemizygotes (*atg1<sup>PZ</sup>/atg18<sup>KG</sup>*) cause a reduction in bouton number to  $59 \pm 5.8$  ( $n = 19$ ),  $54 \pm 4.0$  ( $n = 28$ ), and  $68 \pm 6.3$  ( $n = 28$ ), respectively. These mutations also reduce *spin*-induced overgrowth to  $72 \pm 4.9$  ( $n = 28$ ),  $75 \pm 6.7$  ( $n = 16$ ), and  $76 \pm 5.8$  ( $n = 15$ ), respectively ( $P > 0.05$  compared with wild-type and *atg1<sup>PZ</sup>/atg1<sup>DS</sup>* ANOVA and  $***P < 0.001$  compared with *spin*). (B) Images of the NMJ at muscle 6/7 in segment A3, with nerves shown in magenta (HRP) and boutons in green (syt). (Scale bar, 20  $\mu$ m.) (C) Expression of *hSOD1* in the muscle or nerve partially rescues bouton number in a *spin* mutant to  $103 \pm 3.2$  ( $n = 16$ ) and  $106 \pm 8.0$  ( $n = 16$ ), respectively ( $***P < 0.001$ , ANOVA). (D) Expression of *fos<sup>DN</sup>* in the muscle or nerve partially rescues bouton number in a *spin* mutant to  $120 \pm 8.4$  ( $n = 11$ ) and  $113 \pm 9.5$  ( $n = 9$ ) ( $n = 16$ ), respectively ( $***P < 0.001$ , ANOVA). (E) Expression of *atg5IR* in the muscle or nerve partially rescues bouton number in a *spin* mutant to  $127 \pm 5.57$  ( $n = 16$ ) and  $126 \pm 6.51$  ( $n = 16$ ), respectively ( $***P < 0.001$ , ANOVA).

## Discussion

**OS Contributes to Synaptic Overgrowth and Electrophysiological Deficits in an LSD Model.** Our data show that OS causes growth of the *Drosophila* NMJ and is a contributory factor in the overgrowth seen in *spin* loss-of-function mutants, a model of a neurodegenerative LSD. OS, presence of lipofuscin, and dysregulated autophagy (5–7) are identified cellular hallmarks of LSD; excessive dendritogenesis and synaptogenesis are also observed (2, 3). The “pathogenic signaling cascade” driving synapse growth in LSD has yet to be identified. Lysosomes are rich in transition metals in a low pH and dysregulation in LSD produces an environment conducive for Fenton reactions generating ROS

(1, 27). OS induces autophagy, contributing further to lysosomal build-up of undegraded material. A positively reinforcing cycle ensues: impaired lysosomal degradation increases OS and the induction of autophagy, causing a failure of autophagic clearance. In a model of LSD in *Drosophila* we focus on the effects of OS on synaptic development. We show that the LSD-like mutant *spin* has increased oxidative damage and activation of the OS response. We suggest *spin* lysosomal dysfunction generates OS, as seen in known LSDs (7). Our data support the proposal that OS and the activation of JNK/AP-1 in *spin* mutants promotes the generation of synaptic overgrowth (Fig. S7). We rescue the majority of the synaptic overgrowth in *spin* by reducing the OS burden. Our data are supported by the observation that OS generated independently of lysosomal dysfunction promotes synapse growth. OS also contributes to synaptic dysfunction in *spin* mutants; progressive reduction in EJP amplitudes with high-frequency stimulation is observed (12) (Fig. 1D). This reduction in EJP amplitude is similar in size to that observed in synapses defective in endocytosis, such as *dap160*, or deficient for ATP synthesis (45–47). It has previously been reported that the electrophysiological defect seen in *spin* mutants is a result of altered synaptic vesicle recycling and is inferred from both a reduction in FM1-43 dye uptake and the presence of multilamellar structures in the NMJ (12). The deficit in synaptic transmission observed in *spin* mutants can be fully rescued by expression of either *hSOD1* or *fos<sup>DN</sup>* (Figs. 1D and 3B), suggesting that if OS and resultant *fos* signaling at the NMJ is reduced, normal synapse size and function can be restored.

**OS Induces Growth of the *Drosophila* NMJ.** The synergistic effect on bouton number seen in heterozygotes of *spin* and *sod1* are indicative of a threshold at which overgrowth is induced, indicating these mutations affect a shared pathway. An important suggestion that can be made as a result is that trafficking defects, previously suggested to cause overgrowth through continued expression/disinhibition of signaling pathways of growth signals (9, 48–50), may not be the only mechanism through which lysosomal dysfunction causes synapse overgrowth; OS may contribute to the activation of synaptic growth pathways.

**Components of the JNK/AP-1 Signaling Pathway Are Differentially Required for OS-Induced Growth.** The requirement for JNK, *fos*, and *jun* activity for OS-induced synaptic growth is found to be different between *spin*, *sod1*, *sod2* mutants, and paraquat-treated animals. This finding suggests differential activation of components of the JNK pathway in each condition. In *Drosophila*, *fos* can homodimerize and is known to act independently of *jun* in synapse overgrowth in *hiw* mutants and conditions of cytoskeletal disruption (19, 51). In *sod2* mutants (and paraquat treatment), OS is generated predominantly in mitochondria, and under these conditions we observe a role for *jun* in synapse growth. This observation potentially suggests a context-dependent role for *jun* in the regulation of synaptic growth, which may indicate divergent responses to different cellular sources of OS.

**Autophagy, OS, and Synaptic Growth.** OS is a potent activator of autophagy. Activation of autophagy has been shown to promote synapse growth in *Drosophila* via degradation of an upstream inhibitor of the JNK signaling pathway (20). Autophagic down-regulation of the *hiw* protein has been proposed as a mechanism to regulate synaptic growth (20). The *hiw* protein and its target wallenda (*wnd*) are both found presynaptically (19), but we observe JNK/AP-1 and autophagy activity contributing to synapse overgrowth in both the muscle and nerve. This observation suggests a previously unidentified muscle-derived JNK/AP-1 signal contributing to synapse growth that is likely to be independent of direct *hiw/wnd* regulation. The major identified transcriptional output of the JNK/AP-1 pathway is antioxidant and autophagic responses (24, 25). Autophagy and OS may directly contribute to the regulation of synapse growth mecha-



nisms. The function of the cell-adhesion protein DE-cadherin is sensitive to OS (52), although the generality of this finding has yet to be defined. Autophagy has been observed to selectively phagocytose receptor proteins from an identified synapse (53, 54), suggesting a mechanism where autophagy can directly regulate the function and growth of the synapse independently of highwire degradation.

Taken together, our data suggest that the highly conserved JNK/AP-1 signaling pathway, a well-known mediator of synaptic growth and function, can be activated by OS to induce synaptic overgrowth. Many neurodegenerative disorders generate an OS burden in affected neurons. Investigating the effects of OS on synaptic development and function, as well as identifying signaling pathways, in such a disease context provides a potentially important insight into the pathology of a number of neurodegenerative diseases.

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## Materials and Methods

Wandering third-instar larvae were dissected and stained as described previously (9). Wandering third-instar larvae were dissected in modified HL3 (12). Intracellular recordings were made in muscle 6 from segments A3. Detailed methods can be found in *SI Materials and Methods*.

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