
Figures and figure supplements

Adverse impact of female reproductive signaling on age-dependent neurodegeneration after mild head trauma in *Drosophila*

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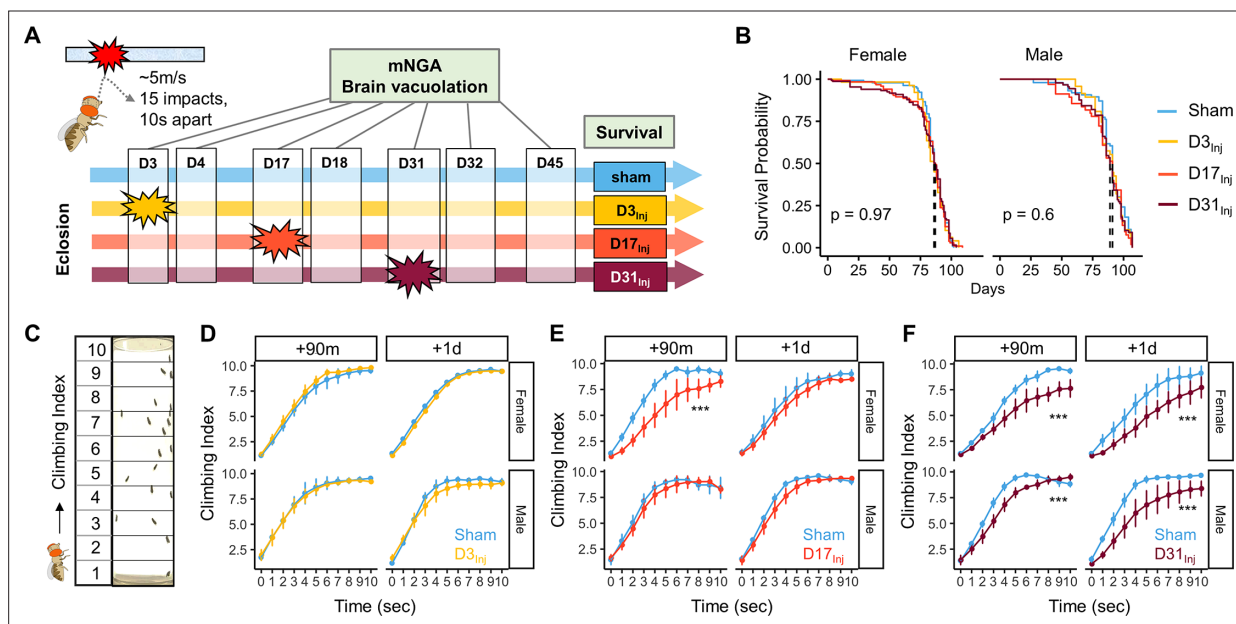


Figure 1. Exposure of adult fruit flies to a very mild form of repetitive head impacts at different ages elicits minimal acute effects. **(A)** Schematic illustration of the experimental design. Male and female flies were exposed to very mild head trauma (vmHT) on D3, D17, or D31 post-eclosion (denoted as D3_{inj}, D17_{inj}, and D31_{inj}), whereas the sham groups did not receive vmHT. Sensorimotor behavior and brain pathology were assessed at 90 min and 1 day after vmHT exposure using modified negative geotaxis assay (mNGA) and imaging-based quantification of vacuolization. **(B)** Survival curves showing no significant alteration in lifespan among different age-at-injury groups and between sexes. Kaplan–Meier p-values were determined using the Mantel–Cox log rank test with Bonferroni correction. Total N = 871, n > 50 flies in each condition. **(C)** Schematic diagram depicting mNGA where the Climbing Index (CI) at each second of the 10 s trial was calculated using the number of flies in each of the 10 height bins (see ‘Materials and methods’ for details). **(D–F)** CI plots depicting acute effects of vmHT on the climbing behavior of D3_{inj}, D17_{inj}, and D31_{inj} cohorts. **(D)** Both male and female D3_{inj} flies showed no deficits in climbing at 90 min and 1 day post-injury. **(E)** Only female D17_{inj} flies exhibited climbing deficits at 90 min post-injury (**p=5.75e-08) but they recovered 1 day post-injury. **(F)** Both male and female D31_{inj} flies exhibited marked decline in climbing ability at both time points (p-values: 5.36e-08 and 6.2e-06 for female + 90m and +1d; 0.00033 and 1.91e-07 for male +90m and +1d). A total of nine videos were used for each condition: three experimental repeats of three trials, number of flies in each video n ≥ 10. Error bars: standard error (±se). Repeated-measures ANOVAs were conducted to examine the effects of injury on climbing indices at each second.

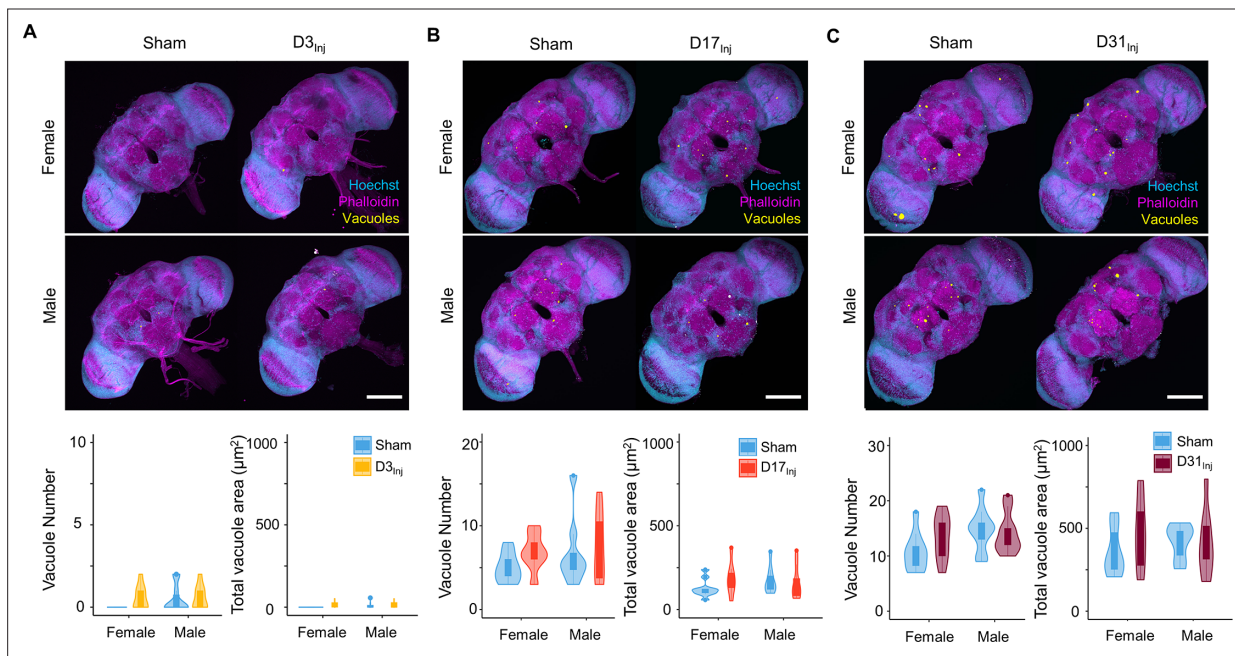


Figure 2. Exposure to very mild head trauma (vmHT) does not acutely increase vacuole formation in the brain. (A–C) Two-photon whole brain imaging and quantification of vacuoles in D3_{inj} (A), D17_{inj} (B), and D31_{inj} (C) flies. Top panels: representative z-projected whole-brain images of different injury groups with their respective sham brains of both sexes. Vacuoles are highlighted by yellow color. Scale bar = 100 μm . Bottom: violin plots and boxplots represent quantification of vacuole number and total vacuole area in each condition. Boxplots whiskers correspond to the maximum 1.5 interquartile range. Two experimental replicates resulting in $n > 10$ brains for all conditions. Stats: nonparametric Wilcoxon rank-sum tests, $p > 0.05$.

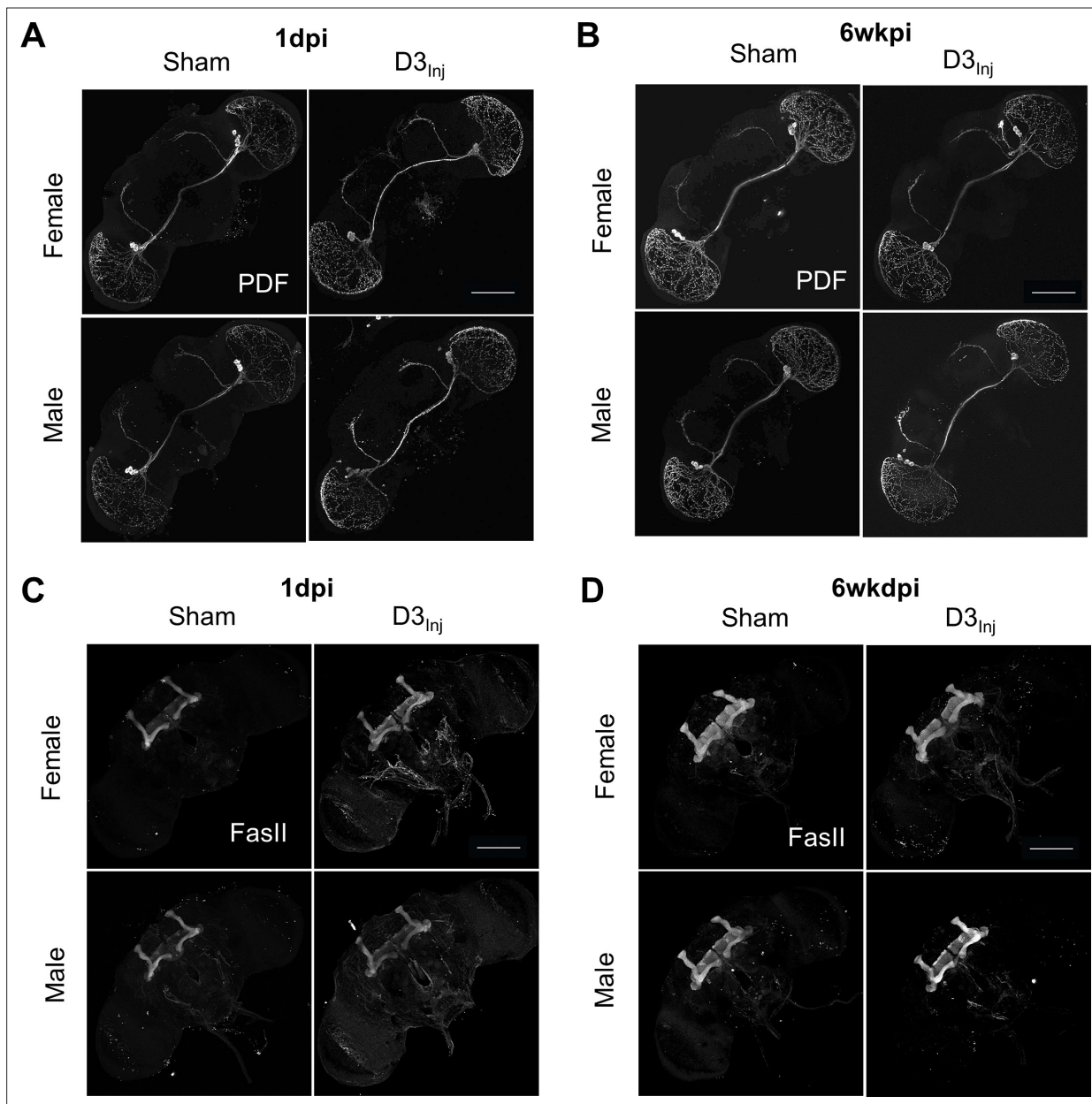


Figure 2—figure supplement 1. Exposure to very mild head trauma (vmHT) on D3 does not elicit gross morphological alterations in pigment dispersing factor (PDF) neurons and mushroom bodies. **(A, B)** vmHT exposure on D3 did not significantly alter PDF neuron axonal projections or dendritic arborizations when assessed 24 hr or 6 weeks after injury. **(C, D)** vmHT exposure on D3 did not visibly alter mushroom body axon bundles when assessed 24 hr or 6 weeks after injury. Scale bar = 100 μ m.

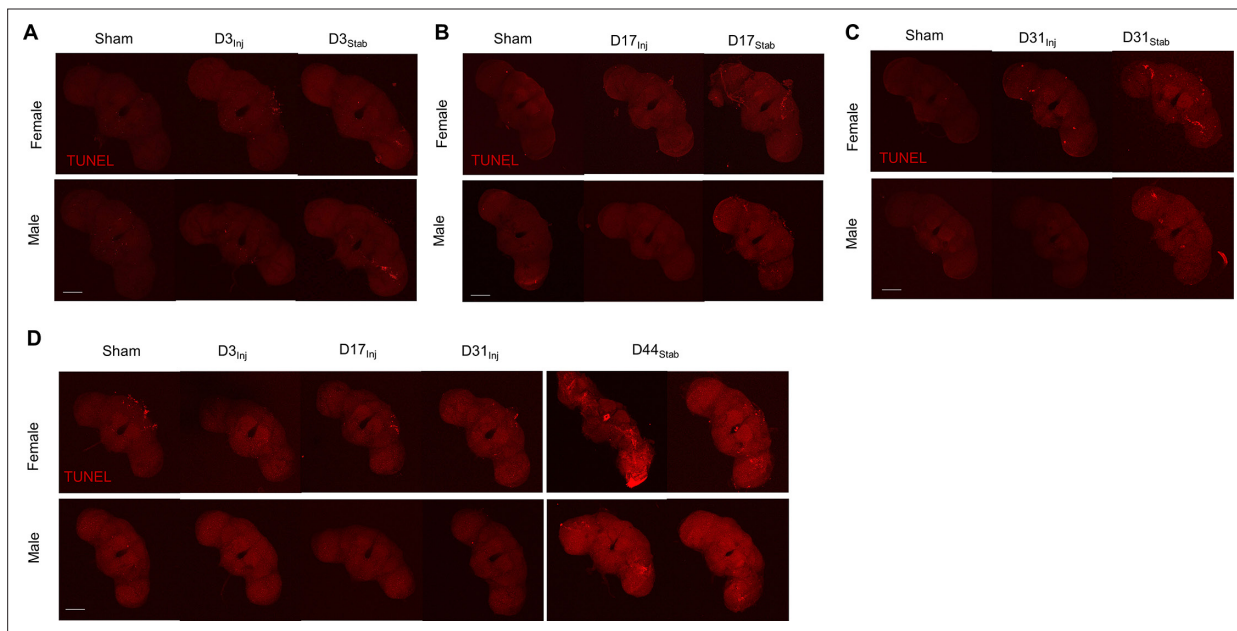


Figure 2—figure supplement 2. Exposure to very mild head trauma (vmHT) does not elicit significant apoptosis. (A–C) Representative images of TUNEL staining in *Drosophila* brains at 1 day post D3_{Inj}, D17_{Inj}, and D31_{Inj}. There was limited TUNEL signal in both sexes and no differences in staining pattern and signal intensity were observed between sham and injured groups. A set of flies were subject to stabbing injuries at the same time and their brains were used as positive controls of apoptosis (right panels). TUNEL staining was visible in the optic lobes or other regions that were penetrated by the fly pin. (D) Representative images of TUNEL staining in sham and injured brains on D45. vmHT were delivered on D3, D17, or D31. Stabbing injuries were performed on D44 (24 hr prior to fixation). No differences in staining pattern and signal intensity were observed between sham and injured groups and between sexes. In comparison, brains of stabbed flies (right panels) exhibit much stronger TUNEL signal intensity and region-specific apoptosis near the location of primary injury. Scale bar = 100 μ m. Each condition contained n = 15 flies.

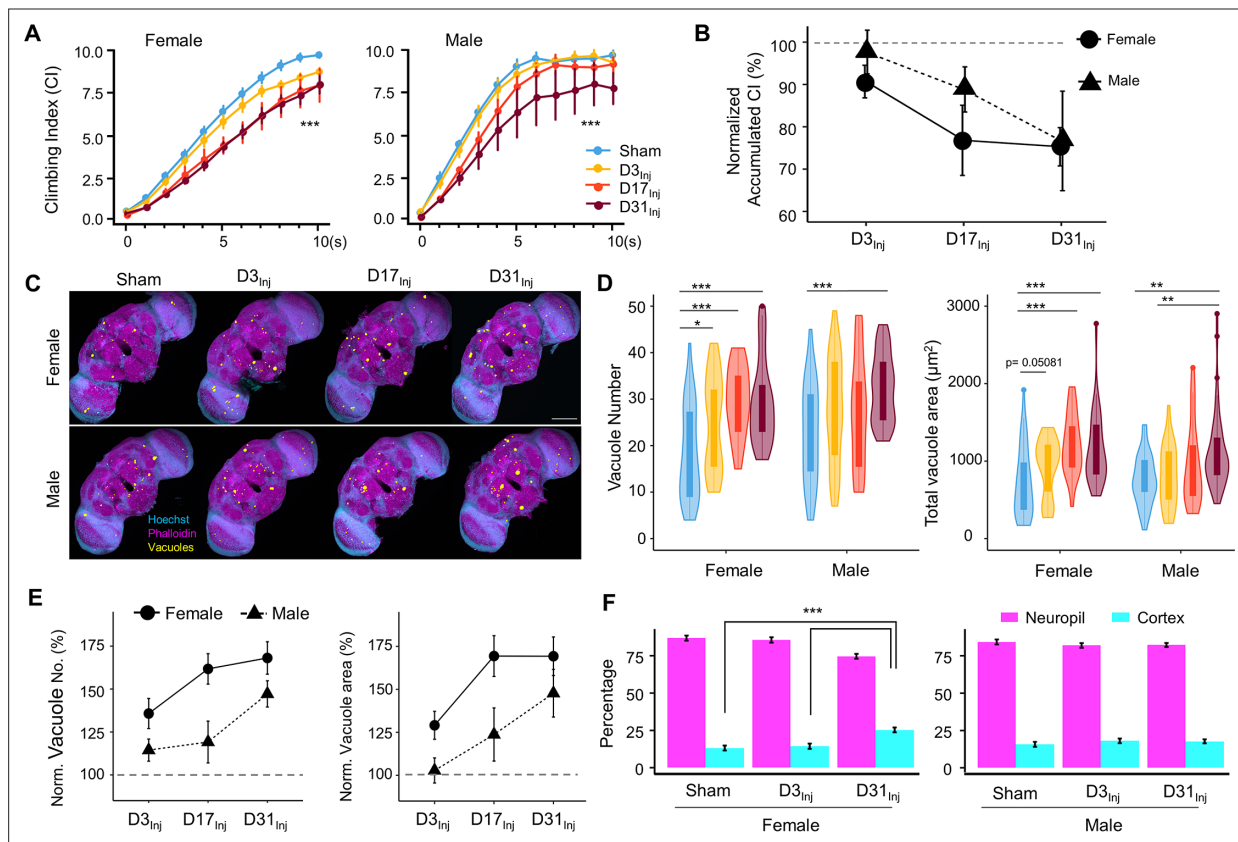


Figure 3. Exposure to very mild head trauma (vmHT) results in late-life brain deficits and neurodegeneration. **(A)** Exposure to vmHT at various ages altered negative geotaxis behavior when assessed on D45. A total of nine videos were used for each condition: three experimental repeats of three trials, number of flies in each trial $n \geq 10$. Error bar: \pm se. Repeated-measures ANOVA and Bonferroni post hoc tests were conducted to examine the effects of injury on climbing indices at each second. Overall, vmHT exposure reduced Climbing Index (CI) for females (***) and males (***) $p=1.2e-11$). See **Supplementary file 1c** for p-values from pairwise comparisons by injury conditions and time. **(B)** Sex differences in vmHT-induced climbing impairment are associated with age-at-injury. D3_{Inj} and D17_{Inj} females exhibit a stronger decline in normalized accumulated CI compared to males. D31_{Inj} females and males, on the other hand, suffered a similar substantial reduction in accumulated CI. Accumulated CI data of injury groups are normalized to their respective sham levels. **(C)** Representative z-projected whole images depicting vacuole formation in each condition (sex/injury group). Scale bar = 100 μ m. **(D)** Quantification of vacuole number and total vacuole area in each condition (total N = 352, $n > 30$ in each condition). Here all sham groups were combined by sex. Boxplots whiskers correspond to the maximum 1.5 interquartile range. Statistics: nonparametric Wilcoxon rank-sum tests. See **Supplementary file 1c** for p-values from pairwise comparisons by injury conditions. **(E)** Sex differences in the increase of brain vacuolation were partially dependent on age-at-injury. Vacuole number and total vacuole area of each injury condition were normalized to their respective sham groups. Overall, females exhibited a higher percentage of increase in vacuolation than males. **(F)** Quantification of the percentage of vacuoles in neuropil and cortex region of the brain in sham, D3_{Inj}, and D31_{Inj} groups of both sexes. Data was from two independent trials of $n = 15$ brains in each condition. Two-sample t-tests were used to compare the percentage of vacuoles in neuropil and cortical region between each injury condition. D31_{Inj} females had significantly less percentage of vacuoles in the neuropil region compared to the sham group (***) $p=2.19493e-6$.

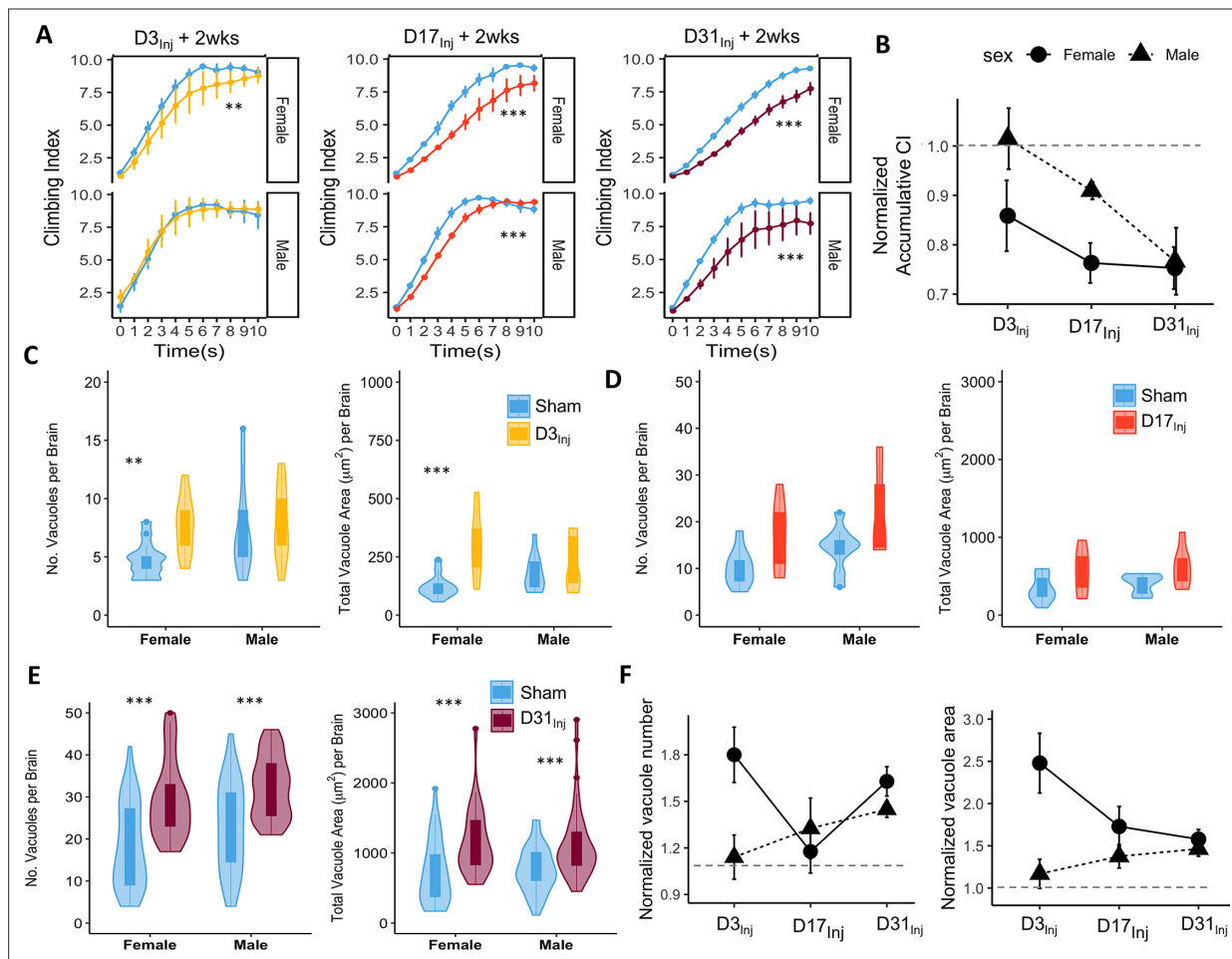


Figure 3—figure supplement 1. Age-at-injury affects post-injury recovery after 2 weeks. Climbing Index (CI) plots depicting sensorimotor behavior assessed two weeks following D3_{Inj}, D17_{Inj}, and D31_{Inj}. Female D3_{Inj} groups exhibited a significant decrease in negative geotaxis when assessed on D17 (** $p=0.00114$), whereas males did not ($p>0.05$). Both female (** $p=1.53\text{e-}10$) and male (** $p=2.47\text{e-}05$) D17_{Inj} groups suffered sensorimotor deficits when assessed on D31. D31_{Inj} flies all exhibited significant detriment to climbing behavior when assessed on D45 (** $p=2\text{e-}16$ for female, *** $p=2.58\text{e-}07$ for male). Repeated-measures ANOVA was used to calculate statistical significance. A total of three experimental repeats and a total of nine videos were used in each condition. $n > 15$ per condition per repeat. Error bar: \pm se. **(B)** In both male and female flies, older age-at-injury is associated with larger decreases in accumulated climbing indices normalized against the respective sham controls. Error bar: \pm se. **(C–E)** Quantification of vacuole formation at 2 weeks following D3_{Inj}, D17_{Inj}, and D31_{Inj}. D3_{Inj} increased vacuole number (** $p=0.0017$) and total vacuole area (** $p=0.00016$) in female brains but not in male brains. D17_{Inj} did not significantly alter vacuole formation in both sexes though there seems to be a trend toward injury increasing both vacuole size and vacuole number. D31_{Inj} increased both vacuole formation in females and males. Two repeats resulting in $n > 12$ brains per condition. Wilcoxon rank-sum tests were used to calculate p-values. From left to right, *** $p=2.6\text{e-}05$, *** $p=4.5\text{e-}05$, *** $p=8\text{e-}05$, and *** $p=0.00048$. **(F)** Vacuole number and total vacuole number in each injury condition were normalized against their respective sham groups. Older age-at-injuries were associated with accelerated vacuole formation in males, but this association was not observed in females. Error bar: \pm se.

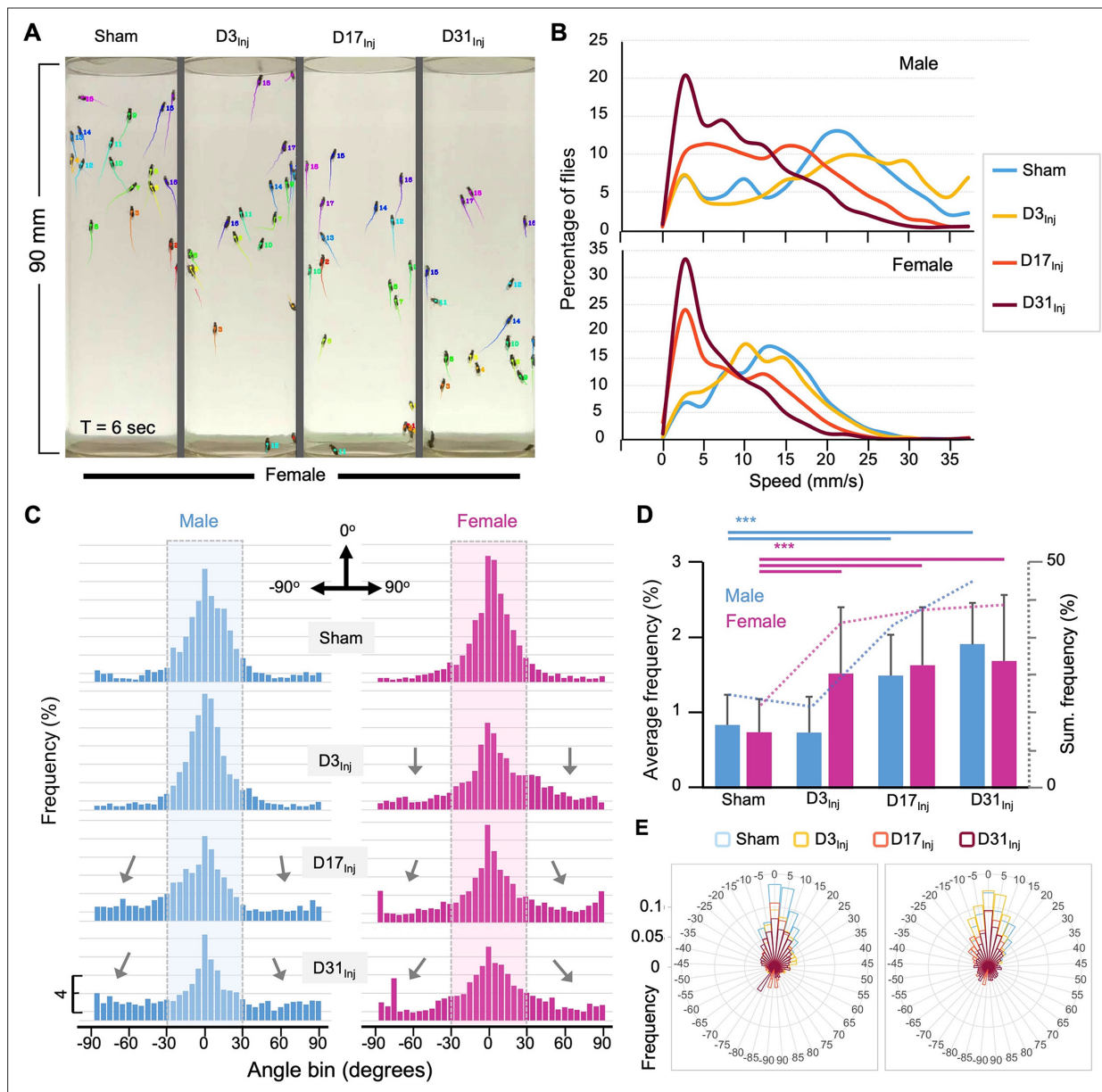


Figure 4. AI-assisted tracking and quantification of individual fly's behavior provides insight into defects in the speed and direction of movement. **(A)** Representative snapshot of idtracker.ai-generated video at T = 6 s. Videos used for analyses were from D45 flies (see **Video 1**). Each tail represents a fly's movement trajectory from the last 30 frames. Color is auto-assigned to individual flies in each trial. **(B)** Histogram plots of the climbing speed of individual flies at a temporal resolution of 1/60 s. The y-axis depicts the percentage of flies with a specific speed (x-axis). **(C)** Angular histograms showing the angle distribution of individual flies during the first 3 s of modified negative geotaxis assay (mNGA) trial. The highlighted sections represent normal fly directional orientation (between -30° and 30° with 0° as vertical). Arrows indicate the increased incidents of climbing angles outside the normal range. **(D)** Average percentages and accumulated percentages of flies with abnormal directional movement ($<-30^{\circ}$ or $>30^{\circ}$) during the first three seconds of NGA trial. Two-sample t-tests, $***p < 0.001$. Error bar: se. **(E)** Rose plot depicting overall frequency distribution of fly orientation during the first 3 s of NGA trial.

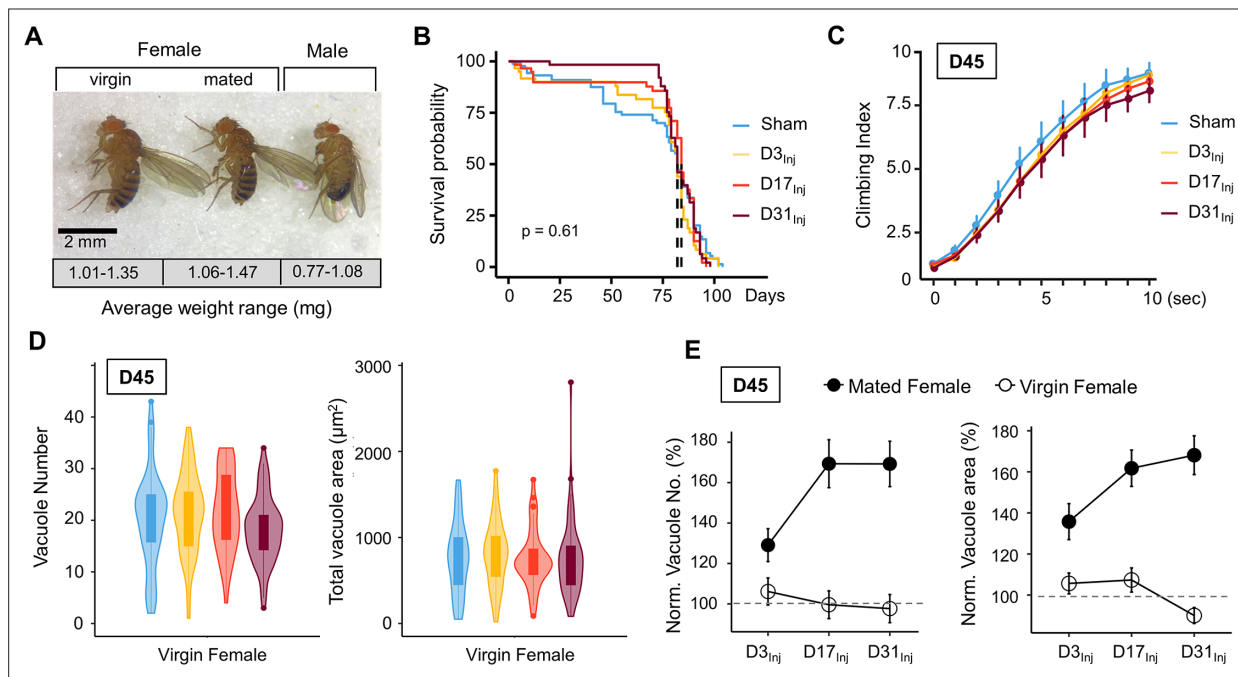


Figure 5. Virgin females do not exhibit neurodegenerative conditions regardless of age-at-injury. **(A)** Comparison of size and mass of virgin female, mated female, and male flies. Three separate trials of $n > 100$ in each condition. Total mass in each trial was averaged over the number of flies to generate a weight range for each condition. **(B)** Very mild head trauma (vmHT) resulted in no significant change to the lifespan of virgin females. Kaplan-Meier p-values were determined using the Mantel-Cox log rank test with Bonferroni correction. Total $N = 271$, $n > 30$ flies in each condition. **(C)** vmHT did not affect climbing behaviors of virgin females on D45 regardless of the age-at-injury. A total of nine videos were used for each condition: three experimental repeats of three trials, number of flies in each trial $n \geq 10$. Repeated-measures ANOVAs were conducted to examine the effects of injury on climbing indices at each second ($p > 0.05$). Error bar: \pm se. **(D)** vmHT did not increase vacuole number or total vacuole area on D45 regardless of the age-at-injury. Total $N = 224$, $n > 25$ for each condition. Statistics: nonparametric Wilcoxon rank-sum tests. **(E)** Plots depicting differences in vacuole formation between virgin and mated females. Vacuole number and total vacuole area of each injury condition were normalized to the respective sham controls. Mated females exhibited much higher percentage of increase in vacuolation than virgin females. Error bar: \pm se.

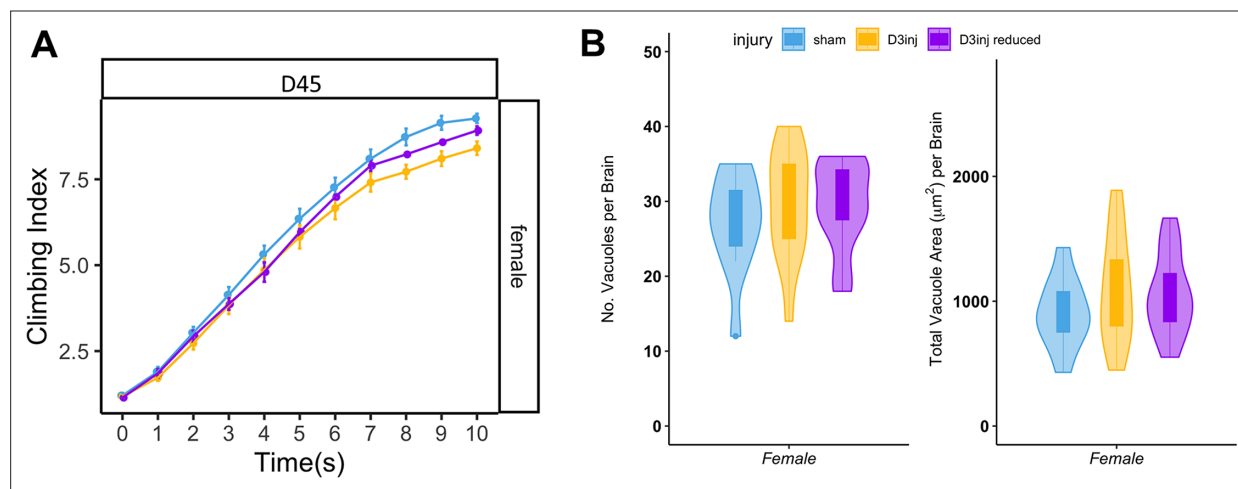


Figure 5—figure supplement 1. Exposure to reduced very mild head trauma (vmHT) on D3 elicited similar late-life behavioral deficits and brain pathology as exposure to regular vmHT. (A) Climbing Index (CI) plots depicting decreases in CI in both regular D3_{inj} and reduced D3_{inj} females when climbing behavior was assessed on D45. Error bar: \pm se. (B) Quantification of vacuole formation on D45. Vacuolation was similarly elevated in reduced D3_{inj} brains and in regular D3_{inj} brains. Only one experiment was performed for reduced D3_{inj} (n = 15). Boxplots whiskers correspond to the maximum 1.5 interquartile range.

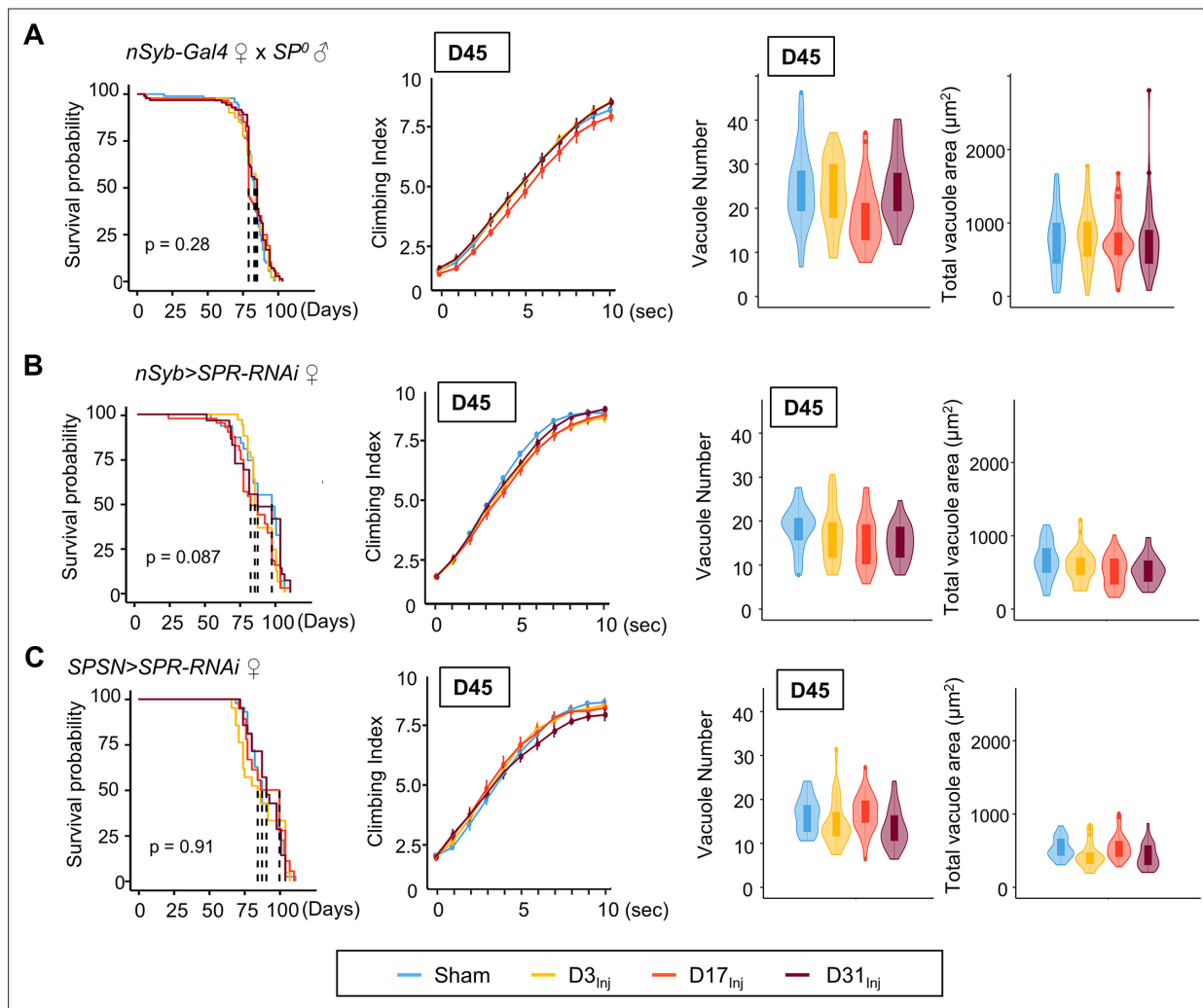


Figure 6. Eliminating SP signaling mitigates the emergence of neurodegenerative conditions in the female. **(A–C)** Survival curves, modified negative geotaxis assay (mNGA) quantification, and vacuole quantification of female flies mated to *SP⁰*-males, females with pan-neuronal RNAi knockdown of SPR mated to wildtype males, and females with SPSN-specific knockdown of SPR mated to wildtype males ($n > 30$ in each condition). No difference was detected between sham and injured groups in all three genotypes. Error bar: \pm se. Boxplots whiskers correspond to the maximum 1.5 interquartile range. Stats: nonparametric Wilcoxon rank-sum tests, $p > 0.05$.

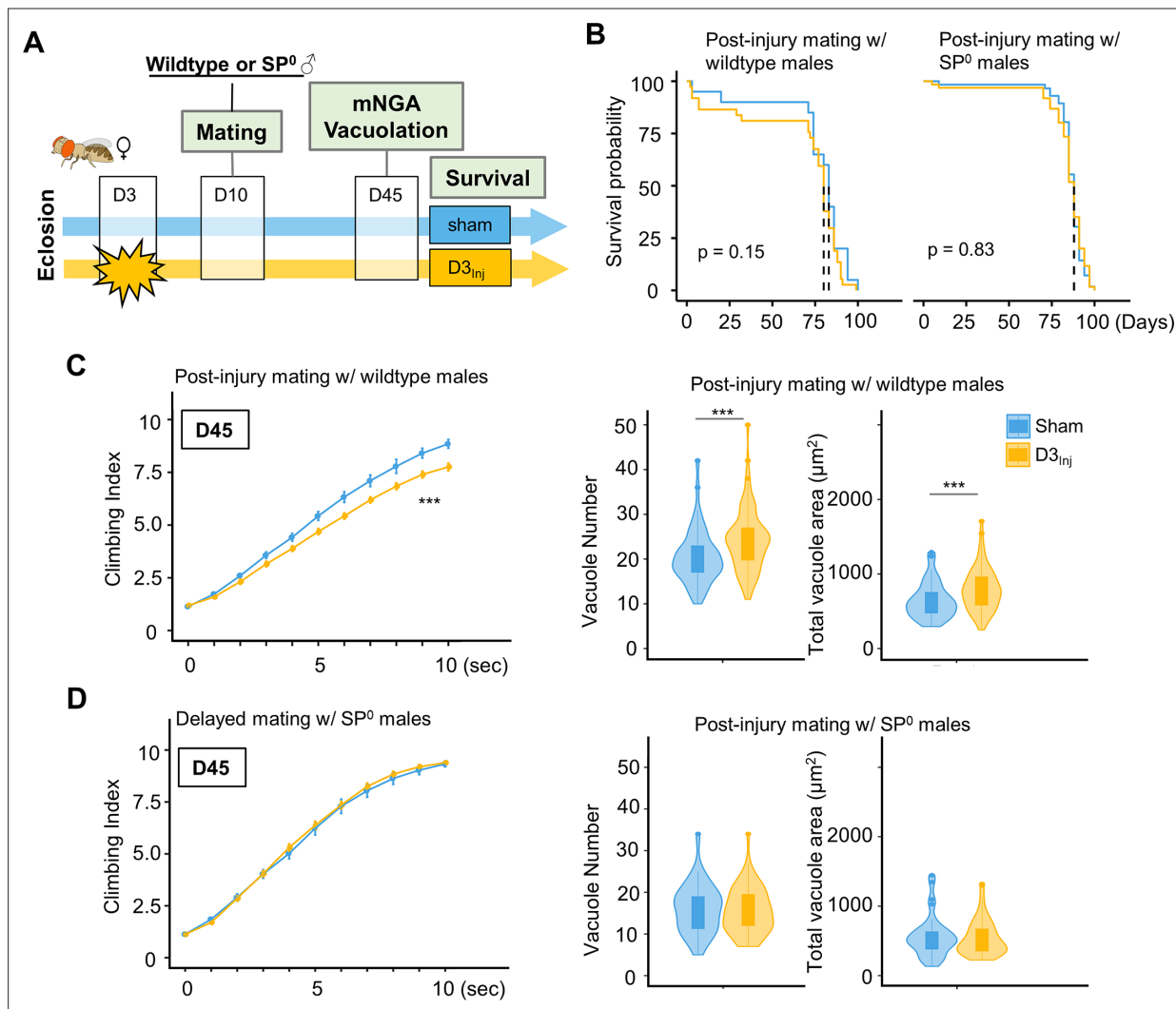


Figure 7. Introducing SP signaling to pre-injured virgin females reinstates neurodegenerative phenotypes. **(A)** Diagram of mating schematic and relevant behavioral and pathological assays. Virgin female flies were subjected to very mild head trauma (vmHT) on D3, whereas sham flies never received an injury. On D10, these females were exposed to either wildtype or SP⁰ males for 24 hr to allow mating. Modified negative geotaxis assay (mNGA) and brain vacuolation were assessed on D45. **(B)** No change in the lifespan was observed for females that were subjected to vmHT as virgin followed by post-injury mating with either male group. N = 182, n > 40 for each condition. **(C)** When assessed on D45, females subjected to post-injury mating with wildtype males exhibited significant sensorimotor deficits (**p=3.4e-15) and vacuole formation (vacuole number: p=0.0004536, vacuole area: p=0.0009563). A total of nine NGA videos were used for each condition: three experimental repeats of three trials, number of flies in each trial n ≥ 10. Error bar: ±se. Repeated-measures ANOVA and Bonferroni post hoc tests were conducted to examine the effects of injury on climbing indices at each second. For vacuole analyses, N = 149, n > 30 in each condition. **(D)** When assessed on D45, females subjected to post-injury mating with SP⁰ male flies did not elicit sensorimotor deficits and vacuole formation. A total of nine NGA videos were used for each condition: three experimental repeats of three trials, number of flies in each trial n ≥ 10. For vacuole analyses, N = 105, n > 20 in each condition.

