Loss of highwire protects against the deleterious effects of traumatic brain injury in Drosophila Melanogaster

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Abstract

Background

Traumatic brain injury (TBI) is a major global cause of death and disability. Axonal injury is a major underlying mechanism of TBI and could represent a major therapeutic target. We provide evidence that targeting the axonal death pathway known as Wallerian degeneration (WD) improves outcome in a Drosophila Melanogaster model of high impact trauma. This cell-autonomous neurodegenerative pathway is initiated following axon injury, and in Drosophila, involves activity of the E3 ubiquitin ligase highwire. In this study we explore the effects of that a loss-of-function mutation in the highwire gene has on a range of outcomes following high impact trauma.

Results

Results demonstrate that a loss-of-function mutation in the highwire gene rescues deleterious effects of a traumatic injury, including - improved functional outcomes including climbing ability and flight maintenance, lifespan, survival of a subset of dopaminergic neurons, and retention of synaptic proteins.

Conclusion

We demonstrate that a loss-of-function mutation in the highwire gene rescues deleterious effects of a traumatic injury. This data suggests that highwire represents a potential therapeutic target in traumatic injury.

Background

Traumatic brain injury (TBI) is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force.[1] It is a major cause of death and disability, and each year in Europe alone ~ 2.5 million people will experience TBI, 1 million of which will be admitted to hospital, and 75,000 will die.[2] Various secondary injury cascades follow the initial primary insult of TBI, promoting ongoing neuronal cell loss. The secondary injury response is multifaceted and comprises a number of processes including Wallerian degeneration (WD).[3]

WD is an active, cell-autonomous death pathway that leads to degeneration of the distal axonal segment following transection.[4] In some neuronal subtypes WD following axonal injury may lead to a progressive dying back of the proximal axon segment and death of the neuronal cell body.[5] A related process, Wallerian-like degeneration, occurs through the same molecular mechanisms as WD but does not require a complete axonal transection and instead is typically characterised by an impairment of axonal transport.[4] WD and Wallerian-like degeneration are both thought to play a role in secondary brain injury following TBI although complete axotomy that occurs at the time of an injury (primary axotomy) is uncommon, and the degree to which sub-axotomy level injuries subsequently impair axonal transport and induce Wallerian-like degeneration remains incompletely characterised.[6–10]
Activation of the WD pathway is fundamentally linked to the nicotinamide adenine dinucleotide NAD synthetic pathway (Supplementary Fig. 1A). Nicotinamide mononucleotide adenylyl transferase (NMNAT) is a key enzyme in the WD/Wallerian-like degeneration pathway, converting nicotinamide mononucleotide (NMN) to nicotinamide adenine dinucleotide (NAD). NMNAT also displays chaperone function during stress responses.[11–15] Following transection, the delivery of the axonal isoform of NMNAT (NMNAT2) from the neuronal cell body to the distal axon is compromised. As NMNAT2 is degraded NMN accumulates and levels of NAD fall.[13, 16–18] This results in activation of the toll-like receptor adaptor protein; sterile motif-containing and armadillo-motif containing protein (SARM1) resulting in axon fragmentation (Supplementary Fig. 1B).[19–22]

Evidence for a role of WD/ Wallerian-like degeneration in TBI has emerged from rodent cortical-contusion injury studies. The first WD gene to be explored in a model of TBI was the slow Wallerian degeneration gene (Wlds). This gene encodes a mutant protein (WLDs) that can substitute for NMNAT, and Wlds expressing mice demonstrate less motor and cognitive impairment following a cortical-contusion injury. [23] Similarly, knockout of SARM1 was associated with reduced neuronal loss and cognitive impairment following CCI.[24] These studies suggest that therapeutic modulation of the Wallerian-like degeneration pathway may be possible, and potentially could improve outcomes from TBI.[3]

Another tractable context to explore the biology of WD is Drosophila Melanogaster. Loss-of-function mutations in the highwire (hiw) gene in D. Melanogaster are associated with a strong delay in axon degeneration following transection both in vitro and in vivo.[25–28] Delayed WD is also seen with mutations in the mammalian ortholog of hiw; PHR1.[4, 29, 30] The hiw gene encodes a large 5233 amino acid protein with E3 ubiquitin ligase activity that modulates levels of dNMNAT.[25, 26, 30, 31] Hiw also has presynaptic regulatory activity that is required to control excess synaptic growth at the neuromuscular junction.[25, 31, 32] To investigate for potential modifiers of brain trauma we utilised a model of high impact trauma (HIT) in D. Melanogaster in which WD pathways show extensive conservation with mammalian species.[4, 19, 20, 30, 33–35] Given the role of hiw in dNMNAT depletion and subsequent axon degeneration, and the assessment that Wallerian-like degeneration may contribute to the secondary brain injury seen in TBI, we hypothesised that flies with a null mutation in hiw (hiwΔN) would show protection against the effects of TBI. We show that hiwΔN flies showed relative protection against long-term mortality and cell death. Brain vacuolation and necrotic cell death occurred regardless of genotype – suggesting that the hiw mutants still underwent a neurodegenerative process but there was reduced presynaptic marker depletion and dopaminergic (PPL1) neuron loss. This suggests that a subset of vulnerable – but functionally important- neurons may be rescued by hiw loss-of-function. This translated to a preservation of normal behavioural measures. These findings suggest that hiw and its mammalian ortholog PHR1 are potential therapeutic targets in experimental and clinical TBI.

Materials And Methods

Drosophila Melanogaster Stocks and Conditions
Hiw mutant hiwΔN and control hiwWT (FRT19AΔn) flies were obtained from Marc Freeman (University of Massachusetts). Newly enclosed flies were collected daily, separated by sex, into vials of 20-35 flies, and aged for experimental use. All experiments were conducted on flies aged 1-4 days unless otherwise stated. All flies were maintained at a constant 25°C temperature and humidity, in glass vials with standard agar/commeal/yeast feed. Flies were exposed to a 12h light-dark cycle. Feed was changed in all vials once every 14 days or sooner as required. All experiments were conducted exclusively on male flies in order to avoid confounding effects relating to the female reproductive cycle.

**High impact trauma device, injury calibration, incapacitation rates, and intestinal barrier dysfunction**

Flies were subjected to a standardised impact with the HIT device. After injury, vials were laid on their side and flies were given a minimum of 10 minutes to recover motility before being transferred to a glass vial containing standardised feed. All polystyrene vials were discarded after a single use. The severity of injury was calibrated in hiwWT flies by assessing the death rates 24 hours following HIT when the angle of initial deflection, and thus recoil force, was adjusted. Incapacitation rates were recorded by assessing the percentage of flies that failed to show signs of purposeful movement within 20 seconds of initial impact. To evaluate intestinal barrier dysfunction following HIT flies were transferred to feed containing dissolved Brilliant Blue FCF dye (#80717, Sigma). After 24 hours the percentage of flies that had blue food dye dispersed outside of the abdominal cavity and proboscis were counted.

**Early death rate and long-term survival assay**

To assess for variation in early death rates we exposed flies to a HIT at a standardized time of day. Any flies dying immediately or within the first 24 hours of a HIT were considered to have died of the undifferentiated primary effects of a HIT. Dead flies were removed and all remaining live flies were transferred to new vials and long-term survival was monitored. A daily count of number of fly deaths was conducted in all vials for the lifetime of all flies. Dead flies were discarded every day.

**Rapid iterative negative geotaxis (RING) assay and flight assay**

A custom made rapid iterative negative geotaxis (RING) device was manufactured and used to measure negative geotaxis/climbing ability as a behavioural measure of motor function.[36, 37] Flies were gently transferred to fresh empty polystyrene vials without anaesthesia with a maximum density of 25 flies per vial. Groups of up to 6 vials were inserted into the RING device, and after 5 minutes for the flies to adjust to the environmental change the device was tapped three times to settle flies to the bottom of the vials. Exactly 5 seconds after the last tap a picture was taken to assess the height climbed. The head of the fly was the reference point for the climbing height achieved. Maximum height achieved was graded into 5mm intervals, flies that climbed less than 5mm were scored zero, and any fly that exceeded 50mm was awarded the maximum score was 5cm. The average height achieved for the vial was calculated. This was repeated 3 times at 60 second intervals and an average score given for that vial. The reduction in climbing ability was calculated on a vial by vial basis by subtracting the baseline height climbed preinjury from the final height climbed at 45 days. For the flight assay, flies were anaesthetised on ice for exactly 5
minutes then the flat of a 30G 1” needle (#Z192368, Sigma) was attached to the anterior notum of a fly just posterior to the neck using clear nail varnish, leaving flight muscles unimpeded. Flies were given 15 minutes to fully recover. Needles were fixed in place under a video microscope. If required then a gentle mouth-blown puff of air was used to stimulate flight and the flying time was recorded for 30 seconds per fly for analysis. This was repeated 3 times per fly and the average of time spent in flight was calculated for each condition. All RING and flight assays were conducted at the same time of day in a quiet room with standardized light and environmental conditions.

**Haematoxylin and eosin histology, and vacuole counting**

Anaesthetised flies were submerged in cold 1x PBS, the proboscis and rostral trachea were removed, and the amputated heads gently rocked in fresh ice cold 4% paraformaldehyde solution for 45 minutes. The tissue was alcohol dehydrated, xylene washed, and embedded in paraffin for serial sectioning with a microtome (Leica RM2235) at a thickness 7μM. Sections were mounted on poly-L-lysine coated slides (P0245, Sigma). Wax was removed with a xylene bath then alcohol washes before haematoxylin and eosin staining, and application of coverslips. After blinding, three representative coronal sections were examined from a central brain region that included the medulla using brightfield microscopy. The average number of ³5μM vacuoles per slice in each brain was calculated.

**Immunohistochemistry**

Fly brains were dissected in cold 1x PBS and fixed in 4% paraformaldehyde-PBS for 30 minutes. Samples were washed in 1x PBS with 0.3% Triton X-100 (#T8787, Sigma) and blocked for 1 hour at room temperature in 1x PBS with 0.3% Triton X-100 and 1% BSA (#9647, Sigma). Brains were incubated in primary antibody diluted with blocking solution for 72 hours. After washing and incubating in a fluorescent secondary antibody solution for 4 hours, samples were washed and mounted between two coverslips in ProLong diamond antifade mountant (#P36965, ThermoFisher). Confocal images were acquired on a Leica imaging system, at z-stack intervals not greater than 0.6μM and blinded for analysis. Primary antibodies used were mouse Tyrosine-Hydroxylase (anti-TH) antibody 1:100 (TH-antibody, #22941, Immunostar Inc.) for the PPL1 cluster. Secondary antibodies were goat anti-mouse IgG (H+L) Alexa Fluor 488 (#A11034, ThermoFisher), and donkey anti-rabbit IgG (H+L) Alexa Fluor 594 (#21207).

**Immunoblotting**

Fifteen whole fly heads were collected and homogenized in Laemmli sample buffer and centrifuged 13,000 rpm for 5 minutes. Supernatant protein lysates were resolved by SDS-PAGE on Mini-protean 4-15% SDS resolving gel (#4561086, Bio-Rad) and transferred to Immobilon-P PVDF membrane (#IPVH00010, Merck). They were blocked in a 1% BSA solution (#9647, Sigma), then probed with the following primary antibodies: Bruchpilot 1:5000 (nc82, #2314866, DSHB), Discs-large 1:10,000 (4F3, #528203, DSHB), Neuroglian 1:5000 (BP104, #528402, DSHB), and β tubulin 1:5000 (E7, #2315513, DSHB). Bands were detected with goat anti-mouse, and goat anti-rabbit horseradish peroxidase-linked secondary antibodies.
(#1706515 and #1721011, Bio-Rad) and Supersignal West Dura extended duration chemiluminescence substrate (#34075, ThermoFisher). Density of bands was quantified using Image J software (v1.51n).

Flow cytometry

Five fly brains were dissected in cold 1xPBS solution and transferred to dissection media containing 7.5mls of DMEM (high glucose, HEPES, phenol-red free, #21063029, ThermoFisher), 2.5mls of 10x trypsin (#15400054, ThermoFisher), and 1% BSA (#9647, Sigma). Brains were washed in trypsin free dissection media, gently triturated using a 200μL pipette 30 times, then filtered through a 70μM strainer. The resulting solution was mixed in a 1:1 ratio with Annexin V & dead cell solution (Annexin V & 7-AAD, #MCH100105, Merck), incubated at room temperature for 20 minutes, then processed on the Muse Cell Analyser (Merck) using inbuilt analysis software. Dilutions in trypsin free dissection media were as required. For positive controls brains were incubated in 200mM of Actinomycin D (#A1410, Sigma) at 37°C for 6 hours before processing. Gating was kept constant for all experiments.

Results

A Drosophila Melanogaster high impact trauma device produces a quantifiable injury that demonstrates genotype-dependent variation in early survival

To model TBI in D. Melanogaster we utilised the HIT device. This consists of a spring-loaded attachment that holds a polystyrene vial of flies and inflicts a rapid acceleration-deceleration impact injury (Supplementary Fig. 2A). The severity of injury correlated with the angle of initial deflection of the device spring. An angle of 90° produced an average death rate of 22% at 24 hours and 30% at 7 days post-injury in wild type (hiwWT) flies (Supplementary Fig. 2B). External injuries were never seen at angles of 90° or less but were common (82%) at an angle of 135°, therefore the 90° was chosen to represent a single severe HIT in subsequent studies. Our choice of HIT severity was also based on the observed incapacitation rate - defined as the proportion of flies that demonstrated a lack of purposeful movement 20 seconds or more following trauma. The incapacitation rate was comparable in both hiwWT and hiwΔN, but when compared to a HIT at 90°, incapacitation rate at 70° was greatly reduced (~ 100% vs. ~50%) and was less consistent (Supplementary Fig. 2C). Given this more consistent evidence of neural injury, and the lack of evidence of systemic injury at 90°, this HIT protocol was therefore chosen to represent a single severe traumatic HIT in subsequent studies.

The hiwΔN flies have previously been reported as having synaptic overgrowth at neuromuscular junctions but are otherwise phenotypically normal.[25, 31, 32, 38] We found that hiwWT and hiwΔN demonstrated similar long-term survival, indicating no significant effect of the hiw null allele on baseline viability. However, we found that both hiwWT and hiwΔN flies demonstrated reduced survival at 24 hours following HIT (Supplementary Fig. 2D). This early death rate was greater in hiwΔN flies. The cause of this small excess burden of death was not determined by this study. This may reflect non-TBI related causes related to the constitutive loss of hiw expression. HIT also resulted in an incapacitation rate that was comparable
in both hiw\textsuperscript{WT} and hiw\textsuperscript{ΔN}. Increased mortality following HIT could also be due to traumatic intestinal barrier dysfunction. To exclude this possibility flies were exposed to coloured food post HIT and examined for leakage of food dye.[39] This demonstrated very low rates of intestinal barrier breakdown regardless of genetic background, supporting the conclusion that the HIT severity we applied did not cause gross thoraco-abdominal trauma (Supplementary Fig. 2E).

**High impact trauma induced long-term mortality is reduced and functional impairment rescued by loss of highwire**

To determine if loss of hiw could protect against long-term effects of TBI we examined survival in animals that lived beyond the initial 24 h post injury period. Injury caused a significant reduction in long-term survival in both hiw\textsuperscript{WT} and hiw\textsuperscript{ΔN} animals compared to uninjured controls. However, hiw\textsuperscript{ΔN} animals demonstrated significantly increased survival following injury compared to hiw\textsuperscript{WT}, particularly from ~20 days post injury (Fig. 1A). To determine if loss of hiw could rescue injury-induced climbing deficits we used the rapid iterative negative geotaxis (RING) assay.[36, 37] This demonstrated a significantly reduced climbing ability after HIT in hiw\textsuperscript{WT} flies, which was attenuated in those with a hiw\textsuperscript{ΔN} deletion (Fig. 1B). We also examined motor function by measuring flying ability. Flight behavior has been shown to vary with the level of protocerebral anterior medial dopaminergic neurons.[40] At 7 days following injury hiw\textsuperscript{WT} show a significant reduction in the percentage of time they spend in flight over a 30 second period when compared to controls of their own genotype. This deterioration in flying activity after injury is strikingly diminished in the hiw\textsuperscript{ΔN} flies (Fig. 1C). Injured flies that do not have flight activity still have healthy looking wings, make frequent spontaneous wing movements, and engage in wing grooming behavior, suggesting that the failure to fly even when provoked by a stimulus of air is not simply as a result of a peripheral wing injury. This is supported by the cases where short periods of flight are initiated but the flies seem unable to maintain for a prolonged duration.

**Loss of highwire reduces neuronal apoptosis**

In order to explore the mechanism of neuronal cell loss following HIT we performed flow cytometry of dissociated fly brains (Fig. 2A&B). This demonstrated low-levels of baseline early apoptosis (Annexin V positive, 7 AAD negative) and late apoptosis/cell death (Annexin V positive and 7 AAD positive) in hiw\textsuperscript{WT} and hiw\textsuperscript{ΔN} uninjured controls (<0.5%). In contrast, 7 days following injury there was a rise in both early apoptosis, and late apoptosis/cell death by flow cytometry. This is direct evidence for brain injury following impact with the HIT device. The levels of early apoptosis were significantly lower in the hiw\textsuperscript{ΔN} flies compared to hiw\textsuperscript{WT} following injury at 7 days, however the absolute difference was small (~0.5%). There were no significant differences in late apoptosis/death at either time point. This suggests that HIT causes a neuronal apoptotic cell death that is rescued by hiw\textsuperscript{ΔN} mutation.

**Dopaminergic neuron loss is attenuated by loss of highwire**
To explore the mechanisms underlying the protective effects of hiw following TBI we performed histological studies on fly brains. We first looked for evidence of injury-induced neurodegeneration by quantifying brain vacuolation.[33, 41–43] Both hiw\textsuperscript{WT} and hiw\textsuperscript{ΔN} flies demonstrated an increase in vacuolation at 28 days following injury, though no attenuation of vacuolation was observed in hiw\textsuperscript{ΔN} flies (Fig. 3A). We next examined a well-characterised subpopulation of dopaminergic neurons (protocerebral posterior lateral 1 cluster, PPL1), which are involved in climbing and flying behaviour (Fig. 3B).[40, 44, 45]

The number of neurons in the PPL1 cluster is remarkably consistent in wild-type flies, with an average of 12 neurons. Immunostaining for tyrosine hydroxylase (TH) demonstrated a significant decrease in the number of neurons in the PPL1 cluster following injury in hiw\textsuperscript{WT} flies. However, no reduction in PPL1 neuron numbers was seen following injury in hiw\textsuperscript{ΔN} flies (Fig. 3C,D).

**Loss of highwire reduces synaptic protein loss following injury**

In order to probe the nature of underlying cellular responses to the HIT we conducted western blots analysis using the pan-neuronal marker Neuroglian (Ngl), the presynaptic marker Bruchpilot (Brp), and the post synaptic maker Disc Large (Dlg) at 24 hours and 7 days following injury. After injury there was a significant reduction in Ngl at 24 hours in hiw\textsuperscript{WT} indicating a generalized loss of neurons. This trend was also seen at 7 days but was no longer significant. In contrast, hiw\textsuperscript{ΔN} flies did not have a reduction in Ngl. Mirroring the Ngl loss, Brp was also reduced following injury at 24 hours, but this reduction persisted at 7 days with some progressive loss. As with ngl, the hiw\textsuperscript{ΔN} flies were protected against Brp loss regardless of timepoint- and a non-significant trend suggesting a possibly increase was seen. Finally, Dlg levels were examined. Again, trends mirrored those of Ngl and Brp, with small falls seen in the hiw\textsuperscript{WT}, and small rises in the hiw\textsuperscript{ΔN} flies, but notably these were not significant in any case (Fig. 4).

**Discussion**

Our results provide support for the hypothesis that a mutation in the hiw gene (hiw\textsuperscript{ΔN}) demonstrates protection against the deleterious effects of HIT. The hiw\textsuperscript{ΔN} mutation results in a complete loss-of-function of highwire, leading to delayed degradation of dNMNAT, a core step in the WD process. The hiw\textsuperscript{ΔN} mutation has previously been shown to strongly delay WD both in vitro and in vivo.[25–28] In D. Melanogaster, the hiw\textsuperscript{ΔN} mutation protected against several deleterious effects of a high-velocity impact trauma model. Deaths were significantly reduced in keeping with longer-term protection against secondary brain injury processes. Flies with hiw\textsuperscript{ΔN} mutation suffer less neurodegeneration following HIT as manifest by reduced PPL1 dopaminergic neuron loss. Dopaminergic neurons have previously been
shown to demonstrate selective vulnerability following brain injury and in various neurodegenerative conditions including Parkinson's disease.[44–47] Given the well-characterised function of highwire in dNMNAT degradation, the mechanisms for the protection seen with hiwΔN mutation are likely to directly involve the Wallerian-like degeneration pathway, specifically through maintenance of dNMNAT levels in the axonal compartment and/or the cell body and delayed neuronal degeneration. As there is only a single dNMNAT isoform in D. Melanogaster, a decline in levels may cause death of both the axon and the cell body. An alternative explanation would be that loss of highwire function is maintaining dNMNAT levels that are then functioning as a molecular chaperone, possibly through alleviation of prototoxic stress.[11, 12, 14, 15, 48] Given the nature of the model system we cannot completely exclude that the rescue of lifespan and behavior are not due to effects outside of the CNS, however, the lack of external injuries, the failure of the model to cause intestinal barrier dysfunction, and the initial period of recovery and normal behavior following injury argue against this. The hiwΔN mutation is constitutive, therefore there may be unrecognized systemic effects beyond those we have characterized in the brain. This could be addressed in future studies by examination of a conditional mutant. We know that hiwΔN flies have previously been reported as having synaptic overgrowth at neuromuscular junctions but are otherwise phenotypically normal.[25, 31, 32, 38] We did not find any alteration in the levels of synaptic proteins in the uninjured hiwΔN brains compared to wild-types in our analysis. This suggests that the synaptic overgrowth effects seen previously at the neuromuscular junction of hiwΔN do not occur in the brain. We demonstrated that HIT trauma resulted in an excess of apoptotic cell death that was partially rescued by hiwΔN. This is an intriguing finding given that WD is a non-apoptotic mechanism of axon degeneration. One possibility is that absence of retrograde delivery of trophic factors by axonal transport could trigger apoptosis of cell bodies- this hypothesis requires further testing. The ability of hiwΔN to prevent injury induced climbing and flying defects may be explained by dopaminergic neuron rescue, and PPL1 neuronal loss has previously linked to climbing deficits.[40, 45] The preferential presynaptic protein loss seen on Western blot analysis is a pattern that is established in neurodegenerative models.[49, 50] dNMNAT has also been shown to maintain presynaptic active zones by directly interacting with bruchpilot.[51] This is consistent with our finding that bruchpilot was relatively preserved after injury in hiwΔN. Together, the finding suggest that HIT causes apoptotic and selective neuronal cell death and presynaptic protein loss, and that highwire loss of function can partially rescue these effects and their functional consequences.

**Conclusion**

Numerous injury cascades result from the initial insult of a TBI. The injury response is complex and multifaceted, and comprises a number of processes including Wallerian degeneration – a self-destruct programme triggered in the axon processes. Our report describes evidence that targeting Wallerian degeneration improved outcome in a Drosophila model of high impact trauma. We demonstrated that by blocking the function of the *highwire* gene we could prevent many of the effects of a traumatic injury. These included preserving the climbing and flying ability of the flies, stopping them from dying prematurely after injury, and preserving a population of dopaminergic neurons that otherwise degenerate
after an injury. These findings suggests that Wallerian degeneration is an important factor in TBI and that *highwire* represents a potential therapeutic target. This opens up a fresh avenue for TBI research and in the future it could lead to new pharmacological treatments.

**Declarations**

- Ethics approval and consent to participate N/A
- Consent for publication N/A
- Availability of data and materials – available from corresponding author
- Competing interests – CSH, AL, JA, DKM, MPC Nil
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- Authors’ contributions - CSH, JS, AL, DKM and MPC conceived the study, designed the experiments, and wrote the manuscript. CSH performed all experiments.
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**Animal welfare and experimental protocol.**

The experimental protocol was approved by the Babraham Institute ethical review board, and all animal work was performed in accordance with the 1986 Animals (Scientific Procedures) Act under Project License PPL 70/7620 following an appropriate ethical review process at the Babraham Institute. No live vertebrates or higher invertebrates were used in this research.

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Figures
Figure 1

Long-term mortality, climbing, and flying ability are relatively preserved in highwire mutants (A) Mortality rates over lifetime of flies in hiwΔN and hiwWT flies. n = 6 vials of 20-35 flies per condition. Statistical analysis was with Log-rank test. **** = p≤0.0001. (B) Reduction in climbing ability compared to baseline in injured versus uninjured hiwΔN and hiwWT flies at 45 days post injury. n = 6 vials of 35 flies per condition. Statistical analysis was with two-way ANOVA test. *= p≤0.05. (C) Percentage of time spent in flight over a 30 second period in injured versus uninjured hiwΔN and hiwWT flies at 7 days post injury. n = 10-12 per condition. Statistical analysis was with two-way ANOVA test. **= p≤0.01. Error bars show standard error of the mean for all experiments.
Figure 2

Flow cytometry of dissociated drosophila brains shows necrosis but low levels of apoptosis after injury (A) Flow cytometry demonstrating the percentage of cells undergoing early apoptosis, and late apoptosis/cell death following injury at 24 hours and 7 days in hiwΔN and hiwWT flies. n=9. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. **= p≤0.01. (B) Graphical representative flow cytometry data.
Injured flies develop brain vacuoles regardless of genotype, but depletion of PPL1 cluster dopaminergic neurons that is prevented by highwire mutation (A) Rates of vacuolation seen by hematoxylin and eosin staining in central brain regions at 28 in hiwΔN and hiwWT flies 28 days following injury. n = 5 per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. *= p≤0.05. (B) Representative hemotoxylin and eosin stained brain sections from uninjured and injured
hiwWT flies. The insert shows a close up of a typical vacuole. A further small vacuole is marked by an asterix. (C) Schematic image and TH stained whole brain mount showing the location of various dopaminergic neuronal groups including the PPL1 cluster. (D) Number of TH-positive PPL1 neurons in hiwΔN and hiwWT flies at 28 days post injury. n = 10-12 clusters per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. **= p≤0.01. (E) Representative PPL1 dopaminergic neuronal clusters showing depleted neuron numbers in injured hiwWT flies (9 neurons) and preserved numbers in injured hiwΔN flies (12 neurons).
Figure 4

Injured flies demonstrate a loss of presynaptic marker protein that is prevented by highwire mutation. Levels of Neuroglian, Bruchpilot, or Disc large relative to β tubulin loading control at 24h and 7 days following injury in hiwΔN and hiwWT flies. Representative blots are displayed. n = 5 per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. *= p≤0.05, **= p≤0.001.

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