## Title

*Highwire* is a novel target that protects against the effects of traumatic brain injury

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## Declaration

I confirm I fulfil all the eligibility criteria for the prize Campbell-Connolly Neurotrauma Prize- including that I have performed the majority (over 50%) of the work here enclosed, and the work was undertaken mainly in the UK.

## Abstract

Traumatic brain injury (TBI) is a major worldwide cause of death and disability. Secondary injury responses occur following TBI and may be suitable targets for therapeutic intervention, however existing targets have failed to translate to clinical benefit. New targets are required. This work demonstrates some of the first evidence that we can improve responses to TBI by targeting the axonal death pathway known as Wallerian degeneration (WD). This cell-autonomous neurodegenerative pathway is initiated following axon injury. It involves activity of the E3 ubiquitin ligase *highwire*. Using an innovative *Drosophila* model of high impact trauma we demonstrate that a loss-of-function mutation in the *highwire* gene rescues deleterious effects of brain injury, including -improved functional outcomes, lifespan, survival of dopaminergic neurons, and retention of synaptic proteins. This data suggests that *highwire* represents a credible novel therapeutic target in TBI – opening up a new vista of secondary brain injury targets relating to axon degeneration.

## Significance statement

Traumatic brain injury is a common event that causes a high burden of mortality and morbidity around the world. There are a lack of disease modifying therapies to improve outcome following injury. This research show for the first time that a loss-of function mutation of the gene *highwire* is able to prevent several deleterious after-effects of brain injury in a *Drosophila* model, including premature death, impaired behaviour, and loss of dopaminergic neurons in the brain. This rescue relate to *highwire*'s role in the axon degeneration pathway 'Wallerian degeneration'. This research suggests that inhibiting axon degeneration, including by targeting of *highwire* homologs, may be a viable therapeutic target in traumatic brain injury.

## Introduction

Traumatic brain injury (TBI) is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force(35). 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(33). Various secondary injury cascades follow the initial primary insult promoting ongoing neuronal cell loss. The secondary injury response is complex and multifaceted, and comprises a number of processes likely to include Wallerian degeneration (WD)(23). WD is an active, cell-autonomous death pathway that leads to degeneration of the distal axonal segment following transection(12). 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 (45,45). A related process, Wallerian-like degeneration, occurs through the same molecular mechanisms as WD but does not require a complete axonal transection. Wallerian-like degeneration is instead typically characterised by an impairment of axonal transport (12). 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(9,34)(8). The degree to which sub-axotomy level injuries subsequently impair axonal transport and induce Wallerian-like degeneration remains incompletely characterised(8,43). TActivation of the WD pathway is fundamentally linked to the NAD synthetic pathway (**Supplementary figure 1A**). Nicotinamide mononucleotide adenylyl transferase (NMNAT) is a key enzyme in the WD/ Wallerian-like degeneration pathway in mammals, converting nicotinamide mononucleotide (NMN) to nicotinamide adenine dinucleotide (NAD). NMNAT also displays chaperone function during stress responses(2,3,7,13,50). 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 (17,19)((13,36). This activates a mechanism that requires sterile motif-containing and

armadillo-motif containing protein (SARM1) for its completion resulting in axon fragmentation (**supplementary figure 1B)** (18,32,39,42). Loss-of-function mutations in the *hiw* gene are associated with a strong delay in axon degeneration following transection both *in vitro* and *in vivo*(38,40,47,48). Delayed WD is also seen with conditional deletion of the mammalian ortholog of *highwire*; *PHR1*(4,12,20). The *highwire* gene encodes a large 5233 amino acid protein with E3 ubiquitin ligase activity that modulates levels of dNMNAT(20,44,47,48)**.** *Highwire* also has presynaptic regulatory activity that is required to control excess synaptic growth at the neuromuscular junction(11,44,47). Evidence for a role of WD/ Wallerian-like degeneration in TBI has emerged from rodent cortical-contusion injury (CCI) studies. The first WD gene to be explored in a model of TBI was the slow Wallerian degeneration gene ($Wld^s$). This gene encodes a mutant protein ($WLD^s$) that can substitute for NMNAT2, and $Wld^s$ expressing mice demonstrate less motor and cognitive impairment following a CCI (15). Similarly, knockout of SARM1 was associated with reduced neuronal loss and cognitive impairment following CCI (22). These studies suggest that therapeutic modulation of the Wallerian-like degeneration pathway may be possible, and potentially could improve outcomes from TBI(23). To investigate for potential modifiers of brain trauma we utilised *Drosophila melanogaster* of high impact trauma (HIT) in which WD pathways show extensive conservation with mammalian species(6,12,14,18,20,28,42). Given the role of *highwire* 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 *highwire* ($hiw^{\Delta N}$) would show protection against the effects of TBI. We found that $hiw^{\Delta N}$ flies showed relative protection against long-term mortality, cell death including dopaminergic neuron loss, and presynaptic marker depletion following TBI, this was reflected by preservation of behavioural measures. These findings suggests that *highwire* is a potential therapeutic target in TBI.

## Results

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

To model TBI in *Drosophila* we utilised the high-impact trauma (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 figure 2A**). The angle of deflection can be used to control the severity of the injury. An angle of 90° produced an average death rate of 22% at 24 hours and 30% at 7 days post-injury in wild type ($hiw^{WT}$) flies (**supplementary figure 2B**). External injuries were never seen at angles of 90° or less, but was common (82%) at an angle of 135°, therefore the 90° was chosen to represent a single severe traumatic high impact trauma (HIT) in subsequent studies. The $hiw^{\Delta N}$ flies have previously been reported as having synaptic overgrowth at neuromuscular junctions but are otherwise phenotypically normal(10,11,44,47). We found that $hiw^{WT}$ and $hiw^{\Delta N}$ demonstrated similar long-term survival, indicating no significant effect of the *highwire* null allele on baseline viability. However, we found that both $hiw^{WT}$ and $hiw^{\Delta N}$ flies demonstrated reduced survival at 24 hours following HIT (**supplementary figure 2C**). This early death rate was greater in $hiw^{\Delta N}$ flies. The cause of this small excess burden of death was not determined by this study. which may reflect non-TBI related causes related to the constitutive loss of *hiw* expression. HIT also resulted in a incapacitation rate- defined as the proportion of flies that demonstrated a lack of purposeful movement 20 seconds or more following trauma- was comparable in both $hiw^{WT}$ and $hiw^{\Delta N}$. At 70° the incapacitation rate was greatly reduced compared to 90° (**supplementary figure 2D**). 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 (the 'smurf' phenotype)(27). This demonstrated very low rates of intestinal barrier breakdown regardless of genetic background supporting the assertion that the HIT we applied did not cause gross thoraco-abdominal trauma (**supplementary figure 2E**).

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

To determine if loss of highwire could protect against long-term effects of TBI we examined survival in animals that lived beyond the initial 24h post injury period. Injury caused a significant reduction in long-term survival in both $hiw^{WT}$ and $hiw^{\Delta N}$ animals compared to uninjured controls. However, $hiw^{\Delta N}$ animals demonstrated significantly increased survival following injury compared to $hiw^{WT}$, particularly from ~20 days post injury (**figure 1A**).

To determine if loss of *highwire* could rescue injury-induced climbing deficits we used the rapid iterative negative geotaxis (RING) assay(16,41). This demonstrated a significantly reduced climbing ability after HIT in $hiw^{WT}$ flies, which was attenuated in those with a $hiw^{\Delta N}$ deletion (**figure 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(1). At 7 days following injury $hiw^{WT}$ 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 $hiw^{\Delta N}$ flies (**figure 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.

**Dopaminergic neuron loss is attenuated by loss of *Highwire***

To explore the mechanisms underlying the protective effects of *highwire* following TBI we performed histological studies on fly brains. We first looked for evidence of injury-induced neurodegeneration by quantifying brain vacuolation (28,29,31,37). Both $hiw^{WT}$ and $hiw^{\Delta N}$ flies demonstrated an increase in vacuolation at 28 days following injury, though no attenuation of vacuolation was observed in $hiw^{\Delta N}$ flies (**figure 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(1,26,46)(**figure 3B**). These cells are reliably stained and easily counted by confocal microscopy. 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 $hiw^{WT}$ flies. However, no reduction in PPL1 neuron numbers was seen following injury in $hiw^{\Delta N}$ flies (**figure 3C,D**).

**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 (**figure 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 $hiw^{WT}$ and $hiw^{\Delta N}$ 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 $hiw^{\Delta N}$ flies compared to $hiw^{WT}$

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*$^{\Delta N}$ mutation.

**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*$^{WT}$ indicating a generalized loss of neurons. This trend was also seen at 7 days but was no longer significant. In contrast, *hiw*$^{\Delta N}$ 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*$^{\Delta N}$ 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*$^{WT}$, and small rises in the *hiw*$^{\Delta N}$ flies, but notably these were not significant in any case (**figure 4**).

## Discussion

Our results provide support for the hypothesis that a mutation in *highwire* (*hiw*$^{\Delta N}$) demonstrates protection against the deleterious effects of HIT. The *hiw*$^{\Delta N}$ mutation results in a complete loss-of-function of the *highwire* gene, leading to delayed degradation of dNMNAT, a core step in the Wallerian degeneration process. In Drosophila, this protected against several deleterious effects of a high-velocity brain impact trauma model. Deaths greater than 24 hours after injury were significantly reduced in keeping with longer-term protection against secondary brain injury processes. As the model is a whole organism impact – it cannot be entirely ruled out that the phenotypic differences seen could reflect preservation of axons, or other cell types, outside the brain. However, even if this were the case the relevance to human TBI is not diminished given the association of TBI with polytrauma. Flies with *hiw*$^{\Delta N}$ 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(24–26,46). Given the well-characterised function of *highwire* in dNMNAT degradation, the mechanisms for the protection seen with *hiw*$^{\Delta N}$ 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. There is only a single dNMNAT isoform in Drosophila, a failure to maintain dNMNAT supply to axons causes their degeneration and this might result in secondary loss of cell bodies through the absence of retrogradely delivered trophic factors. 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(2,3,7,30,50). We demonstrated that HIT trauma resulted in an excess of apoptotic cell death that was partially rescued by *hiw*$^{\Delta N}$. This is an intriguing finding given that WD is clearly not apoptotic. However, it is possible 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 *hiw*$^{\Delta N}$ 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(1,46).The preferential presynaptic protein loss seen on Western blot analysis is a pattern that is established in neurodegenerative models(5,21). dNMNAT has also been shown to maintain presynaptic active zones by directly interacting with bruchpilot(49). This is consistent with our finding that bruchpilot was relatively preserved after injury in *hiw*$^{\Delta N}$. 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. This establishes *highwire*, and the associated Wallerian degeneration pathway as a potential therapeutic avenue in brain trauma.

## Experimental procedures

### Drosophila Stocks and Conditions

Highwire mutant *hiw*$^{\Delta N}$ and control *hiw*$^{WT}$ (FRT$^{19A}$;;) flies were obtained from Marc Freeman (OHSU, Portland, Oregon). 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/cornmeal/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 on male flies only.

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

Flies were subjected to a standardised impact with the HIT device causing a single severe high impact trauma. 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 *hiw*$^{WT}$ 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. 24 hours later the percentage death was recorded. 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. Deaths within the first 24 hours following injury were excluded from the long-term survival analysis.

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

A custom made RING device was manufactured and used to measure negative geotaxis/climbing ability as a behavioural measure of motor function(16,41). 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 animal 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 ≥5μM 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% Trition 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 we made 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.

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## Figures

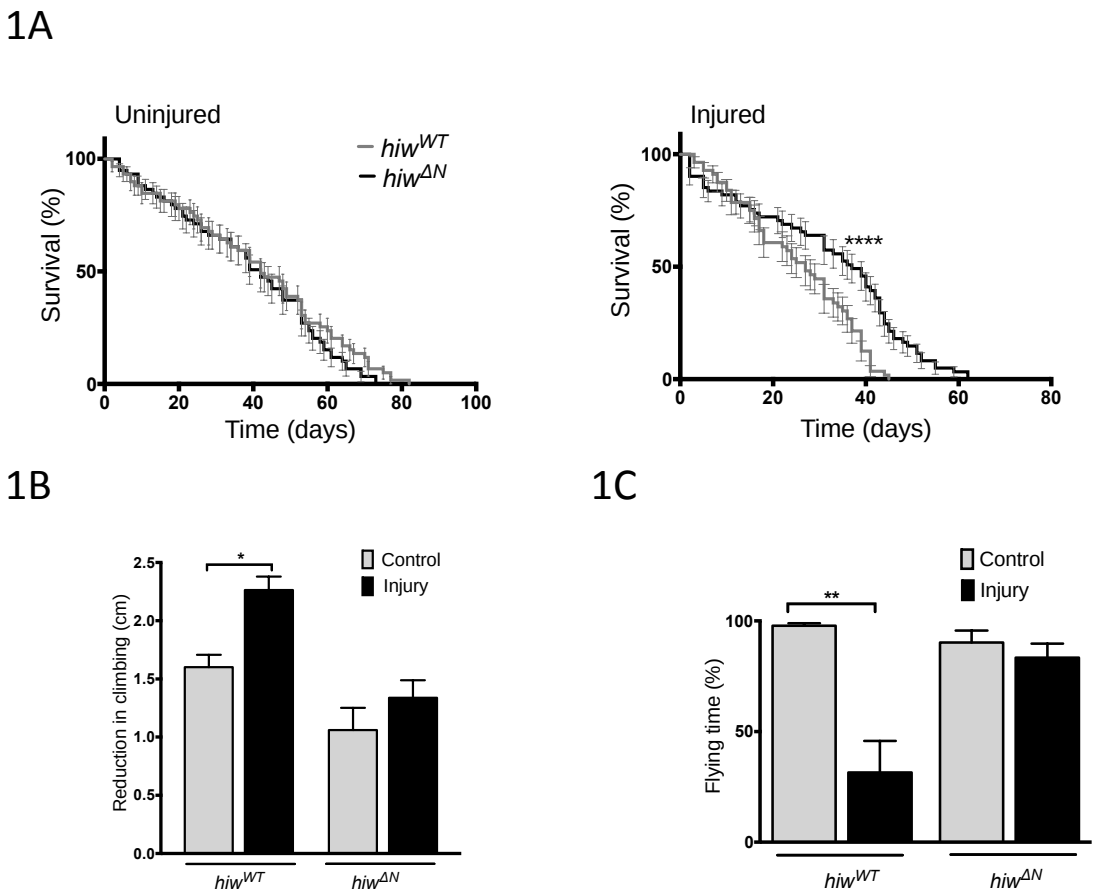
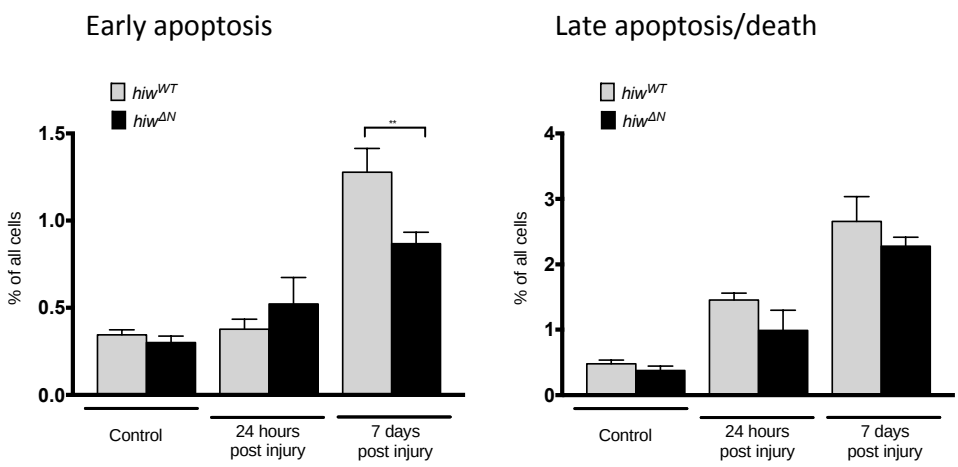



**Figure 1**
**Long-term mortality, climbing, and flying ability are relatively preserved in *highwire* mutants**
**(A)** Mortality rates over lifetime of flies in $hiw^{\Delta N}$ and $hiw^{WT}$ flies. n = 6 vials of 20-35 flies per condition. Statistical analysis was with Log-rank test. **** = $p \leq 0.0001$. **(B)** Reduction in climbing ability compared to baseline in injured versus uninjured $hiw^{\Delta N}$ and $hiw^{WT}$ flies at 45 days post injury. n = 6 vials of 35 flies per condition. Statistical analysis was with two-way ANOVA test. *= $p \leq 0.05$. **(C)** Percentage of time spent in flight over a 30 second period in injured versus uninjured $hiw^{\Delta N}$ and $hiw^{WT}$ flies at 7 days post injury. n = 10-12 per condition. Statistical analysis was with two-way ANOVA test. **= $p \leq 0.01$. Error bars show standard error of the mean for all experiments.

2A




2B

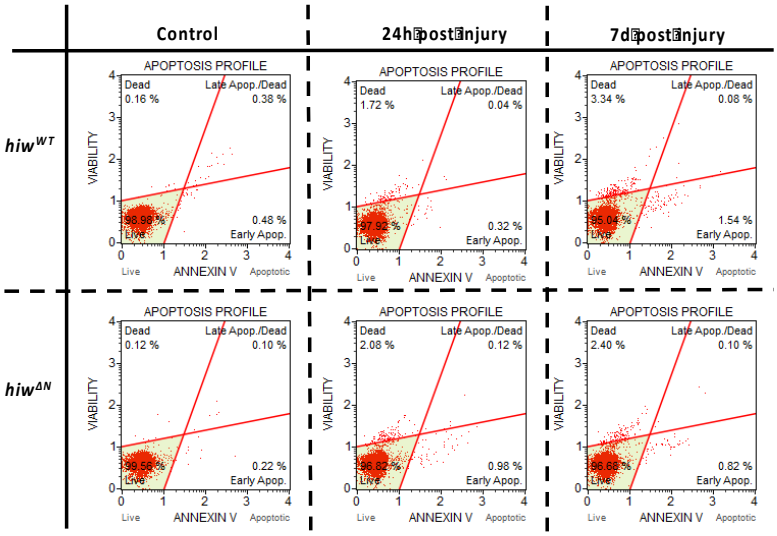
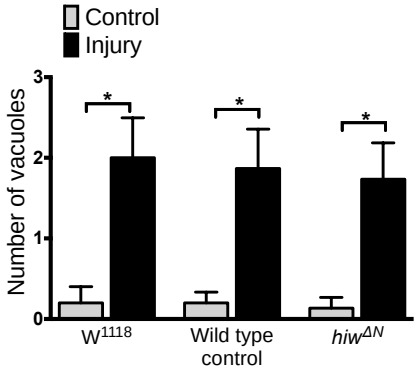



**Figure 2**

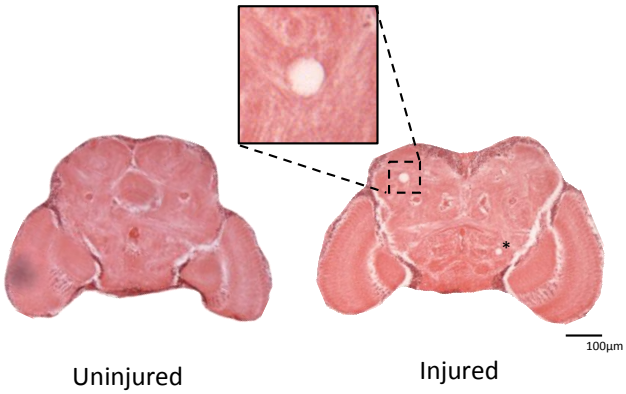
**Flow cytometry of dissociated drosophila brains shows necrosis but low levels of apoptosis after injury**

**(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 *hiw*$^{\Delta N}$ and *hiw*$^{WT}$ 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.

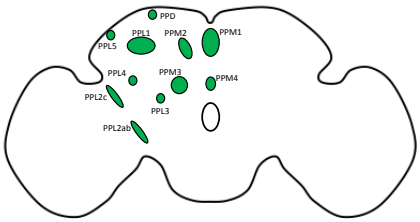
3A

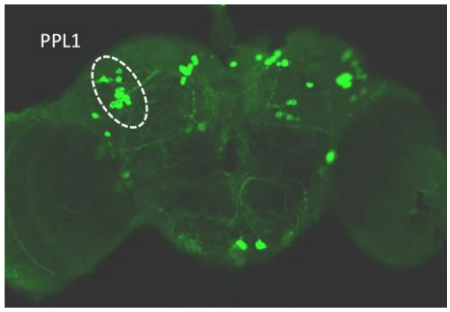

Control
Injury
Number of vacuoles
3
2
1
0
*
*
*
W1118
Wild type control
hiwΔN

3B

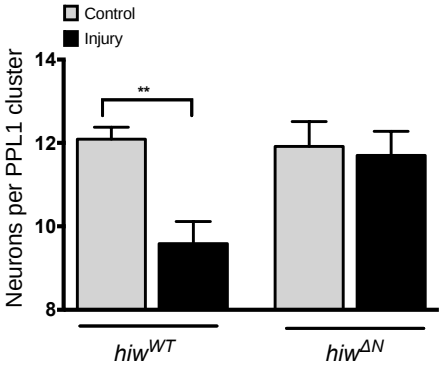

100µm
Uninjured
Injured

3C


PPD
PPL1
PPM2
PPM1
PPL5
PPL4
PPM3
PPM4
PPL2c
PPL3
PPL2ab


PPL1

3D


Control
Injury
Neurons per PPL1 cluster
14
12
10
8
**
hiwWT
hiwΔN

3E

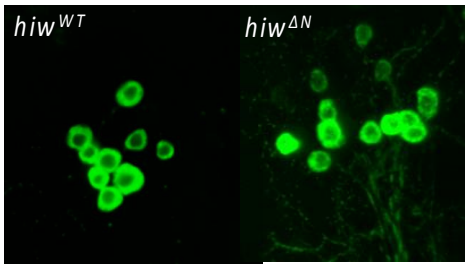

hiwWT
hiwΔN

**Figure 3**

**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 $hiw^{\Delta N}$ and $hiw^{WT}$ 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. *= $p \leq 0.05$. **(B)** Representative hemotoxylin and eosin stained brain sections from uninjured and injured $hiw^{WT}$ flies. The insert shows a close up of a typical vacuole. A further small vacuole is marked by an *. **(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 $hiw^{\Delta N}$ and $hiw^{WT}$ 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. **= $p \leq 0.01$. **(E)** Representative PPL1 dopaminergic neuronal clusters showing depleted neuron numbers in injured $hiw^{WT}$ flies (9 neurons) and preserved numbers in injured $hiw^{\Delta N}$ flies (12 neurons).

4

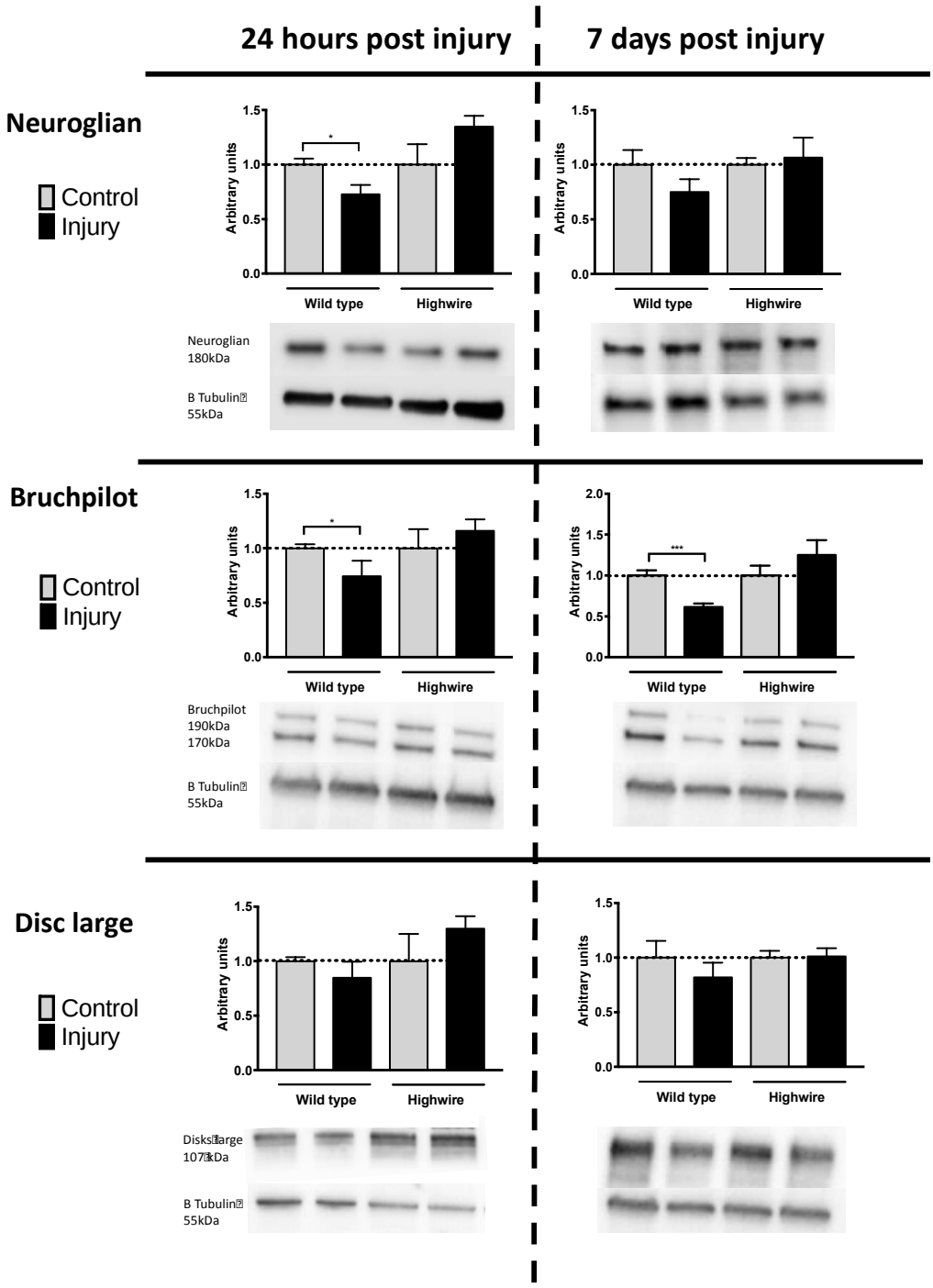


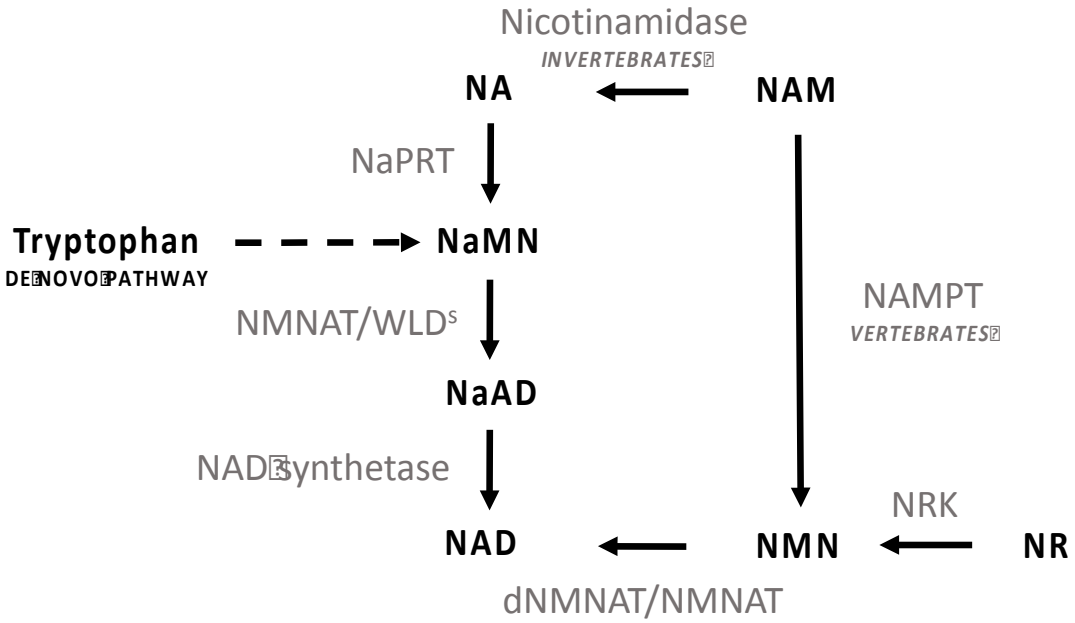

**Figure 4**

**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 $hiw^{\Delta N}$ and $hiw^{WT}$ 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. *= $p \leq 0.05$, ***= $p \leq 0.001$.

## Supplementary Figures

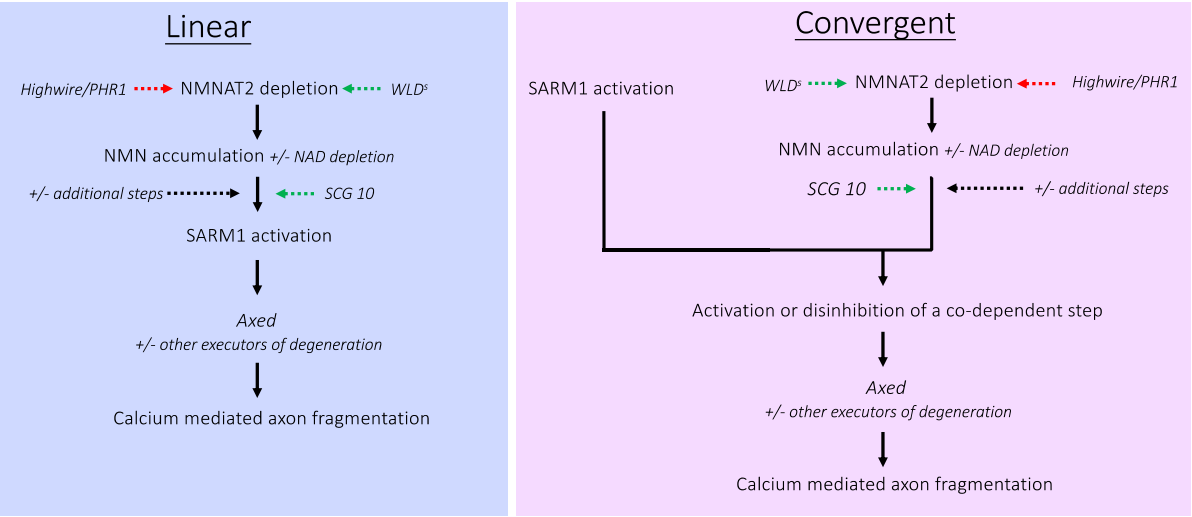
Supplementary 1A




**The NAD synthesis pathway**

The *de novo* NAD synthesis pathway generates NAD from the essential aromatic amino acid tryptophan. Nicotinic acid mononucleotide (NaMN) is converted to nicotinic acid adenine dinucleotide (NaAD) by nicotinamide mononucleotide adenylyltransferase (NMNAT). NaAD is then converted to NAD by NAD synthetase. There are three additional thee salvage pathways. Nicotinamide mononucleotide (NMN) is produced by nicotinamide phosphoribosyltransferase (NAMPT) from nicotinamide (NAM), and by nicotinamide riboside kinase (NRK) from nicotinamide riboside (NR). NMN is then converted to NAD by NMNAT. Alternatively NAM is converted to nicotinic acid (NA) by nicotinamidase, and then NA is converted to NaMN by nicotinc acid phosphoribosyltransferase (NaPRT)

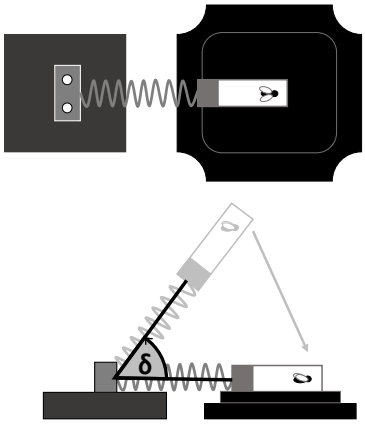
# Supplementary 1B



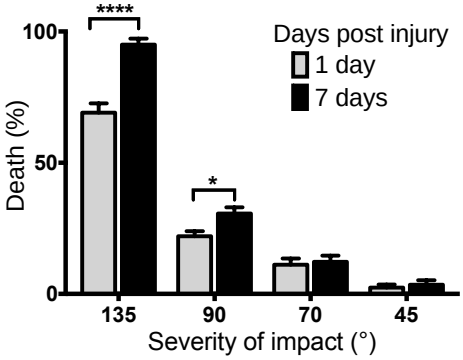

**Molecular control pathways of Wallerian degeneration**

Experimental evidence has thus far not been able to definitively differentiate between the possibilities of a linear or a convergent Wallerian degeneration pathway. Steps in WD include NMNAT2 depletion, NMN accumulation, prodegenerative SARM1 activation with subsequent Axed activation (in Drosophila), and finally calcium mediated axon dismantling. SARM1 activation may be a linear downstream consequence of NMN accumulation/a changing ratio of NMN/NAD, or SARM1 may function in parallel with a NMNAT2 depletion dependent process to converge on a co-dependent step. **Black arrows** signify the core pathway, **red arrows** signify pro-degenerative modulating factors, while **green arrows** represent factors that delay Wallerian degeneration. It is likely that this picture is incomplete and additional steps will be elucidated in the future. Adapted from Conforti *et al* 2014 & Gilley *et al* 2015.

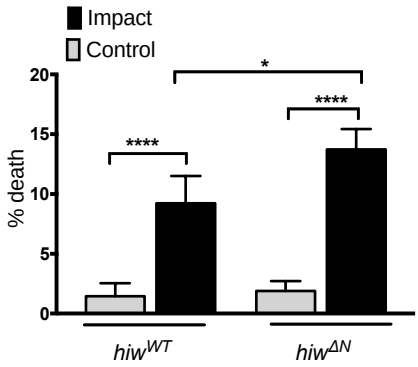
Supplementary 2A



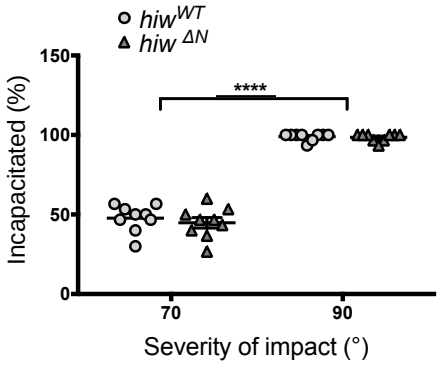

Supplementary 2B



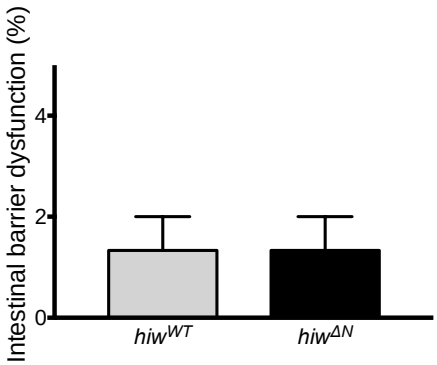

Supplementary 2C




Supplementary 2D




Supplementary 2E




**Rates of death following injury relate to the severity of a single impact as determined by the initial angle of impact device spring deformation and by genotype, incapacitation rates vary with force but not genotype, and the intestinal barrier is rarely disrupted following a single impact**

**(A**) A top-down and lateral schematic of the high-impact trauma (HIT) device. Varying the angle of initial deflection (δ) alters the amount of stored energy and hence the restoring force upon release of the spring. When the released vial strikes the impact board the flies are subjected to a rapid deceleration force proportional to δ. **(B)** Percentage survival one day and seven days post injury was assessed in relation to the severity of a single impact. Severity was altered by adjusting the degree of initial spring deformation calculated from the horizontal in the HIT. n = 9 vials of 20-35 flies per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. *= $p \leq 0.05$, **** = $p \leq 0.0001$. **(C)** All genotypes tested showed a significant increase in mortality 24 hours following a single severe TBI compared to matched controls. 24 hour mortality was greatest in the injured $hiw^{\Delta N}$ flies, which were significantly more likely to die than injured $hiw^{WT}$ flies. n = 6 vials of 20-35 flies per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. *= $p \leq 0.05$, **** = $p \leq 0.0001$. **(D)** Incapacitation rates were determined by subjecting flies to a single impact and assessing the percentage making no purposeful movement 20 seconds later. Each symbol represents the average incapacitation rate for one vial of flies following an impact at either 70◦ or 90◦ (70◦ v 90◦, **** $p \leq 0.0001$). n = 9 vials of 20-35 flies per condition. Error bars show standard error of the mean. Statistical analysis was with two-way ANOVA test. **(E)** Following a single impact flies were fed standard feed containing blue food dye. In the event of intestinal barrier dysfunction the dye would be expected to extend beyond the gut and proboscis, manifesting as a diffusely blue 'smurf' fly. n = 9 vials of 20-35 flies per condition. Error bars show standard error of the mean. Statistical analysis was with Mann-Whitney U test.