Physiological, anatomical, and behavioral changes after acoustic trauma in Drosophila melanogaster

Kevin W. Christie
University of Iowa

Elena Sivan-Loukianova
University of Iowa

Wesley C. Smith
University of Iowa

Please see article for additional authors.

DOI: https://doi.org/10.1073/pnas.1307294110
Title

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Authors

Kevin W. Christie, Elena Sivan-Loukianova, Wesley C. Smith\textsuperscript{a}, Benjamin T. Aldrich, Michael A. Schon, Madhuparna Roy\textsuperscript{b}, Bridget C. Lear, and Daniel F. Eberl\textsuperscript{1}

Affiliations

Biology Department, University of Iowa, Iowa City IA 52242, USA

\textsuperscript{a}Current Address: Neuroscience Interdepartmental Program, University of California, Los Angeles, CA 90095, USA

\textsuperscript{b}Current Address: Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA.

\textsuperscript{1}To whom correspondence should be addressed. E-mail: daniel-eberl@uiowa.edu
Abstract

Noise-induced hearing loss (NIHL) is a growing health issue, with costly treatment and lost quality-of-life. Here we establish *Drosophila melanogaster* as an inexpensive, flexible, and powerful genetic model system for NIHL. We exposed flies to acoustic trauma, and quantified physiological and anatomical effects. Trauma significantly reduced sound-evoked potential (SEP) amplitudes and increased SEP latencies in control genotypes. SEP amplitude but not latency effects recovered after 7 d. While trauma produced no gross morphological changes in the auditory organ, Johnston’s Organ (JO), mitochondrial cross-sectional area was reduced 7 d after exposure. In *nrv3* heterozygous flies, which slightly compromise ion homeostasis, trauma had exaggerated effects on SEP amplitude and mitochondrial morphology, suggesting a key role for ion homeostasis in resistance to acoustic trauma. Thus *Drosophila* exhibit acoustic trauma effects resembling those found in vertebrates, including inducing metabolic stress in sensory cells. This first report of noise trauma in *Drosophila* is a foundation for studying molecular and genetic sequelae of NIHL.

Significance

NIHL is an important health issue, yet its mechanisms and potential treatments remain unclear. We present the first study of NIHL in the fruit fly *Drosophila*, which has many advantages as a NIHL model. We examined auditory function and structure after exposing flies to acoustic trauma. Trauma impairs auditory
system function and changes neural mitochondria size, suggesting metabolic stress. In mutant flies with a reduced ability to cope with such stresses, the responses to trauma were more severe and recovery delayed or impaired.
Introduction

Noise-induced hearing loss (NIHL) is a pervasive and growing health issue arising from occupational and recreational hazards, with significant costs in healthcare and personal quality of life. Despite this, the molecular and physiological mechanisms involved in the etiology or recovery from injury are not yet fully understood. Importantly, intense acoustic trauma can induce permanent damage - unlike other vertebrates, mammals cannot regenerate auditory hair cells (1,2). NIHL associated with permanent changes in auditory sensitivity causes multiple consistent effects: stereocilia bundle disruption, inner (IHC) and outer hair cell (OHC) death or damage, supporting cell tissue disruption, and eventual spiral ganglion cell damage or loss (3-7). Most studies to date used mammalian model organisms such as mice (8,9), rats (10), and guinea pigs (11-14). These animals have difficult access to the inner ear inside the temporal bone, and high maintenance costs coupled with relatively long generation times.

*Drosophila* is a compelling alternative model system with strong genetic tools, inexpensive production of large numbers of animals, and an accessible auditory system that is becoming better understood genetically and physiologically. During courtship, *Drosophila* males vibrate their wings to produce a courtship song composed of pulse and sinusoidal components (15,16). This song facilitates species identification and mate selection (16,17). *Drosophila* males and females detect airborne vibrations via Johnston’s Organ (JO) in the second antennal segment (18). The JO is an array of chordotonal...
mechanoreceptors (or scolopidia – see Fig 1A-C). Via the aristae, acoustic energy is transformed to rotational movement of the third antennal segment, activating mechanosensitive channels on JO neuron dendrites. Like vertebrate hair cells, JO neurons are ciliated and respond to mechanical stimulation. While JO has morphologically diverged from hair cells in the human inner ear, the genetic program for its development shares a strong homology (19,20). For example, the *Atoh1* gene required for vertebrate auditory hair cell specification was found by direct homology to the fly *atonal* gene required for JO specification and *atonal/Atoh1* genes can be functionally exchanged between mice and flies (21,22). The advantages of studying hearing in *Drosophila* are that the genome is fully sequenced, genetic tools for extensively manipulating the genome are at hand, genetic background effects can be effectively eliminated, and large numbers of individuals can be tested.

In this study, we establish *Drosophila* as an inexpensive and flexible model system for genetic and physiological study of NIHL. We exposed two control strains (Canton-S, 40AG13) to acute acoustic trauma, and examined physiological, behavioral and anatomical effects. Our findings show immediate effects on auditory function, with reduced and delayed evoked activity. While evoked potential amplitudes were restored after 7 d, the latency of these potentials did not fully recover and we found significant changes in JO neural mitochondrial morphology. We also tested mutant flies with a reduced copy number of *nervana 3 (nrv3)* encoding a Na⁺/K⁺ ATPase β subunit expressed in JO neurons (23). We hypothesized that compromised JO ionic homeostasis
would confer susceptibility to noise trauma. Indeed, *nrv3* heterozygotes showed increased sensitivity to trauma and a significantly reduced auditory functional recovery.
Results

Physiological/Behavioral Responses to Trauma in Control Strains

Electrophysiology of non-traumatized wildtype Canton-S (CS) and 40AG13 flies (Fig 2A,B, dark bars) showed clear responses to the pulse stimuli, with sound-evoked potential (SEP) amplitudes similar to previous studies (18). We focused on test stimuli presented at 85 dB SPL (14.2 mm/s PV), as this most closely approximates the levels heard naturally during courtship interactions (24). Responses increased monotonically with increasing stimulus SPL from 65 dB (0.5 mm/s PV) to 85 dB (Fig S1A-C, dark bars). Increasing the SPL to 95 dB (40.7 mm/s PV) resulted in similar or slightly decreased responses (Fig S1D, dark bars), also matching previous behavioral results (25). Untreated CS flies showed larger SEP amplitudes than 40AG13 flies at all time-points and for all stimulus levels (Fig 2, Fig S1, dark bars), reflecting differences in strain genetic backgrounds.

SEPs of flies exposed to acoustic trauma showed highly significant decreases immediately after trauma cessation (Fig 2A,B, 0d; p<0.001, t-test). SEP amplitudes for CS and 40AG13 flies were reduced 18.5% and 15.8% from non-traumatized groups, respectively. Reductions were significant across stimulus SPL range (Fig S1A-D, 0d), with trauma-induced SEP reductions generally larger in response to lower SPL stimuli. At 7 d post-trauma, no significant differences were seen between treatment groups, regardless of genotype or SPL (Fig 2A,B, Fig S1A-D, 7d; t-test), indicating that JO can recover from traumatic sound.
Trauma also affected song-induced courtship behavior in male 40AG13 flies, as revealed in a male auditory courtship assay (25). This strain was chosen for behavioral study for its robust activity in this assay. Immediately after trauma, males showed significantly reduced courtship behavior compared to control flies (Fig 2C, 0d). At 7 d after trauma, courtship responses were not significantly different between control and traumatized flies (Fig 2C, 7d). To ensure that directed courtship activity differences were not due to underlying changes in baseline locomotor behavior, we quantified locomotor activity for 1 hr after cessation of trauma (a time span much longer than the duration of courtship behavioral experiments). Trauma had no noticeable effect on overall locomotor behavior (Fig 2D).

Acoustic trauma also affected timing of SEP responses to song pulses. Immediately post-trauma, SEPs to the second stimulus pulse (Fig 3A #2, grey box) of non-traumatized flies had the lowest latencies. In traumatized flies, these latencies depended similarly on pulse number, but were significantly delayed compared to controls by ~44-52 µs (Fig 3A, white boxes; \(p<0.0001\), Welch’s \(t\)-test). At 7 d after trauma (Fig 3B), latencies also increased with pulse number. Increased latencies were still seen in traumatized flies compared to controls (Fig 3B, white boxes; \(p=0.0337-0.0066\), Welch’s \(t\)-test), but latency differences were reduced to ~29-37 µs for subsequent pulses. Importantly, this incomplete recovery of latency is unlike SEP amplitudes, which recover to levels indistinguishable from controls after 7 d.

*Physiological Response to Trauma in nrv3 Mutants*
In mammals, acoustic trauma induces metabolic stress in cochlear hair cells and spiral ganglion neurons, changing mitochondrial fission/fusion dynamics, and with sufficiently intense trauma, inducing apoptosis pathways (5,26,27). To test whether flies with slightly compromised physiological components would be sensitized to acoustic trauma, we exposed flies heterozygous for a mutation in the *nervana 3* (*nrv3*) gene, encoding a Na$^{+}$/K$^{+}$ ATPase $\beta$ subunit specifically expressed in auditory neurons (23; Fig S5), to acoustic trauma and measured SEPs. The Na$^{+}$/K$^{+}$ ATPase is expressed in JO scolopidia where it likely regulates ion homeostasis (23). We hypothesized that mutant heterozygote JO neurons would be more severely affected by constitutive activity driven by the trauma stimulus, and demonstrate a higher sensitivity to trauma-induced metabolic stress.

Compared with 40AG13/CyO sibling control flies, untreated *nrv3*/40AG13 heterozygotes had similar SEP amplitudes at all time-points (Fig 4A, B, dark bars). Immediately after trauma, however, control and mutant flies had significant SEP reductions (Fig 4A,B, light bars, 0d; 40AG13/CyO: $p<0.0001$, *nrv3*/40AG13: $p<0.0001$, t-test), with control and *nrv3* heterozygotes showing 44.4% and 48.4% reductions respectively. One day post-trauma, treated 40AG13/CyO and *nrv3*/40AG13 SEPs had partially recovered, but were still significantly reduced – with the *nrv3* heterozygotes showing a larger deficit - from controls (by 20.7% and 29.8%, respectively). Seven days after trauma, control fly SEPs were not significantly different from traumatized flies (Fig 4A, 7d; t-test), while *nrv3* heterozygotes (Fig 4B, 7d) had not fully recovered, with SEP values significantly
lower than non-traumatized flies ($p=0.0311$, t-test; a 16.4% reduction).

**Anatomical Effects of Trauma in Control and nrv3 Mutants**

Using TEM, we examined the JO scolopidia of traumatized and control flies (40AG13, and nrv3 heterozygotes) for anatomical differences at 0, 1 and 7 d post-trauma (for scolopidal morphology see Fig 1A-C). No consistent gross morphological differences were found due to genotype or trauma condition (Fig S2-4). All scolopidia showed normal structure in both cross- and longitudinal sections. These results suggest robust homeostatic mechanisms that maintain the auditory organ’s physiological competence even under high duty-cycle metabolic stress.

Compared to untreated controls, traumatized 40AG13/CyO and their sibling nrv3/40AG13 mutant heterozygous flies also displayed no gross JO anatomical differences at 0, 1 or 7 d post-treatment. At the sub-cellular level, mitochondria in JO neurons appeared smaller 7 d post-trauma. To quantify this effect, JO neuron mitochondrial cross-sectional areas were measured for each genotype/treatment group 0, 1, and 7 d after trauma cessation. Immediately and 1d after trauma, 40AG13/CyO control mitochondria did not show significant reduction in area (Fig 5A 0d, 1d), while 7d post-trauma a 13.8% decrease in area was found (Fig 5A 7d; $p<0.001$, Kruskal-Wallis). Non-exposed nrv3/40AG13 mutant heterozygotes (Fig 5B, grey bars) generally had smaller cross-sectional areas than untreated 40AG13/CyO control flies. Acoustic trauma had a similar but more immediate and pronounced effect on nrv3/40AG13 flies as on the 40AG13/CyO controls.
Neurons showed marked reductions in mitochondrial cross-sectional area at all 3 time-points (Fig 5B, 0d: ~28% decrease, \(p<0.001\); 1d: 14% decrease, \(p<0.01\); 7d: 25.1% decrease, \(p<0.001\), Kruskal-Wallis). These results support the hypothesis that acoustic trauma induces metabolic stress on the fly’s auditory system, thereby affecting mitochondrial function.

**Effect of Acoustic Trauma on Circadian Locomotor Behavior**

Acoustic trauma, in addition to sensorineural effects, may affect the animals’ gross behavior and activity levels, which in turn may manifest as changes to mitochondrial morphology. To control for this indirect effect, we exposed several fly lines to sham and noise trauma, and monitored circadian locomotor behavior (see SI). In the first experiment (Fig S7) we found no obvious differences in locomotion or circadian effects for flies of Canton-S or 40AG13 control strains at 1 h resolution (Fig S7A-2), and both sham and noise-treated flies of both genotypes show expected crepuscular activity peaks during L:D conditions (Fig S7A, days 1-5, alternating light/shaded regions). Decreasing temporal resolution to 12 and 24 hr (Fig S7B1-2, C1-2) also revealed no significant acoustic trauma effects.

In the second experiment, we tested 40AG13, 40AG13/CyO, and \(nrv3/40AG13\) flies (Fig S8). Again, no qualitative differences in hourly locomotion due to noise trauma were observed in any genotype (Fig S8A1-3), with no significant trauma effects at the 12 or 24 hr resolution (Fig S8B1-3 and C1-3). Our results are consistent with the idea that the decreased mitochondrial areas
seen in 40AG13/CyO and nr\textsuperscript{v}3/40AG13 require normal auditory function, rather than arising as a by-product of altered locomotor behavior post trauma treatment.
In this study we establish that *Drosophila* react to acute acoustic trauma with significant changes in auditory physiological response magnitude and latency, auditory behavior and mitochondrial size. In studies using cats (28), gerbils (29), and guinea pigs (12,13), auditory nerve (AN) compound action potential (CAP) reductions are on the same order as the *Drosophila* SEP attenuation. The acoustic test stimulus and primary test level (85 dB SPL, 14.2 mm/s PV) was chosen for ethological relevance (15,16) and to ensure a robust physiological response. Most acoustic trauma studies focus on changes in auditory thresholds and CAP/single fiber tuning curves (6,14,29-31) by systematically varying test stimulus SPL and frequency; similar studies in *Drosophila* may be needed to better compare trauma effects by expressing changes in auditory sensitivity in terms of acoustic energy.

Importantly, our results show that this physiological effect has significant consequences on whole organism behavior. That traumatized 40AG13 males show reduced courtship behavior (Fig 2C) suggests that the reduction in auditory system function has a real effect on mating behavior. We currently cannot determine whether the reduction in courtship is attributable only to a sensory deficit in the auditory system or there are also effects of trauma on courtship circuits downstream of hearing. However, noise trauma did not significantly change fly locomotor behavior immediately after (Fig 2D) or 1 week post-trauma (Fig S7-8), suggesting there is no general effect of trauma on behavior.
Canton-S SEPs showed larger non-traumatized SEP responses across all SPLs used (Fig 2, Fig S1) and demonstrated slightly smaller reductions in SEP magnitudes in response to trauma than did 40AG13, suggesting that genetic background differences affect hearing sensitivity and response to physiological perturbation. Genomic differences have significant effects on the susceptibility and response to interventions in NIHL and age-related hearing loss in mice (32,33) and humans (34). A strength of *Drosophila* is that rapid generation times, large number of lines, and diverse molecular and genomic tools enable large scale and fine resolution genetic studies, making it an excellent system to study interactions between genetic background effects and NIHL.

Increased SEP latencies with trauma ~40-50 µs for SEP pulses 2-5 resemble delays observed in other systems. CAP latencies (measured at the N1 peak) similarly increased in cats after acute acoustic trauma (28). Gunshot noise trauma delayed guinea pig CAPs by ~50-200 µs compared to controls (13).

Conversely, other studies found reduced latencies of single AN fibers in response to acute noise (28,30,31,35). Latency reductions are probably due to the particular mammalian cochlea mechanical tonotopy and tuning properties.

Increasing probe SPLs to elicit a response in a tonotopic region with trauma-induced threshold shift may activate fibers with a much higher center frequency (CF) – which are more basal in the cochlea and have a smaller traveling wave delay and shorter latency (11,36). Basilar membrane mechanical property changes from OHC damage are also implicated in latency reduction (35,36). As the JO lacks the cochlear structure and traveling wave dependence, it is
unsurprising that this response to noise trauma is absent.

As with SEP amplitudes, it is difficult to compare recovery timecourses between studies using different species and experimental parameters.

Nonetheless, *Drosophila* hearing recovery resembles vertebrate acoustic trauma responses in some aspects. At 7 d post-trauma, SEP amplitudes for both CS and 40AG13 flies were not significantly different from unexposed controls, while SEP latencies remained elevated. Previous studies show that not all noise-induced physiological changes recover at identical rates. In gerbils exposed to noise trauma, CAP (*N*₁) amplitudes had not returned to pre-trauma levels, even if CAP thresholds recovered (29). In guinea pigs (14) and mice (9) large losses in AN fibers or IHCs lead to long-lasting reductions in CAP or auditory brainstem response (ABR), which remain even after behavioral or physiological thresholds return to normal. Often the nominal thresholds ‘mask’ permanent damage caused by trauma, which may involve ongoing degeneration of neuronal elements (9).

One striking result of our study is the lack of JO gross morphological changes after trauma. In vertebrates, acoustic trauma often correlates with stereocilia disruption (37), OHC and IHC damage or loss (4,6,7,26,37), spiral ganglion cell loss (6,7,14,37), and damage to supporting tissue and non-sensory cochlear cell types (6,7). While *nompC* is an important mechanosensitive channel in *Drosophila* auditory function (18,38,39), the identity, arrangement and structure of other mechanotransduction system elements (additional mechanosensitive ion channels, support and connector proteins, etc.) in JO sensory neuron cilia are unclear (40). Thus, we cannot rule out that noise trauma
mechanically disrupts this system as observed in vertebrate IHC stereocilia (41).

As models of *Drosophila* transducer function suggest similar properties as vertebrate hair cells (40), similarity in trauma-induced damage patterns would not be surprising. Previous studies showed changes in auditory epithelial cell-cell junctions after trauma, suggesting these junctions (and their molecular components) as noise-induced damage targets (42,43). In the alligator, noise trauma sufficient to cause only temporary threshold changes induced transient microlesions in hair cell plasma membranes, allowing abnormal Lucifer Yellow dye diffusion into hair cell cytoplasm (4). Post-trauma, hair cell appearance and CAP responses were grossly normal. JO scolopidial integrity may also be transiently compromised during trauma, and thus appear normal on subsequent histological analysis.

Anatomical structures and functions of auditory systems undergoing acoustic trauma in *Drosophila* and vertebrates are sufficiently different that the mechanisms responsible for SEP or CAP decreases may not be shared. It is currently thought that reduced CAP after intense sound trauma is caused by reduced AN fiber recruitment (due to damage or death of hair cells and/or spiral ganglion neurons) and from a 'broadening' of the response due to de-synchronization of afferents with similar CFs (11,36). Also, hair cells or AN fiber death or degeneration may occur over days to months (6,9). We investigated physiological and anatomical status immediately after, 1 d and 7 d post-trauma. While 7 d is a significant portion (~10%) of average *D. melanogaster* lifespan, molecular and cellular changes responsible for cellular damage and
degeneration may still require longer post-trauma survival times to manifest in sensory cell loss.

In contrast to gross morphology, we found sub-cellular trauma-induced changes. Traumatized flies showed consistent and robust decreases in mitochondrial cross-sectional area in JO neurons, and these changes were not associated with any changes in circadian locomotor behavior (Fig S7-8). After trauma, mitochondrial dysfunction has been implicated as a cause of auditory pathology (44,45) and a consequence of acoustic trauma via oxidative stress and reactive-oxygen species (ROS) production (27,46-48). Reduced mitochondrial size is often associated with disrupted balance of ongoing mitochondrial fission/fusion, biasing the system towards fission. Increased fission is also associated with cellular metabolic stress, and may activate apoptotic pathways (49,50). Elevated metabolic stress may arise from excitotoxicity (46,47), and/or disruption of ionic homeostasis (27,46). The latter is supported by *nrv3* heterozygote responses to noise trauma. The reduced Na⁺/K⁺-ATPase β subunit in JO neurons (Fig S5) is sufficient to maintain normal sensory function (Fig 4B) under non-traumatized conditions, but not with increased demands under noise trauma. This manifests in an exaggerated immediate hearing detriment, lack of full recovery 7 d after trauma (Fig 4B), and exaggerated mitochondrial size reduction in mutant heterozygotes (Fig 5B). Thus, the acoustic trauma protocol can be used to reveal cryptic or dominant phenotypes in animals with compromised genotypes whose hearing appears normal under unstressed conditions. The result is particularly striking, as the mutants still had one
Notably, the processes underlying the physiological and anatomical deficits seem to be somewhat 'decoupled', or acting on different time scales. In both 40AG13/CyO and nrv3/40AG13 flies, the physiological deficit from trauma is apparent immediately, but after 7 d control flies show recovery, while nrv3 heterozygotes still have reduced function. Trauma-induced differences in mitochondrial morphology show a different pattern: in control flies, they are only detectable after 7 d (by which time SEP amplitudes have recovered), while in nrv3 heterozygotes they are significant at all time points. This makes the relationship between physiological and anatomical effects of trauma unclear.

Over-stimulating the JO neurons could result in multiple metabolic stressors (ROS generation, molecular depletion, ionic imbalances, etc.) any of which could initiate reduction (and subsequent recovery) of stimulus-driven activity and could also initiate mitochondrial morphological change. The different time scales of mitochondrial effects in nrv3 heterozygotes may reflect chronic subphenotypic stress that sensitizes mitochondria to the effects of noise trauma. Genetic and molecular signaling cascades that may be involved in trauma-initiated physiological and anatomical changes have yet to be determined, and further gene-transcriptional analysis is required.

We have shown that several acoustic trauma effects observed in vertebrates manifest in a Drosophila model, including reductions in evoked response magnitude, increased response latency, and mitochondrial changes indicative of cellular stress. This suggests similarity in trauma-induced changes in
cellular physiology and gene expression in *Drosophila* and vertebrate models.
Materials and Methods

Genetic strains

Adult wild-type Canton-S and control 40AG13 (described in 25) flies were used. In addition, we crossed nrv3^{15}/CyO (23) with 40AG13 flies, and resultant nrv3^{15}/40AG13 heterozygous flies were tested vs. 40AG13/CyO sibling controls.

Flies were reared on a yeast-cornmeal-agar medium at 22-25°C, and age-matched at 0-4 d post-eclosion.

Acoustic Trauma

Flies anesthetized with CO\textsubscript{2} were placed into custom-made plastic vials made by fusing the top halves of two 15 ml centrifuge tubes (Corning). The tube was coated with a strip of agarose/sucrose/apple juice gel running the tube’s inside length to provide nutrition and humidity. A small square of fine nylon mesh placed over one end of the tube was held in place by screwing a fitted cap with a large hole in the center. The other end was screwed into one of six caps embedded in a thick plastic 'plug' inserted into the narrow end of a plastic funnel (20.3 cm base, 9.53 cm height). Each of these caps also had central holes covered by nylon mesh. As *Drosophila* respond to the air particle-velocity (PV) component, both mesh-covered open tube ends allowed sound-driven air to pass longitudinally though the tube with minimal restriction. The funnel base was coupled to an 8-in (20.3 cm) 8Ω speaker (Radio Shack) so that air PV was efficiently transmitted through the tubes.

Trauma stimulus was a continuous 250 Hz tone, approximating the *D.*
*melanogaster* pulse-song carrier frequency (15) and which consistently produces maximal SEP amplitudes. The tone, stored on a G3 iMac computer, was fed to an Optimus MPA-40 amplifier (Radio Shack) driving the speaker. The speaker and tube apparatus was installed in a modified incubator (68.9 cm x 46.7 cm x 45.7 cm interior volume) lined with acoustic foam (Auralex Acoustics), at 22-25°C, humidified with a water pan on the incubator floor.

Flies were exposed to trauma stimulus for 24 hr at ~120 dB SPL, with PV values ranging from 340-336 mm/s within the tubes. Non-traumatized control flies were placed in identical tubes and kept for 24 hr at the same temperature as exposed flies. After each treatment period, flies from each trauma condition (trauma or sham) were placed in food vials until used in neurophysiological recordings.

**Electrophysiology**

Electrophysiology was performed as described (18); for details see SI. Synthetic acoustic stimuli for electrophysiology resembled the pulse components of *Drosophila* courtship song (15): 5 pulses, each 5 ms in duration, with an inter-pulse interval of 35 ms, for a total stimulus duration of 165 ms. Pulse trains were repeated 10 times, with an inter-stimulus interval of 425 ms. Stimulus trains were presented to the fly at 4 sound pressure levels: 65, 75, 85, and 95 dB SPL, with corresponding PVs of 0.5, 4.2, 14.2, and 40.7 mm/s. All acoustic stimuli (trauma and neurophysiological) were calibrated with a digital SPL meter (CEL-240, Casella CEL Inc.), and a custom-built calibrated PV microphone with pre-
amplifier (gift of M. Göpfert).

Recordings were performed a few hours after cessation of acoustic trauma (0 d time post-trauma) for some flies, 1 d and 7 d later for others. The genotype/trauma treatment group identities were blinded to the experimenter and used in random order. Equal numbers of male and female flies were used in each recording session. Averaged SEPs were calculated and analyzed off-line using numerical analysis software (GNU Octave). For SEP magnitudes, differences between control and traumatized groups were tested at all time-points and SPLs using Student's t-test (two-tailed; α = 0.05), with Welch's correction if data were heteroscedastic. SEP latencies were defined as the time elapsed between the first positive peak of each stimulus pulse to the first large negative peak of the associated SEP (Fig 1D). Because these times incorporate the sound particle velocity travel time from the speaker to the fly and because the 'zero' point is arbitrary, the absolute values of the calculated SEP latencies are less important than the values relative to each other. Timing data were similar for both genotypes (determined using histograms and Bartlett's test for homogeneity of variance), so SEP latency data were pooled for CS and 40AG13 flies. Post-stimulus peak latencies were determined for pulses 2-5 in the train. Analysis of the first pulse was omitted due to hardware-induced variability in the pulse shape. Statistical differences were tested using Welch's modified t-test (two-tailed; α = 0.05), due to sample heteroscedasticity.

Courtship Behavior Analysis
The courtship behavior of traumatized and control 40AG13 flies was examined using established protocols (25), with the following modifications. Synthetic pulse stimuli ranged from 76-78 dB SPL (0.9-2.0 mm/s PV) within the behavior chamber. Courtship behavior was scored during 1 min of silence followed by 1 min of synthetic pulse song, and normalized courtship index (CI) scores were calculated as a % increase in directed courtship behavior by dividing the CI scores for males presented song to those during the preceding silence. Significance was tested with Student's t-test (two-tailed; α = 0.05) after verifying normality and homoscedasticity.

Circadian locomotor analysis

Circadian rhythm analysis was performed as described (51). Locomotor activity of control and noise-exposed adult male 40AG13 flies (3-5d post eclosion) was monitored with Trikinetics Activity Monitors for 8 d of LD (12 hr/12hr; see Fig S8A1-3) at 25°C. Data were analyzed off-line using numerical analysis software (GNU Octave) and statistical software (Prism, GraphPad). Activity levels were quantified as averaged number of crossings per unit time (Figs S7-8). Differences between traumatized and control flies were tested via Student's t-test (two-tailed; α = 0.05). See SI for additional details.

Transmission electron microscopy

Heads of flies were dissected 0,1, and 7 d after trauma and fixed overnight at 4°C in 2.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, then rinsed in PB. They were postfixed in 1% OsO$_4$ in PB for
1 hr, dehydrated and processed for embedding in Epon 812. Ultrathin sections
(75 nm) were stained with aqueous uranyl acetate and lead citrate and examined
with a Jeol 1230 electron microscope.

Mitochondrial Measurements

Mitochondrial cross-sectional areas were measured from 10 neurons in
each of 3 male nrv3/40AG13 heterozygotes and 40AG13/CyO sibling controls at
0, 1, and 7 d after sham or trauma treatment. Total mitochondria for each
genotype/treatment group ranged from 473 to 865, with a total of 7685
measurements. To ensure comparable results between genotypes and
treatments, we restricted our analysis to longitudinal TEM sections of JO sensory
neurons containing a large and well-defined nucleus profile (Fig. 1C) and
measured all visible mitochondria. Measurements were made using a Bamboo
tablet (Wacom Inc.) and ImageJ (NIH) on calibrated digital TEM images. Due to
non-normal shapes of the data distributions, we used the natural log of cross-
sectional area, resulting in quasi-normal data amenable to statistical testing.
Differences were tested using the Kruskal-Wallis test (α=0.05), followed by
Dunn's test of multiple comparisons.
Acknowledgments

We thank Julie Jacobs, Steven Green, and Paul Abbas for technical advice and comments. We thank Jeremy Richardson and Hanh Nguyen-Kratz for material aid in equipment design and construction. We are grateful to Martin Göpfert for providing a calibrated PV microphone. This work was supported by the National Institutes of Health grant R21 DC011397 (to DFE) and facilitated by P30 DC010362 (to Steven Green) supporting the Iowa Center for Molecular Auditory Neuroscience.
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evening pacemaker neurons to regulate multiple features of circadian behavior.

Figure Legends

Fig 1. Organization of Drosophila Johnston’s Organ (JO) and physiological response to sound. A) Deconvolution micrograph of labeled scolopidia in JO. The actin scolopale rods are labeled with phallloidin (magenta), mitochondria in some JO neurons are labeled with mito-GFP (green), nuclei are labeled with TOPRO-3 (blue). B) Schematic diagram of an individual scolopidium, oriented and colored similar to scolopidia in A. C) Approximately longitudinal section of JO in an untraumatized control 40AG13 fly. Labels: m – membranous structure; N – nuclei of JO neurons; ScN – nuclei of scolopale cells; bb - basal bodies; cd – ciliary dilation; cap – dendritic cap; mt – mitochondria; t - trachiole. Scale bar = 1 µm. D) Example of sound-evoked potentials (SEPs) recorded in response to acoustic stimulation. The top trace is the synthetic courtship song pulse stimulus; immediately below is the resulting SEP, with the analyzed amplitude and latency parameters indicated. Bottom: multiple SEPs from a wild-type fly in response to a pulse train.

Fig 2. Acoustic trauma reduces sound-evoked potentials and courtship behavior, without affecting circadian locomotor activity. A-B) Average SEP amplitudes are shown for flies of two lab strains, either exposed (white bars) or not (black bars), to acoustic trauma at two times post trauma. A) SEPs of Canton-S flies. N=69 (sham), 72 (trauma) for 0d; 42 (sham), 39 (trauma) for 7d. B) SEPs of 40AG13 flies. N=66 (sham), 75 (trauma) for 0d; 39 (sham), 37 (trauma) for 7d. C) Mean
normalized courtship index (CI) scores for sham (black bars) and traumatized (white bars) 40AG13 male flies 0 d and 7 d after treatment, N=12 for sham and 16 for traumatized flies at each time-point. For A-C, horizontal bars and asterisks show significant differences (t-test; *, p<0.05; **, p<0.01; ***, p<0.001) and error bars indicate SEM. D) Average activity (measured in crossings/s) of sham (solid line) and trauma-exposed (dashed line) 40AG13 male flies at 1 min intervals for 1 h after cessation of trauma treatment. N=17 flies per treatment.

Fig 3. Post-stimulus SEP response latency increases with acoustic trauma. Plots show distributions of SEP peak latencies (measured from the first pulse stimulus peak to the large negative peak of the subsequent SEP; see Fig 1D) for the second to fifth SEPs. Box and whisker plots show medians as central vertical lines, boxes represent 25-75% quartiles, and whiskers show 5-95 percentiles. Grey bars represent non-traumatized flies, white bars traumatized. A) SEP latencies immediately after treatment (0 d). For sham flies, N=133 (P2), 134 (P3-5). For trauma flies, N=113 (P1), 140 (P2), 139 (P3), 140 (P4), 141 (P5). B) Latencies 7 d post-trauma. For sham flies, N=78 (P2), 79 (P3-5); for trauma flies, N=76 (P2-5). Vertical brackets and asterisks between each sham/trauma pair indicate significant differences (Welch's t-test; *, p<0.05; **, p<0.01; ****, p<0.0001). Note: X-axis absolute values are irrelevant as they depend on tubing length and sound propagation time during stimulus delivery, but this was constant for all measurements; relative values are informative.
Fig 4. Reduced Na\(^+\)/K\(^+\) ATPase \(\beta\) subunit gene dosage sensitizes flies to acoustic trauma. Mean SEPs for control (black bars) and traumatized (white bars) flies. A) 40AG13/Cyo control SEPs. \(N=20-22\) for 0d, 23-28 for 1d, 16-18 for 7d. B) nrv3/40AG13 heterozygote SEPs. \(N=16-23\) for 0d, 23-25 for 1d, 21 for 7d. Horizontal bars and asterisks show significant differences (t-test; *, \(p<0.05\); ***, \(p<0.001\); ****, \(p<0.0001\)). Error bars indicate SEM.

Fig 5. Acoustic trauma reduces mitochondrial size. Natural log of mitochondrial cross-sectional areas calculated from A) control flies (40AG13/Cyo, left bars), and B) nrv3/40AG13 heterozygous mutants (right bars) 0, 1, and 7 d after acoustic trauma. Grey bars correspond to non-traumatized groups; white bars represent flies exposed to trauma. For control flies, \(N\) (no trauma) = 616 for 0d, 504 for 1d, and 581 for 7d; \(N\) (trauma) = 474 for 0d, 473 for 1d, and 529 for 7d. For mutants, \(N\) (no trauma) = 650 for 0d, 810 for 1d, and 810 for 7d; \(N\) (trauma) = 772 for 0d, 865 for 1d, and 601 for 7d. Horizontal bars and asterisks indicate significantly different pairs (Kruskal-Wallis; ns, not significant, **, \(p<0.01\), ***, \(p<0.001\)). Boxes represent 25-75 percentiles from the median value (middle line). Whiskers indicate 5-95 percentiles.
Ln (mitochondria area [$\mu$m$^2$])

A  40AG13/CyO

ns  ns  ***

B  nrn3/40AG13

***  **  ***
**Supplemental Materials and Methods**

**Electrophysiology**

Sound-evoked potentials (SEPs) were recorded as previously described (S1-3). Each fly was mounted in a trimmed 200 µl pipette tip with its head protruding and held in place with modeling clay. The tip was then placed on a custom holder mounted to a movable stage. Guided via a dissecting microscope, two electrolytically sharpened tungsten electrodes were inserted—a recording electrode into the joint between the 1\textsuperscript{st} and 2\textsuperscript{nd} antennal segments, and a reference electrode into the head capsule near the left supraorbital bristles. Electrode signals were passed to and amplified by a DAM50 differential amplifier (World Precision Instruments), then digitized and normalized via an instruNet 100B Analog/ Digital Data Acquisition I/O system (G.W. Instruments) onto a G3 Macintosh computer using SuperScope II (G.W. Instruments) software.

Acoustic stimuli were converted to an analog signal using the Data Acquisition I/O system, fed to a PCA4 amplifier (Pyle Audio Inc.) connected to an 8-inch (20.3 cm) 8Ω speaker (Radio Shack) placed in an acoustic foam-lined box. Sound was delivered to the fly via 0.25 inch (63.5 mm, inner diameter) Tygon tubing with one end mounted close to, but not touching, the speaker cone. A plastic pipette tip, cut to a 5 mm circular opening and plugged loosely with cotton to reduce echo, was inserted into the other end and mounted close to the fly; antennae were kept within the hemisphere circumscribed by the pipette opening to maintain near-field acoustic conditions (S4).

**Circadian locomotor analysis**

Circadian rhythm analysis was performed as described (S5). In the first experiment, locomotor activity of control and noise-exposed adult male and female Canton-S and 40AG13 flies (3-5d post eclosion) was monitored with Trikinetics Activity Monitors for 5 d of LD (12 hr/12hr; see Fig S7A1-2) followed by 7 d of DD at 25ºC. To eliminate the effect of behavioral disruption from placement in the apparatus and acclimatization, all data taken from the first 14 hr were discarded, as were data from flies that died during the course of the experiment. In the second experiment, activity of control and noise-exposed adult male 40AG13, 40AG13/CyO, and nrv3/40AG13 flies (3-5d post eclosion) were studied with the above apparatus for 8 d of LD (12 hr/12hr; see Fig S8A1-3), starting immediately after trauma ended. Data were analyzed off-line using numerical analysis software (GNU Octave) and statistical software (Prism, GraphPad). Activity levels were quantified as averaged number of crossings per unit time (Fig S7-8). For the first experiment, data for males and females were pooled, and differences between traumatized and control flies tested via Student's \(t\)-test (two-tailed; \(\alpha = 0.05\)) for data binned at 12 h and 1 d for the first 7 d of the experiment. In the second, male-only, study we also discarded any data from flies that died during behavioral monitoring. Differences between genotype/treatment groups were tested with 1-way ANOVA (\(\alpha = 0.05\)) with Bonferroni post-hoc comparisons between control and noise-exposed animals within each genotype.

**Nrv3 Immunostaining**

Antennae were dissected in PBS, fixed in 4% paraformaldehyde in PBS for 30min, embedded in O.C.T. and then cut into 25µm sections in a cryostat. Antennae were stained with polyclonal antibodies against Nrv3 (generated in guinea pig; (1:200 in PBT+ BSA; S6) and incubated overnight. After primary antibody staining, antennae were washed in PBT+BSA for an hour before FITC (1:200) secondary antibody incubation. Confocal images were taken using a Leica SP2 Confocal Microscope. The Nrv3 antibody was a gift from Dr. Greg Beitel, at Northwestern University.
Supplementary References


Supplemental Material Figures

Figure S1. Physiological effects of acoustic trauma are evident across a broad range of test stimulus sound pressure levels. Mean sound-evoked potentials (SEPs) with varying stimulus sound-pressure level (dB SPL) versus trauma treatment and days post treatment with varying stimulus sound-pressure level (dB SPL). (A) 65 dB. Canton-S flies: \( N = 65 \) (sham), 68 (trauma) for 0d; 40 (sham), 39 (trauma) for 7d. 40AG13 flies: \( N = 65 \) (sham), 73 (trauma) for 0d; 38 (sham), 37 (trauma) for 7d. (B) 75 dB. Canton-S flies: \( N = 67 \) (sham), 67 (trauma) for 0d; 41 (sham), 39 (trauma) for 7d. 40AG13 flies: 65 (sham), 75 (trauma) for 0d; 39 (sham), 37 (trauma) for 7d. (C) 85 dB (data presented in fig. 1). Canton-S flies: \( N = 69 \) (sham), 67 (trauma) for 0d; 42 (sham), 39 (trauma) for 7d. 40AG13 flies: 66 (sham), 75 (trauma) for 0d; 39 (sham), 37 (trauma) for 7d. (D) 95 dB. Canton-S flies: \( N = 66 \) (sham), 66 (trauma) for 0d; 41 (sham), 39 (trauma) for 7d. 40AG13 flies: 64 (sham), 76 (trauma) for 0d; 40 (sham), 37 (trauma) for 7d. Significance tested by t-test (*, \( p<0.05 \); ***, \( p<0.001 \); **** \( p<0.0001 \)). Error bars indicate SEM. For additional description of graph features, see Fig. 1.
Figure S2. Canton-S flies do not exhibit gross JO abnormalities immediately post-trauma. Longitudinal TEM sections of JO in sham (A,B) and trauma-exposed (C,D) Canton-S flies. All scale bars = 1 mm.
Figure S3. Acoustic trauma does not produce JO morphological defects seven days post-trauma. (A1) Longitudinal and (A2) cross-sectional TEMs of sham 40AG13/CyO flies. (B1) Longitudinal and (B2) cross-sectional TEMs of traumatized 40AG13/CyO flies. (C1) Longitudinal and (C2) cross-sectional TEMs of sham nrv3/40AG13 flies. (D1) Longitudinal and (D2) cross-sectional TEMs of traumatized nrv3/40AG13 flies. All scale bars = 1 mm.

Figure S4. JO neurons show no gross morphological changes seven days post-trauma. Example TEM sections from (A) Sham 40AG13/CyO flies. (B) Trauma-exposed 40AG13/CyO flies. (C) Sham nrv3/40AG13 flies. (D) Trauma-exposed nrv3/40AG13 flies. All scale bars = 1 mm.
Figure S5. Nrv3 protein expression is limited to JO sensory neurons. Confocal image of a cryosection through the antenna showing Nrv3 labeling in the plasma membrane of JO neurons (solid arrow), in the cytoplasm of the neuronal soma (asterisk) and in the inner dendritic segment (dashed arrow). The nerve from the third antennal segment (labeled by AN) also shows Nrv3 expression.
Figure S6. Noise trauma changes mitochondrial morphology. Example TEM sections with highlighted mitochondria (purple) and mitochondria alone from sham (A,C) and traumatized (B,D) 40AG13/CyO flies. The reductions in mitochondrial size between sham (E,G) and noise-exposed (F,H) nrv3/40AG13 flies are even more prominent. All scale bars = 1 μm.
Figure S7. Exposure to acoustic trauma does not greatly affect circadian locomotor patterns. Activity levels pooled in 1 h bins show behavior of Canton-S (A1) and 40AG13 (A2) flies during 5 d of LD (shaded rectangles), and 2 d of DD. Both control (blue lines) and noise-exposed (red lines) show similar anticipatory ‘morning’ and post-dark phase ‘evening’ activity peaks. Activity levels measured in 12 hr (B1, B2) and 1 d (C1, C2) intervals were not significantly different between control (dark bars) and traumatized (white bars) flies. Combined N for Canton-S: 27 flies each for control and traumatized groups; for 40AG13: 23 control flies and 26 traumatized flies. Error bars represent mean count numbers ± SEM.
Figure S8. Circadian behavior of nrv3 heterozygote flies is not affected by exposure to noise trauma. Activity levels pooled in 1 h bins show behavior of 40AG13 (A1), 40AG13/CyO (A2), and nrv3/40AG13 flies during 7 d of LD (shaded rectangles). Both control (blue lines) and noise-exposed (red lines) show similar anticipatory ‘morning’ and post-dark phase ‘evening’ activity peaks. Activity levels measured in 12 hr (B1-3) and 1 d (C1-3) intervals were not significantly different between control (dark bars) and traumatized (white bars) flies. Total N values: 40AG13 - 17 control, 17 traumatized flies; 40AG13/CyO - 18 control, 17 traumatized flies; nrv3/40AG13 - 17 control, 18 traumatized flies. Error bars represent mean count numbers ± SEM.