Mechanically induced damage of lateral-line hair cells

Mechanical overstimulation causes acute injury and synapse loss followed by fast recovery in lateral-line neuromasts of larval zebrafish

Melanie Holmgren¹, Michael E. Ravicz⁴,⁵, Kenneth E. Hancock⁴,⁵, Olga Strelkova⁴,⁵, Artur A. Indzhykulian⁴,⁵, Mark E. Warchol¹,³, Lavinia Sheets¹,²*

¹. Department of Otolaryngology, Washington University School of Medicine, St. Louis, MO, USA
². Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA
³. Department of Neuroscience, Washington University School of Medicine, St. Louis, MO, USA
⁴. Eaton-Peabody Laboratory, Massachusetts Eye and Ear, Boston, Massachusetts, USA
⁵. Department of Otolaryngology–Head and Neck Surgery, Harvard Medical School, Boston, Massachusetts, USA

* Corresponding author
Lavinia Sheets: sheetsl@wustl.edu

Abstract
Excess noise damages sensory hair cells, resulting in loss of synaptic connections with auditory nerves and hair-cell death. The cellular mechanisms underlying mechanically induced hair-cell damage and subsequent repair are not completely understood. Hair cells in neuromasts of larval zebrafish are structurally and functionally comparable to mammalian hair cells but undergo robust regeneration following damage. We therefore developed a model for mechanically induced hair-cell damage in this highly tractable system. Free swimming larvae exposed to strong water current for 2 hours displayed mechanical injury to neuromasts, including afferent neurite retraction, damaged hair bundles, and reduced mechanotransduction. Synapse loss was observed in apparently intact exposed neuromasts, and this loss was exacerbated by inhibiting glutamate uptake. Mechanical damage also elicited an inflammatory response and macrophage recruitment. Remarkably, neuromast morphology, synapse number, and hair-cell function fully recovered within 2 days following exposure. Our results indicate functional changes and synapse loss in mechanically damaged lateral-line neuromasts that appear similar to damage observed in noise-exposed mammalian ear yet are completely repaired.

Keywords: Hair cell, mechanical damage, ribbon synapse, inflammation, regeneration
Introduction

Hair cells are the sensory receptors of the inner ear and lateral-line organs that detect sound, orientation, and motion. They transduce these stimuli through deflection of stereocilia, which opens mechanically-gated cation channels (LeMasurier & Gillespie, 2005; Qiu & Muller, 2018) and drives subsequent transmission of sensory information via excitatory glutamatergic synapses (Glowatzki & Fuchs, 2002). Excessive mechanical stimulation, such as loud noise, can damage hair cells and their synaptic connections to afferent nerves. The degree of damage depends on the intensity and duration of the stimulus, with higher levels of traumatic noise leading to hair-cell loss (Cho et al., 2013; Nordmann, Bohne, & Harding, 2000) and lower levels leading to various pathologies, including damage to the hair-cell mechanotransduction complex/machinery and stereocilia (Gao, Ding, Zheng, Ruan, & Liu, 1992; Husbands, Steinberg, Kurian, & Saunders, 1999), misshapen hair cells (Bullen, Anderson, Bakay, & Forge, 2019), synaptic terminal damage, neurite retraction, and hair-cell synapse loss (Fernandez et al., 2020; Henry & Mulroy, 1995; Kujawa & Liberman, 2009; Puel, Ruel, Gervais d’Aldin, & Pujol, 1998). In addition to directly damaging hair cells, excess noise also initiates an inflammatory response (Hirose, Discolo, Keasler, & Ransohoff, 2005; Kaur et al., 2019). Such inflammation is mediated by macrophages, a class of leukocyte that responds to injury by clearing cellular debris and promoting tissue repair (Wynn & Vannella, 2016).

The variety of injury and range of severity suggests multiple signaling pathways are involved in hair-cell damage associated with exposure to strong mechanical stimuli. Also unknown are the cellular processes mediating repair following such trauma. While hair cells show a partial capacity for repair of stereocilia and synaptic connections, some sub-lethal damage to hair cells is permanent. Numerous studies of the mammalian cochlea suggest that a subgroup of inner hair-cell synapses are permanently lost following noise exposure (Cho et al., 2013; Hickman, Smalt, Bobrow, Quatieri, & Liberman, 2018; Kujawa & Liberman, 2009; Shi et al., 2013). Glutamate excitotoxicity is likely the pathological event that initiates noise-induced hair-cell synapse loss (Hu, Rutherford, & Green, 2020; Kim et al., 2019; Puel et al., 1998), but the downstream cellular mechanisms are still undefined. Further, the cellular mechanisms that promote hair-cell synapse recovery following damage-induced loss are also not understood.

Zebrafish have proven to be a valuable model system for studying the molecular basis of hair-cell injury and repair. Zebrafish sensory hair cells are structurally and functionally homologous to mammalian hair cells (Coffin, Kelley, Manley, & Popper, 2004; Kindt & Sheets, 2018; Sebe et al., 2017). In contrast to other vertebrate model organisms, zebrafish hair cells
Mechanically induced damage of lateral-line hair cells are optically accessible in whole larvae within the lateral-line organs. These sensory organs, called neuromasts, contain clusters of ~14 hair cells each and are distributed throughout the external surface of the fish to detect local water movements. Zebrafish can regenerate complex tissues, including lateral-line organs (Kniss, Jiang, & Piotrowski, 2016; Xiao et al., 2015). This capacity for organ repair combined with optical accessibility allows us to study cellular and synaptic damage and repair in vivo following mechanical trauma.

In order to model mechanical damage in zebrafish lateral-line organs, we developed a protocol to mechanically stimulate the lateral line of free-swimming 7-day-old larvae. Using this protocol, we were able to induce mechanical injury to lateral-line organs that resembled the trauma observed in the mammalian cochlea following acoustic overstimulation. We observed synapse loss and enlargement of postsynaptic densities (PSDs) in all exposed neuromasts as well as hair-cell loss and afferent neurite retraction in a subset of neuromasts. Hair cell mechanotransduction, as measured by uptake of the cationic dye FM1-43, was significantly reduced after mechanical injury. We also observed an inflammatory response similar to that observed in the mammalian cochlea after noise trauma. Remarkably, mechanically induced lateral-line damage appeared to rapidly recover; hair-cell number and morphology returned to normal within 4 - 8 hours following exposure, concurrent with clearance of cellular debris by macrophages. Additionally, neuromasts showed partial recovery of afferent innervation, FM1-43 uptake, and hair-cell synapse number within 2 hours following exposure, and completely recovered by 2 days post-exposure, indicating mechanically injured neuromasts fully repair following damage.

Results

Mechanical overstimulation of zebrafish lateral-line hair cells

To mechanically damage hair cells of lateral-line organs in free-swimming 7-day-old zebrafish, we developed a stimulation protocol using an electrodynamic shaker to create a strong water current (Fig. 1A). The frequency used for mechanical stimulation was selected and further verified (see Method Details) based on previous studies showing 60 Hz to be within the optimal upper frequency range of mechanical sensitivity of superficial posterior lateral-line neuromasts (which respond maximally between 10-60 Hz), but a suboptimal frequency for hair cells of the anterior macula of the inner ear (Levi, Akanyeti, Ballo, & Liao, 2015; Trapani & Nicolson, 2010; Weeg, Fay, & Bass, 2002). Dorsal-ventral displacement of a 6-well dish at 60 Hz and acceleration of 40.3 m/s² (+/-0.5m/s²) created water flow and disturbance of the water surface that was strong enough to trigger ‘fast start’ escape responses—a behavior mediated in
Mechanically induced damage of lateral-line hair cells

part by zebrafish lateral-line organs to escape predation (Fig. 1 B inset) (McHenry, Feitl, Strother, & Van Trump, 2009; Nair, Azatian, & McHenry, 2015). 'Fast start' escape responses can be activated by stimulating hair cells of the lateral line and/or the posterior macula in the ear (Bhandiwad, Zeddies, Raible, Rubel, & Sisneros, 2013). To verify that the observed escape responses were mediated predominantly by flow sensed by lateral-line hair cells rather than hair...
Mechanically induced damage of lateral-line hair cells

cells of the macula, we exposed a group of larvae to low dose (3µM) copper sulfate (CuSO₄) for 1 hour to specifically ablate lateral-line hair cells, but leave hair cells of the ear intact (Olivari, Hernandez, & Allende, 2008). Following a 2 hr recovery after CuSO₄ treatment, we recorded fish behavior with a high-speed camera during the stimulus (1000 fps for 10 s) and compared the responses of fish with lesioned lateral-line organs with those of untreated control siblings (Fig. 1 B,C; Movie S1,2). When subjected to intense water flow, we found that ‘fast start’ responses—defined as a c-bend of the body occurring within 15 ms followed by a counter-bend (Burgess & Granato, 2007; McHenry et al., 2009)—occurred significantly less frequently in larvae with ablated lateral line organs than in siblings with intact lateral line (Fig. 1 C,E; avg. ‘fast start’ responses: 0.4 (± 0.1)/s in lateral-line ablated vs 1.5 (± 0.1)/s in control; 3 trials, 10 s per trial; **P=0.0043). Accordingly, some CuSO₄-treated fish were unable to escape the current and were swept into the waves (Fig. 1 E; arrowheads; Movie S2). These observations indicate that the strong current generated by our device is stimulating lateral-line hair cells and evoking a behaviorally relevant response.

A subset of mechanically injured neuromasts undergo physical displacement that is position dependent but does not require mechanotransduction

To induce mechanical damage to lateral line organs, larvae (10-15 per well) were exposed to an initial 20 minutes of strong water current stimulus followed by 10 minutes of rest, then 2 hours of continuous stimulus (Supplemental Fig. 1A). The ten-minute break in exposure was introduced early on when establishing the stimulus duration because it appeared to enhance larval survival; it was therefore maintained throughout the study for consistency. Fish were euthanized and fixed immediately after exposure, then processed for immunohistofluorescent labeling of hair cells and neurons. Unexposed sibling fish served as controls. As posterior lateral-line (pLL) neuromasts have been shown to specifically initiate escape behavior in response to strong stimuli (Liao & Haehnel, 2012), analysis of the morphology of pLL neuromasts L3, L4, and L5 was conducted for exposed and control larvae (Fig. 2 A). Initially, we divided the observed neuromast morphology into two categories: “normal”, in which hair cells were radially organized with a relatively uniform shape and size, and “disrupted”, in which the hair cells were misshapen and displaced to one side, with the apical ends of the hair cells localized anteriorly (Fig. 2 B, C; see Methods Details for measurable criteria). Position of the neuromast along the tail was also associated with vulnerability to disruption; we observed a gradient of damage in the pLL from rostral to caudal i.e. L5 was more susceptible to disruption than L4, which was more susceptible to disruption than L3 (Fig. 2 F; Repeated measure One-way ANOVA *P=0.0386, **P=0.0049, ***= 0.0004).
Additionally, we compared mechanical stress from our sustained exposure to an exposure protocol that delivered intermittent pulses of stimulus (“periodic exposure”; Supplemental Fig.1 A). We observed neuromast disruption less frequently with periodic exposures vs. sustained exposure of the same intensity (Supplemental Figure 1 B; Unpaired t-test **P=0.0034), supporting that displacement of neuromasts is a consequence of mechanical injury. Additionally,
Mechanically induced damage of lateral-line hair cells

we examined hair cell morphology in the ears of larvae exposed to sustained stimulus and observed no apparent damage (Supplemental Fig. 2), indicating our overstimulation protocol produces mechanical damage specifically to lateral-line organs.

To determine if hair-cell activity plays a role in the displacement of neuromasts, we exposed *lhfp5b* mutants—fish that have intact hair cell function in the ear, but no mechanotransduction in hair cells of the lateral line—to sustained stimulation (Erickson, Pacentine, Venuto, Clemens, & Nicolson, 2019). We observed comparable morphological disruption of neuromasts lacking mechanotransduction (Fig. 2G), suggesting that displacement of lateral-line hair cells is due to physical damage from the stimulus. Further, we observed the adjacent supporting cells in neuromasts with disrupted hair-cell morphology appeared similarly displaced and elongated (Fig. 2 E; white arrows), indicating that mechanical injury disrupts the structural integrity of the entire neuromast organ.

**Hair-cell loss and loss of afferent nerve contacts correspond to neuromast disruption**

Moderate noise exposures can cause damage or loss of cochlear hair-cell synapses, including swelling and retraction of afferent nerve fibers, while more extended and/or severe exposures lead to hair-cell loss. To address whether mechanical damage in the lateral line produced similar morphological changes, we surveyed the number of hair cells per neuromast and the percentage of neuromast hair cells lacking afferent innervation in fish immediately following exposure to sustained stimulation (Fig. 3). We observed a reduction in the number of hair cells per neuromast immediately following exposure (Fig. 3 D; Unpaired t-test, *P* = 0.0125; n = 114-149 L3-L5 neuromasts; N = 13 trials) as well as a significant reduction in the percentage of hair cells per neuromast innervated by afferent neurons (Fig. 3 E; One sample Wilcoxon test, ****P < 0.0001; n = 76-103 neuromasts; N = 12 trials).

As described in Fig. 2 F, we found on average ~half of L3-L5 neuromasts examined showed “disrupted” hair-cell morphology immediately following sustained stimulus exposure. To define the associations between overall neuromast morphology and specific structural changes in mechanically injured neuromasts, we examined the numbers of hair cells and the percent of hair cells contacted by afferent fibers in exposed neuromasts parsed into “normal” and “disrupted” morphologies. With hair cell number, we observed significant loss specifically in “disrupted” neuromasts, while “normal” neuromast hair-cell number appeared comparable to control (Fig. 3 D; Ordinary one-way ANOVA, *P* = 0.9995 (normal), ****P < 0.0001 (disrupted)). With hair-cell afferent contacts we observed a similar trend i.e., a significant number of hair cells lacked afferent innervation in stimulus exposed neuromasts with “disrupted” morphology, but not...
Mechanically induced damage of lateral-line hair cells

“normal” neuromasts (Fig. 3 E’; One sample Wilcoxon test, P>0.9999 (normal), ****P<0.0001 (disrupted)).

Figure 3: Hair-cell loss and de-innervation is specific to “disrupted” neuromasts.
(A-C) Representative maximum intensity projection images of control (A), or exposed lateral-line neuromasts with “normal” (B) or “disrupted” (C) morphology immediately post exposure (0 h). Synaptic ribbons (magenta; Ribeye b) and hair cells (blue; Parvalbumin) were immunolabeled. Afferent neurons were expressing GFP.

(D) Hair-cell number per neuromast (NM; L3-L5) immediately following sustained strong wave exposure. A moderate but significant reduction in hair cell number was observed (*P=0.0125) and was specific to “disrupted” neuromasts (P=0.9995 norm, ****P<0.0001 dis). Scale bar: 5µm (E)

Percentage of neuromast hair cells innervated by afferent nerves. Each symbol represents a single neuromast; symbols in Ctrl appear as one because of overlap at 100% innervated (n=76). A significant portion of neuromast hair cells lacked afferent innervation following exposure; ****P<0.0001 sustained). Hair cells lacking afferent innervation were specifically observed in disrupted neuromasts (P>0.9999 normal, ****P<0.0001 disrupted). Pink symbols on graphs (Exp) represent pooled exposed neuromasts, while gray (Norm) and red (Dis) represent neuromasts parsed into normal and disrupted groups. Error Bars = SEM

Mechanically overstimulated neuromasts with “normal” morphology lose a greater number of hair-cell synapses when glutamate clearance is inhibited

Hair cells contain electron-dense presynaptic specializations—called dense bodies or synaptic ribbons—apposing afferent PSDs which constitute afferent synaptic contacts (Davies, Tingley, Kachar, Wenthold, & Petralia, 2001; Sheets, Trapani, Mo, Obholzer, & Nicolson, 2011). In the larval zebrafish lateral line, afferent nerve fibers innervate multiple hair cells per neuromast forming ~3-4 synaptic contacts per hair cell. To determine whether strong water current exposure contributed to hair-cell synapse loss, we counted the number of intact synapses (ribbons juxtaposed to PSDs; Fig. 4 A-C) in control and exposed larvae. We observed
Mechanically induced damage of lateral-line hair cells

Figure 4: Synapse loss is observed in hair cells following mechanical overstimulation and exacerbated by blocking glutamate uptake. A-C) Representative maximum intensity projection images of unexposed (A), or wave stimulus exposed lateral-line neuromast with “normal” (B) or “disrupted” (C) morphology. Synaptic ribbons (magenta; Ribeye b), PSDs (green; MAGUK) and hair cells (blue; Parvalbumin) were immunolabeled. Scale bar: 5µm (D) Intact synapses per neuromast hair cell. Each symbol represents an individual neuromast (L3-5). Pink symbols on graph (Exp) represent pooled exposed neuromasts, while gray (Norm) and red (Dis) represent neuromasts parsed into normal and disrupted groups. The number of intact synapses per hair cell was significantly reduced following exposure (Unpaired T-Test **P=0.0036) and was somewhat more pronounced in “normal” neuromasts than “disrupted” (One-way ANOVA, *P=0.0213 normal, P=0.0685 disrupted). (E) The number of intact synapses per hair cell in larvae co-treated with TBOA (to block glutamate uptake) or drug carrier alone during exposure. Synapse loss was significantly greater in “normal” NMs co-exposed to TBOA (Two-way ANOVA. ****P<0.0001). Error Bars = SEM

194 a significant reduction in the number of intact synapses per hair cell following sustained
195 exposure (Fig. 4 D; Unpaired t-test, **P=0.0036). When we compared “normal” and “disrupted”
196 neuromasts following exposure, we observed a loss of intact synapses per hair cell in all
197 exposed neuromasts, with significantly fewer synapses in “normal” exposed neuromasts relative
198 to control (Fig. 4 E; Ordinary one-way ANOVA, *P=0.0213 (normal), P=0.0685 (disrupted)).
Additionally, significant synapse loss was observed in the neuromasts of fish exposed to the less mechanically damaging “periodic” stimulus (Supplemental Fig. 1 F’). Taken together, these data revealed that synapse loss was observed in all mechanically overstimulated neuromast hair cells, including neuromasts that were not mechanically disrupted.

Previous studies indicate that excess glutamate signaling may be a key factor driving inner hair-cell synapse loss following exposure to damaging noise (Chen, Kujawa, & Sewell, 2010; Kim et al., 2019). We therefore inhibited glutamate clearance from neuromast hair-cell synapses by pharmacologically blocking uptake with the glutamate transporter antagonist Threo-beta-benzyloxyaspartate (TBOA) during sustained stimulus exposure. We observed a significantly greater degree of hair-cell synapse loss in stimulated neuromasts with “normal” morphology co-treated with TBOA than in stimulated neuromasts co-treated with drug carrier alone (Fig. 4 E; Two-way ANOVA ****P<0.0001 “normal” TBOA), suggesting glutamate excitotoxicity contributes to hair-cell synapse loss observed in mechanically overstimulated neuromasts with intact morphology.

To further characterize synapse loss in relation to afferent neurite retraction, we examined fish in which we immunolabeled synaptic ribbons, PSDs, afferent nerve fibers, and hair cells (Fig. 5 A,B). We then quantified instances where synapses, i.e. juxtaposed pre- and postsynaptic components associated with hair cells, were no longer adjacent to an afferent nerve terminal. As these synapses appeared detached and suspended from their associated neurons, making them no longer functional, we refer to them as synaptic debris. While we rarely observed synaptic debris in unexposed neuromasts, we observed a greater relative frequency of synaptic debris in neuromasts exposed to strong water current stimulus (Fig. 5 C; One sample Wilcoxon test, P=0.1250 (control), *P=0.0313(normal), **P=0.0039(disrupted)).

Cumulatively, we observe two distinct types of morphological damage to afferent synapses in mechanically overstimulated neuromasts: loss of synapses that is exacerbated when glutamate uptake is blocked and a higher incidence of synaptic debris that appear detached from afferent neurites.

Mechanically overstimulated lateral line neuromasts show signs of hair-cell injury and macrophage recruitment

The inner ears of birds and mammals possess resident populations of macrophages, and additional macrophages are recruited after acoustic trauma or ototoxic injury (Warchol, 2019). A similar macrophage response occurs at lateral line neuromasts of larval zebrafish after neomycin ototoxicity (Warchol, Schrader, & Sheets, 2021). Analysis of fixed specimens, as well as time-lapse imaging of living fish (e.g., Hirose, Rutherford and Warchol, 2017), has
Mechanically induced damage of lateral-line hair cells
demonstrated that macrophages migrate into neomycin-injured neuromasts and actively phagocytose the debris of dying hair cells. To determine whether a similar inflammatory response also occurs after mechanical injury to the lateral line, we characterized macrophage behavior after sustained 60 Hz stimulation. These studies employed Tg(mpeg1:yfp) transgenic fish, which express YFP in all macrophages and microglia. Fish were fixed immediately after exposure, or allowed to recover for 2, 4 or 8 hours. Control fish consisted of siblings that received identical treatment, but were not exposed to mechanical stimulation. Data were obtained from the two terminal neuromasts from the pLL of each fish (Fig. 6 A). In agreement
with data shown in Fig. 3 D, we observed a modest but significant decline in hair-cell number in specimens that were examined immediately and at 2 hours after sustained exposure (Fig. 6 B; **P<0.003). Consistent with earlier studies (Hirose, Rutherford, & Warchol, 2017), 1-2 macrophages were typically present within a 25 µm radius of each neuromast (Fig. 6 C). In
Mechanically induced damage of lateral-line hair cells

uninjured (control) fish, those macrophages remained outside the sensory region of the
neuromast and rarely contacted hair cells. However, at 2, 4 and 8 hours after sustained
stimulus, we observed increased macrophage-hair cell contacts (Fig. 6 D; *P=0.024), as well as
the presence of immunolabeled hair-cell debris within macrophage cytoplasm (suggestive of
phagocytosis, Fig. 6 A, 4 h., arrow). Macrophage-hair cell contact and phagocytosis peaked at 2
hours after exposure (Fig. 6 E; **P= 0.0013 (2 h)). Notably, the numbers of macrophages within
a 25 µm radius of each neuromast remained unchanged at all time points after exposure,
suggesting that the inflammatory response was mediated by local macrophages and that
mechanical injury did not recruit macrophages from distant locations (Fig. 6 C). This pattern of
injury-evoked macrophage behavior is qualitatively similar to the macrophage response
observed in the mouse cochlea after acoustic trauma (Hirose et al., 2005; Kaur et al., 2019;
Kaur et al., 2015).

Mechanically injured neuromasts fully repair following exposure

To determine if damage to mechanically injured neuromasts was progressive, persistent,
or reversible, we characterized neuromast morphology, hair-cell number, innervation, and
synapse number at both 2 hours and 48 hours following sustained exposure to strong current
stimulus. We observed a decrease in the percentage of neuromasts showing “disrupted”
morphology 2 hours following exposure, relative to fish fixed immediately following exposure
(Fig. 7 A; 54% disrupted (0 h) vs. 32% disrupted (2 h); N=4 trials), suggesting that physical
disruption of neuromast morphology following mechanical injury is rapidly reversible. Consistent
with this observation, the average hair-cell number per neuromast at 2 hours post-exposure
appeared to recover (Fig. 7 B; Unpaired t-test, P=0.3011; n=63-75 neuromasts; N=7 trials).
Recovery of hair-cell number occurred within 2-4 hours (Fig. 6 A, B) and corresponded with
macrophages infiltrating neuromasts and phagocytosing hair-cell debris (Fig. 6 A, E) as well as
a slight increase in supporting cell proliferation (Supplemental Figure 3 A,C). We also observed,
compared to immediately following exposure, a lesser degree of afferent fiber retraction (Fig. 7
C; One sample Wilcoxon test, ***P=0.0010; n=38-44 neuromasts; N=6 trials) and some synaptic
recovery 2 hours following mechanical injury (Fig. 7 D, Unpaired t-test, *P=0.0271; n=37-47
neuromasts; N=6 trials) indicating partial recovery of innervation and synapse number.

A recent study characterized zebrafish lateral-line hair-cell damage induced by exposure
to ultrasonic waves and reported a delayed hair-cell loss 48-72 hours following exposure (Uribe
et al., 2018). To determine if lateral line neuromasts damaged from strong water current
stimulus generated by our apparatus either continued to recover or underwent further delayed
damage, we examined hair-cell morphology, number, and innervation 48 hours following
Mechanically induced damage of lateral-line hair cells

sustained stimulus exposure. Nearly all exposed neuromasts examined showed “normal” HC
morphology (Fig. 7 E) with no difference in hair-cell number (Fig. 7 F; Unpaired t-test, P=0.1253; 
n=49-57 neuromasts; N=5 trials). Hair-cell innervation after 48 hours was comparable to control
fish (Fig. 7 G; One sample Wilcoxon test, P=0.9999; n=24-28 neuromasts; N=5 trials). We also
surveyed whether synapse loss resulting from overstimulation persisted in exposed neuromasts

Figure 7: Mechanically overstimulated neuromasts recover hair-cell morphology, innervation,
and synapse number by 48 hours following sustained exposure. (A,E) Average percentage of
exposed neuromasts with “normal” vs. “disrupted” morphology following exposure. Each dot
represents the percentage of disrupted neuromasts (L3-L5) in a single experimental trial; lines
connect data points from the same cohort of exposed fish following 2 hours (A) or 48 hours (E)
recovery. (B,F) Hair-cell number per neuromast was nearly comparable to control following 2 hour (B,
P=0.3011) and 48 hour recovery (F, P=0.1253). (C,G) The percentage of neuromast hair cells lacking
afferent innervation was still significant following 2 hour recovery (C, ***P=0.0010), but was fully
recovered by 48 hours (G, P>0.9999). (D,H) Significantly fewer intact synapses per hair cell were
observed 2 hours following exposure (D, *P=0.0271), but appeared to recover by 48 hours (H,
P=0.7044). Each pink symbol on graphs (Exp) represent an individual exposed neuromast; both
normal and disrupted were pooled for the analysis. Error Bars = SEM
and observed synapse number per hair cell also appeared to fully recover (Fig. 7 H; Unpaired t-test, $t^{0.7044}; n=23-24$ neuromasts; N=5 trials).

![Image](https://example.com/image.jpg)

**Figure 8:** Changes in synaptic ribbon and PSD sizes following sustained mechanical overstimulation. (A-A”) Representative images of control (A) and exposed (A’, A”) neuromasts. Synaptic ribbons (magenta; Ribeye b), PSDs (green; MAGUK), and hair cells (blue, Parvalbumin) were immunolabeled. Scale bars: 5µm (main panels), 1µm (insets). (B-E) Box and whisker plots of relative synapse volumes normalized to 0h control. Whiskers indicate the min. and max. values; "*" indicates the mean value, horizontal lines indicate the relative median value of the control. (B) Ribbon volume appeared comparable to control immediately following exposure but was reduced 2 hours after exposure ($P=0.0195$). (C) Significant reduction in ribbon size relative to control was specific to disrupted neuromasts (Kruskal-Wallis test: $***P=0.0004$ (2h)). (D) Significantly larger PSDs were observed both immediately and 2 hours following exposure ($****P<0.0001$). (E) Enlarged PSDs were present in both “normal” and “disrupted” exposed neuromasts, with a greater enlargement observed 0h post-exposure (Kruskal-Wallis test: $****P<0.0001$ (0h); $***P=0.0001$, $**P=0.0024$ (2h)).

**PSDs are enlarged in all neuromasts following mechanical overstimulation**

Previous studies in mice and guinea pigs indicate moderate noise exposures modulate the size of synaptic components (Kim et al., 2019; Song et al., 2016). To determine if pre- and postsynaptic components were also affected in our model, we compared the relative volumes of neuromast hair-cell presynaptic ribbons and their corresponding PSDs in control and stimulus exposed larvae. We observed a moderate reduction in synaptic-ribbon size following exposure; ribbon volumes were significantly reduced relative to controls following 2 hours recovery (Fig. 8...
Mechanically induced damage of lateral-line hair cells

Mechanically induced damage of lateral-line hair cells

B; Kruskal-Wallis test *P=0.0195; N=3 trials), and this reduction was specific to “disrupted” neuromasts (Fig. 8 C).

While the changes in ribbon volume we observed were modest and delayed in onset, we saw dramatic enlargement of PSDs immediately and 2 hours following exposure (Fig. 8 D; Kruskal-Wallis test ****P<0.0001; N=3 trials). In contrast to the observed reduction in ribbon size, relative PSD volumes were significantly enlarged in all exposed neuromasts regardless of whether neuromast morphology was “normal” or “disrupted” (Fig. 8 E). These data reveal enlarged PSDs as the predominant structural change in mechanically overstimulated neuromast hair-cell synapses.

Figure 9: Scanning electron microscopy imaging of tail neuromasts following mechanical injury confirms the damage is more prominent for posterior neuromasts. (A-C) Representative images of tail neuromasts of control fish larvae, presented as they are positioned on the larva: L1, L2 and terminal neuromasts. Each hair cell carries a tubulin-based primary cilium (kinocilium), which is thicker than the multiple actin-filled, mechanosensitive stereocilia arranged in a staircase. (D-G) Examples of tail neuromasts immediately following sustained stimulus exposure, presented as they are positioned on the larva: L2, L4, and two terminal neuromasts highlighting different levels of damage, with much more pronounced damage evident on terminal neuromasts. Scale bars: 1 µm.

Mechanically injured neuromasts have damaged kinocilia, disrupted hair-bundle morphology, and reduced FM1-43 uptake

An additional consequence of excess noise exposure in the cochlea is damage to mechanosensitive hair bundles at the apical end of hair cells and, correspondingly, disruption of mechanotransduction (Wagner & Shin, 2019). Larval zebrafish lateral-line hair cells each have a hair bundle consisting of a single kinocilium flanked by multiple rows of actin-rich stereocilia (Kindt, Finch, & Nicolson, 2012). To determine if our exposure protocol damaged apical hair-cell
Mechanically induced damage of lateral-line hair cells structures, we used confocal imaging and scanning electron microscopy (SEM) to assess hair bundle morphology in both unexposed control larvae and larvae fixed immediately following sustained exposure. All neuromasts throughout the fish were evaluated, but to remain consistent with our fluorescence imaging results, we closely assessed the appearance of the caudal pLL neuromasts. We found the caudal neuromasts to be more damaged than the ones positioned more rostrally: the frequency of neuromasts with apparently disrupted appearance

Figure 10: Scanning electron microscopy imaging of neuromasts following mechanical injury reveals disorganized hair cell stereocilia bundles and damaged kinocilia. (A-E) Representative images of tail neuromasts of control fish larvae. Each hair cell carries a kinocilium, which is visibly thicker than its neighboring actin-filled, mechanosensitive stereocilia: see panel C featuring both structures at higher magnification (the kinocilium diameter is 220 nm, while stereocilia measured 90-110 nm). The kinocilia of control neuromasts are long (10-15 µm) and bundled together, while the stereocilia bundles have an apparent staircase arrangement. (F-K) Representative images of damaged tail neuromasts immediately following noise exposure featuring short (F-H, yellow arrows), disorganized (G, H), and swollen (I-K, yellow arrowheads) kinocilia, and disorganized stereocilia. (K) Same stereocilia bundle as in J marked with an asterisk at higher magnification to highlight the difference in the diameter of the kinocilium (360 nm) and neighboring stereocilia (85-100 nm) for noise exposed hair cells, as compared to the control hair cells in C. Scale bars: A, B, D-J – 2 µm; C, K – 500 nm. (L-M) Kinocilia diameter at bundle level (L: Mann Whitney test ***P=0.0007) and 3-5 µm above bundle level (M: Unpaired t-test *P=0.0317). Exposed NM data in L were not normally distributed (D'Agostino-Pearson test ****P<0.0001). Error Bars = SD
Mechanically induced damage of lateral-line hair cells

increased the closer its position to the tail (Fig. 9). This is consistent with our fluorescence
observations (Fig. 2 F; Supplemental Fig. 4 B) in which L5 neuromasts were more likely to be
disrupted than more anteriorly positioned L3. In a few extreme cases, terminal neuromasts
appeared engulfed by the surrounding skin, with only the bundle of kinocilia visible through the
sleeve-like opening of the skin (Fig. 9 F,G).

A closer examination of neuromast morphology revealed a difference of the kinocilia
length and bundling. The neuromasts of the control fish carry a bundle of long (10-15 µm),
uniformly shaped kinocilia (Fig. 10 A-E; Supplemental Fig. 4 A). In contrast, the neuromasts of
the fish fixed immediately after sustained exposure often appear to carry much shorter kinocilia
(Fig. 10 F-H, yellow arrows), which lack bundling and, in some cases, pointing to different
directions (Fig. 10 G,H; Supplemental Fig. 4 B). The apparent kinocilia length difference
between control and overstimulated neuromasts suggests at least some kinocilia could be
undergoing a catastrophic damage event at the time of stimulation, as their distal parts break off
the hair cells. This is further supported by some examples of kinocilia with thicker, ‘swollen’
proximal shafts closer to the cuticular plate of the cell, some of which extend into a thinner distal
part while others appear to lack the distal part completely (Fig. 10 I-K, yellow arrowheads).
Accordingly, the average diameter of kinocilia at the level of the hair bundle (L2-5) was
significantly larger than control, with a few kinocilia showing dramatically thicker widths ~2x
greater than the thickest control (Fig. 10 L; ***P=0.0007). When measured ~3-5 µm above the
bundle, the exposed neuromast kinocilia have a somewhat larger average diameter relative to
control, but not as dramatic as observed at the base (Fig. 10M; *P=0.0243). Stereocilia bundles
from both groups of animals carried tip links, but we were unable to systematically evaluate and
quantify their abundance. However, we observed signs of damaged bundle morphology
following overstimulation, as they often appeared splayed, with gaps between the rows of
stereocilia (Fig. 10 I, J).

To determine the effect of mechanical overstimulation on hair-bundle function, we
assessed mechanotransduction by briefly exposing larvae to the mechanotransduction-channel
permeable dye FM1-43 (Toro et al., 2015). While most hair cells showed a dye uptake in both
experimental groups, we observed a significant reduction in the relative intensity of FM1-43
immediately following exposure (Fig. 11 A-C, G; ****P<0.0001). Reduction of FM1-43 uptake
appeared to correspond with the degree of mechanical displacement of neuromasts, as we
observed notably less relative fluorescence in “disrupted” neuromasts compared to neuromasts
with “normal” morphology (Fig. 11 H; ****P<0.0001 (disrupted), ***P=0.0009 (normal)).
Mechanically induced damage of lateral-line hair cells

Corresponding to what we observed with hair-cell morphology and number (Fig. 7 A,B), mechanotransduction appears to recover rapidly; 2 hours post exposure FM1-43 uptake is less reduced (Fig. 11 D-G; **P=0.0043) and nearly fully recovered in exposed neuromasts with “normal” morphology (Fig. 11 H; ****P<0.0001 (disrupted), P=0.3846 (normal)). We further observed that FM1-43 uptake after 48 h recovery was comparable to that observed in undamaged (control) fish (Fig. 12) supporting that hair-cell morphology and function fully recover.

Discussion

To model mechanical injury resulting from noise trauma in the zebrafish lateral line, we describe here a method to mechanically overstimulate neuromasts of the posterior lateral line. Using this method, we observed: i) hair-cell synapse loss and enlarged postsynaptic densities in

Figure 11: Hair-cell mechanotransduction is reduced following mechanical overstimulation. (A-F)
Representative images of FM1-43FX fluorescent intensity immediately (A-C) or 2 hours (D-F) following exposure. Note that fluorescent intensity appears visibly and dramatically reduced in “disrupted” neuromasts (C,F) and somewhat reduced in “normal” neuromasts 0h post exposure (B). (G) Quantified FM1-43FX fluorescence intensity measurements indicate recovery of mechanotransduction in exposed neuromasts (Unpaired t-test: ****P<0.0001 (0h), **P=0.0043 (2h)). (H) FM1-43FX fluorescence in “normal” exposed neuromasts is significantly reduced at 0h but appears to fully recover by 2h post-exposure. By contrast, fluorescence intensity in “disrupted” neuromasts modestly recovered 2h post-exposure. (Ordinary one-way ANOVA ****P<0.0001, ***P=0.0009, **P=0.0078 (0h); ****P<0.0001 (2h)).
Mechanically induced damage of lateral-line hair cells

all stimulus exposed neuromasts, ii) morphological displacement, hair-cell loss, and afferent
neurite retraction in a subset of mechanically disrupted neuromasts, iii) an inflammatory
response that peaked 2-4 hours following stimulus exposure, iv) kinocilia and hair bundle
damage, and v) reduced FM1-43 uptake, suggesting damage to mechanotransduction
machinery. Remarkably, mechanically injured neuromasts rapidly recover following exposure;
neuromast morphology, innervation, synapse number, and mechanotransduction showed partial
recovery within hours and were completely recovered by 2 days post exposure.

Zebrafish lateral-line as a model for sub-lethal mechanical damage and noise-induced
synapse loss

Mechanical damage to the zebrafish lateral line induced by strong water current stimulus
is observable immediately following exposure, is specific to the lateral-line organ, and appears
to be rapidly repaired. These observations contrast with a recently published noise damage
protocol for larval zebrafish which used ultrasonic transducers (40-kHz) to generate small,
localized shock waves (Uribe et al., 2018). They reported delayed hair-cell death and modest
synapse loss that was not apparent until 48 hours following exposure, was not accompanied by
decreased mechanotransduction, and was observed in the inner ear as well as the lateral-line
organs. Some of the features of the damage they observed—delayed onset apoptosis and hair-
Mechanically induced damage of lateral-line hair cells

cell death—may correspond to lethal damage following blast injuries. We propose features of the damage we observe with our stimulus protocol—reduced mechanotransduction, hair synapse loss, and rapid inflammatory response—may correspond to sub-lethal noise-induced damage of hair-cell organs. Because of differences in the nature of the stimuli in these two studies, it is difficult to directly compare the pathological outcomes. Mechanical overstimulation in the Uribe et al., study was induced using ultrasonic (40 kHz) actuators. Such high frequencies are far outside those that are detected by lateral line neuromasts (Levi et al., 2015; Trapani & Nicolson, 2010) supporting that cavitation in the water medium is likely causing the observed damage. In contrast, our study delivered a stimulus of high intensity 60 Hz water waves directly to the fish. This frequency is within the range of sensitivity of lateral line neuromasts of larval zebrafish and evoked a lateral-line mediated behavior (Fig 1B, C) suggesting that the hair cells were being directly stimulated by the water motion. The present method more closely resembles the techniques that are typically used to study noise damage in the mammalian cochlea, where high intensity acoustic energy causes hair cell and synaptic injury in specific regions of the cochlea that are best-responsive to the frequency of the stimulus. This idea is further supported by the observation that synapse loss in hair cells exposed to strong current stimulation is greater when glutamate uptake is blocked (Fig. 4 E), suggesting a shared mechanism of glutamate excitotoxicity between noise-exposed mammalian ears and strong water current stimulus exposed lateral-line organs (Kim et al., 2019; Sebe et al., 2017).

Disruption of neuromast morphology is a consequence of mechanical injury

Strong water current exposure produced a percentage of pLL neuromasts that were morphologically “disrupted”. Several observations support that such “disrupted” neuromasts represent mechanical injury to neuromast organs. One is that lhfpl5b mutant neuromasts, which lack mechanotransduction specifically in lateral-line hair cells, were comparably vulnerable to physical disruption as their wild-type siblings (Fig. 2 G;(Erickson et al., 2019)). This finding supports that hair-cell activity during stimulation does not underly the physical displacement of hair cells observed following strong water current stimulus. Additionally, physical disruption of the neuromast affects the whole organ—hair cells and their adjacent supporting cells (Fig. 2 D). This observation contrasts with what is observed in mammalian ears exposed to high intensity noise, where mechanical injury to outer hair cells is localized to stereocilia disruption (H. Wang, Yin, Yu, Huang, & Wang, 2011). Speculatively, displacement of hair cells in mechanically injured neuromasts may be due to loss of structural support from displaced supporting cells. As the lateral-line organs are superficially localized on the surface of the skin, some of the intense mechanical tension applied across the tail is likely coupled to neuromasts leading to the
Mechanically induced damage of lateral-line hair cells

physical displacement of a subset of exposed neuromasts. Finally, we observed greatly reduced
FM1-43 uptake in “disrupted” neuromasts and measurable changes kinocilia likely reflecting
mechanical damage (Fig. 10; (Wagner & Shin, 2019)). At this time, we are unable to directly
correlate the bundle or kinocilia phenotype as seen on SEM of “disrupted” vs. “normal”
neuromasts to their function as assessed by FM1-43 loading experiments. Future work on
mechanical damage will focus on a close evaluation of the FM1-43 uptake and subsequent
SEM imaging of the same neuromast immediately after stimulus exposure and following
recovery over time.

Hair-cell overstimulation and synapse loss

We observed significant loss of hair-cell synapses in neuromasts that were exposed to
strong water currents but not mechanically disrupted. Two notable observations were made in
exposed neuromasts regarding synapse loss. First, loss of synapses in “normal” exposed
neuromasts was markedly more severe when synaptic glutamate clearance was inhibited (Fig. 4
E), suggesting that synapse loss may reflect moderate hair-cell damage resulting from
overstimulation and excess glutamate accumulation. Involvement of glutamate signaling as a
key mediator of noise-induced synapse loss has recently been reported in mice; loss of
glutamate signaling prevents noise-induced synapse loss and pharmacologically blocking
postsynaptic Ca\(^{2+}\) permeable AMPA receptors protects against cochlear hair-cell synapse loss
from moderate noise exposure (Hu et al., 2020; Kim et al., 2019). Second, synapse loss
occurred in neuromasts that appeared to be fully innervated (Fig. 3 B, Fig. 4 B). This
observation was somewhat more surprising given that pharmacologically activating
evolutionarily conserved Ca\(^{2+}\) permeable AMPARs has been shown to drive afferent neurite
retraction in the zebrafish lateral line (Sebe et al., 2017). We propose that subtle damage to
afferents in “normal” exposed neuromasts may accompany synapse loss but not be apparent as
neurite retraction, as single afferent processes innervate multiple hair cells of the same polarity
within an individual neuromast (Dow, Jacobo, Hossain, Siletti, & Hudspeth, 2018; Faucherre,
Pujol-Martí, Kawakami, & Lopez-Schier, 2009). Additionally, glutamate signaling may not be the
only driver of synapse loss resulting from excess stimulation; studies in zebrafish and mice
support that mitochondrial stress also contributes to hair-cell synapse loss (X. Wang et al.,
2018; Wong et al., 2019). Future work using this model to examine the effect of excess
mechanical stimulation on mutants with reduced glutamate release or impaired glutamate
 clearance (to elevate or reduce glutamate in the synaptic cleft, respectively) combined with
modified mitochondrial function may define the relative roles of glutamate excitotoxicity and hair-
cell mitochondrial stress to synaptic loss.
Role of inflammation following mechanical injury to lateral-line organs

Our results indicate that mechanical injury to neuromasts evokes an inflammatory response. Prior studies of larval zebrafish have shown that macrophages reside near the borders of uninjured neuromasts and migrate into neuromasts after ototoxic injury (Hirose et al., 2017). We found that macrophages migrate into neuromasts within ~2 hours of mechanical injury, where they contact hair cells and, in some cases, engulf hair-cell debris. Although this macrophage response is similar to that which occurs after ototoxic injury to neuromasts (Carrillo et al., 2016; Hirose et al., 2017; Warchol et al., 2021), the extent of hair cell loss after mechanical overstimulation is much less than the injury that occurs after ototoxicity. We observed macrophage entry in 30-40% of exposed neuromasts, despite modest hair cell loss (Fig. 6 B,D). It is possible that the morphological changes characteristic of mechanically injured neuromasts are accompanied by the release of macrophage chemoattractants. In addition, studies of noise exposure to the mammalian cochlea indicate that high levels of synaptic activity (without accompanying hair cell loss) can evoke macrophage migration to the synaptic region (Kaur et al., 2019). In either case, the signals responsible for such recruitment remain to be identified. The observation that macrophages had internalized immunolabeled hair-cell material further suggests that recruited macrophages engage in the phagocytosis of hair cell debris, but it is not clear whether macrophages remove entire hair cells or target specific regions of cellular injury (e.g., synaptic debris; Fig. 5). In any case, our data indicate that the macrophage response to mechanical injury of zebrafish lateral-line neurons is similar to that which occurs after noise injury to the mammalian cochlea (Warchol, 2019) and suggests that zebrafish may be an advantageous model system in which to identify the signals that recruit macrophages to sites of excitotoxic injury.

Hair-cell synapse morphology following mechanical overstimulation

Immediately following mechanical overstimulation, the most pronounced morphological change we observed in hair-cell synapses was significantly enlarged PSDs (Fig. 8 D, E). Speculatively, PSD enlargement may be a consequence of reduced glutamate release from hair cells following sustained intense stimulation. Mice and zebrafish fish lacking hair-cell glutamatergic transmission have enlarged postsynaptic structures (Kim et al., 2019; Sheets, Kindt, & Nicolson, 2012), indicating that glutamate may regulate postsynaptic size. While our data do not directly support this idea, we speculate that reduced glutamatergic transmission in mechanically overstimulated neuromasts may be a consequence of damaged hair bundles and impaired mechanotransduction (Zhang et al., 2018). Alternatively, cholinergic efferent feedback, which has been shown to hyperpolarize lateral-line hair cells, may reduce hair-cell excitability.
Mechanically induced damage of lateral-line hair cells during sustained strong current exposure to protect against excess glutamate release, excitotoxic damage, and synapse loss (Carpaneto Freixas et al., 2021). Interestingly, presynaptic ribbons were not similarly enlarged, but instead showed a modest reduction in size following mechanical injury. Functional imaging of zebrafish lateral line has shown that a subset of hair cells in each neuromast are synaptically silent, and these silent hair cells can become active following damage (Zhang et al., 2018). As ribbon size has been observed to correspond with synaptic activity (Merchan-Perez & Liberman, 1996; Sheets et al., 2012), reduction in ribbon size may reflect recruitment of synaptically silent hair cells following mechanically-induced damage. Future functional studies are needed to determine if mechanical overstimulation recruits more active hair-cell synapses, and to verify whether glutamate release from active synapses is reduced following mechanical overstimulation.

Lateral-line neuromasts fully recover following mechanical damage

Previous studies indicate mammalian cochlear hair cells have some capacity for repair following sub-lethal mechanical damage, including tip-link repair and regeneration of a subset of ribbons synapses (Indzhykulian et al., 2013; Jia, Yang, Guo, & He, 2009; Kim et al., 2019). But such ability is limited, and our understanding of hair-cell repair mechanisms is incomplete. In some mammalian models, synaptic ribbon repair in the cochlea following noise trauma has been reported as partial but permanent, i.e. a subset of synapses are lost and do not recover. In contrast, we observe complete recovery of hair cell-afferent synapses following mechanical trauma to the zebrafish lateral line. The cellular mechanisms responsible for such repair are not fully defined, but may involve regulation of neurite growth, glutamate signaling, inflammation, and/or neurotrophic factors (Kaur et al., 2019; Kim et al., 2019; Wan, Gomez-Casati, Gigliello, Liberman, & Corfas, 2014). Further study using this zebrafish model of mechanical overstimulation may provide insights that will assist in the development of methods for promoting complete synapse repair following damaging stimuli to the mammalian cochlea.

In addition to hair bundle damage and loss of synapses in morphologically intact neuromasts, we also found that mechanical trauma resulted in a small degree of hair-cell loss in disrupted neuromasts (Fig. 3D), but that hair-cell numbers had recovered after 2 hours (Fig. 7). The mechanism that mediates this recovery is not clear. Hair-cell regeneration in the inner ear can occur via direct phenotypic conversion of a supporting cell into a replacement hair cell (e.g., Warchol, 2011), and it is conceivable that such transdifferentiation could occur within 2 hours of injury to recover lost hair cells. While the short recovery time is not consistent with the longer process of renewed proliferation and hair-cell differentiation that underlies most hair-cell regeneration in zebrafish and birds (Harris et al., 2003; Ma, Rubel, & Raible, 2008; Romero-
Mechanically induced damage of lateral-line hair cells

Carvajal et al., (2015), these studies were focused on recovery following profound damage and complete hair-cells loss. It is also notable that hair cell synapse number fully recovers in the lateral line following mechanical injury. The recovery we observe may reflect the rapid recovery from mild to moderate neuromast damage that occurs in this externally localized sensory organ that is directly exposed to the fish’s environment and therefore continuously vulnerable to injury.

In summary, our data show that exposure of zebrafish lateral-line organs to strong water current results in mechanical injury and loss of afferent synapses, but that these injuries recover within 48 hr. Our next steps will be to define the time course for morphological and functional recovery, and to determine how lateral-line mediated behavior is affected by mechanically induced damage. Sub-lethal overstimulation of hair cells in the zebrafish lateral line provides a useful model for defining mechanisms of damage and inflammation and for identifying pathways that promote hair-cell repair following mechanically-induced injury.

Declaration of interests:
The authors declare no competing financial or non-financial interests.

Acknowledgments: This work was supported by the National Institute on Deafness and Other Communication Disorders R01DC016066 (L.S.), R01DC017166 (A.A.I), and R01DC006283 (M.E.W.), Washington University Dept. of Otolaryngology (L.S.), and the Amelia Peabody Charitable Fund (L.S.). We would like to thank Valentin Militchin (WashU) and Evan Foss (Mass Eye and Ear) for engineering support and Mark Rutherford for thoughtful feedback on the manuscript.
Mechanically induced damage of lateral-line hair cells

Method Details

Ethics Statement

This study was performed with the approval of the Institutional Animal Care and Use Committee of Washington University School of Medicine in St. Louis and in accordance with NIH guidelines for use of zebrafish.

Zebrafish

All zebrafish experiments and procedures were performed in accordance with the Washington University Institutional Animal Use and Care Committee. Adult zebrafish were raised under standard conditions at 27-29°C in the Washington University Zebrafish Facility. Embryos were raised in incubators at 29°C in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgCl₂; (Nüsslein-Volhard & Dahm, 2002)) with a 14 h:10 h light:dark cycle. After 4 dpf, larvae were raised in 100-200 ml E3 media in 250-ml plastic beakers and fed rotifers daily. Sex of the animal was not considered in our studies because sex cannot be predicted or determined in larval zebrafish.

The transgenic lines TgBAC(neurod1:EGFP) (Obholzer et al., 2008), Tg(tnks1bp1:EGFP) (Behra et al., 2012), Tg(-6myo6b:βactin-EGFP) (Kindt et al., 2012) and Tg(mpeg1:YFP) (Roca & Ramakrishnan, 2013) were used in this study. Fluorescent larvae were identified at 3 dpf without anesthesia in E3 media. The mutant line lhfl5bvo35vo35 was also used (Erickson et al. 2020).

Genotyping

To genotype lhfl5bvo35vo35 larvae and siblings after mechanical stimulation and immunohistochemical labeling, ~1 mm tail tissue was excised, and genomic DNA was extracted by incubation in a lysis buffer (10 mM Tris pH 8.0, 50 mM KCl, 0.3% NP-40, 0.3% Tween-20). A genomic region of lhfl5b was amplified by PCR using forward primer GCGTCATGTGGGCAGTTTTC and reverse primer TAGACACTAGCGGCCTTGC. The lhfl5bvo35 mutation disrupts a MluCI restriction site (AATT), so PCR products were digested with MluCI, and homo- and heterozygotes were resolved by differences in band size on a 1-1.5% agarose gel.

Experimental Apparatus

Multi-well plates containing larvae were clamped to a custom magnesium head expander (Vibration & Shock Technologies, Woburn, MA) on a vertically-oriented Brüel+Kjær LDS Vibrator, V408 (Brüel and Kjær, Naerum, Denmark). An additional metal plate was fitted to the bottom of the multi-well dish to fill a small gap between the bottoms of the wells and the
Mechanically induced damage of lateral-line hair cells

Mechanically overstimulation of lateral-line organs in free swimming larvae

At 7 dpf, free-swimming zebrafish larvae were placed in untreated 6-well plates (Corning, Cat# 3736; well diameter: 34.8 mm; total well volume: 16.8 ml) with 6 ml E3 per well, pre-warmed to 29°C. Up to 15 larvae were placed in each well. Individual wells were sealed with Glad® Press ‘n Seal plastic food wrap prior to placing the lid on the plate. An additional metal plate was fitted to the bottom of the multi-well dish to fill a small gap between the bottoms of the wells and the head expander.

Mechanical water displacement exposures (stimulus parameters: 60 Hz, 40.3 ± 0.5 m/s²) were conducted at room temperature (22-24°C) up to 2 hours after dawn. The frequency selected for mechanical overexposure of lateral-line organs was based on previous studies showing 60 Hz to be within the optimal upper frequency range of mechanical sensitivity of superficial posterior lateral-line neuromasts (Weeg and Bass 2002, Trapani et al, 2009, Levi et al, 2015). To confirm that 60 Hz was the optimal frequency to induce damage, we tested 45, 60, and 75 Hz at comparable intensities. We observed at 75 Hz no apparent damage to lateral line neuromasts while 45 Hz at a comparable intensity proved toxic i.e. it was lethal to the fish.

Exposures consisted of 20 minutes of stimulation followed by a 10-minute break and 2 hours of uninterrupted stimulation. We also tested periodic exposures that consisted of a series of short pulses spanning 2 hours total: 2 20-minute exposures each followed by 10 minutes of rest, followed by 30 minutes of stimulation, a 10-minute break, and a final 20 minutes of stimulation. During the entire duration of exposure, unexposed control fish were kept in the
Mechanically induced damage of lateral-line hair cells

same conditions as exposed fish i.e. placed in a multi-well dish and maintained in the same room as the exposure chamber. For experiments pharmacologically blocking glutamate uptake, fish were co-exposed to 10 µM DL-TBOA (Tocris; Cat. No.1223) + 0.1% DMSO or 0.1% DMSO alone. After exposure, larvae were either immediately fixed for histology, prepared for live imaging, or allowed to recover for up to 2 days in an incubator at 29°C.

Ablation of lateral-line organ with CuSO₄

Free-swimming larvae were exposed to freshly made 3 µM CuSO₄ solution in E3 for 1 hour, then rinsed and allowed to recover for 2 hours to ensure complete ablation of the lateral-line neuromasts. Neuromast ablation was confirmed by immunofluorescent labeling of hair cells. The effects of low-dose copper exposure are likely specific to lateral-line organs; a previous study in zebrafish determined exposure to low-dose CuSO₄ for 1 hour did not alter the acoustic escape response, which is similar to the fast start response we observed but evoked by higher frequency stimulation (100-500 Hz) of the anterior macula of inner ear (Buck, Winter, Redfern, & Whitfield, 2012).

Fast-start escape response behavior assay

Images of larval swimming behavior (1000 frames per second) were acquired with an Edgertronic SC1 high-speed camera (Sanstreak Corp). Image acquisition began 10 seconds following stimulus onset. All subsequent analysis was performed using ImageJ. To track swimming behavior, images were initially stabilized using the Image Stabilizer Plugin. In stabilized images, the position of individual larval heads (located via the pigmented eyes) in each frame were tracked using the Manual Tracking Plugin. Larvae were tracked over 10 seconds (10,000 frames total) per trial. ‘Fast start’ responses—defined as a c-bend of the body occurring within 15 ms followed by a counter-bend— were identified manually.

Whole-mount immunohistochemistry

For visualization of zebrafish lateral-line hair cells, neurons, and synapses: 7-9 dpf larvae were briefly sedated on ice, transferred to fixative (4% paraformaldehyde, 1% sucrose, 37.5 µM CaCl₂, 0.1 M phosphate buffer) in a flat-bottomed 2 ml Eppendorf tubes, and fixed for 5 hours at 4-8°C. Fixed larvae were permeabilized in ice-cold acetone for 5 minutes, then blocked in phosphate-buffered saline (PBS) with 2% goat serum, 1% bovine serum albumin (BSA), and 1% DMSO for 2-4 hours at room temperature (RT; 22-24°C). Larvae were incubated with primary antibodies diluted in PBS with 1% BSA and 1% DMSO overnight at 4-8°C, followed by several rinses in PBS/BSA/DMSO and incubation in diluted secondary antibodies conjugated to Pacific Blue (1:400), Alexa Fluor 488 (1:1000), Alexa Fluor 555 (1:1000), Alexa Fluor 647 (1:1000; Invitrogen), or DyLight 549 (1:1000; Thermo-Fisher) for 2 hours at RT. In some
experiments, fixed larvae were stained with 2.5 ug/ml 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) diluted in PBS to label all cell nuclei. Larvae were mounted on glass slides with elvanol (13% w/v polyvinyl alcohol, 33% w/v glycerol, 1% w/v DABCO (1,4 diazobicylo[2,2,2] octane) in 0.2 M Tris, pH 8.5) and #1.5 cover slips. For visualization of inflammation and macrophage recruitment: 7 dpf larvae were sedated on ice, transferred to 4% paraformaldehyde fixative in PBS, then fixed overnight at 4-8°C. The next day larvae were rinsed in PBS and blocked in PBS with 5% normal horse serum (NHS), 1% DMSO, and 1% Triton x-100 for 2 hours at RT. Larvae were incubated with primary antibodies diluted in PBS with 5% NHS and 1% Triton-x 100 overnight at RT, rinsed several times in PBS, then incubated in diluted secondary antibodies listed above for 2 hours at RT. Larvae were mounted on glass slides with glycerol/PBS (9:1); coverslips were sealed with clear nail polish.

**Primary antibodies**

The following commercial antibodies were used in this study: GFP (1:500; Aves Labs, Inc; Cat# GFP-1020), Parvalbumin (1:2000; Thermo Fisher; Cat# PA1-933), Parvalbumin (1:2000; Abcam; Cat# ab11427), Parvalbumin (1:500; Sigma-Aldrich; Cat# P3088), MAGUK (K28/86; 1:500; NeuroMab, UC Davis; Cat# 75-029), Otoferlin (1:500; Developmental Studies Hybridoma Bank/ HCS-1). Custom affinity-purified antibody generated against *Danio rerio* Ribeye b (mouse IgG2a; 1:2000; (Sheets et al., 2011)) was also used.

**Live hair-cell labeling**

To selectively label hair-cell nuclei, live zebrafish larvae were incubated with DAPI diluted 1:2000 in E3 media for 4 minutes. Larvae were briefly rinsed 3 times in fresh E3 media, then immediately exposed to mechanical overstimulation or drug. To label with FM1-43 vital dye (n-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)-styryl) pyridinium dibromide; ThermoFisher)), free-swimming larvae were exposed to FM 1-43 at 3 µM for 20 seconds, then rinsed 3 times in fresh E3 as previously described (Toro et al., 2015). Live imaging of FM1-43 did not provide the temporal resolution needed to compare relative uptake immediately and 2 hours following exposure. We therefore examined FM1-43 uptake using the fixable analogue. Free-swimming larvae were exposed to FM 1-43FX at 3 µM for 20 seconds, then rinsed 3 times in fresh E3 and immediately fixed (4% paraformaldehyde, 4% sucrose, 150 µM CaCl₂, 0.1 M phosphate buffer). Relative labeling of hair cells at 1-3 hours appeared comparable between live FM1-43 and FM 1-43FX. We also verified loss of FM 1-43FX uptake in larvae following brief treatment with 5 mM BAPTA to disrupt tip links (Kindt et
Mechanically induced damage of lateral-line hair cells

al., 2012). FM 1-43 and FM 1-43FX mean signal intensity from maximum projection images was calculated using ImageJ as the integrated pixel intensity divided by the area of the neuromast region of interest.

**EdU labeling and quantification**

To label proliferating cells, we used the Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 555 dye (Invitrogen). Following mechanical overstimulation, larvae were incubated in 500 µM EdU with 0.5% DMSO in E3 for 4 hours at 28°C then fixed in 4% paraformaldehyde in PBS overnight at 4°C. Larvae were washed in 3% bovine serum albumin in PBS then permeabilized with 0.5% Triton-X in PBS. GFP signal in Tg[tnks1bp1:GFP] fish was amplified using anti-GFP primary antibody (Aves), followed by a secondary antibody conjugated to Alexa Fluor 488. The EdU detection reaction was performed according to manufacturer guidelines; larvae were incubated in a reaction cocktail (4 mM CuSO4 and Alexa Fluor 555 azide in 1X Click-iT reaction buffer with reaction buffer additive) for 1 hour at 25°C. Larvae were washed, counterstained with Hoechst 33342, and mounted on slides with elvanol. Confocal images of neuromasts were acquired using an LSM 700 laser scanning confocal microscope with a 63x 1.4 NA Plan-Apochromat oil-immersion objective (Carl Zeiss). The numbers of EdU+ cells per neuromast were quantified in ImageJ.

**Confocal imaging (fixed specimens)**

Images of fixed samples were acquired using an LSM 700 laser scanning confocal microscope with a 63x 1.4 NA Plan-Apochromat oil-immersion objective (Carl Zeiss). Confocal stacks were collected with a z step of 0.3 µm over 7-10 µm with pixel size of 100 nm (x-y image size 51 x 51 µm). Acquisition parameters were established using the brightest control specimen such that just a few pixels reached saturation in order to achieve the greatest dynamic range in our experiments. These parameters including gain, laser power, scan speed, dwell time, resolution, and zoom, were kept consistent between comparisons.

**Confocal imaging (live)**

Live zebrafish larvae were anesthetized with 0.01% tricaine in E3, then mounted lateral-side up on a thin layer of 1.5-2% low-melt agarose in a tissue culture dish with a cover-glass bottom (World Precision Instruments) and covered in E3 media. Z-stack images with a z step of 0.5 µm were acquired via an ORCA-Flash 4.0 V3 camera (Hamamatsu) using a Leica DM6 Fixed Stage microscope with an X-Light V2TP spinning disc confocal (60 micron pinholes) and a 63x/0.9 N.A. water immersion objective. Z- acquisition parameters w/ X-light spinning disc:
Mechanically induced damage of lateral-line hair cells

488 laser “20% power”, 150 ms per frame. Image acquisition as controlled by MetaMorph software.

Confocal image processing and analysis

All analysis was performed on blinded images. Digital images were processed using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). In order to quantitatively measure sizes and fluorescent intensities of puncta, raw images containing single immunolabel were subtracted for background using a 20-pixel rolling ball radius and whole neuromasts were delineated from Parvalbumin-labeled hair cells using the freehand selection and “synchronize windows” tools. Puncta were defined as regions of immunolabel with pixel intensity above a determined threshold: threshold for Ribeye label was calculated using the Isodata algorithm (Ridler, 1978) on maximum-intensity projections, threshold for MAGUK label was calculated as the product of 7 times the average pixel intensity of the whole neuromast region in a maximum-intensity projection. Particle volume and intensity were measured using the 3D object counter (Bolte & Cordelieres, 2006) using a lower threshold and a minimum size of 10 voxels. To normalize for differences in staining intensity across experimental trials, all volumes were divided by the median control volume in each trial for each individual channel. The number of particles above lower threshold was quantified using the ImageJ Maximum Finder plugin with a noise tolerance of 10 on maximum-intensity projections. Intact synapses were manually counted and defined as adjoining or overlapping maxima of Ribeye and MAGUK labels. The number of synapses per hair cell was approximated by dividing the number of intact synapses within an neuromast by the number of hair cells in the neuromast.

Quantitative data on macrophage response to mechanical injury were collected from the two caudal-most (‘terminal’) neuromasts. Confocal image stacks were obtained using a Zeiss LSM700 microscope and visualized using Volocity software. These image stacks were used to derive three metrics from each neuromast. First, the number of macrophages within 25 µm of a particular neuromast was determined by inscribing a circle of 25 µm radius, centered on the neuromast, and counting the number of macrophages that were either fully or partially enclosed by this circle. Next, the number of macrophages contacting a neuromast was determined by scrolling through the x-y planes of each image stack (1 µm interval between x-y planes, 15 µm total depth) and the counting macrophages that were in direct contact with Otoferlin-labeled hair cells. Finally, the number of macrophages that had internalized Otoferlin-labeled material (hair-cell debris) were counted and were assumed to reflect the number of phagocytic events. For each metric, the recorded number reflected the activity of a single macrophage, i.e., a
Mechanically induced damage of lateral-line hair cells

A macrophage that made contacts with multiple hair cells and/or had internalized debris from several hair cells was still classified as a single ‘event.’

Subsequent image processing for display within figures was performed using Photoshop and Illustrator software (Adobe).

**Scanning electron microscopy**

To image hair-cell bundles, zebrafish larvae were exposed to strong water current stimulus, then anesthetized in 0.12% tricaine in E3 and immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) supplemented with 2 mM CaCl$_2$. Larvae were shipped overnight in fixative, then most of the fixative (~90-95%) was removed, replaced with distilled water, and samples were stored at 4°C. Next, larvae were washed in distilled water (Gibco), dehydrated with an ascending series of ethanol, critical point dried from liquid CO$_2$ (Tousimis Aurosamdri 815), mounted on adhesive carbon tabs (TedPella), sputter coated with 5 nm of platinum (Leica EM ACE600), and imaged on Hitachi S-4700 scanning electron microscope. Kinocilia diameter measurements were performed using ImageJ.

**Statistical analysis**

All statistical analyses were performed using Prism 8 (Graphpad Software Inc). Where appropriate, datasets were confirmed for normality using the D'Agostino-Pearson test. Statistical significance between multiple conditions with one independent variable was determined by one-way ANOVA (normal distribution of data) or by a Kruskal-Wallis test (non-normal distribution of data) and appropriate post-hoc tests. Statistical significance between multiple conditions with two independent variables was determined by two-way ANOVA. Statistical significance between two conditions was determined by an unpaired Student’s $t$ test or a Mann–Whitney U test, as appropriate. The one-sample Wilcoxon test was used to calculate the difference between observed and default value. Mixed model analysis was used to compare time-series data. Based on the variance and effect sizes reported previously studies, the number of biological replicates were suitable to provide statistical power to avoid both Type I and Type II error (Sebe et al., 2017; Uribe et al., 2018).
Mechanically induced damage of lateral-line hair cells

References


Mechanically induced damage of lateral-line hair cells


Mechanically induced damage of lateral-line hair cells
Mechanically induced damage of lateral-line hair cells


Mechanically induced damage of lateral-line hair cells


Supplemental Figure 1: Fish exposed to periodic stimulus have less mechanical damage to neuromasts, but still show significant synapse loss. (A) Schematic of the two exposure protocols. Sustained exposure was a 20 min. pulse followed by 120 minutes uninterrupted mechanical overstimulation; periodic exposure was 90 min. exposure with intermittent 10 min. breaks totaling 120 minutes. (B) Periodic stimulus causes less neuromast disruption. Immediately following sustained exposure, 46% of exposed neuromasts showed a “disrupted” phenotype, whereas following a periodic exposure only 17% of the neuromasts appeared “disrupted” (Unpaired t-test **P=0.0034). (C) Position of the neuromast along the tail was also associated with vulnerability to disruption with both sustained and periodic stimulation. (D-D’) Hair cell number per neuromast following exposure. With periodic exposure, the number of hair cells was comparable to control (Unpaired t-test, *P=0.0125 sustained, P=0.9916 periodic). (E-E’) % of neuromast hair cells innervated. There was no significant reduction in afferent innervation of neuromast hair cells (One sample Wilcoxon test, ****P<0.0001 sustained, P=0.0625 periodic). (F-F’) Number of intact synapses per neuromast. There is significant loss of synapses with both periodic and sustained exposures (*P=0.0245 periodic; **P=0.0036 sustained).
Mechanically induced damage of lateral-line hair cells

Supplemental Figure 2: Hair cell organs of the ear appeared undamaged in larvae exposed to sustained stimulus. Representative maximum intensity images of hair cell organs in the ears of control (A-C) and larvae exposed to sustained strong current stimulus (A’-C’). Hair cells in A-B were immunolabeled with an antibody against Otoferlin; posterior macula in C were immunolabeled with antibodies against Parvalbumin to label hair cells and CtBP to label synaptic ribbons. Scale bars: 10µm
Supplemental Figure 3: Exposed neuromasts show a modest increase in supporting cell proliferation following sustained stimulation. (A,B) Representative cross-section images of EdU (magenta) labeling of proliferating neuromast cells. Fish were exposed to EdU for 4 hours following exposure. (A) EdU labeling of neuromast hair cell not co-localized with GFP. (B) EdU labeling colocalized with supporting cell GFP label. Scale bars: 5µm (C-D) Average number of EdU+ hair cell (C) or supporting cells (D) per neuromasts. The average number of EdU+ supporting cells were significantly greater than 0 in all neuromasts, with a modest increase in exposed neuromasts (Wilcoxon Rank Test: **P=0.0078, ***P=0.0005).
Supplemental Figure 3: Exposed neuromasts show a modest increase in supporting cell proliferation following sustained stimulation. (A,B) Representative cross-section images of EdU (magenta) labeling of proliferating neuromast cells. Fish were exposed to EdU for 4 hours following exposure. (A) EdU labeling of neuromast hair cell not co-localized with GFP. (B) EdU labeling colocalized with supporting cell GFP label. Scale bars: 5µm (C-D) Average number of EdU+ hair cell (C) or supporting cells (D) per neuromasts. The average number of EdU+ supporting cells were significantly greater than 0 in all neuromasts, with a modest increase in exposed neuromasts (Wilcoxon Rank Test: **P=0.0078, ***P=0.0005).
Mechanically induced damage of lateral-line hair cells

**Supplemental Movies**

Swimming behavior of 7-day-old larvae during exposure to the strong water current stimulus shown in Figure 2 over 500ms (1000 fps/ 500 frames).

**Movie S1:** Swimming behavior of control fish with intact lateral line organs. Magenta circle indicates a fish prior to a “fast escape” response.

**Movie S2:** Swimming behavior of larvae whose lateral-line neuromasts were ablated with CuSO₄. Magenta circles indicate larvae that were swept into the waves and could no longer tracked.
"Mechanical overstimulation causes acute injury and synapse loss followed by fast recovery in lateral-line neuromasts of larval zebrafish"

Response to Preprint Peer Review comments

We would like to thank the reviewers for their thoughtful and thorough critique of our manuscript. In this resubmission, we added important additional data and restructured our manuscript to reflect as many of the recommendations as possible. Additionally, we have added experiments to define the cellular mechanisms underlying observed damage following mechanical injury.

The most significant additions of new data include:

- Further experiments demonstrating block of glutamate clearance exacerbates stimulus-induced hair-cell synapse loss.
- Analysis of neuromast disruption in lhfpl5b mutant null larvae showing mechanical displacement. Lhfpl5b mediates mechanosensitivity in lateral-line hair cells, allowing us to determine whether mechanotransduction is required for mechanical disruption of neuromasts.
- Testing the vibratory stimulus at various frequencies to confirm the optimal frequency to induce acute, generally sub-lethal damage to lateral-line hair cells is 60 Hz.
- Assessment of neuromast supporting cell and hair cell proliferation following mechanical overstimulation.
- Quantitative analysis of kinocilia SEM and confocal images of hair bundles in control and stimulus exposed fish.

Individual comments are addressed as outlined below.

Reviewer 1:

- The authors use a vertically-oriented Brüel+Kjær LDS Vibrator to deliver a 60 Hz vibratory stimulus to damage lateral line hair cells. It is not made clear on why this frequency was selected. Did the authors choose this frequency because they screened a number of frequencies and this is the one that did the most damage to hair cells or was it chosen for another reason? Or, do all frequencies do the same amount of damage? The authors should screen a number of frequencies and choose the stimulus that does the most damage to hair cells. This would set the field in the best direction, should members of the community attempt this new technique. It is not necessary to repeat all of the experiments, but the authors should show which frequencies are best for inducing damage.

The frequency selected for mechanical overexposure of lateral-line organs was based on previous studies showing 60 Hz to be within the optimal upper frequency range of mechanical sensitivity of superficial posterior lateral-line neuromasts, with maximal response between 10-60 Hz, but a suboptimal frequency for hair cells of the anterior macula in the ear (Weeg and Bass 2002, Trapani et al, 2009, Levi et al, 2015). To confirm that 60 Hz was the optimal frequency to induce damage, we tested 45, 60, and 75 Hz at comparable intensities. We observed at 75 Hz no apparent damage to lateral
Mechanically induced damage of lateral-line hair cells

line neuromasts while 45 Hz at a comparable intensity proved toxic i.e. it was lethal to the fish. We have updated the Results and Method Details to include our rationale for choosing 60 Hz.

- The SEM images of the hair bundle are beautiful and do show damage to the hair bundle, but historically speaking older studies in mammals have shown that the actin core of the stereocilia is damaged. It would be critical to know if this was the case. Showing damage to the kinocilium and stereocilia splaying is a start, but readers of eLife would need to know if the actin cores are damaged. So, TEM should be used to find damage to the actin cores of stereocilia.

Our main goal of this initial manuscript was to survey morphological and functional changes in mechanically injured lateral line organs with an emphasis on inflammation and synapse loss. We agree TEM studies showing damage to the actin core of the stereocilia will be important to determine whether mechanical damage to neuromast hair bundles fully mimics mammalian stereocilia damage, but these experiments will require significant time to perform and optimize. We have expanded our analysis of hair-bundle morphology in this study and intend to pursue deeper analysis of hair bundle damage, i.e. examination of the stereocilia actin core, in future follow-up studies.

- I think the use of "Noise-exposed lateral line" as a term for mechanically overstimulated lateral line hair cells is not correct and could be misleading. The lateral line senses water motion not sound as the word noise would imply. Calling the stimulus "noise" should be removed throughout.

We have removed the term “noise” throughout the manuscript and replaced it with either “strong water current stimulus” or “mechanical overstimulation” where appropriate.

- Decreases in mechanotransduction are shown by dye entry. These results should be strengthened using microphonic potentials to determine the extent of damage. This experiment is not necessary but would improve the quality of the document.

While we agree that microphonic recordings would provide further support for reduced mechanotransduction, quantitative FM1-43 uptake in zebrafish lateral line hair cells is a well-established proxy for microphonic measurements. In a previous study using the same protocol utilized in our manuscript, FM1-43 labeling intensity was shown to directly correspond with microphonic amplitude (Toro et al, 2015). Moreover, the fixable analogue of FM1-43 (FM1-43FX) gave us comparable relative measurements of uptake as live FM1-43 and provided the additional advantage of high temporal resolution and the ability to simultaneously assay entire cohorts of control and overstimulated fish (which is not possible with microphonic measurements or live FM1-43 imaging), as we could expose groups of fish briefly to the dye at determined time intervals following overstimulation, then immediately place in fixative.

- In figure (2), PSD labeling is not clear.

We assume the reviewer meant PSD labeling in Figure 4 and we agree it is difficult to discern. We have changed the hair-cell label from gray to blue in the images so that the green PSD labeling is clear.
Mechanically induced damage of lateral-line hair cells

Minor comments

- Page 2: "They transduce these stimuli though deflection of stereocilia...". Change "though" to "through".
- Figure 1 legend add "be" between longer and tracked "could no longer tracked".

Thank you for identifying these typos. They have been corrected.

Reviewer 2:

1. While the findings are carefully measured and described, the effects of insult on hair cells are relatively minor, with a change in hair cell number, extent of innervation or synapses per hair cell (Figs 3 and 4) in the range of 10% reduction compared to control. One potential value of the model would be to use it to discover underlying pathways of damage or screen for potential therapeutics. However with these modest changes it is not clear that there will be enough power to determine effects of potential interventions. One advantage of the zebrafish model is the ability to overstimulate large cohorts of larvae, thereby providing enough power to uncover modest but significant changes resulting from moderate damage to hair cells. While not as well suited for unbiased large-scale screens of therapeutics, our overexposure protocol provides the opportunity to determine the role of specific cellular pathways (e.g. metabolic stress, inflammation, and glutamate excitotoxicity) in hair-cell damage and synapse loss following mechanically-induced damage via genetic or pharmacological manipulation of these pathways. Additionally, as the hair cell synapses fully repair following stimulus-induced loss, the zebrafish model has the potential for identifying novel pathways for repair through transcriptomic profiling (for an example, see Mattern et al, Front. Cell Dev. Biol., 2018). Cumulatively, these future experimental directions will provide important mechanistic information that could be used toward the development of targeted therapeutic interventions.

2. The most dramatic phenotype after shaking is a physical displacement of hair cells, described as disrupted morphology. However it is not clear what the underlying cause of this change. Are only posterior neuromasts damaged in this way? Is it a wounding response as animals are exposed to an air interface during shaking? It is also not clear to what extent this displacement reveals more general principles of the effects of noise on hair cells. Additional discussion of underlying causes would be welcome.

We agree that the underlying causes of the physical displacement of posterior lateral-line neuromasts warranted further investigation and we have expanded appropriate sections of the results. To determine if excessive hair-cell activity plays a role in the displacement of neuromasts we have exposed lhfpl5b mutant—fish that have intact hair cell function in the ear, but no mechanotransduction in hair cells of the lateral line—to mechanical overstimulation. We observed comparable disruption of neuromasts lacking mechanotransduction, supporting that displacement of lateral-line hair cells is due to mechanical damage and does not require intact mechnotransduction. Further, when examining the adjacent supporting cells in disrupted neuromasts, we observed they are similarly displaced and elongated. We conclude that observed disruption of hair cells is a consequence of mechanical displacement of the entire neuromast organ. We have
Mechanically induced damage of lateral-line hair cells

added additional discussion of this phenomenon to the Results and Discussion sections of the manuscript.

3. Because afferent neurons innervate more than one neuromast and more than one hair cell per neuromast, measurements of innervation of neuromasts (Figure 3) or synapses per hair cell (Fig 4) cannot be assumed to be independent events. That is, changes in a single postsynaptic neuron may be reflected across multiple synapses, hair cells, and even neuromasts. This needs to be accounted for in experimental design for statistical analysis.

We agree that changes in single postsynaptic neurons, which innervate groups of hair cells of the same polarity within a neuromast, could be reflected across multiple synapses. Additionally, it is plausible that excitotoxic events at the postsynapse, while not contributing to apparent neurite retraction, could be contributing to synapse loss across multiple innervated hair cells. We have updated the manuscript to reflect the potential contribution of postsynaptic signaling to synapse loss and added experiments pharmacologically blocking glutamate uptake.

4. The SEM analysis provides compelling snapshots of apical damage, but could be supplemented by quantitative analysis with antibody staining or transgenic lines where kinocilia are labeled. The amount of reduced FM1-43 labeling is one of the more dramatic effects of the shaking insult, suggesting widespread disruption to mechanotransduction that could be related to this apical damage. Further examination of the recovery of mechanotransduction would be interesting.

To supplement the SEM snapshots of severe apical damage, we have expanded the SEM image analysis with quantitative data on kinocilia morphology. We have also added confocal images of hair bundles using antibody labeling of acetylated tubulin in a transgenic line expressing β-actin-GFP in hair cells. We agree that correlative studies of mechanotransduction recovery relative to hair-bundle morphology would be interesting, and we intend to examine this question in a future follow-up study.

5. A previous publication by Uribe et al.2018 describes a somewhat similar shaking protocol with somewhat different results - more long-lasting changes in hair cell number, presynaptic changes in synapses, etc. It would be worth discussing potential differences across the two studies.

We agree we did not adequately address the considerable differences between our mechanical damage protocol for the zebrafish lateral line and the damage protocol described by Uribe et al, 2018. We have provided a more direct comparison in the Results section and addressed the differences in our protocols in-depth in the Discussion section.

Our damage protocol uses a stimulus within the known frequency range of lateral-line hair cells (60 Hz) that is applied to free-swimming larvae and evokes a behaviorally relevant response (fast start response). The damage is observable immediately following noise exposure, is specific to posterior lateral-line neuromasts, and appears to be rapidly repaired. Some features of the damage we observe—reduced mechanotransduction and hair-cell synapse loss—may correspond to mechanically induced damage of hair cell organs in other species. Notably, hair cell synapse loss in
Mechanically induced damage of lateral-line hair cells

seemingly intact neuromasts is exacerbated by pharmacologically blocking synaptic glutamate clearance, supporting that the 60 Hz frequency stimulus is overstimulating neuromast hair cells directly and suggesting that the mechanism of synapse loss may be similar to inner hair cell synapse loss reported in mice following moderate noise exposures.

By contrast, the damage protocol published by Uribe et al used ultrasonic transducers (40-kHz) to generate small, localized shock waves rather than directly stimulate neuromast hair cells. The damaged they reported—delayed hair-cell death and modest synapse loss with no effect on hair-cell mechanotransduction—was not apparent until 48 hours following exposure and not specific to the lateral-line organ. Some of the features of the damage they observed—delayed onset apoptosis and hair-cell death—may correspond to damage reported in mice following blast injuries.

Minor Comments:

6. Copper is potentially non-specific, described as neurotoxic. Are there other tests for lateral line-dependent effects on swimming/escape responses?

A previous study comparing the effect of various ototoxins in zebrafish determined exposure to low-dose CuSO4 for 1 hour did not alter the acoustic escape response, which is similar to the fast start response we observed but evoked by higher frequency stimulation (100-500 Hz) of the anterior macula of inner ear (Buck et al, Hearing Research, 2012) We have added this information and citation to the manuscript.

7. Statistics for quantified differences in behaviors in Figure 1 are missing.
We have added the statistics to the manuscript.

8. Micrograph examples of neuromast displacement in addition to the schematic would be helpful in describing the phenotype.
We have added representative confocal images of neuromast morphology to Figure 2. In addition, we’ve supplemented our images of neuromast hair cells additional labeling which shows similar displacement of the supporting cells in disrupted neuromasts.

9. Define error bars in figure legends. What are additional points in 3E?
The error bars have been defined as either standard error of the mean (SEM) or standard deviation (SD) in the figure legends. In 3E each point represents the percentage of hair cells with afferent innervation in a single neuromast; multiple individual points in the Control and Normal Exposed appear as one because the symbols all overlap at 100% innervated. This information has been added to the Figure legend.

10. Are differences in hc number in Fig 6 significant?
The differences in hair-cell number in Fig 6 were significant at 0 and 2 hours post exposure (Mixed-effects analysis: **P<0.003). The appropriate statistical analysis has been added to this figure and to the text.

11. Were hair cell changes in the ear observed, as reported by Uribe et al?
We did not observe hair cell changes in either the posterior or anterior macula in the ears of overexposed larvae. An additional supplemental figure (Supplemental Fig. 2) and a description in the results have been added to the manuscript.
Reviewer 3:

As the authors point out, zebrafish hair cells can be regenerated. With that in mind, and to make the relevance for mammalian hair cell repair clear, a clear distinction between mechanisms mediated by "repair" or "regeneration" needs to be made. The authors discuss that proliferative hair cell generation can be excluded based on the short time period, but suggest that transdifferentiation might be involved. Recovery of neuromast hair cell number occurs within the same 2 hour period in which neuromast morphology and hair cell function improved, making it difficult to determine the extent to which "regeneration" contributed to the recovery. The amount of transdifferentiation has to be shown experimentally (lineage tracing?).

We agree that the distinction between "repair" and "regeneration" needs to be made when discussing this model of mechanical damage to zebrafish hair cell organs. We have tried to clarify that most of what we observe regarding recovery—restoration of neuromast shape, mechano-transduction, afferent contacts, and synapse number—reflect mechanisms of repair following mechanical damage (and, in the case of synapse loss, overstimulation) rather than regeneration. However, one feature of damage that may reflect rapid regeneration is restoration of hair cells number following mechanical injury. To experimentally determine whether proliferation contributed to hair cell generation, we assessed the incorporation of the thymidine analog EdU during a 4 hour recovery following mechanical overexposure in a transgenic line expressing GFP in neuromast supporting cells and observe a modest but not statistically significant increase in the number of proliferating supporting cells in neuromasts exposed to strong current stimulus, suggesting recovery of lost hair cells is not primarily due to renewed proliferation.

The number of hair cells that are lost and recover within several hours are low, i.e., typically ~1 hair cell/neuromast. We observed this consistently in all of our experiments, but the mechanisms responsible are not clear. Based on previous studies of hair cell regeneration in the lateral line, the recovery time appears too rapid to be caused by renewed proliferation, a notion that is further supported by our Edu studies. On the other hand, it is possible that a few supporting cells may undergo the initial phases of phenotypic change into hair cells during this short time period, and we speculate that such transdifferentiation may be responsible for the observed recovery. We should emphasize that this is a new observation and, at present, we do not fully understand the underlying mechanism. However, the focus of the present study is on mechanical damage, synaptic loss, and subsequent repair. We believe that it is important to report our consistent findings of low level hair cell loss and recovery, but a detailed characterization of the mechanism would require considerable effort and would best be the topic of a future study.

The classification of "normal" vs "disrupted" is vague and not quantitative. The examples shown in the paper seem to be quite clear-cut, but this reviewer doubts that was the case throughout all analyzed samples. Formulate clear benchmarks and criteria for the disrupted phenotype (even when blind analysis is performed).

We have defined measurable criteria for "normal" vs "disrupted" neuromasts that we have added to the Method Details section: “We defined exposed neuromast morphology
as “normal” when hair cells appeared radially organized with a relatively uniform shape and size, with ≤7 µm difference observed when comparing the lengths from apex to base of an opposing pair of anterior/posterior hair cells. Length was measured from a fixed point at the center of the hair bundle to the basolateral end of each opposing hair cell. We defined neuromasts as “disrupted” when hair cells appeared elongated and displaced to one side, with >7 µm difference observed when comparing the lengths of an opposing pair of anterior/posterior hair cells. Generally, the apical ends of the hair cells were displaced posteriorly, with the basolateral ends oriented anteriorly.

- **Sustained and periodic exposure:** These two exposure protocols not only differ with respect to sustained vs periodic, they also differ in total exposure time (Fig 2B). This complicates the interpretation, especially considering the authors own finding that a pre-exposure is protective.

To clarify—pre-exposure was not protective to hair-cell survival. Rather, in preliminary experiments, pre-exposure appeared to reduce larval mortality, and we have clarified that observation in the text of the Results and the Methods Details sections. We agree with the reviewer that comparing the two protocols based on differences in time distribution is complicated in that they also differ in total exposure time. For the purpose of clarity, we now focus on the sustained exposure in the main figures and created supplemental figures for the reduced damage still observed using periodic exposure, specifying that reduced damage may be the result of periodic time distribution of stimulus and/or less cumulative time exposed to the stimulus.

- **The data on the mitochondrial ROS aspect seems not well integrated into the overall story.**

We agree that the ROS story was not well integrated and incomplete. We have removed the data describing mpv17-/- mutants and mitochondrial disfunction from this manuscript. A more comprehensive report of mpv17-/- mutant mitochondrial function and morphological analysis of neuromasts following noise exposure will be described in a follow-up manuscript.

- **It is surprising that the hair bundle morphology was not assessed after recovery. This is crucial. Overall, it would be good to see some quantification of the SEM data, e.g. kinocilia length and number of splayed bundles.**

We have expanded the SEM image analysis to quantitatively access kinocilia morphology following exposure. We agree that assessment of recovery using live-imaging of hair bundles paired with subsequent SEM analysis will be informative, and we intend to perform those experiments in a future study.

- **Behavioral recovery (measured as number of "fast start" responses) was also not assessed. This is essential for determining the functional relevance of the recovery.**

We attempted to measure behavior recovery of lateral-line function by measuring “fast-start” responses immediately and several hours after recovery, and discovered that i) strong water current provided stimulation that was too intense to reveal subtle behavioral changes following lateral-line damage and recovery, and ii) when testing larvae immediately following sustained strong current exposures, it was difficult to
Mechanically induced damage of lateral-line hair cells

discern if fewer “fast-start” responses were due to lateral-line organ damage or larval fatigue.

We agree that behavioral recovery is important to assay but acknowledge assessing lateral-line mediated behavior following mechanical damage will require a more sensitive testing paradigm that stimulates the lateral-line sensory organ with a relatively gentle, calibrated water flow stimulus. We are currently performing a follow-up study to this paper using a testing paradigm developed by a postdoctoral associate in our lab (Kyle Newton) that analyses subtle changes in larval orientation to water flow (rheotaxis) mediated by the lateral-line organ. Using this behavior paradigm, we will directly correlate morphological and functional recovery over time.

• This reviewer is not yet convinced that this damage model displays enough commonalities to mammalian noise damage to justify the ubiquitous use of the term "noise" throughout the manuscript. It would be more prudent to use a more careful term along the lines of "mechanical overstimulation-induced damage".

We have removed the term “noise” throughout the manuscript and replaced it with either “strong water current stimulus” or “mechanical overstimulation” where appropriate.

• How was afferent innervation quantified? Just counting GFP labeled contacts to hair cells?

Innervation of neuromast hair cells was quantified during blinded analysis by scrolling through confocal z-stacks of each neuromast (step size 0.3 µm) containing hair cell and afferent labeling and identifying hair cells that were not directly contacted by an afferent neuron i.e. no discernable space between the hair cell and the neurite. Hair cells that were identified as no longer innervated showed measurable neurite retraction; there was generally >0.5 µm distance between a retracted neurite and hair cell. We have added this information to the Methods Detail section.

There was also inconsistency in the use of two variations of the mechanical damage protocol, the time points at which repair was assessed, and whether the damage was quantified in all neuromasts or in normal vs. disrupted neuromasts separately, making the data difficult to interpret.

We have revised our figure legends to clearly indicate when we are assessing damage in all exposed neuromasts (pooled) to control vs. comparative analysis of normal vs. disrupted neuromasts relative to control. In addition, we now focus on the sustained exposure in the main figures, which was the exposure protocol used for the time points in which repair and recovery were assessed.

Minor comments:

• Please discuss the implications of why you think there was only a significant reduction in synapses only in "normal" neuromasts and not "disrupted". Maybe because more cell death occurred in disrupted neuromasts, especially in cells with more synapse loss?

We believe the synapse loss we observe in "normal" neuromasts is a consequence of hair-cell overstimulation, which is supported by our observation that blocking glutamate uptake significantly worsens synapse loss specifically in neuromasts with overall intact
Mechanically induced damage of lateral-line hair cells

morphology. Speculatively, the mechanical damage observed in “disrupted” neuromasts (damaged kinocilia, splayed hair bundles, severely reduced FM1-43) may result in correspondingly impaired synaptic activity, thereby providing protection from synapse loss driven by excess stimulation. 

• Clarify whether data in figures 7 and 8 were derived from sustained noise protocol. We have clarified that the data in figures 7 and 8 were derived from sustained noise protocol in the figure legends and the manuscript.

• Why was the sustained protocol used for experiments looking at repair? Wouldn't it be more clear to use the periodic protocol, which results in less hair cell death, in which case most of the repair seen would be due to direct repair, rather than transdifferentiation of SCs to replace damaged hair cells? We chose to focus on sustained mechanical exposure for this initial study characterizing mechanical injury to the lateral line because it produced more severe neuromast damage with mostly comparable phenotypes to the periodic protocol.

• A few representative images demonstrating the presence of tip links in both groups would be important to support the authors claims. Quantification may be difficult, but it would be surprising if tip links weren't damaged immediately following mechanical damage. One of the major issues we encountered when preparing our samples for SEM was the removal of the gelatinous cupula that covers the apical end of the neuromasts. We discovered that higher doses of the fish anesthetic Tricaine (0.12%) had the effect of dissolving the cupula but also made intact hair bundles more difficult to identify. Below (pg. 52) are examples of lower dose tricaine-treated neuromast hair bundles in control fish—notice the gelatinous gunk covering the stereocilia. On the lower right are arrows indicating what may be tip links. We hope to further investigate tip link loss and recovery, but it will first require a careful titration of tricaine concentration that would yield a higher tip link presence in control samples.
Mechanically induced damage of lateral-line hair cells

- Display statistical analysis in graphs in Figure 2.
  Statistical analysis in Figure 2 F have been added.

- Describe statistics used to analyze data presented in Figure 6 in results section and
display the results of this analysis in the figure.
  We used mixed-effects modeling to analyze inflammation time series data. The results
  of these analyses are now displayed in Figure 6.

- Display statistical analysis in graphs in Figure 7.
  Statistical analysis is displayed in graphs in Figure 7.

- Discuss differences between mammals and fish. Speculate about why fish might be
  more efficient than mammals at repairing sublethal damage when damaged cells could
  be easily replaced. Is this surprising?
  We include in the Discussion section that the rapid recovery we observe in mechanically
  injured lateral-line organs may reflect the recovery from mild to moderate neuromast
  damage that occurs in this externally localized sensory organ that is directly exposed to
  the fish’s environment and therefore continuously vulnerable to injury. The superficial
  lateral line’s direct exposure to the environment may require more robust mechanisms
  for repair, in contrast to hair cell organs of mammals which are encased in bone.

- Discuss in more depth the debate about synapse repair in mammals and whether the
  results in this manuscript are similar to those studies showing repair in rodents.
  While we did not go into depth on the debate about synaptic repair in different
  mammalian model systems, we did acknowledge ribbon synapse repair in the cochlea
  following noise trauma has been reported as partial in some mammalian models (i.e. a
Mechanically induced damage of lateral-line hair cells

subset of synapses are permanently lost) and the underlying mechanisms of repair appear to involve glutamate signaling, inflammation, and neurotrophic factors. As we observe complete hair-cell synaptic recovery, our reported zebrafish model of hair cell mechanical overstimulation may provide further insight into how to promote complete synapse repair following damaging stimuli.

• More clearly indicate which experiments took into account the position of neuromast, as this seemed to be important in determining the extent of damage.

We have indicated in each Figure Legend the position of the neuromasts analyzed.

• Live cell video of macrophages engulfing HCs (and possibly other repair-related processes) would be nice and would highlight the power of the zebrafish system.

We agree that live imaging of neuromast damage and recovery will take full advantage of the zebrafish model and is a goal of future studies. One caveat to capturing damage and repair to neuromasts in mechanically exposed larvae is paralyzing, stabilizing, and illuminating fluorescent indicators in larvae immediately following exposure induces additional stress to physically traumatized tissue, which can confound interpretation of observed pathological processes and requires careful controls and optimization. We are therefore optimizing for a future study our live-imaging protocols such that we accurately capture damage and subsequent repair following mechanical exposure.