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Research Article

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Noise-Induced Hypersensitization of the Acoustic Startle Response in Larval Zebrafish

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ABSTRACT

Overexposure to loud noise is known to lead to deficits in auditory sensitivity and perception. We studied the effects of noise exposure on sensorimotor behaviors of larval (5–7 days post-fertilization) zebrafish (*Danio rerio*), particularly the auditory-evoked startle response and hearing sensitivity to acoustic startle stimuli. We observed a temporary 10–15 dB decrease in startle response threshold after 18 h of flat-spectrum noise exposure at 20 dB re·1 ms^{-2}. Larval zebrafish also exhibited decreased habituation to startle-inducing stimuli following noise exposure. The noise-induced sensitization was not due to changes in absolute hearing thresholds, but was specific to the auditory-evoked escape responses. The observed noise-induced sensitization was disrupted by AMPA receptor blockade using DNQX, but not NMDA receptor blockade. Together, these experiments suggest a complex effect of noise exposure on the neural circuits mediating auditory-evoked behaviors in larval zebrafish.

KEYWORDS: inner ear, damage, behavior, hearing, prepulse inhibition

INTRODUCTION

Overexposure to loud noise can cause temporary or permanent hearing loss (Davis 2017; Ryan et al. 2016). Damage to the peripheral auditory system from noise overexposure can result in hair cell death (Dinh et al. 2016; Kurabi et al. 2017) and ultimately lead to permanent hearing loss and auditory neuropathy. These detrimental effects of noise overexposure on the peripheral auditory system often lead to subsequent changes in the morphology, physiology, and function of auditory processing pathways (Eggermont 2015; Rubel and Fritzsch 2002; Wang et al. 2002). Changes in auditory function due to noise overexposure have been described in several taxa including rodents (Carder and Miller 1972; Chen et al. 2013), marine mammals (Finneran 2012), birds (Ryals et al. 1999), and fish (Smith 2012) suggesting that this noise-induced effect is common in many vertebrates.

Noise-induced changes in auditory function can lead to perceptual abnormalities, such as loss of frequency discrimination (Suta et al. 2015), and changes in sensorimotor behaviors. For example, mice exposed to noise levels of 94–100-dB SPL for 2 h show marked increases in thresholds for acoustic startle responses, prepulse inhibition, and auditory CNS activation as well as behavioral hyperactivity (Hickox and Liberman 2014). While rodents such as mice have been a good species to investigate the effects of noise on the auditory system, other non-mammalian species have recently become attractive study systems to investigate the effects of noise on

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the auditory system and inner ear development (Whitfield et al. 2002; Monroe et al. 2015).

One such non-mammalian species used to investigate hearing and inner ear development in vertebrates is the zebrafish (*Danio rerio*), which has several advantages for studies including genetics, embryology, in vivo visualization, and behavioral hearing assays. Larval zebrafish demonstrate a robust acoustic startle response that is easy to measure and quantify (Bhandiwad and Sisneros 2016), and the auditory pathway of this acoustic startle response is thought to be less complex than that in mammals. Auditory afferents from the statoacoustic ganglion synapse onto the lateral dendrites of the Mauthner cell (M-cell), a large command neuron that initiates the characteristic "C-start" startle response in zebrafish (Korn and Faber 2005). The all-or-none response of the M-cell can also be directly activated by exogenous electric field potentials, allowing for the separation of the sensory and motor components of the startle response (Tabor et al. 2014). Furthermore, zebrafish at 5 days post-fertilization possess a fully functioning auditory system with homologies to the mammalian auditory pathway (Vanwalleghem et al. 2017) and only ~80 hair cells in the saccule, the main organ of hearing (Inoue et al. 2013). Together, these features of the zebrafish auditory system provide a tractable preparation that can be readily used to examine the effects of noise on the inner ear and behavioral response pathways.

Previous studies in adult goldfish, a related species, have shown that 24 h of flat-spectrum 170 dB (re·1 μPa) noise exposure results in an 83 % loss of hair cell bundles, accompanied by a temporary decrease in auditory sensitivity of 10–20 dB as measured by the auditory brainstem response (Smith et al. 2006). Auditory thresholds partially recover after hair cell regeneration, but do not return to pre-exposure levels (Smith et al. 2004; Smith et al. 2006). However, the effects of noise on auditory sensitivity and sensorimotor behaviors in fish during early functional development are not well understood.

The objective of this study was to investigate the effects of long-term noise exposure on loudness perception in larval zebrafish. We hypothesized that, similar to studies in mice, overexposure to loud noise would induce sensitization to startle-inducing acoustic stimuli and induce temporary threshold shifts. We describe a perceptual condition in larval zebrafish whereby startle sensitivity is greatly enhanced temporarily after noise exposure and then recovers to pre-exposure levels after 8 h. We also explore physiological changes in response to noise and the role of AMPA in mediating this hypersensitive state.

METHODS

Animals

Larval zebrafish (5–7 days post-fertilization (dpf)) were used for all experiments, a stage where sex is indeterminate. Wild-type (AB) zebrafish were mated and eggs were collected according to standard procedures (Westerfield 2000). After staging, eggs were raised in petri dishes ($n < 50$ larvae per dish) and housed in incubators at 28.5 °C. After 4 dpf, fish were fed live rotifers daily and transferred to fresh embryo medium using glass wide-bore pipettes to minimize shearing damage to the mechanosensory hair cells of the superficial neuromasts. Fish were also fed and monitored for changes in behavior or stress during the noise exposure protocol. All experimental and animal care procedures were approved by the University of Washington Animal Care and Use committee.

Behavioral Testing

Startle response and PPI experiments were performed as described in a previous study (Bhandiwad et al. 2013). Briefly, a 96-well plate was secured to an acrylic platform that was mounted on to a vertically oriented Bruel-Kjaer Type 4810 shaker. An accelerometer (PCB model 355B04) was also attached to the acrylic plate to measure the acoustic particle motion levels of the auditory stimulus. Each well was filled with ~400 μL of embryo medium and the system was calibrated at 30, 45, 60, 75, 90, 190, 310, and 410 Hz. These frequencies were empirically determined, produced minimal or no resonance sound stimulus artifact, and had negligible off-axis components of the particle motion z-axis stimulus (i.e., minimal particle motion in the x- or y-axes), providing a reliable and repeatable stimulus (Bhandiwad et al. 2013). Acoustic stimuli were generated using MATLAB software and relayed to the shaker via a Tucker-Davis System III (Tucker-Davis Technologies, Alachua, FL). The sound-producing shaker apparatus was placed on a vibration isolation table to minimize exogenous vibratory stimuli in a sound-attenuating chamber.

All fish were allowed to acclimate to temperature (28.5 ± 1 °C) and lighting conditions for 15 min before each test. A single replicate consisted of 24 fish in the central 6 × 4 wells, which were presented with randomized pure-tone stimuli of the frequencies listed above. Sound stimuli of 14 to − 16 dB re·1 ms^{-2} in 6-dB steps were used for the startle response experiments. For prepulse experiments, prepulse stimuli of − 34 to − 16 dB re·1 ms^{-2} in 6-dB steps were paired with startle pulses of 800 Hz at 14 dB (re·1 ms^{-2}). The startle pulse used was chosen because it reliably elicits a response probability of 0.85

(Bhandiwad et al. 2013). Because the decibel scale is logarithmic, a 6-dB increase represents a doubling of stimulus amplitude. The resulting behavioral responses were recorded for 50 ms after stimulus presentation using a Photron Fastcam 1024PCI at 1000 frames/s. Intertrial intervals were randomized at 70 ± 10 s in order to minimize habituation effects. Positive responses were defined as a startle response that was initiated 5 ms from the end of the stimulus ramp and a bend angle of the animal's body (measured as the angle between the head, midpoint, and tail) of less than 30°. Responses were recorded as a binary variable (1 for response and 0 for no response) and group-level data (response percentage) were fit to a Weibull cumulative distribution curve using a maximum likelihood method with sound level as the dependent variable for each frequency tested. The resulting model fit was interpolated to find the sound level at which the percent response reached 5 % (Bhandiwad et al. 2013).

Potential differences in startle responses could also result from generalized hyperactivity. In order to test for generalized hyperactivity, noise-exposed and control fish were individually placed in wells of a 96-well plate recorded for 30 min at 25 °C in the absence of auditory stimuli. All noise-exposed fish were tested within 1 h after cessation of noise exposure. Videos were analyzed using Ethovision XT (Noldus Technologies) and total distance moved and time spent moving were measured for each fish.

Noise Exposure

Noise exposure was conducted in the same apparatus used for behavioral testing. Cohorts of 24 fish were placed individually in wells of the 96-well plate with ~400 µL embryo medium. Using the one-dimensional shaker, the fish were presented with a flat spectrum "white noise" (1–10,000 Hz) stimulus at 20 dB re·1 ms^{-2}. A single cohort was exposed to noise for 1, 8, 12, 18, or 24–36 h. Due to instrumental constraints, the noise stimulus was a looped 1-s sound with a 20-ms cosine gate. Sound level was measured using the accelerometer and was calibrated for the 1-s period. During the sound exposure protocol, fish were monitored and fed every 4–6 h and embryo medium was added when necessary. In order to control for the potential effects of habituation, noise exposure experiments were repeated with control fish that were placed in the sound-isolation chamber for 18 h, but were not exposed to noise. For all experiments, fish were removed from the shaker system after noise exposure and allowed to reacclimatize for ~15 min before further testing.

In order to test for recovery from noise exposure, fish were exposed to 18–24 h of 20 dB (re·ms^{-2}) noise and allowed to rest in a quiet environment (average amplitude of − 55 dB re·1 ms^{-2}) for 1, 8, or 12 h after noise exposure. After the rest, fish were tested using the startle response assay as described above.

Electrical Stimulation

Direct electric field (EF) stimulation was used to investigate the effects of noise on M-cell excitability using a protocol similar to Tabor et al. (2014). Groups of three fish (5–7 dpf) were placed in a 3-cm diameter circular arena and illuminated from below using an LED array. A single sinusoidal EF pulse (1-ms period) was generated using MATLAB software and amplified through a Bruel-Kjaer Type 4810 amplifier. These pulses were presented via silver wire electrodes placed 3 cm apart across the center of the arena.

Excitatory EF pulse-induced startle responses were recorded at 1000 fps using the high-speed camera apparatus described above. Direct stimulation of the M-cell is dependent on the orientation of the animal with reference to the anode and is very sensitive to orientation. Therefore, stimuli were delivered only when at least one fish was within 30° off-axis from the anode-cathode axis, determined by visual inspection. Orientation angles were confirmed post hoc and only fish that were within 30° off-axis were used in the analysis. Fish were tested initially with randomized EF-pulse amplitudes of 0.25, 0.75, 1, and 1.5 V/cm, each repeated 10 times. After exposure to 18 h of 20-dB (re·ms^{-2}) noise, fish were tested again using the same stimuli presented in the same randomized order as the prenoise condition. The effect of noise was calculated as the difference in response percentage to EF pulses using a repeated measures design.

DNQX and APV Treatment During Noise Exposure

The excitatory pathway of the startle response is mediated through glutamatergic pathways; therefore, all pharmacological treatments were conducted before behavioral experiments to control for this confound. Studies in mice have shown that startle hyperexcitability is mediated by AMPA receptors (Hickox and Liberman 2014). In addition, NMDA receptors have been implicated in changes in startle excitability in zebrafish (Burgess and Granato 2007; Best et al. 2008; Bergeron et al. 2015) and habituation (Roberts et al. 2011). The glutamatergic antagonists DNQX (an AMPA receptor antagonist) and APV (an NMDA receptor antagonist) were used in this study. Concentrations of 20 µM DNQX (6,7-dinitroquinoxaline-2,3-dione, Sigma) and 25 µM APV (2-amino-5-phosphonovaleric acid, Sigma) were dissolved in embryo medium with 20 µM DMSO.

These concentrations were empirically determined as the highest concentrations that did not cause any mortality or any observable behavioral deficits after 18 h of exposure. Fish were immersed in drug solutions (20 μM DNQX, 25 μM APV, or 20 μM DMSO as a control) and allowed to acclimate 15 min before noise exposure onset. Fish were maintained in these solutions for the duration of the 18 h noise exposure protocol. Control fish were immersed and maintained in these drug solutions, but kept at ambient noise levels (−35 to −40 dB re·1 ms^{-2}) for the duration. After the noise exposure protocol, all fish were washed in fresh embryo medium three times and allowed to acclimate 15–30 min in fresh embryo medium before behavioral testing.

Statistical Analysis

All data were analyzed using MATLAB 2009B. Response data collected from behavioral experiments were analyzed by fitting a Weibull curve fitting using a maximum-likelihood method. Thresholds were interpolated from the curve at each frequency and were defined as the stimulus level at which the startle response could be elicited in 5 % of trials. For the prepulse inhibition experiments, a similar curve fitting procedure was used. Threshold was defined as the prepulse stimulus level that inhibited the startle response to the "catch" stimulus by 5 %.

Differences in startle response thresholds between control and noise-exposed fish were analyzed using nonparametric methods, due to the heteroscedasticity of the threshold data. Friedman tests were used to compare startle thresholds after noise and recovery experiments. Individual tests for differences at specific frequencies were carried out using post hoc pairwise Wilcoxon rank sum tests. Tests were adjusted for multiple comparisons using a Bonferroni adjustment where appropriate.

The habituation and direct electrical activation experiments were analyzed using linear regression to account for a continuous dependent variable. All locomotor experiments were analyzed using independent samples *t* tests with Bonferroni adjustment.

RESULTS

Behavioral Thresholds to Acoustic Stimuli: Control vs. Noise-Exposed

Moderate exposure to flat-spectrum noise at 20 dB (re·1 ms^{-2}) for 18 h led to profound decreases in startle response threshold (Fig. 1). Startle response thresholds decreased by 8–14 dB (re·1 ms^{-2}) in noise-exposed fish compared to controls ($X^2(1) = 27.2$, $p < 0.001$, $n = 10$ groups of 24 fish), with the greatest difference at 90 Hz between control (median (Mdn) = 0 dB, interquartile range (IQR) 3 to −7 dB re·ms^{-2}) and noise-exposed fish (Mdn = −15 dB, IQR −9 to −21 dB re·ms^{-2}).

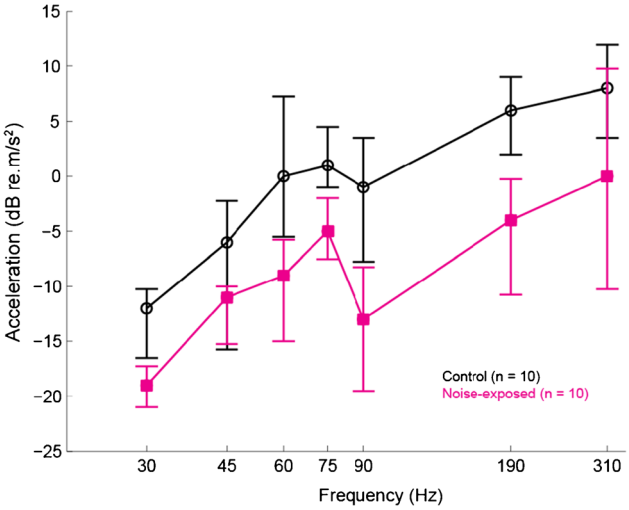

Fig. 1. Noise exposure results in decreased startle thresholds. Startle response thresholds to particle motion stimuli in 18-h white noise-exposed (magenta squares) and control (black circles) conditions ($n = 10$ groups of 24 fish). Thresholds were defined as at a 5 % startle response level. Data presented as median ± 1 quartile, and more negative numbers indicate higher sensitivities

Noise-induced startle sensitization also depended on the duration of noise exposure (Fig. 2). In fish tested before and after noise exposure, significant decreases in overall threshold were observed after 12 h of 20-dB re·ms^{-2} noise exposure ($X^2(1) = 8.35$, $p = 0.003$, $n = 8$ groups of 24 fish). There were no differences between noise exposures of 12 and 24–36 h, suggesting an asymptotic effect of noise exposure (($X^2(1) = 0.01$, $p = 0.9$, $n = 8$ groups of 24 fish). There were no overall differences in startle thresholds between control fish and fish exposed to 1 h of noise ($X^2(1) = 1.21$, $p = 0.27$, $n = 8$ groups of 24 fish) or 8 h of noise ($X^2(1) = 1.8$, $p = 0.18$, $n = 8$ groups of 24 fish).

Recovery from noise exposure was also time-dependent (Fig. 3). Immediately after 18–24 h of noise exposure, startle thresholds were significantly lower at all frequencies ($X^2(1) = 6.9$, $p < 0.001$, $n = 10$ groups of 24 fish). After 1 h of recovery in an environment with mean sound levels < −60 dB (re·1 ms^{-2}), startle thresholds were not significantly different from startle thresholds measured immediately after noise exposure (data not shown in figure). However, after 8 h of recovery, startle thresholds returned to pre-noise exposure levels and were not significantly different from those measured before noise exposure ($X^2(1) = 0.37$, $p = 0.54$, $n = 7$ groups of 24 fish). Further recovery (12 h) led to a significant increase in startle thresholds compared to controls ($X^2(1) = 7.53$, $p < 0.01$, $n = 7$). However, this increase

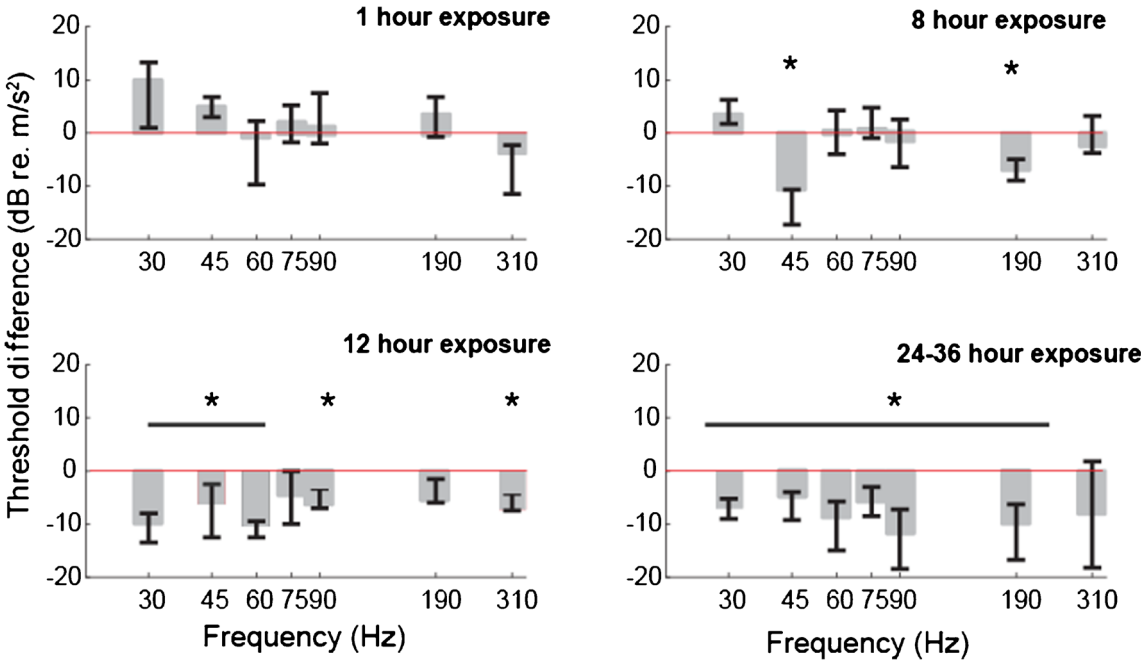

FIG. 2. Noise-induced sensitization is time dependent. Differences between prenoise exposure and post-noise exposure startle thresholds at 1 h noise exposure (top left, $n = 8$ groups of 24 fish), 8 h noise exposure (top right, $n = 8$ groups of 24 fish), 12 h noise exposure (bottom left, $n = 8$ groups of 24 fish), and 24–36 noise exposure (bottom right, $n = 8$ groups of 24 fish) tested at seven frequencies. Note that no change in startle response threshold is equal to a threshold difference of 0 (horizontal red line)

was driven primarily by a difference in startle threshold between noise-exposed (Mdn = – 5 dB, IQR 8 to 1 dB re·ms^{-2}) and control fish (Mdn = – 5 dB, IQR – 3 to – 10 dB re·m/s^2) at 30 Hz ($U = 29$, $p < 0.005$, $n = 7$).

To test whether decreases in startle threshold after noise exposure were due to generalized increase in locomotor activity, noise-exposed (18 h at 20 dB re·m/s^2) and control fish were tracked for 30 min within 1 h of noise-exposure cessation and locomotor activity was recorded. The small difference in locomotor activity (measured as the time spent moving during the 30-min period) observed between noise-exposed (546 ± 38 s SEM) and control fish (647 ± 50 s SEM) failed to reach statistical significance ($t(58) = -2.16$, $p > 0.05$, $\alpha = 0.025$, $n = 30$; Fig. 4). However, the total distance moved was significantly reduced in noise-exposed fish (1030 ± 68 mm SEM) compared to the control group (1379 ± 66 mm SEM) ($t(58) = -3.7$, $p < 0.001$, $n = 30$). Together, these data indicate that noise-exposed fish were slightly less active than the control group in normal locomotor activity.

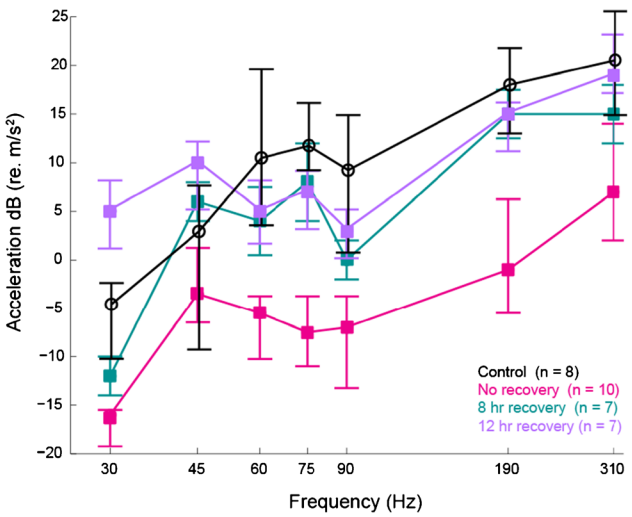

Fig. 3. Recovery from noise-exposure is time dependent. Startle thresholds to pure-tone stimuli after noise exposure immediately after 18 h noise exposure (magenta squares, $n = 10$), 8 h recovery (green squares, $n = 7$ groups of 24 fish), and 12 h recovery (purple squares, $n = 7$ groups of 24 fish). Control (no noise exposure) startle thresholds are plotted as black circles ($n = 8$ groups of 24 fish). Thresholds are reported as medians ±1 quartile

Our previous work showed that the PPI assay was a more sensitive measure than the startle response assay for measuring absolute auditory thresholds (Bhandiwad et al. 2013), motivating us to assess PPI following noise exposure (Fig. 5). Prepulse inhibition thresholds were not significantly different between noise-exposed and control fish ($X^2(1) = 0.08$, $p = 0.77$, $n = 8$ groups of 24 fish). In addition, post-noise prepulse inhibition thresholds were also not significantly different from post-noise startle thresholds at both 30 Hz (Mdn prepulse – 26 dB, IQR – 34 to – 21 dB re·m/s^2; Mdn startle – 21 dB, IQR – 24 to – 18 dB re·ms^{-2}) and 90 Hz (Mdn prepulse – 25 dB, IQR – 31 to – 18 dB re·ms^{-2}; Mdn startle – 18 dB, IQR – 22 to – 14 dB re·ms^{-2}). Visual observations con-

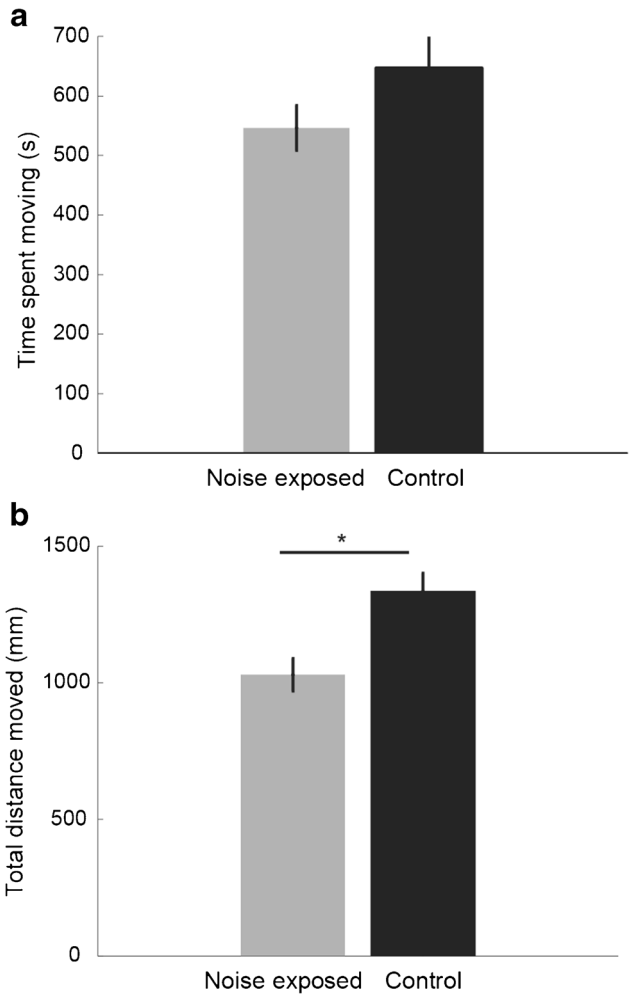

Fig. 4. Overall locomotor activity is not higher in noise-exposed fish. Movement in noise-exposed (gray, $n = 30$) and control (black, $n = 30$) individual fish. Animals were motion-tracked for 30 min in the absence of auditory stimuli and total time spent moving (**a**) and total distance moved (**b**) were measured. Total distance moved was significantly lower in noise-exposed fish compared to that in controls ($p < 0.001$), whereas total time spent moving was not significant overall

firmed that prepulse stimuli would occasionally result in a startle response in noise-exposed fish (at levels below our 5 % response threshold).

The prepulse inhibition paradigm utilized a design in which a "no prepulse" trial was compared with a paired "prepulse" to estimate the prepulse effect. As shown in Fig. 6, analysis of only responses in the no prepulse trials throughout the experiment showed a dramatic decrease in response percentage from the initial trial (84 ± 5 %, mean ± SEM) to the last trial (53 ± 2 %), likely due to habituation to the stimulus ($\beta = -0.015$, $p < 0.001$, $r^2 = 0.77$, $n = 8$ groups of 24 fish). However, in noise-exposed fish, this response decrease was not present for the duration of the experiment ($\beta = -0.001$, $p = 0.31$, $r^2 = 0.77$, $n = 8$ groups; Fig. 6). Furthermore, the initial stimulus

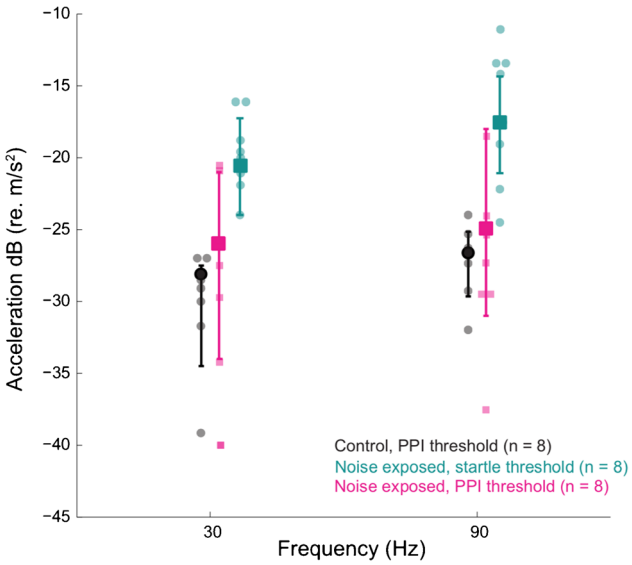

Fig. 5. Prepulse inhibition thresholds are not changed after noise exposure. Auditory thresholds measured using a PPI assay showed no differences between control (black circles, $n = 8$ groups of 24 fish) and noise-exposed (magenta squares, $n = 8$ groups of 24 fish) fish. PPI thresholds in noise-exposed fish were also not significantly different from startle thresholds in noise-exposed fish (green squares, $n = 8$ groups of 24 fish)

presentations resulted in higher response percentage in noise-exposed fish (98 ± 1 %) compared with controls (84 ± 5 % ($U = 139.5$, $p < 0.001$)).

Prepulse inhibition is dependent on the interstimulus interval (ISI) between the prepulse and the startle-inducing stimulus (Burgess and Granato 2007;

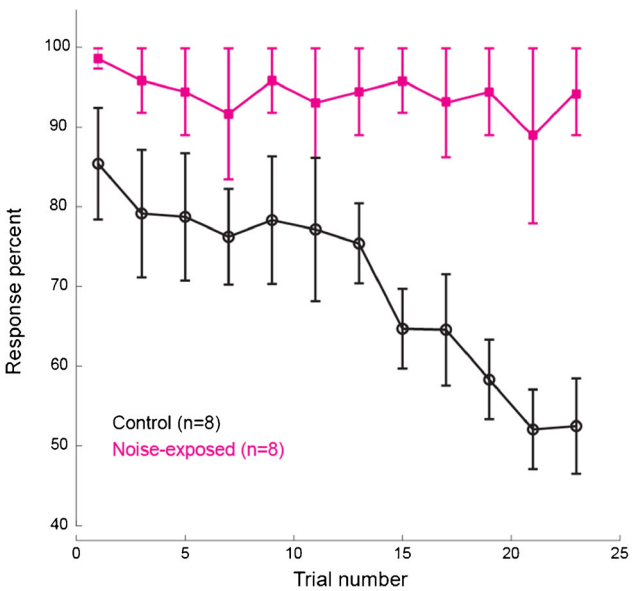

Fig. 6. Habituation to startle-inducing stimuli is reduced after noise exposure. Response probability to the startle-inducing "catch" stimuli used in the PPI experiments is plotted by trial number in noise exposed (magenta squares, $n = 8$ groups of 24 fish) and controls (black circles, $n = 8$). Control fish have significantly lower response probability at the first stimulus presentation and have a steeper decline than noise-exposed fish

Bergeron et al. 2015). To test whether noise exposure changed prepulse inhibition at different ISIs, we tested fish with a paradigm in which the prepulse (90 Hz, −16 dB re·1 ms^{-2}) and startle stimulus were kept constant, but the ISIs were varied between 10 and 290 ms (Fig. 7). The PPI effect was defined as a decrease in response percentage. Noise-exposed fish showed profound decreases in prepulse inhibition compared to controls above 10 ms ISI ($X^2(1) = 20.46$, $p < 0.001$, $n = 6$ groups; Fig. 7). However, at 10 ms ISI, there were no differences in prepulse inhibition effect between noise exposed (Mdn 0.26, IQR 0.17 to 0.29, $n = 6$ groups) and control fish (Mdn 0.2, IQR 0.18 to 0.22, $n = 6$ groups) ($U = 49$, $p = 0.13$).

Electrical Stimulation

Direct stimulation of the M-cells using electrical pulses showed a positive linear relationship between increasing stimulus voltage and response probability in both prenoise and post-noise conditions ($\beta = 0.54$, $p < 0.001$; Fig. 8). There were no significant differences in startle probability between pre- and post-noise exposure fish ($F(3,28) = 1.1$, $p = 0.36$, $n = 8$ groups of 3 fish).

APV and DNQX Treatment During Noise Exposure

We tested whether NMDA receptors were involved in noise-induced hypersensitization in zebrafish. Startle thresholds in zebrafish treated with 25 µM APV were not significantly different from noise-exposed fish treated with DMSO vehicle ($X^2(1) = 0.53$, $p = 0.47$, $n = 6$ groups of 24 fish; Fig. 9). In addition, fish treated with APV but not exposed to noise showed similar thresholds to non-treated quiet controls ($X^2(1) = 2.22$, $p = 0.13$, $n = 6$ groups of 24 fish), indicating that the APV alone did not lead to hyper- or hyposensitization of the startle response. We also tested whether AMPA receptors were involved in noise-induced hypersensitivity by bath application of the AMPA receptor antagonist, DMQX. Startle thresholds in fish treated with 20 µM DNQX showed a significant increase compared to noise-exposed fish treated with vehicle ($X^2(1) = 33.34$, $p < 0.001$, $n = 6$ groups of 24 fish; Fig. 10). Startle responses of DNQX-treated fish exposed to noise were not significantly different from startle thresholds of both DNQX-treated fish not exposed to noise and control (vehicle, no noise) fish ($X^2(1) = 0.27$, $p = 0.61$, $n = 6$ groups of 24 fish).

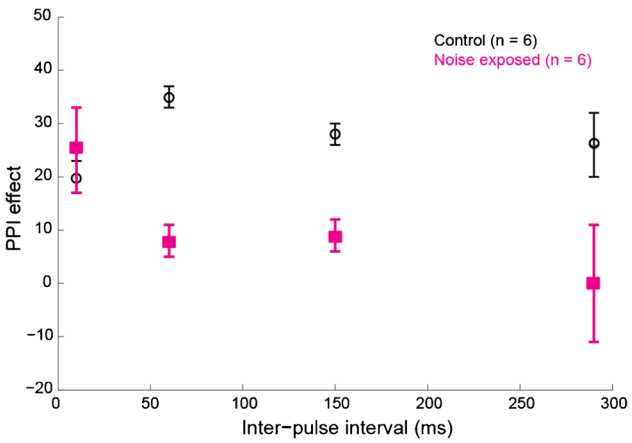

Fig. 7. Prepulse inhibition effect is reduced at longer inter stimulus intervals. Prepulse inhibition effect, measured as the total decrease in percent response to a 90 Hz, −16 dB re·1 m/s^2 prepulse, plotted against increasing interstimulus intervals for noise-exposed (magenta squares, $n = 6$ groups of 24 fish) and control (black circles, $n = 6$ groups of 24 fish) conditions. Noise-exposed fish had significantly lower prepulse inhibition at all interstimulus intervals except 10 ms. Interstimulus intervals were defined as the time between the end of the prepulse stimulus and the beginning of the startle-inducing "catch" stimulus

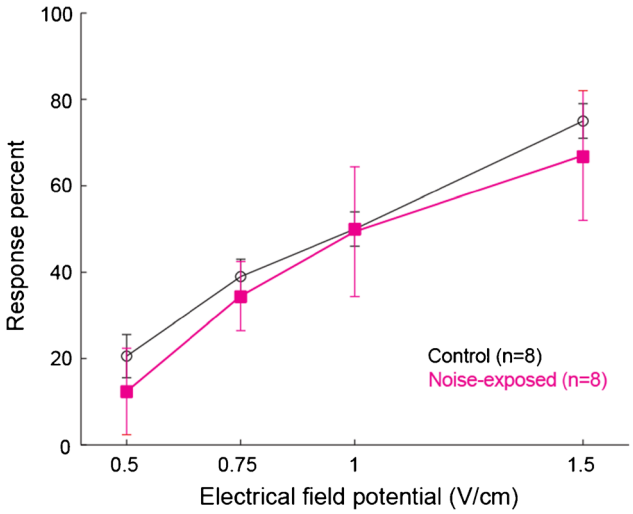

Fig. 8. Startle responses to electrical field stimuli are not affected by noise exposure. Response percentages to direct electrical field pulses in prenoise (black circles, $n = 8$ groups of 3 fish) and post-noise (magenta squares, $n = 8$ groups of 3 fish) conditions. Positive responses were defined as M-cell-mediated startle responses in animals aligned within 30° of the anode-cathode axis. There were no significant differences in responsivity between prenoise and post-noise conditions

DISCUSSION

The goal of this study was to investigate the effects of noise exposure on loudness perception in zebrafish and test the hypothesis that acoustic overexposure would alter sensitization to startle-inducing stimuli. We demonstrated that exposure to 18 h of flat spectrum loud noise induced a temporary condition in larval zebrafish in which acoustic startle thresholds decreased by 10–15 dB, then returned to pre-

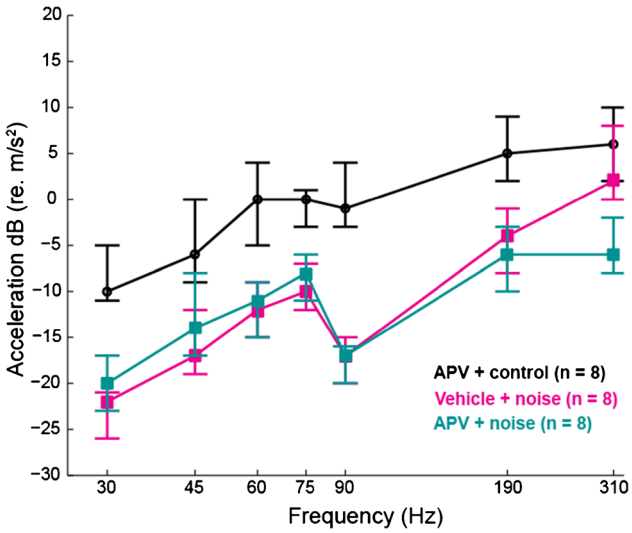

Fig. 9. NMDA receptor blockade does not disrupt noise-induced startle sensitization. Startle response thresholds in groups of animals treated with a bath-applied NMDA receptor antagonist, APV. Animals treated with 25 μM APV during noise exposure (green squares) had significant decreases in startle threshold compared with animals treated with 25 μM APV kept in quiet (black circles). Groups treated with 25 μM APV did not have significantly different thresholds from animals treated with the vehicle, DMSO, and exposed to noise (magenta squares)

exposure thresholds after 8 h of recovery in a quiet environment. Our experiments also demonstrated that the changes leading to this hypersensitization were not due to overall changes in locomotor

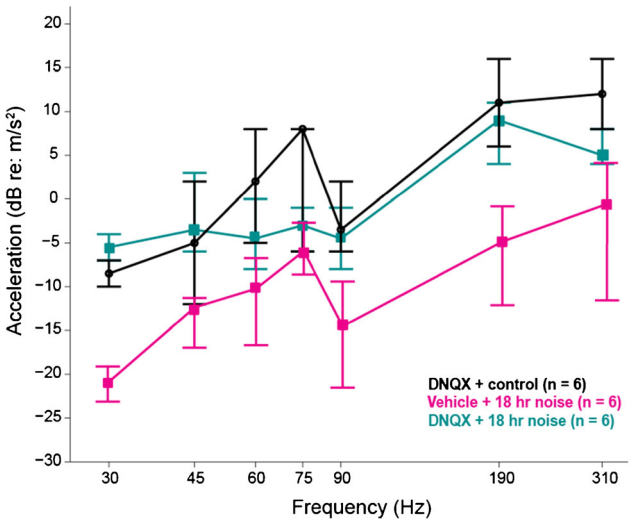

Fig. 10. AMPA receptor blockade results in disruption of noise-induced startle sensitization. Startle response thresholds in groups of animals treated with a bath-applied AMPA receptor antagonist, DNQX. Animals treated with 20 μM DNQX during noise exposure (green squares) were not significantly different compared with animals treated with 20 μM DNQX and kept in quiet (black circles) and from animals treated with the vehicle and not exposed to noise (data not shown). However, animals treated with the vehicle and exposed to noise (magenta squares) had significant decreases in startle threshold compared to both DNQX-treated groups

behavior (i.e., increased overall activity levels) or excitability of Mauthner cells. Auditory sensitivity measured by the PPI thresholds did not significantly change after noise exposure, suggesting that overall hearing sensitivity of 5–7 dpf zebrafish was not affected by this noise exposure paradigm.

The increased startle responsiveness seen in larval zebrafish is similar to startle-inducing hypersensitivity observed in rodents after noise exposure (Hickox and Liberman 2014; Chen et al. 2013). Although startle responses in rodents are measured using startle amplitude (the force generated by the animal onto a pressure plate) rather than a change in probability of response, as with zebrafish in this study, the increase in startle sensitivity is greatest near the threshold for both rodents and zebrafish. This suggests that the effect of noise is a change in the dynamic range of startle-inducing stimuli. Our data also agree with a previous study in adult three-spined stickleback fish (*Gasterosteus aculeatus*), which demonstrated a twofold increase in the number of observed startle responses to broadband acoustic stimuli after noise exposure (Purser and Radford 2011), indicating that noise-induced startle hypersensitivity may be a common effect found in both fish and mammals.

Startle threshold sensitivity following 20 dB (re·1 ms^{-2}) noise exposure returned to prenoise levels within 8 h of recovery, indicating a temporary effect of noise exposure. Studies in human subjects (Melnick 1991) and chinchillas (*Chinchilla* sp.; Carder and Miller 1972) have shown that temporary threshold shifts (decreased sensitivity) are also exposure-dependent and rapidly increase toward an asymptotic threshold shift that is reached within 8–10 h of exposure. Similarly, recovery from asymptotic threshold shifts of ~10 dB can require at least 48 h after noise exposure to return to prenoise levels in human subjects (Melnick 1976). Our data show that startle threshold shifts in larval zebrafish require a longer exposure time and recover to pre-exposure levels in a shorter time period when compared to mammalian studies (Figs. 2 and 3). The recovery data shows a subsequent increase in startle threshold after 12 h at 30 Hz, the lowest frequency tested. This suggests a potential desensitization or rebound to startle-inducing stimuli at low frequencies after the 12-h recovery period.

In contrast to the observed increase in startle sensitivity, the lack of a threshold change using the PPI assay is different from previous auditory evoked potential (AEP) studies in adult zebrafish (Smith et al. 2011) and other adult cyprinid species, including goldfish (*Carassius auratus*; Smith et al. 2004) and minnows (*Pimephales promelas*; Scholik and Yan 2001). These investigations report auditory threshold increases of up to 30 dB (re·1 μPa) after long-term

noise exposure at the best frequency of hearing (Amoser and Ladich 2003). Furthermore, noise exposure in adult zebrafish and goldfish results in hair cell death and full recovery from noise exposure can take up to 14 days after exposure (Amoser and Ladich 2003; Smith et al. 2006). These apparent differences could result from multiple factors. First, adult otophysan fish, such as zebrafish and goldfish, have specialized auditory accessory structures called the Weberian ossicles that connect the swim bladder to the inner ear, thereby increasing the sensitivity and frequency range of hearing (Popper and Fay 1993). These structures may, in turn, exacerbate the effect of noise, leading to saccular damage and physical damage to the auditory apparatus (Smith et al. 2006; Casper et al. 2013). At 5–7 dpf in zebrafish, the Weberian ossicles and its connection between the inner ear and swim bladder are not developed. This immaturity may limit the damage in larval zebrafish. Second, hair cells in the juvenile zebrafish inner ear have "immature" biophysical properties compared to adult zebrafish (Olt et al. 2014), which may affect hair cell survival in the presence of loud noise and lead to no overall change in auditory sensitivity. Future work is needed to investigate the ontogenetic effects of noise exposure on hair cell death in the zebrafish inner ear to resolve these differences.

Although there were no significant differences in PPI thresholds after noise exposure, the effect of a fixed prepulse was diminished at most interpulse intervals (Fig. 7), suggesting a decrease in the prepulse inhibition effect above threshold. These data are in contrast with rodent studies that consistently show an increase in PPI effect (i.e., more inhibition of the startle response to the same magnitude prepulse tone) after noise or salicylate administration (Hickox and Liberman 2014; Rybalko et al. 2011; Sun et al. 2009; Yang et al. 2007) and decreases in PPI with startle habituation (Blumenthal 1997). These differences in PPI between zebrafish and rodents may be due to the differences in mechanisms of PPI in fish and mammals. In zebrafish, PPI is thought to be mediated by populations of GABAergic and glycinergic interneurons in the hindbrain including the PHP cell that inhibit firing of the Mauthner cell at the spike initiation site (Weiss et al. 2008; Faber and Korn 1989). These interneurons receive direct input from primary afferents of the VIIIth nerve. Furthermore, ablation of *gsx1* expressing glutamatergic interneurons in the hindbrain of larval zebrafish has shown a differential effect of short ISI and long ISI PPI (Bergeron et al. 2015). Noise exposure could potentially affect only the neurons mediating long ISI PPI. In contrast, auditory PPI in mammals is thought be mediated by the central auditory system, primarily the inferior colliculus (Fendt et al. 2001; Li et al. 2009). Due to the mechanistic differences in PPI circuitry and limitations with our behavioral paradigm, we cannot exclude the possibility of changes in central auditory processing and PPI in larval zebrafish after noise exposure.

In order to determine whether the observed changes in startle sensitivity after noise exposure were specific to the acoustic startle response pathway, we measured startle responses that were evoked by direct stimulation of the Mauthner cells using electric field pulses and found no difference between noise-exposed and control fish. This finding suggests that startle sensitization is not likely due to changes in Mauthner cell membrane potential and excitability. Therefore, the locus of action is likely at the presynaptic terminal of the Mauthner cell lateral dendrite, which receives input from saccular afferents, or in the auditory pathway. We also measured overall locomotor activity in noise-exposed and non-exposed animals and observed that noise-exposed fish showed a significant decrease in the total distance moved in a 30-min period and there was no significant difference in the time spent moving compared to control fish (Fig. 4). These data are consistent with previous observations in larval zebrafish (Yokogawa et al. 2012) and adult stickleback (Purser and Radford 2011) of reduced locomotor activity after noise exposure and suggest that increased startle responsivity to auditory stimuli is not likely due to generalized hyperactivity.

Together, our results suggest a complex effect of noise exposure on the acoustic startle pathway in larval zebrafish. Studies on hair cell death and regeneration in the zebrafish saccule have shown that acoustic trauma can cause hair cell death (Smith et al. 2006; Schuck and Smith 2009), which can result in significant changes in AEP thresholds (Uribe et al. 2013).

Hair cell regeneration in the saccule requires at least 2 days of recovery after noise exposure (Schuck and Smith 2009), suggesting that the temporary change in startle threshold after noise exposure is not due to hair cell death and regeneration. However, it should be noted that noise-induced damage to the cochlear hair cells and the primary afferents of the VIIIth nerve in mammals has been associated with both hyperacusis (Hickox and Liberman 2014) and loudness recruitment, perceptual phenomena where thresholds remain unchanged, but stimuli above threshold are perceived as abnormally loud (Pickles 2012). Measurement of physiological and structural changes in the saccule could help resolve the mechanisms by which noise exposure influences the auditory startle pathway in larval zebrafish.

Blockade of the AMPA receptors using DNQX during noise exposure resulted in a reduction of

startle response sensitization. AMPA is directly involved in mediating the startle response in the mammalian caudal pontine reticular nucleus (Krase et al. 1993) and is involved in noise-induced cochlear synaptopathy (Liberman et al. 2011) and subsequent increase in startle responses (Hickox and Liberman 2014). Studies in adult goldfish show that AMPA receptors play a major role in signal transduction at the M-cell lateral dendrite (Mirjany and Faber 2011). AMPA signaling at the inner ear and lateral line hair cell synapses have also been shown to regulate function and to be involved in excitotoxic damage (Liberman et al. 2011; Trapani and Nicolson 2011; Sebe et al. 2017). The effects of noise exposure could affect either or both peripheral and central synapses. Furthermore, DNQX can also bind to the kainate receptor with similar affinity (Honore et al. 1988); and therefore, some of these effects could potentially be kainate mediated, as previously shown in guinea pigs and rats (Pujol et al. 1985). Therefore, the mechanism(s) by which AMPA receptor blockade affects startle sensitivity remains unclear.

Habituation to a strong startle-inducing stimulus (10 dB re·1 ms^{-2}) decreased after noise exposure. This effect is consistent with previous observations in rats (Davis 1974). However, this habituation-suppression is confounded by noise-induced sensitization. Electrical stimulation of the reticular formation in rats leads to a sensitization effect, whereas direct stimulation of the dorsal cochlear nucleus leads to sensitization followed by rapid habituation (Davis et al. 1982), which suggests that these two processes have distinct processes in mammals. In zebrafish, rapid habituation to a startle-inducing stimulus is thought to occur primarily at the lateral dendrite of the M-cell mediated by NMDA receptors (Roberts et al. 2011; Wolman et al. 2011) and glycinergic feedforward inhibition through interneurons (Marsden and Granato 2015; Koyama et al. 2016). In this study, we observed that NMDA receptor blockade using bath application of APV does not affect sensitization due to noise exposure, suggesting that habituation and hypersensitization of the startle may be mediated through different processes, as predicted by the dual theory of habituation (Groves and Thompson 1970). Future work will be required to elucidate the different mechanisms that mediate habituation and hypersensitization of startle responses induced by noise overexposure in zebrafish.

Non-auditory effects, such as stress and fear potentiation, have been suggested as a mediator for changes in the startle response of mammals (Davis 2006) and in zebrafish (Griffiths et al. 2012). Noise exposure has been shown to transiently increase plasma cortisol levels in adult goldfish within 10 min of exposure (Smith et al. 2004), although these effects are not seen with long-term noise exposure. Therefore, the observed changes in startle response may also be influenced centrally by other neuromodulators induced by noise overexposure that have yet to be described. Future research that investigates stress-related and auditory-related effects due to noise may provide insight on how these two processes might be related, and how these processes may lead to auditory-related changes such as hyperacusis. Thus, zebrafish may provide a new and tractable model to investigate novel treatments for noise-induced perceptual disorders and the mechanisms of noise-induced changes in the auditory system that may be conserved across vertebrates.

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