The effects of noise exposure and genetic background on auditory-evoked behaviors in larval zebrafish

Ashwin A. Bhandiwad

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2016

Reading Committee:

Joseph Sisneros, Chair

David Raible

Edwin Rubel

Michael Beecher

Program Authorized to Offer Degree:

Psychology

University of Washington

Abstract

The effects of noise exposure and genetic background on auditory-evoked behaviors in larval zebrafish

Ashwin A. Bhandiwad

Chair of Supervisory Committee:
Professor Joseph Sisneros
Department of Psychology

Auditory sensitivity and perception can be influenced by extrinsic factors, such as environmental noise, and intrinsic factors, such as genes and arousal. How do these factors interact and influence auditory-related behaviors? I addressed this question in the larval zebrafish (*Danio rerio*). Zebrafish have been a powerful genetic model species used in the study of development of auditory structures and exhibit a well-studied and robust escape response when presented with a loud acoustic stimulus. I used psychophysical methods to measure auditory sensitivity and perception by observing changes in this behavioral response. In chapter 2, I describe a novel paradigm using prepulse inhibition as a tool to measure auditory sensitivity in larval zebrafish. I show that this paradigm is more sensitive than other behavioral measures used in zebrafish research. Next, in chapter 3, I demonstrate that loud noise exposure leads to a temporary

hypersensitivity toward startle-inducing stimuli, whereas auditory sensitivity, as measured using the prepulse inhibition paradigm, is unchanged. I use pharmacological and physiological measures to explore potential mechanisms of this hypersensitization effect. Finally, in chapter 4, I investigate effects of genetic background on auditory sensitivity and susceptibility to noise exposure between closely related individuals and across multiple zebrafish lines. The results of these experiments show large-scale differences in startle sensitivity between wild-type zebrafish lines. I discuss the implications of this data, summarize these findings in a larger context, and discuss future studies in chapter 5.

TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY

AB – AB zebrafish line

AB/WIK – Hybrid zebrafish from AB and WIK lines

ABR – Auditory brainstem response

AEBR – Acoustically evoked behavioral response

AEP – Auditory evoked potential

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APV - (2*R*)-amino-5-phosphonovaleric acid

ASR – Acoustic startle response

DASPEI - 2-(4-(dimethylamino)styryl)-N- ethylpyridinium iodide

dB - decibels

DNQX - 6,7-dinitroquinoxaline-2,3-dione

dpf – Days post-fertilization

EF – Electrical field

FFT - Fast Fourier Transform

hpf - hours post-fertilization

Hz - Hertz

IQR - Interquartile range

M-cell – Mauthner cell

MS222 - Tricaine methanesulfonate

NMDA – N-methyl-D-aspartate

PPI – Prepulse inhibition

RMS - Root mean-squared

TL - Total length*

TL – Tubingen longfin zebrafish line*

TTS – Temporary threshold shift

TU – Tubingen zebrafish line

WIK – WIK zebrafish line

*TL is used as “Tubingen Longfin” only in Chapters 4 and 5.

PREFACE

Sections from Chapter 1 have been adapted from the following book chapter:

Bhandiwad AA, Sisneros JA (2016) Revisiting psychoacoustic methods in fish hearing. In: Advances in Experimental Medicine and Biology 877: 157-84

Chapter 2 has been previously published as:

Bhandiwad AA, Zeddies DG, Raible DW, Rubel EW, Sisneros JA (2013). Auditory sensitivity of the larval zebrafish (*Danio rerio*) measuring using a behavioral prepulse inhibition assay. J Exp Biol 216: 3504-3513

Chapter 3 has been prepared for publication format. As such, there may be some redundancies in the text.

ACKNOWLEDGEMENTS

First and foremost, I am deeply thankful to my parents, brother, grandparents, and all the members of my family who have helped and supported me. I would not be here if not for your love and support. I love you all very much and hope to continue making you proud.

I also thank my advisor, Joe Sisneros. Joe, you've been an amazing mentor and contributed so much to my development as a scientist. Your teaching, advice, and your willingness to try zebrafish studies have been invaluable these past six years, and I am honored to have been your student.

I'm also thankful to Dave Raible and Ed Rubel, who have given me every opportunity to succeed. Dave and Ed, your help and advice over my graduate career is something I truly cherish. I would also like to thank others who have been mentors during my graduate career: Mike Beecher and Marti Bosma, my committee members, and Katie Peichel. I can never forget the wisdom and insights you've given me. I would also like to thank my previous advisors, Sal Genovese and Sonke Johnsen, without whom I would not be in graduate school.

A very special thank you to David Zeddies and Dick Fay, who provided me with the kernel of the idea that became this dissertation. I am truly fortunate to have worked with you both and hope I have done this work as half as well as you both would.

I am grateful for the support, advice, and friendship of all the members of the Sisneros lab, past and present, throughout my graduate career: Liz Whitchurch, Peter Alderks, Rob Mohr, Nick Lozier, Ruiyu Zeng, and Orphal Colleye. I would also like to thank members of the Raible/Rubel labs, Allison Coffin, Arminda Suli, Joy Sebe, Dale Hailey, Robert Esterberg, Tor Linbo, Tammy Stawicki, and Kelly Owens, for advice, help, and support with the zebrafish work. I look forward to continued collaborations and friendships with you all.

I am also extremely thankful for all the friends I have made throughout my graduate career, who are too many to mention. I especially thank Teri Kirby, Valerie Tryon, Joyce Yang, Kristine Thimm, Jessica Thomas, and Thomas Edwards. You're all such incredible people who have made living and working here an absolute joy.

I am also thankful to the Psychology department's support and administrative staff, in particular Jeanny Mai and Susan Carpenter-Brandt, without whom nothing would ever have gotten done. Thanks for cheerfully making literally everything work smoothly.

Last, but certainly not least, I would like to thank Sarah Dygert and Bjorn Hubert-Wallander. Your love and support throughout my time in Seattle have helped make me who I am today. I love you both so very much.

This work was funded by grants from the National Institutes of Health, National Science Foundation, and University of Washington, to the Sisneros and Raible labs, and to the University of Washington Auditory Neuroscience Training Grant, University of Washington Top Scholar fellowship, and the GO-MAP Stroum Fellowship for financial support.

DEDICATION

This work is dedicated to my grandfather, Dr. PK Nargund, who continues to inspire me to be a great person as well as a great scientist.

Chapter 1. BACKGROUND AND SIGNIFICANCE

Auditory plasticity can be defined as the ability to change neuronal function to adapt to external and internal changes. Auditory plasticity can occur at every level of the auditory pathway, from the middle ear and cochlea (reviewed in Liberman and Mulroy 1982) to the auditory cortex (Willott et al. 1993). This plasticity can result in gain of function, for example in auditory learning (Buonomano and Merzenich 1998, Gao and Suga 2000, Knudsen 1999) and ontogeny (Rubel 1978), or loss of function, as a result of damage to auditory structures (Liberman and Dodds 1984, Salvi et al. 2000).

Extrinsic influences on auditory plasticity include environmental noise (Wang et al. 2002, Kujawa 2015, Eggermont 2013), ototoxic drugs (Harris et al. 2003, Hawkins 1973, Kiang et al. 1976) and experience-dependent changes (Gao and Suga 2000, Thiel et al. 2002, Kilgard and Merzenich 1998). Intrinsic influences on auditory plasticity include inter-individual differences such as genetic variability (as reviewed in Gorlin et al. 1995), development (Rubel 1978, Ruben and Rapin 1980), and age-related changes (Frisina 2009), and state-dependent effects within individuals, such as attention (Picton and Hillyard 1974, Fritz et al. 2007). This dissertation focuses on the interaction between extrinsic and intrinsic effectors of auditory-evoked behaviors in fishes. Here, I show how external influences, such as noise exposure, and internal influences, such as genetic background, can change and shape hearing in fish.

1.1 HEARING IN FISHES

There are more than 30000 known species of teleost fish, which live in diverse and variable acoustic environments. This has led to anatomical specializations for hearing across teleost taxa

(Retzius 1881, Platt and Popper 1981). Fishes vary in their hearing range, from very low frequencies (100-300 Hz) in hearing non-specialists (reviewed in Popper et al. 2003 and Popper and Fay 1993) to ultrasonic hearing in clupeid fishes (Mann et al. 1997).

Interest in fish hearing dates back to Aristotle's observation that "fishes undoubtedly hear…For they are observed to run away from any loud noise, such as would be made by the rowing of a galley, so as to become easy of capture in their holes; for though a sound be very slight in the open air, it has a loud and alarming resonance to creatures that hear under water" (for translation of Aristotle's original text see Barnes 1984). Parker (1903) was the first to quantitatively show that these inner ears were functional and that fish possess a sense of hearing. Working with the killifish (*Fundulus heteroclitus*), Parker performed a relatively simple experiment in which he attached a viol string to an aquarium wall, vibrated the string, and observed that fish responded to acoustic stimuli by moving their pectoral fins in 96% of the trials. These movements were also observed when the lateral line nerves were cut (94%), but rarely when the acoustic division of the VIIIth nerve was cut (18%).

The study of hearing in fishes allows us to understand common motifs in the structure and function of the auditory system across vertebrates. Anatomical structures and genetic basis of hearing and balance is remarkably conserved from fishes to mammals (Nicolson 2005, Whitfield et al. 2002). However, it should be noted that the sense of hearing in fishes is different from mammalian hearing in a few critical ways. First, all teleost fish respond to the particle motion, or near field, component of sound, whereas mammals respond to the pressure, or far-field component of sound (deVries 1950). Second, the auditory pathways in fish are similar to other

animals primarily in the periphery (which I will discuss later in this chapter), and diverge from mammalian, avian, and anuran hearing with respect to central auditory processing.

1.2 MEASUREMENT OF HEARING IN FISH

1.2.1 *Electrophysiological measures*

In general, electrophysiology has been the most commonly used technique during the past 30 years to assess the auditory capabilities of fishes. Techniques such as single neuron recordings, auditory evoked potential (AEPs, also referred to in previous literature as the auditory brainstem response, or ABR) and microphonic potential recordings have been instrumental in understanding various auditory capabilities of fishes including temporal encoding (Fay 1977, Fay and Coombs 1983, Carr 1986, Bodnar and Bass 1997, Kozloski and Crawford 2000, Bodnar and Bass 2001), frequency selectivity (Fay and Edds-Walton 1997, Weeg et al. 2002), auditory plasticity (Sisneros and Bass 2003, Sisneros 2009), directional sensitivity (Enger et al. 1973, Fay 1979, Lu et al. 1996, Fay and Edds-Walton 2000, Edds-Walton and Fay 2003), and the role of inhibition in shaping frequency tuning properties and phase locking ability (Fay 1978, Fay 1995, Lu et al. 1996, Kawasaki and Guo 1998, McKibben and Bass 1999, Maruska and Tricas 2009). These methods are rapid and precise compared to behavioral methods and as a result, they have been the technique of choice to investigate the hearing abilities of fish since the early 1960s (Enger 1963, Furukawa and Ishii 1967).

Although electrophysiological methods are critical in the study of fish hearing, they do have some limitations. First, some methods, like single auditory neuron and microphonic potential recordings, are technically difficult to perform, can involve invasive surgeries, and require physical restraint of the animal. Even non-invasive methods such as the AEP recording

technique, which has been used in over 100 fish species (Ladich and Fay 2013), requires the animal to be physically restrained. Unfortunately, some fish species are difficult to test using electrophysiological methods due to a reduced tolerance for restraint, surgery or other invasive methods. Second, electrophysiological methods are often difficult to perform on small animals, especially those that are early in development and less stress-tolerant. Although microphonic potentials have been recorded from larval fish (Lu and DeSmidt 2013, Inoue et al. 2013), most other electrophysiological recording methods are technically challenging to perform on embryonic and larval fishes. This limitation reduces the ontogenetic stages and the age/size ranges that can be compared and makes developmental physiology studies more difficult to perform. Third, electrophysiological methods such as the AEP recording technique that are used to measure auditory thresholds are difficult to compare to behavioral measures of hearing and are even more difficult to interpret in the context of natural auditory driven behaviors (Ladich and Fay 2013, Sisneros et al. 2016). Microphonic potentials of the fish inner ear, for example, can only inform us of hair cell activity, but not whether this activity results in an auditory percept for these fishes. Thus, there is a gap in our understanding of the relationship between behavioral and electrophysiological thresholds; although some correlations have been described for electrophysiological and behavioral thresholds, these correlations have only been made for goldfish (*Carassius auratus*) with no single representative relation between behavioral and physiological measures of auditory sensitivity (Ladich and Fay 2013). Variation of auditory thresholds obtained by electrophysiological measures can often be related to such factors such as electrode placement, morphology of the inner ear and skull, and the threshold criteria used.

1.2.2 *Behavioral measures*

Why are behavioral methods important in understanding auditory function in fishes? Because hearing capability is often directly related to a behaviorally relevant function to that animal, it should follow that any stimulus that can evoke a behavioral change is a 'relevant' stimulus to that animal. When considering the use of behaviorally relevant stimuli, researchers must use auditory stimulus parameters that take into account the hearing range of the species of interest. For example, in determining an audiogram for a fish species with no known hearing specializations, the use of stimulus frequencies greater than 2 kHz (roughly the upper frequency limit of sound-pressure sensitive fish) would often be superfluous, except in cases where fishes might be sensitive to ultrasound (>20 kHz). As Popper and Fay (1993) stated in their influential review on sound detection and processing in fish, "…we could say that all objects that may produce or scatter sound simultaneously are equally 'biologically significant', in the sense that no source can be identified or localized without significant processing of the simultaneous sounds from the other sources."

Behavioral methods are often a preferred method used to measure auditory capabilities in fishes because they are non-invasive. In some cases, behavioral methods may be the only means available to study hearing because alternative methods such as auditory electrophysiology require surgical preparation; this preparation prevents the use of many species that are sensitive and less stress tolerant to the surgery required for invasive auditory physiology experiments. Another advantage of using non-invasive behavioral methods is that they can be used to test hearing capabilities of fish in longitudinal studies, which is useful in determining the onset and development of hearing in a given species. Finally, auditory evoked behaviors require the

integration of multiple circuits and higher order auditory processing to produce a reliable and behaviorally relevant response. Thus, the use of auditory evoked behaviors provides an inherently sensitive way to assess hearing.

Auditory reflexive behaviors are involuntary movements in response to a sensory stimulus. Innate reflex responses are often used in psychoacoustic studies of hearing because they are stereotyped, repeatable and do not require conditional behavioral training in order to evoke them. Furthermore, these conserved innate responses serve a behaviorally relevant function, and are therefore robust and can be elicited easily. This allows for very fast and efficient measures of auditory capability.

1.3 ZEBRAFISH

The zebrafish (*Danio rerio*) is an excellent model in the study of fish hearing. Zebrafish are a tropical freshwater species in the family Cyprinidae and like all cyprinids, zebrafish have a well-developed auditory system, with specializations called Weberian ossicles, which connect the gas-filled swimbladder to the inner ear (Weber 1820). This specialization allows adult zebrafish to encode the pressure component of sound as well as the particle motion component, leading to a greater sensitivity to sound and a larger frequency range of hearing.

Zebrafish have also been a powerful model system to understand early development of the auditory system. Zebrafish embryos are mostly transparent and follow a highly stereotyped developmental timeline. The precursor to the inner ear, the otic placode, develops around 16 hours post-fertilization (Haddon and Lewis 1996). The first hair cells begin to appear around 18 hpf, followed by otolith formation around 20 hpf (Riley and Phillips 2003). The embyros hatch

between 48-72 hpf, and are free-swimming larvae with fully functional auditory systems at 4 days post-fertilization (dpf).

1.3.1 *Inner ear*

The inner ear of 5 dpf larval zebrafish contains three fluid-filled semicircular canals and two otolithic membranes, cumulatively called the otic vesicle (Whitfield et al. 2002). Sensory structures of the otic capsule respond to both vestibular and auditory stimuli. The base of each of the three semicircular canals contains a sensory epithelium called the crista. Each of these cristae contains sensory hair cells with large kinocilia oriented in a preferred and orthogonal direction (Whitfield et al. 1996). Head movements in any of these axes results in a deflection of these cristae and encodes for head direction and acceleration. Zebrafish mutants, such as the circler mutants (Nicolson et al. 1998), *dog-eared* (Kozlowski et al. 2005), and *chameleon* (Hammond et al. 2003), have mutations in the formation of semicircular canals or hair cell function and development and therefore result in a loss of vestibular function. These mutants show unusual circling swimming behavior and an inability to balance.

The two otolithic sensory epithelia, called the saccule and utricle, are the main organs of hearing in larval zebrafish (Figure 1.1). These membranes are attached to large calcium carbonate structures called the otoliths. As sound passes through these fish, the otoliths, which are much denser than the fish, accelerate away from the hair bundles of the sensory epithelia due to inertial forces and cause depolarization (deVries 1950, Popper and Fay 2011). Otolithic endorgans can therefore be thought of as 'biological accelerometers' which encode the particle motion component of sound. The saccule and utricle are both thought to be involved with hearing. However, a study in larval zebrafish demonstrated that vibration-evoked microphonic potentials

decrease much more drastically when the saccule is removed compared to the utricle (Inoue et al. 2013), suggesting that the saccule is the main organ of hearing in the larval zebrafish. Auditory afferent neurons synapse with the hair cells in the saccule and transmit auditory information to the statoacoustic ganglion.

Most of the studies in zebrafish have investigated hair cell development and function (Whitfield et al. 2002), hair cell death and regeneration (Harris et al. 2003, Owens et al. 2009, Namdaran et al. 2012), and mutations that lead to loss of hearing and balance (Nicolson 2005). However, less is known about the auditory percept in larval zebrafish. In this dissertation, I have developed a method to test behavioral reflex responses to auditory stimuli using the acoustic startle response in order to investigate how genetic and environmental influences on hearing in larval zebrafish.

1.4 ACOUSTIC STARTLE RESPONSE IN ZEBRAFISH

The most common reflex response described across multiple species is the auditory startle response (ASR). The ASR has been used most prominently in studies of the development of hearing in larval zebrafish (Kimmel et al. 1978, Zeddies and Fay 2005), but in most other studies it has served only as a test to determine whether or not the auditory system is functional. Zeddies and Fay (2005) were the first to use acoustic startle-like responses to construct audiograms in larval zebrafish. In this study, the authors stimulated 5 dpf larval zebrafish using a one-dimensional shaker (Figure 5) and measured responses using a standard video camera. Using the shaker, the authors were able to provide pure-tone particle motion stimuli and measure the acoustically evoked behavioral responses (AEBR) to the particle motion stimuli. The AEBRs were defined as any acoustically-mediated event that resulted in the movement of the fish and a difference in pixel distribution after frame subtraction in two consecutive video frames; if the

number of differing pixels were two standard deviations above pixel differences during a no-stimulus trial, the fish was determined to have performed an AEBR. AEBRs served as proxies for the ASR when using a non-high speed camera to record the responses because the ASR occurs on the time scale of 5-10 ms and standard (30 frames per second) cameras have a temporal resolution of approximately 33 ms. The authors were able to use this technique to show group-level absolute thresholds for larval zebrafish in early development (from 5-26 days dpf).

1.4.1 *Mauthner cell*

Although variants of the ASR have been described since Aristotle, Wilson (1959) was the first to show that the 'tail-flip' startle response in fish was driven by Mauthner cells (M-cells), giant neurons found in the fourth segment of the reticulospinal formation of the hindbrain (R4). This stereotyped startle response is described in mammals (Parham and Willott, 1988), anurans (Cioni et al. 1989) and urodeles (Marini et al. 1991). In fish, as well as anurans and urodeles, the M-cell circuitry is relatively simple (Zottoli and Faber 2000; Figure 1.2). Briefly, afferent neurons of the VIIIth nerve synapse onto the lateral dendrite of the M-cell at a mixed synapse called the club ending (Lin and Faber 1988, Yao et al. 2014). The M-cells cross over and innervate the motor neurons on the contralateral side of the fish. When activated, an M-cell fires a single spike that activates all the motor neurons on the contralateral side of the fish, causing the fish to bend and accelerate away from the direction of the stimulus. Subsequent input from the spiral ganglion neurons results in a refractory bend and propel the fish forward (Korn and Faber 2005). The startle-escape response was later formalized as the ‘C-start’ response, so called because of the conformation of the body to from a “C” at the apex of the response when all the muscles of that side are contracted (Kimmel et al. 1974). The authors used an experimental paradigm in which they dropped a metal ball into the tank containing zebrafish from varying heights (a greater

height would correspond to a larger intensity) and recorded the startle behavior of the fish using a video camera. Using this, they were able to show that the startle response is present in both larval and adult zebrafish, it could be elicited with auditory or tactile stimuli, and it could be described using a psychometric function. The latter finding is important, because it shows that the M-cells have intensity-dependent firing probability (Neumeister et al. 2000). This property allows for model fitting of this response to a psychometric function, and allows for interpolation of startle response threshold from discrete binomial responses.

The M-cell has been well-studied with respect to neural plasticity. M-cells are regulated by a set of inhibitory hindbrain interneurons (Weiss et al. 2008, Marsden et al. 2015) and are highly plastic. Internal state, such as serotonergic and dopaminergic signaling, can have a large effect on M-cell firing (Korn and Faber 2005). Interestingly, serotonin increases the firing probability of glycinergic inhibitory interneurons (Gotow et al. 1990), whereas dopamine increases the efficacy of electrical and chemical signaling in the excitatory pathway (Pereda et al. 1992). M-cells also receive inputs from visual and tactile stimuli through the medial dendrite, and these non-auditory inputs can influence the M-cell spike probability to auditory stimuli (Zottoli et al. 1995). External influences like repeated stimuli can change the spike probability of the M-cell and lead to habituation (Roberts et al. 2011, Marsden et al. 2015). However, if the stimuli are tetanic and below M-cell firing threshold, these stimuli can lead to potentiation (Yang et al. 1990).

1.5 Effects of noise on hearing

Hearing loss as a result of noise exposure has been well studied across vertebrate species. Noise overexposure can be acute (eg. impact or blast noise) or chronic. Acute overexposure is

commonly associated with damage to the tympanic membrane (Kerr 1980, DePalma et al. 2005) and organ of corti (Roberto et al. 1989, Hamernik et al. 1984) in mammals and damage to the saccule (Popper and Hastings 2009) and gas-filled swimbladders in fish (Yelverton et al. 1975). Chronic noise exposure has been associated with hearing loss (Tarter and Robins 1990), acceleration of age-related hearing loss (Kujawa and Liberman 2006, Bergstrom and Nystrom 1986), and increased stress in humans (Evans et al. 1995), rodents (Alario et al. 1987, Manikandan et al. 2006) and fish (Purser and Radford 2011). In this dissertation, I focus on the effects of chronic noise exposure on the larval zebrafish.

The mechanisms of noise-induced changes in the auditory system are well-documented. Noise exposure leads to hair cell loss in the organ of corti through mechanical damage to the sensory epithelia and supporting structures (Spoendlin 1971, Hamernik et al. 1974, Hawkins et al. 1976) and through metabolic oxidative stress (reviewed in Le Prell et al. 2007). Noise-exposure can also lead to structural changes in the hair cells in the absence of hair cell death, including damage to the tip links (Husbands et al. 1999, Clark and Pickles 1996), shortening of the stereociliar rootlets (Tilney et al. 1982), and fracture and fusion of the stereocilia (Engstrom et al. 1983, Liberman 1987).

Noise-exposure can also affect the afferent neurons innervating hair cells, even the absence of hair cell loss. Studies in the mouse have shown that noise exposure can lead to deafferentation (Liberman and Kiang 1978) and dendritic swelling of the afferent boutons (Robertson 1983, Pujol et al. 1993). These effects are thought to be mediated by glutamate excitotoxicity and can lead to a loss of signal transmission in the auditory nerve of up to 70 dB (Salvi et al. 1979).

Furthermore, these effects can lead to long-term changes in cochlear nerve survival (Kujawa and Liberman 2006, 2009).

These changes in the periphery lead to long-term changes in central auditory processing. Noise exposure has been shown to enhance auditory-evoked responses in the inferior colliculus in the rat (Salvi et al. 1990), which has been behaviorally linked to changes in the ASR and prepulse inhibition of the startle response (Willott and Turner 2000). Noise exposure has also been shown to lead to enhanced responses in the auditory cortex (Syka et al. 1994) and lead to changes in auditory cortex development in rats (Chang and Merzenich 2003).

Changes in the peripheral and central auditory system lead to dysfunction of auditory perception. The effects of noise on permanent hearing loss are well-known, but noise can also lead to temporary threshold shifts (Melnick 1991). Increased activity in the central auditory system after noise has been associated with disorders such as tinnitus (Hazell and Jastreboff 1990), hyperacusis (Sun et al. 2012, Katzenell and Segal 2001), loudness recruitment (Cai et al. 2009), and misophonia (Jastreboff and Jastreboff 2015). These perceptual changes often lead to a loss of quality of life in patients undergoing hearing loss and pose major problems in the understanding and treatment of hearing loss.

1.6 QUESTIONS ADDRESSED IN THIS WORK

How is auditory sensitivity maintained and how do intrinsic and extrinsic factors change auditory evoked behaviors? In this work, I investigate how long-term noise exposure and genetic background can influence both auditory sensitivity and startle responsivity in larval zebrafish. Using novel behavioral techniques, I have explored the following questions:

1.6.1 *What is the behavioral limit of hearing for larval zebrafish?*

I address this question in chapter 2 by developing a novel behavioral paradigm using prepulse inhibition of the startle response to measure the limit of hearing in larval zebrafish. This behavioral measure provides a valuable tool to investigate plasticity in the zebrafish in subsequent chapters.

1.6.2 *How does noise exposure affect auditory sensitivity and loudness perception?*

I address this question in chapter 3 by observing the effects of long-term noise exposure on the startle response and prepulse inhibition thresholds in larval zebrafish and describe how noise exposure leads to a hyperacusis-like state in these fish. I use pharmacological and electrophysiological methods to determine the mechanism of action for noise in the startle response pathway.

1.6.3 *What are the effects of genetic background and inter-strain variability in auditory evoked startle behaviors?*

I address this question in chapter 4 by investigating the effects of genetic background on startle responses in larval zebrafish. Using inbred and hybrid wild-type zebrafish lines, I show that genetic background plays a major role in startle sensitivity. Furthermore, I investigate individual differences within the AB line with respect to startle sensitivity and susceptibility to noise exposure.

These chapters will explore how a relatively simple sensorimotor behavior can be influenced by noise-exposure and how it can vary within the same species with genetic background. The experiments outlined in these chapters will investigate how the auditory system can be plastic in

a larval fish, and points toward future research in other influences that can influence these behaviors. One potential avenue is the interaction of neuromodulators, such as hormones and monoamines, on noise-induced changes in startle sensitivity. Future studies could also explore genetic and environmental influences on auditory development in zebrafish.

1.7 FIGURES

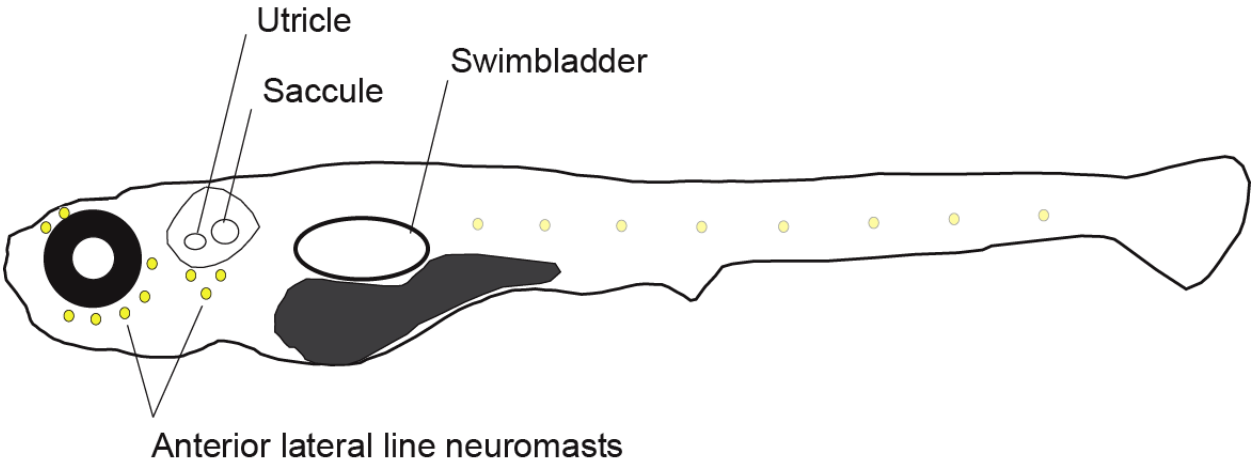

Figure 1.1. **Auditory and mechanosensory structures of a 5 dpf zebrafish larva**. The inner ear contains two otolithic endorgans, the saccule and utricle, which encode auditory stimuli. The mechanosensory lateral line neuromasts (yellow circles) are found on the surface of the animal and encode flow and vibratory stimuli. The swimbladder is an accessory structure in adults and transmits pressure information to the inner ear via Weberian ossicles. However, at 5 dpf, these ossicles are not yet present.

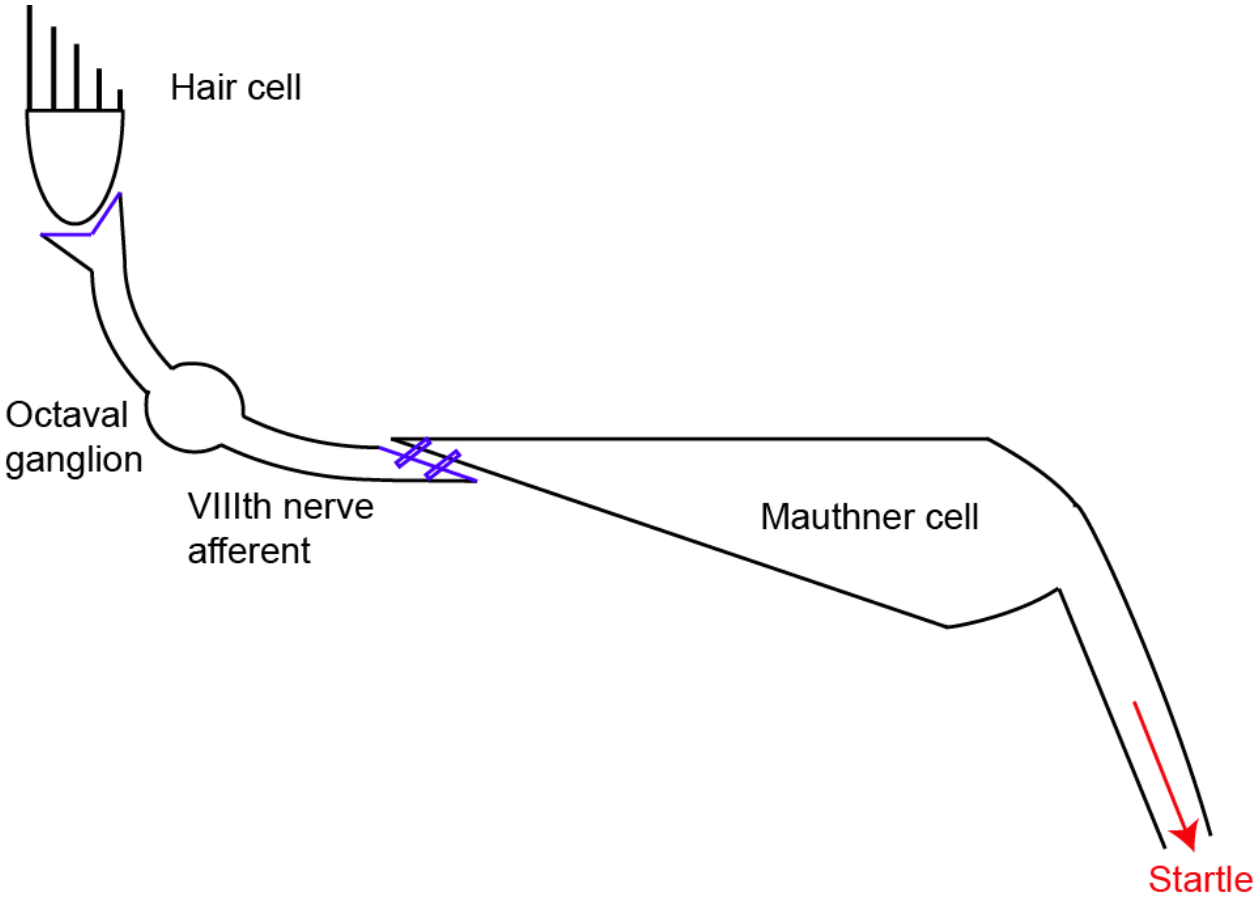

Figure 1.2. **Schematic representation of the excitatory pathway of startle**. Auditory stimuli activate hair cells of the inner ear, which are innervated by afferents of the octaval ganglion. These neurons synapse onto the lateral dendrite of the Mauthner cell at a mixed synapse. A single Mauthner cell action potential propagates to the motor neurons on the contralateral side of the animal and results in a synchronous contraction in the direction opposite from the perceived stimulus.

Chapter 2. BEHAVIORAL MEASUREMENT OF AUDITORY SENSITIVITY IN LARVAL ZEBRAFISH

2.1 SUMMARY

Zebrafish (*Danio rerio*) have become a valuable model for investigating the molecular genetics and development of the inner ear in vertebrates. In this study, we employed a prepulse inhibition (PPI) paradigm to assess hearing in larval wild-type (AB) zebrafish during early development at 5-6 days post fertilization (dpf). We measured the PPI of the acoustic startle response in zebrafish using a 1-dimensional shaker that simulated the particle motion component of sound along the fish's dorsoventral axis. After determining the thresholds to startle-inducing stimuli, the hearing sensitivity of 5-6 dpf zebrafish was characterized using the thresholds of prepulse tone stimuli (90 to 1200 Hz) that inhibited the acoustic startle response to a reliable startle stimulus (820 Hz at 0 dB re. 9.8 m/s^2). Hearing thresholds were defined as the minimum prepulse tone level required to significantly reduce the startle response probability as compared to the baseline (no-prepulse) condition. Larval zebrafish showed greatest auditory sensitivity from 90 Hz to 310 Hz with corresponding mean thresholds of -39 dB to -30 dB re: 1g, respectively. Hearing thresholds of prepulse tones were considerably lower than previously predicted by startle response assays. The PPI assay was also used to investigate the relative contribution of the lateral line to detect acoustic stimuli. After aminoglycoside-induced neuromast hair-cell ablation, we found no difference in PPI thresholds between treated and control fish. We propose that this PPI assay can be used to screen for novel zebrafish hearing mutants and to investigate the ontogeny of hearing in zebrafish and other fishes.

2.2 INTRODUCTION

Zebrafish (*Danio rerio*, Hamilton 1822) have become a valuable model for investigating the development and molecular genetics of the vertebrate inner ear (Whitfield et al. 2002, Nicolson, 2005). The early development of the zebrafish inner ear is similar to that of other vertebrates (Bang et al., 2001; Whitfield et al., 2002; Riley and Phillips, 2003) and its sensory hair cells are homologous to those found in mammals (Coffin et al., 2004). Over 50 genes are known to impact the zebrafish auditory inner ear and/or vestibular system (Granato et al., 1996; Whitfield et al., 1996 and 2002; Riley and Phillips, 2003, Starr et al., 2004; Nicolson, 2005) and many of these genes are conserved and affect the inner ear development and function in other vertebrates, including humans (Nicolson et al., 1998; Moorman et al., 1999; Riley and Moorman, 2000; Busch-Nentwich et al., 2004; Kappler et al., 2004; Kozlowski et al., 2005). However, unlike mammals, zebrafish develop from eggs *ex utero* and are transparent during the first few weeks of life. These characteristics coupled with the animal's rapid generation time, ease of maintenance and accessibility of the inner ear make this animal an attractive genetic model to investigate inner ear development and hearing.

Despite the enormous potential of the zebrafish model to investigate the functional effects of genes on hearing, few behavioral hearing assays have been developed for zebrafish. The most commonly used behavioral measure of auditory function in larval zebrafish is the startle response (Bang et al., 2000 and 2002). It is an innate, reliable, and robust behavior elicited by fast, high intensity stimuli. The startle response is mediated by Mauthner cells (M-cells), which are large reticulospinal neurons that receive information from ipsilateral sensory afferents and synapse to contralateral spinal motor neurons (Eaton et al., 2001; Weiss et al., 2006). When activated, all of

the motor neurons fire synchronously, causing the fish to bend into a characteristic 'C' shape away from the stimulus direction, which is easy to detect and differentiate from normal swimming motion. However, the use of the startle response only tests the grossest aspects of hearing and cannot be used to characterize differences in frequency selectivity or other auditory capabilities. Comparison of startle response thresholds to auditory evoked potential thresholds reveals a large difference in detection sensitivity between these two measures, which likely indicates that the startle response assay has a high rate of Type II error; i.e., the auditory stimulus is detected but is too weak to elicit a startle response. The development of acoustically evoked behavioral responses to pure tones in zebrafish has also been studied from 5 dpf to adults (Zeddies and Fay, 2005) and a positive reinforcement conditioning assay has been developed recently for the assessment of hearing in adult zebrafish (Cervi et al., 2012).

The focus of this study was to develop a prepulse inhibition (PPI) paradigm to assess hearing in wild-type AB zebrafish during early larval development at 5-6 dpf. Prepulse inhibition is a well-studied phenomenon whereby a startle reflex elicited by a strong stimulus is inhibited by the prior presentation of a weaker stimulus (Hoffman and Ison, 1980). Prepulse inhibition and other behavioral suppression techniques have been used to investigate responses to acoustic stimuli since Yerkes (1905), who showed that a pairing of tactile and acoustic stimuli elicited greater response than a tactile stimulus alone; by systematically decreasing the intensity of acoustic stimuli and measuring response intensity, a behavioral hearing range could be constructed. Reflex inhibition and suppression methods have since been used to determine auditory sensitivity in rodents (Ison, 1982; Young and Fechter, 1983; Willott et al., 1994), chickens (Gray and Rubel, 1985), and humans (Ison and Pinckney, 1983). A PPI paradigm is advantageous over

other behavioral techniques because it takes advantage of an innate response that does not need to be learned or conditioned, and the degree of inhibition has been shown to be proportional to the stimulus intensity (Young and Fechter, 1983; Neumeister et al., 2008).

Sound can be quantified in descriptive terms including pressure and particle motion. Most terrestrial ears respond to pressure which is a scalar measure of sound that contains no directional information. In most cases, sound pressure can be readily measured using microphones or hydrophones. In contrast, particle motion is a vector measure of sound that includes directional cues and can be measured with accelerometers (or calculated from pressure gradient measurements). The inner ears of teleost fishes consist of one or more otolithic end organs that respond directly to particle motion and essentially function as accelerometers (Fay, 1984; Hawkins, 1993). Some fish, including adult zebrafish, have specialized adaptations that also allow them to sense the pressure component of sound; however, developmental studies have shown that 5 dpf larval zebrafish lack these adaptations and would therefore only be sensitive to acoustic particle motion at this developmental stage (Higgs et al. 2003; Kimmel et al.,1995).
In this study, we assess the acoustic (particle-motion) sensitivity of the inner ear in 5-6 dpf larval, wild-type (AB) zebrafish using a PPI assay not been previously used with fish. The Mauthner-cell mediated startle response of zebrafish to an acoustic stimulus is modified by the prior presentation of a lower level acoustic stimulus. We show that the PPI assay is a more sensitive measure of the zebrafish auditory capability than the standard acoustic startle response assay. We also use the PPI assay to investigate the relative contribution of the lateral line to acoustic stimulus detection in 5-6 dpf larval wild-type (AB) zebrafish and show that the lateral line is not involved in the encoding acoustic stimuli at the tested frequencies.

2.3 MATERIALS AND METHODS

2.3.1 *Animals and care*

Wild-type (AB) 5-6 dpf zebrafish larvae (*Danio rerio*) were obtained from an adult zebrafish colony housed at the University of Washington. Mating and egg collection were performed according to Westerfield (2000). Fertilized eggs from mated adults were staged according to Kimmel et al. (1995) and raised in petri dishes (densities $\leq$ 50 larvae/dish) housed in incubators at 28.5 ˚C. After 4 dpf, zebrafish larvae were fed live rotifers and then transferred to fresh embryo medium. At 4-6 dpf, larvae were transported to the testing facility in an insulated container, and were then tested the same day. All fish were transferred between containers and to the experimental apparatus using wide-bore pipettes in order to minimize damage of the lateral line neuromasts. Larvae were allowed to acclimate to the experimental lighting and temperature (27 ± 1 °C) conditions for 30 minutes before the experiments were conducted. Animal rearing and experimental procedures were approved by the University of Washington Animal Care and Use Committee.

2.3.2 *Experimental setup*

Sound produced by conventional speakers contains both acoustic pressure and particle motion. The use of a shaker allows for the fine stimulus control of acoustic particle motion in a single direction. The experimental apparatus consisted of a 96 square well plate (containing 3.2 mm diameter wells) secured to a 0.635 cm thick acrylic platform that was mounted on to a vertically-oriented Bruel-Kjaer Type 4810 shaker (Figure 2.1). The apparatus was similar to that of Zeddies and Fay (2005). Although the plate contained 96 wells, only a maximum of 36 central wells that formed a 6x6 array were used during the experiments due to the optical limitations of the high

speed camera. Individual fish with approximately 400 µL embryo medium were placed in each of the central test wells. The experimental apparatus was housed inside a sound attenuation chamber (Industrial Acoustics, New York, NY USA) on a vibration-isolation air table, in order to minimize external vibratory noise. A TDT System III (Tucker Davis Technologies, Alachua, FL) and a PC computer running a custom Matlab stimulus generation program were used to relay the stimulus signal to a Bruel & Kjaer Type 2710 amplifier (Bruel & Kjaer, Naerum, Denmark) that drove the shaker and produced controlled vibratory stimuli along the dorsoventral axis of the fish within the well. An accelerometer (model 355B04, PCB Piezotronics Inc, Depew, NY) was mounted onto the acrylic platform in order to measure the acoustic particle acceleration of the fish in the plate wells. The output of the accelerometer was amplified (Model 482A PCB amplifier) and then relayed to the A-D input of the TDT System III. Stimulus generation, capture, and TDT System III were controlled using Matlab and ActiveX software (Microsoft Corp, Redmond, WA).

The zebrafish behavioral responses were recorded using a Photron Fastcam 1024PCI (Photron USA, San Diego, CA) at 1000 fps (512 x 512 pixel resolution) synchronized to the vibratory stimulus via a TTL pulse. TTL pulses from the camera were recorded at each frame-capture using the System III and were later synchronized to the stimulus onset for analysis. All trials were illuminated from above using an LED array.

2.3.3 *Acoustic stimuli*

Acoustic stimuli were 24 ms cosine-squared gated 100 ms tones. Tonal stimuli of 90, 210, 310, 410, 540, 820, 1070, and 1200 Hz were created using Matlab 2009b (Mathworks Inc, Natick, MA) and sampled at 100 kHz. These frequencies were empirically determined during set up and

initial testing to minimize distortion and motion in the non-vertical axes (i.e., X and Y). The particle motion component of sound was measured using an accelerometer attached to the platform of the shaker system as a means to characterize acceleration in the experimental wells. During set up and initial testing acceleration along the X,Y, and Z axes was measured using a PCB model 034K20 three-dimensional accelerometer, amplified using a Model 482A6 signal conditioner and then relayed to the TDT System III. The accelerometer output was calibrated to the 355B04 accelerometer output prior to testing. Frequencies greater than 1200 Hz were not tested due to voltage and current input limitations of the shaker (Zeddies and Fay, 2005). Before each experiment acoustic stimuli were calibrated for frequency and amplitude. The 6x6 array of central wells were filled with approximately 400 µL embryo medium. The RMS accelerometer voltage output was acquired for each input amplitude. These outputs were checked for linearity and, as expected, the doubling of the stimulus levels resulted in a doubling of the measured acceleration (i.e., the slopes of plots are 6 dB per stimulus level doubling) at all the frequencies tested (Fig. 1). The sensitivity of the accelerometer, calibrated by the manufacturer, was 1 V output/1 g (9.8 m/s^2) acceleration and the particle acceleration levels were determined using the formula

$$dB = 20 * \log\left(\frac{RMS\ Accelerometer\ Output}{1V}\right)$$

Samples of acoustic stimuli at the highest levels used to characterize the acoustic startle response were recorded. The rise-fall times of the acoustic stimuli were empirically determined and chosen as the shortest time that preserved the stimulus envelope. Figure 2 shows the time waveform of the particle motion stimuli (see insets) and the Fast-Fourier transform (FFT) of the stimuli for 90, 410, and 1070 Hz. The stimuli used contained little harmonic distortion. In all

cases, the largest harmonic was attenuated at least 50 dB (re: 1g, or 9.8 m/s^2) relative to the fundamental frequency tested. At the highest levels used to characterize the acoustic startle response, significant particle motion was measured in the orthogonal (x and y) axes. However, this artifact of orthogonal motion in x and y during vertical (z) axis stimulation was not observed at or near levels used to characterize the thresholds for the prepulse inhibition of acoustic startle responses, since most of the x and y acceleration was at or below the measurable limit of the system (approximately -56 dB re. 1g).

2.3.4 *Acoustic startle response characterization*

A characterization of the acoustic startle response was performed in order to differentiate the Mauthner-mediated C-start response from other non-startle behaviors reported for zebrafish. There exist a large number of behaviorally interesting non-startle behaviors, such as the burst swim, J-bend turn, and routine locomotion (see table 1 in Wolman and Granato, 2012), but these behaviors are not associated with a positive C-start response. Previous studies have shown two different startle responses based on different latencies: a Mauthner-mediated (short-latency) startle response occurs on the order of 5-7 ms while a long-latency startle occurs on the order of > 16 ms (Burgess and Granato, 2007; Kohashi and Oda, 2008). For all experiments, only the short-latency startle was used to define a positive response.

For acoustic startle characterization, individual larvae (n = 9; 3x3 array) were presented with either 90 Hz at - 6 dB (re: 1 g) or 90 Hz at -12 dB pure tone stimuli with a 15 ms cosine gated ramp and the behavioral responses were filmed for the first 100 ms after the presentation of the stimulus. The video was then analyzed using a motion tracking Matlab script developed by Hedrick (2008). Four points on the fish (the two eyes, the caudal edge of the swim bladder, and

the caudal fin) were tracked for the duration of the response (Fig. 3B). These points were used to calculate two metrics: Euclidean length or distance between the head (defined as the midpoint between the two eyes) and tail, and the body angle (defined as the angle between the head, midpoint and tail). These two metrics were used to quantify C-start responses, non-startle responses, and other behavioral responses. Positive startle responses were defined as responses that displayed a mean reduction in the Euclidean distance between the head and tail greater than 50% during the time period from the fish's initial movement to the apex of the C-bend of the fish's body, and reached maximum C-bend within 8 ms after onset of the startle response. The duration of startle response was defined as the time of the initial movement of the fish to maximum flexion of the body C-bend. The latency of the startle response was defined as the time between the stimulus onset and initial movement of the fish; the stimulus onset was defined as the endpoint of the cosine gated ramp of the acoustic stimulus. Because latency was variable, only startle responses that occurred within < 50 ms after stimulus onset were considered as part of the criteria for positive acoustic startle responses. These characterizations were tested on >20 previously untested responses to validate accuracy and efficacy of the characterization parameters and then used to differentiate startle responses from non-startle responses in subsequent experiments.

2.3.5 *Acoustic startle and prepulse inhibition experiments*

For the acoustic startle threshold experiments, each replicate (defined as one plate containing 24 fish arranged in a 6x4 array) consisted of stimuli at the frequencies mentioned above, and at intensities from -6 dB to -30 dB re: 1g (varied in steps of 6 dB). That is, each plate of fish was presented with 45 stimuli presented in a repeated measures design. These trials were separated by a randomized inter-trial interval of 70 $\pm$ 10 s based on preliminary data in order to reduce

habituation. The behavioral responses were measured for the duration of the stimulus (100 msec). The C-start occurred at approximately 18 ms after stimulus onset while the long-latency startle was not characterized in this study. The videos were then analyzed using the criteria determined previously (above). For each trial, responses were coded binomially (1 for response, 0 for non-response). Plates that exhibited no responses were coded as having a threshold of 0 dB, one step (6 dB) above the highest presented stimulus level. After precise determination of the startle thresholds, prepulse experiments were conducted.

The experimental procedure for the PPI experiments was similar to that for the startle response experiments except that a frequency of 820 Hz at 0 dB re: 1g was used as a universal startle-stimulus. Each replicate consisted of 32 trials with four sound levels for each frequency presented in random order. These sound levels were empirically determined as the four largest sub-startle threshold levels. That is, these four levels were the highest levels presented that did not elicit a startle response. Additionally, three sound levels at or below the noise floor were tested to confirm reliability of the response and to ensure that PPI did not extend to sound levels below the detection level of our system. A PPI trial consisted of a 50 ms randomized prepulse stimulus with a 24 ms ramp time followed by the startle stimulus. The inter-pulse interval, or the time between the end of the prepulse tone and the beginning of the startle tone, was 70 ms, which was empirically determined in preliminary experiments. Each PPI-startle stimulus presentation (trial) was preceded by a no prepulse 'catch' trial in order to determine baseline startle response probability (Figure 4). The catch trial also controlled for possible habituation to the stimuli. The PPI effect was calculated as the difference between the percent response to the prepulse trial (T_n)

and the mean response probability of the catch trials immediately preceding (T_{n-1}) and following the prepulse trial (T_{n+1}) using the formula

$$PPI\ Response = T_n - \frac{T_{n-1} + T_{n+1}}{2}$$

For all prepulse experiments, plates of fish were presented with no more than 16 total (prepulse and catch) stimuli in order to minimize habituation. After 16 presentations, fish were replaced with naïve fish from the same cohort. Thus, for each dataset a total of 96 fish were used.

2.3.6 *Lateral line ablation experiments*

In order to assess the relative contribution of the lateral line in acoustic detection, the lateral line was ablated by aminoglycoside exposure. Five dpf larvae were exposed to 400 μM neomycin in embryo medium for 1 h and then immediately rinsed four times in fresh embryo medium (Harris et al., 2003; Murakami et al., 2003; Owens et al., 2009; Namdaran et al., 2012). Neomycin exposure at concentrations greater than 100 μM is known to significantly reduce swimming speed in larval zebrafish (Buck et al., 2012). Startle percentage to catch stimuli were measured after neomycin exposure and responses returned to baseline ~3-4 hours post-exposure. To account for any additional effects, larvae were allowed to recover in fresh embryo medium for 6-12 hours before experimentation. This recovery period is not long enough for hair cell regeneration to occur (Ma et al., 2008). After recovery, the fish were tested as described above for the prepulse experiments. Five larvae from each cohort of aminoglycoside exposure were used to assess the efficiency of the aminoglycoside treatment.

To assess efficiency of aminoglycoside ablation, lateral line superficial hair-cell neuromasts were labeled with the fluorescent vital dye DASPEI (2-(4-(dimethylamino)styryl)-N- ethylpyridinium

iodide; 0.005% final concentration in embryo medium) for 15 min. Larvae were then rinsed twice in fresh embryo medium and anesthetized in 10 μg/mL MS222 (Tricaine methanesulfonate, Sigma). Larvae were visualized using an epifluorescent dissecting microscope with 450–490 nM laser. Ten neuromasts were evaluated per fish: supraorbital (SO1,SO2), infraorbital (IO1–4), mandibular (M2), middle (MI1,MI2), and otic (O2) (Raible and Kruse, 2000). Each neuromast was assigned a score of 0–2: 0 (little/no staining), 1 (reduced staining), and 2 (normal staining) for a combined score between 0 and 20 per fish.

2.3.7 *Data Analysis*

The binomial response data collected from each plate was analyzed using a curve-fitting procedure. For each frequency, responses at each stimulus level were converted to a response percentage. Assuming that response percentage for a set of fish was a good measure of probability of eliciting a response from any given fish, the thresholds for each frequency were determined by fitting the response percentages with a Weibull cumulative distribution curve using a maximum likelihood method (Wichmann and Hill, 2001; Treutwein 1995). These curves show the best fit model to the data and are most accurate for the sound levels tested (Fig. 6); note that extrapolation of the curve's upper limits beyond the highest levels tested may not accurately reflect the expected startle response probabilities. Because we did not observe a response in the absence of stimuli, the startle response threshold was conservatively defined as the stimulus level at which the startle response could be reliably elicited > 5% of the time. A startle response probability of 5% represents the 95% 'confidence limit' of the baseline no response condition.

In the PPI experiments, a similar curve-fitting method was used to determine the threshold for the inhibition of the startle response. The binomial response data was converted into response

percentage, as in the case of the startle experiments. However, this response percentage was subtracted from the mean of the paired no-prepulse catch trials before and after stimulus presentation. This yielded a difference in startle probability from the expected value. This difference was then fitted to a cumulative Weibull distribution. The threshold for the inhibition of the startle response was determined for each prepulse frequency tested and was defined as the stimulus level that elicited a 5% reduction of the probability of startle response between PPI trials and the paired catch trials.

A one-way ANOVA was used to test for differences in startle duration between sound stimuli. Startle response data was heteroscedastic (Bartlett's test, $p < 0.01$ for all frequencies), and therefore non-parametric methods were used to analyze all startle and PPI response data. Differences between thresholds as determined by the startle response paradigm and prepulse inhibition paradigm were analyzed using a Friedman test (nonparametric equivalent of a repeated measures 2-way ANOVA; Zar, 1984), and frequency-specific differences between the two paradigms were analyzed using a post-hoc pairwise Mann-Whitney U test, as described by Siegel and Castellan (1988). To assess differences in the lateral line ablation experiments, a Friedman test between treatment and frequency was conducted. All statistical analyses were conducted using Matlab 2009b.

2.4 RESULTS

2.4.1 *Acoustic startle response characterization*

Acoustic startle responses to pure tone stimuli were observed in 5 dpf or older zebrafish and not in fish younger than 5 dpf (data not shown) at the stimulus levels and frequencies tested in this study. Startle responses consisted of an initial quick C-bend of the body, followed by the

refractory bends of the tail and head in alternating directions (Fig. 3A). Analysis of the high speed kinematic data of the evoked startle behavior revealed a highly stereotyped and reliable acoustic startle response. Figure 5 shows representative examples of the kinematic data for both startle and non-startle responses. Positive startle responses consisted of a mean reduction in the Euclidean distance between the head and tail by 72% $\pm$ 8% SD (n = 18), which occurs during the time from the fish's initial movement to the apex of the C-bend of the fish's body. During this initial phase of the startle response, the head-midpoint-tail angle also decreased from 180° (initial) to a mean angle of 45° $\pm$ 5°SD. Changes in the Euclidean distance between head and tail and the head-midpoint-tail angle were highly correlated (r = 0.83; n = 18 responses) for the initial bend during stage I of startle (Foreman and Eaton 1993). In contrast, changes in the Euclidean distance between head and tail (<40%) and the head-midpoint-tail angle (<60%) from the initial position for non-startle responses were much smaller.

The mean latency of the short-latency startle response, defined as the time between the stimulus onset (end of the cosine gated ramp) and initial movement of the fish that met the startle response criteria, was 3.9 $\pm$ 2.8 ms SD (n = 15 responses). The mean duration of startle response from the initial movement of the fish to maximum flexion of the body C-bend was 7.1 $\pm$ 0.74 ms SD (n = 18 responses) and was not different at the sound levels tested (ANOVA, $F(1,35) = 0.05$, $p = 0.82$), indicating that the duration of the startle response was not dependent on stimulus level. The latency and duration of non-startle responses were much longer (> 50 ms) and more variable in duration and were not quantified.

2.4.2 *Acoustic startle thresholds*

The percentage of startle responses between the lowest and highest stimulus intensity increased at each frequency. Figure 7 shows the startle response thresholds for acceleration as a function of frequency. Startle response thresholds were lowest at the lowest frequency tested, 90 Hz (median = -24 dB, interquartile range (IQR): -30 dB to -18 dB re. 1 g) and 210 Hz (-16 dB, IQR: -23 dB to -11.5 dB re: 1 g). Startle thresholds above 210 Hz gradually increased, plateaued between 310 and 410 Hz (-11 dB, IQR: -14 dB to -10 dB re: 1g), decreased slightly between 540 Hz (-15 dB, IQR: -20 dB to - 9 dB re: 1g) and 820 Hz (-16 dB, IQR: -23 dB to -13 dB re: 1g), and then increased rapidly from 1070 Hz (-5 dB, IQR: -7 dB to -3 dB re: 1g) to 1200 Hz (0 dB re: 1g). In general, startle sensitivity decreased with increasing frequency from 90-1200 Hz with the exception of a slight increase between 540 and 820 Hz. At the highest frequency tested, 1200 Hz, only 5 positive responses were observed at any stimulus level.

2.4.3 *Prepulse inhibition thresholds of the startle response*

Prepulse inhibition thresholds were defined as the intensity of the pre-pulse stimulus at each frequency that resulted in a >5% reduction in the probability of a startle response to the standardized startle stimulus (820 Hz at 0 dB re. 1g). As with the startle response data, the PPI response data from 10 plates of 24 fish were fitted with a Weibull distribution. The resulting PPI response profiles at each prepulse frequency are shown in Fig. 8. Figure 7 compares the median PPI thresholds and startle response thresholds in terms of acceleration as a function of prepulse frequency. In general, the PPI audiogram showed a steep increase in thresholds from 90 Hz (-39 dB, IQR: -43 dB to -36 dB re: 1g) to 210 Hz (-35 dB, IQR: -37 dB to -32 dB re: 1g), followed by a gradual threshold increase up to 820 Hz (-23 dB, IQR: -27 dB to -19 dB re: 1g), and then a rapid increase in thresholds up to 1200 Hz (0 dB, IQR: -11 dB to 0 dB re: 1g).

The PPI response is similar in shape to the startle response except that the PPI thresholds were significantly lower compared to the startle response thresholds (Friedman $X^2(1) = 72$, $p < 0.001$). Post-hoc Mann-Whitney U tests showed that these differences were frequency dependent (Figure 7). Prepulse inhibition thresholds were significantly lower than startle thresholds at 90-1070 Hz ($p < 0.01$ for all frequencies), but differences between prepulse and startle response thresholds at the highest frequencies tested (1200 Hz) were not significant ($p=0.2$). The greatest threshold difference was at 310 Hz in which the PPI threshold was approximately 21 dB lower than the startle threshold. Prepulse inhibition thresholds differed more from startle thresholds at lower frequencies (~11-21 dB from 90 to 540 Hz, respectively) than at higher frequencies (~ -7 to 0 dB from 820 to 1200 Hz, respectively).

The degree of habituation was measured by 25 repeated presentations of a no-prepulse catch stimulus (820 Hz at 0 dB re. 1g) with a 70 s inter-stimulus interval. Response percentages followed a biphasic linear decrease, with the inflection point occurring around the 12-15th stimulus presentation. The mean difference in response between the first presentation and the 15th presentation was 19% + 3% SE (n = 5 plates), whereas the mean difference in response between the first and 17th presentation was 33% $\pm$ 4% SE. However, between the 17th presentation and 25th presentation, mean difference in response percentage was only 3% $\pm$ 4% SE.

2.4.4 *Effect of lateral line ablation on PPI thresholds*

In order to determine the relative contribution of the lateral line to acoustic stimulus detection, 5-6 dpf zebrafish were exposed to 400 uM neomycin for 30 minutes. Neomycin exposure resulted

in a high incidence of hair cell death in the superficial neuromasts (DASPEI score: 1.2 $\pm$ 0.19, n = 8, Mean $\pm$ SE) compared to control fish which showed DASPEI scores of 16 $\pm$ 0.9 SE. The PPI assay was conducted at a subset of frequencies (90 Hz, 210 Hz, 820 Hz) used to construct the PPI audiogram. The PPI thresholds for neomycin exposed fish did not differ from the control fish at any of the tested frequencies (Friedman test interaction, $X^2(2) = 0.12$, $p = 0.25$; $n = 10$; Fig. 9). Thus, these results indicate that the mechanosensory superficial neuromasts do not contribute to auditory detection at the tested frequencies during this stage of development. Interestingly, the response probability for the no-prepulse catch trials was greatly decreased after neomycin exposure (81% $\pm$ 3% SD for controls, 65% $\pm$ 5% SD for neomycin exposed fish), which suggests a potential negative effect of neomycin on the locomotor behavior of larval zebrafish (Buck et al., 2012) that persists 6-12 hours after exposure.

2.5 DISCUSSION

To our knowledge, this study is the first to provide a behavioral audiogram for wild-type (AB) zebrafish during early larval development at 5-6 dpf. Our goal was to determine the acoustic sensitivity of larval wild-type zebrafish using the behavioral PPI assay, which quantifies the hearing thresholds of larval zebrafish to prepulse tones (90-1200 Hz) that inhibit the innate acoustic startle response to a reliable acoustic startle stimulus (820 Hz at 0 dB re. 1g). Our results demonstrate that larval zebrafish are most sensitive to low frequency acoustic stimuli from 90 Hz (lowest frequency tested) to 310 Hz and that the hearing thresholds established from the PPI audiograms were considerably lower than those previously obtained from startle audiograms. In addition, we provide evidence that the lateral line mechanosensory superficial neuromasts do not contribute to the detection of acoustic stimuli from 90 to 820 Hz during early development.

We found fast, C-start responses were reliably evoked by pure tone acoustic stimuli in 5-6 dpf wild-type (AB) zebrafish, whereas this behavior was absent in fish < 5 dpf (i.e., 4 dpf zebrafish) over the range tested (up to - 6 dB re. 1g for frequencies 90-1200 Hz). The variability in latency between the responses and the discrepancy between our measurement of latency and previously published accounts of startle latency (Burgess & Granato, 2007; Kohashi and Oda, 2008) was attributed to the ramped stimulus. In fact, some startle responses were observed to onset before the end of the ramp. Zeddies and Fay (2005) found a similar timing of onset of the expression of acoustically evoked behavioral responses to pure tones in 5dpf zebrafish but could not characterize the startle response type due to equipment limitations. Earlier work by Eaton et al. (1977) suggests that the development of the startle response (Mauthner-initiated C-start response) occurs very early in development. This fast startle behavior can be evoked by tactile stimulation as early as 44 hours post fertilization (hpf) (Eaton et al., 1977) and by visual stimuli 68-79 hpf (Easter and Nicola, 1996). Although Burgess and Granato (2007) reported occasional startle responses to uncalibrated broadband acoustic stimuli at 3 dpf, the reliable onset of acoustically-evoked startle responses to pure tone stimuli appears to occur at 5 dpf. This discrepancy in startle response onset may be due to the nature of the stimulus; the use of uncalibrated stimuli might contain low frequency elements that activate both the inner ear and the lateral line, leading to a higher activation of the M-cell at an earlier observed stage.

Acceleration thresholds from the pure-tone startle audiogram (Fig. 7, open circles) indicate that 5-6 dpf zebrafish are most sensitive to low frequencies < 310 Hz and that startle responses occur up to 1200 Hz (the highest frequency tested). The 820 Hz tone at 0 dB (re. 1g) had the highest

response rate and was subsequently used as the startle-inducing stimulus for the prepulse inhibition experiments. The gated acoustic stimuli used here contained little distortion arguing that the fish were responding specifically to the nominal stimulus frequencies.

Habituation to the startle-inducing catch stimulus was negligible in terms of its influence on the PPI response. Even though there was a small drop in the response percentage from the first presentation to the 16th presentation, since the PPI effect was measured relative to the response percentage of the catch trial, the effects of habituation were ameliorated. Habituation to the catch stimulus was also short-lived and in agreement with previous studies (Roberts et al., 2011). Retesting fish greater than 6 hours after initial habituation experiments showed no long-term effects of the stimulus presentations, and fish responded to catch stimuli at the same percentages as naive fish. In rodents, PPI assays for auditory sensitivity are conducted over multiple days to protect against habituation effects (Young and Fechter, 1983). A similar protocol may need to be developed for future comparative studies using the PPI assay with other fish species.

Response thresholds measured using the PPI experimental paradigm for 5-6 dpf zebrafish were lower than startle response thresholds. These PPI thresholds represent the lowest sound levels for the prepulse test tones that are required to effectively inhibit or modify the Mauthner-cell mediated startle response to a loud acoustic stimulus. Between 90 Hz and 540 Hz the PPI thresholds were ~11-21 dB lower than the startle thresholds and between 820 Hz and 1200 Hz the PPI thresholds were < 7 dB lower than startle thresholds (Fig. 7, closed circles). The lowest PPI threshold was -39 dB re 1g at 90 Hz, which is similar to particle motion thresholds for single unit saccular afferent recordings in other fishes, such as toadfish (*Opsanus tau;* range: -107 to -

57 dB re. 1g at 100 Hz; Fay et al., 1994), sturgeon (*Acipenser fulvescens* ; -110 dB to -53 dB re. 1g at 100 Hz; Meyer et al. 2010), and goldfish (*Carassius auratus*; -110 dB to -28 dB re. 1g at 140 Hz; Fay 1984).

Auditory thresholds derived from the PPI assay are known to be similar to those derived from electrophysiological methods. Young and Fechter (1983) found PPI thresholds in rats to be similar to auditory brainstem-evoked potential (ABR) thresholds, while Walter et al. (2012) found PPI thresholds were 10-15 dB SPL more sensitive than ABR thresholds in Mongolian gerbils. Our findings indicate that larval zebrafish have significant auditory capacity below levels that cause startle responses and the hearing threshold levels determined using the PPI paradigm are similar to auditory evoked potential (AEP) thresholds previously characterized for another otophysan fish, the goldfish (Radford et al., 2012). These findings suggest that the auditory system of 5 dpf larval zebrafish is relatively sensitive and functional during early development and that the PPI procedure described here provides a good measure of hearing threshold levels in larval zebrafish. Due to the non-invasiveness of this technique, the auditory sensitivity of larval or juvenile fish (and their cohorts) can be tracked throughout their development, and in future studies allow researchers to compare auditory thresholds as measured by PPI and other electrophysiological methods (eg., AEPs or auditory single unit recordings) within a species.

Responses measured using the PPI assays were most likely mediated by the saccule. The fish inner ear consists of three otolithic end organs: the saccule, utricle, and lagena (Popper and Fay, 1993). The upper frequency range (820-1200 Hz) suggests that PPI response is via the saccule because it is the only inner-ear end organ known to respond at these frequencies in otophysan

fishes (Fay 1981). The findings by Bang et al. (2002) are also consistent with saccule-mediated sound detection in that most mutations found to affect the startle response (to a 400 Hz tone) had morphological defects associated with the saccular auditory pathway. The lagena of wild-type zebrafish does not develop until 12 dpf (Riley and Moorman, 2000) and is thus nonfunctional in fish 5-6 dpf. While the utricle may serve an auditory role in adult sleeper gobies (*Dormitator latifrons*), its sensitivity is approximately 30 dB less than the saccule (Lu et al. 2004). However, studies on adult goldfish have shown that the utricular and saccular afferents are equally sensitive to particle motion stimuli (Fay, 1984; Fay and Olsho, 1979). Future studies that investigate the functional role of the three different end organs (saccule, lagena and utricle) in 5 dpf zebrafish would be instrumental in determining whether each end organ differentially detects and encodes acoustic particle motion and pressure.

Indirect stimulation of the zebrafish inner ear by sound pressure can occur via gas-filled bladders in close proximity to the end organs and/or by means of skeletal adaptations such as the Weberian ossicles in zebrafish and other otophysan fishes that link the swim bladder to the inner ear (Higgs et al., 2003; Popper and Fay, 2011). However, in zebrafish the Weberian ossicles are not fully formed or ossified until approximately 36-37 mm total length (TL) or ~56 dpf (Grande and Young, 2004). Higgs et al. (2003) showed, using AEP, no difference in sound pressure sensitivity of zebrafish during development from 10 to 45 mm TL. However, these authors showed that detectable frequencies >2000 Hz coincided with increases in body size (at approx.17-20 mm TL), swim bladder size, and connectivity of the Weberian elements, which is consistent with the hypothesis that the Weberian apparatus and swim bladder are responsible for transmitting higher frequency information to the inner ear (von Frisch, 1938; Fay and Popper,

1974). Although the swim bladder is inflated and clearly visible in 5 dpf zebrafish (~ 3.5 mm TL), the deflation of the swim bladder at this developmental stage does not affect the acoustically evoked behavioral response thresholds (Zeddies and Fay, 2005). These results suggest that 5-6 dpf zebrafish do not respond to sound pressure, but instead respond exclusively to particle motion and direct acceleration of the inner ear otolithic organs. As a result, it is not surprising that at this early stage of development, the audiogram resembles that of fish that do not have specialized adaptations for detecting pressure.

Ablation of the mechanosensory superficial neuromasts using aminoglycosides had no effect on the hearing sensitivity of larval zebrafish at frequencies of 90, 210, and 820 Hz, which is consistent with AEP studies from adult goldfish (Higgs and Radford, 2013). At this stage of development, 5-6 dpf zebrafish are only known to have superficial neuromasts capable of detecting vibrational stimuli up to 50 Hz (Liao et al., 2012). Lateral line canal neuromasts, which are capable of encoding frequencies up to 200 Hz (Kalmijn, 1988; Montgomery et al., 1995), do not develop until ~32 dpf (~10 mm TL) (Webb and Shirey, 2003). Future studies are needed to determine if there is multimodal overlap between the mechanosensory superficial neuromasts and the inner ear in 5-6 dpf zebrafish at frequencies < 50 Hz.

The PPI assay described here could be used as a valuable tool to screen for novel compounds that protect inner ear hair cells from noise-induced damage and investigate the molecular genetic basis of hearing in larval zebrafish. Molecular genetic studies on zebrafish hearing have thus far focused on unresponsive/deaf mutants with mutations affecting inner ear anatomical development (and/or its associated structures) or a loss of hair cell mechanotransduction

(Nicolson, 2005). A PPI assay could be developed to screen for hearing phenotypes, such as those with reduced auditory sensitivity or frequency selectivity, and ultimately used to investigate the genetic basis of auditory processing during early zebrafish development.

2.6 FIGURES

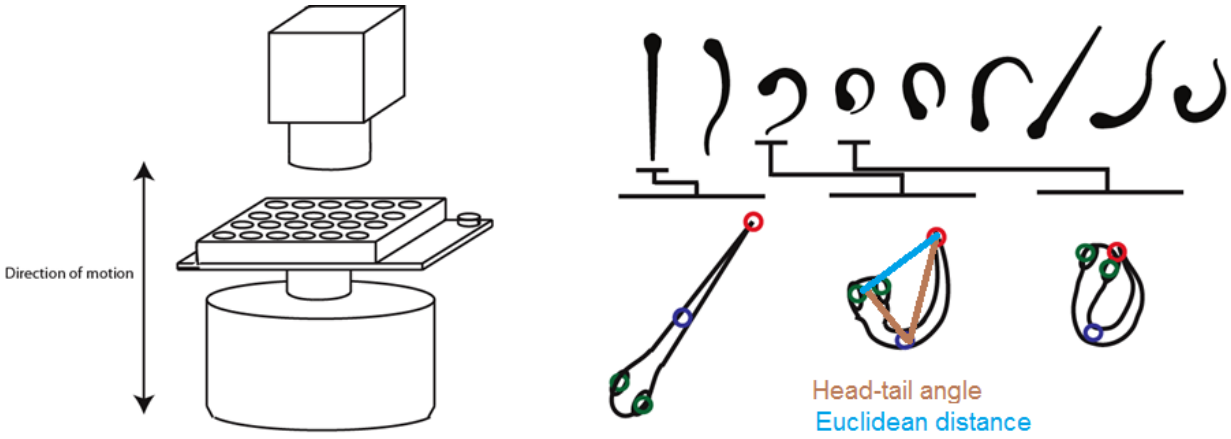

Figure 2.1. **Schematic diagram of shaker system used to deliver particle motion stimuli**. A one-dimensional shaker (Bruel-Kjaer Type 4810) oriented in the dorsoventral axis of the fish delivered pure tone particle motion stimuli. Fish were placed in the central wells of a 96 well plate coupled to the shaker and an accelerometer measured sound levels delivered to the animals. A high-speed camera (Photron FastCam) was used to record behavioral output of groups of zebrafish to presented stimuli.

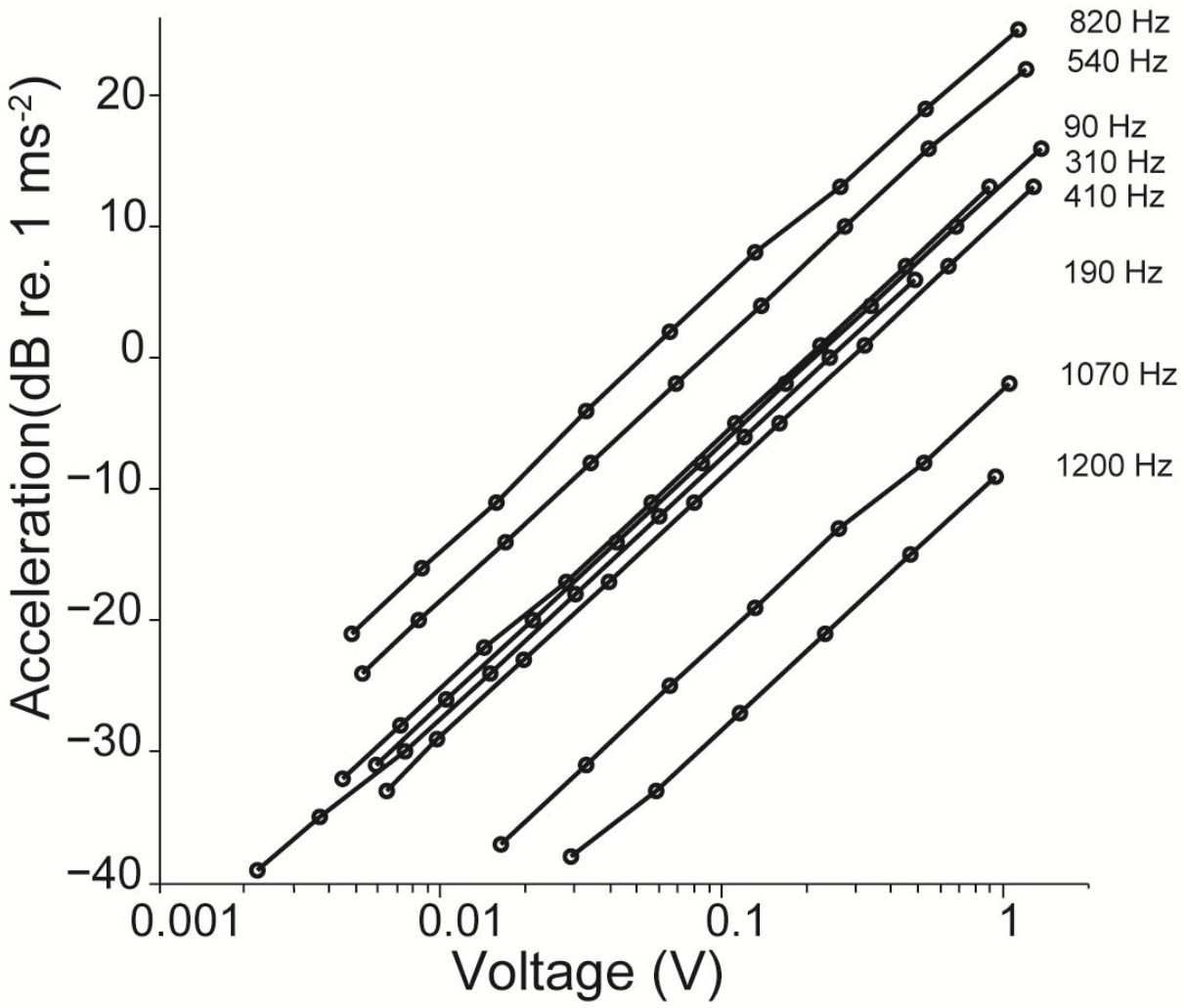

Figure 2.2. **Predictability of acceleration output of the shaker system**. Loglinear representation of the RMS acceleration output of the shaker system (dB re: 1g) as a function of input voltage (V) by frequency with 6x6 array of wells in 96 well plate were filled with 400 µL water. Each line indicates a separate frequency tested. Note that a doubling of the input voltage resulted in a doubling of the measured acceleration (6 dB increase).

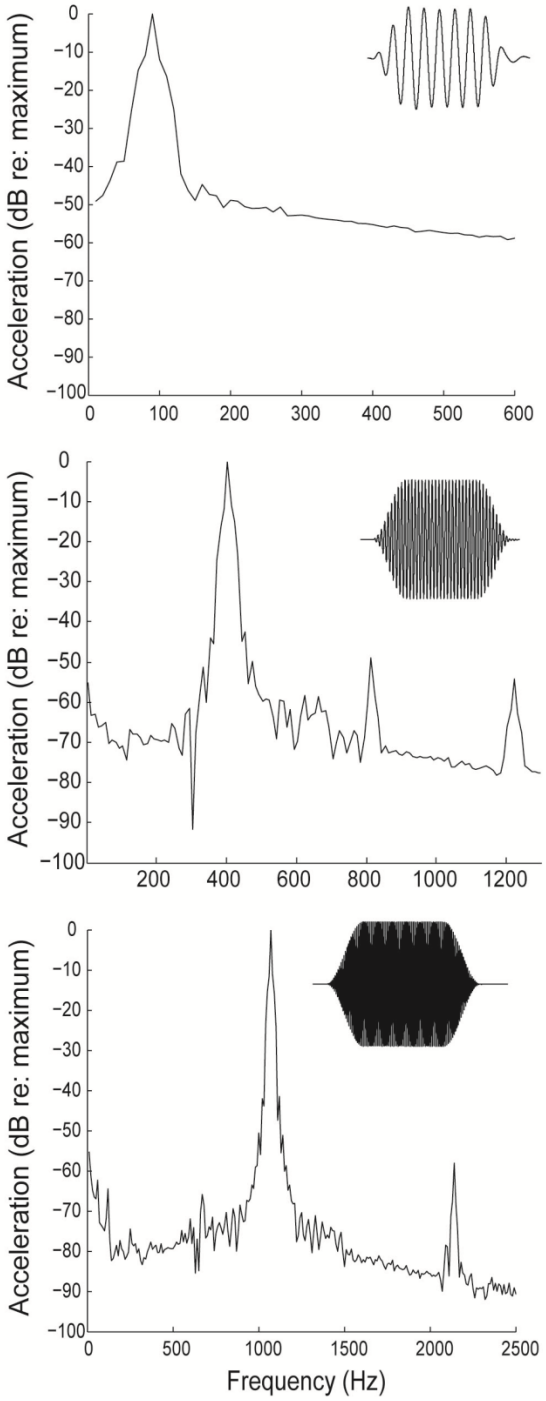

Figure 2.2. **Power spectra of shaker stimuli.** Representative power spectrum of a subset (90 Hz, 410 Hz, 1070 Hz) of sound stimuli used in pure tone startle stimuli and prepulse stimuli measured at the highest level used (-6 dB re: 1g). Data are normalized to a relative value of 0 dB assigned to the maximum sound level for the fundamental frequency tested. [Insets] Time courses of pure tone stimuli used for startle response assay at - 6 dB (re: 1g).

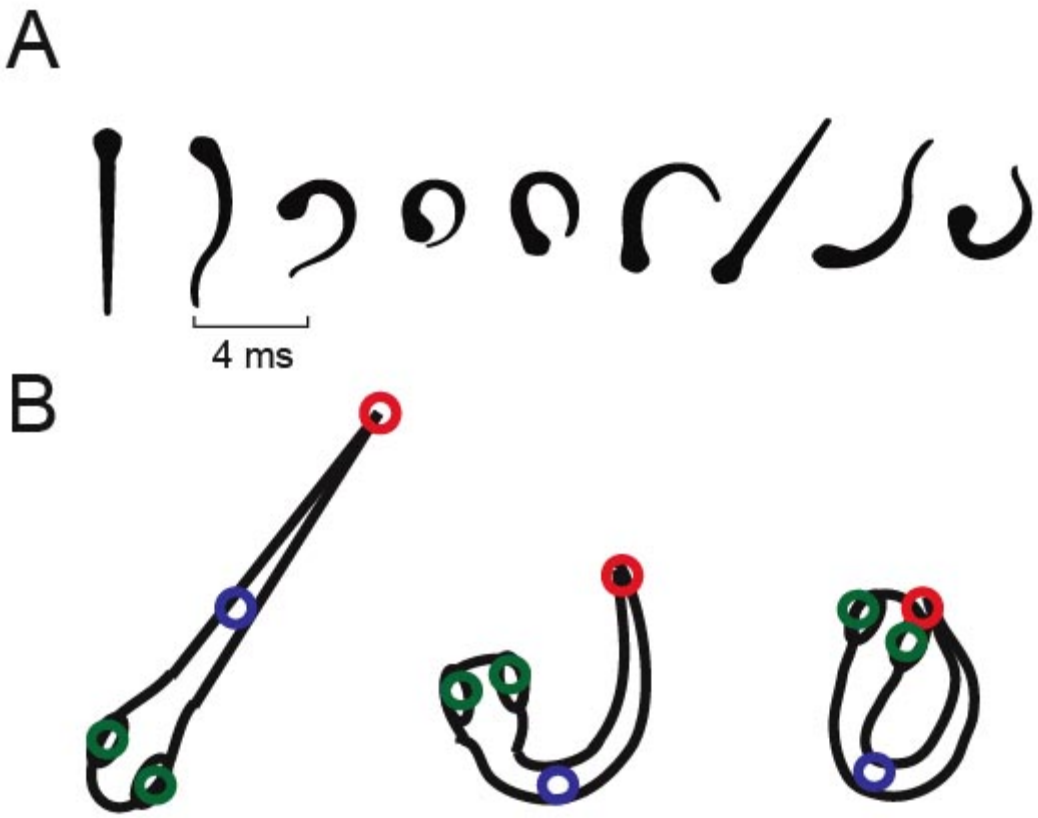

Figure 2.3. **Schematic representation of the startle response.** (A) Diagram of time course of acoustic startle response of 5-6 dpf zebrafish, digitized from a representative positive response fish. The response is characterized by a fast, coordinated contraction on one side of the body, forming a distinctive C shape (Frame 4). Successive frames are 4 ms apart. (B) Diagrammatic representation of the four points marked throughout startle characterization: two eyes (green), caudal edge of the swimbladder (blue), and caudal fin (red). Point tracking was used to measure head-tail Euclidean length and head-midpoint-tail angle throughout responses.

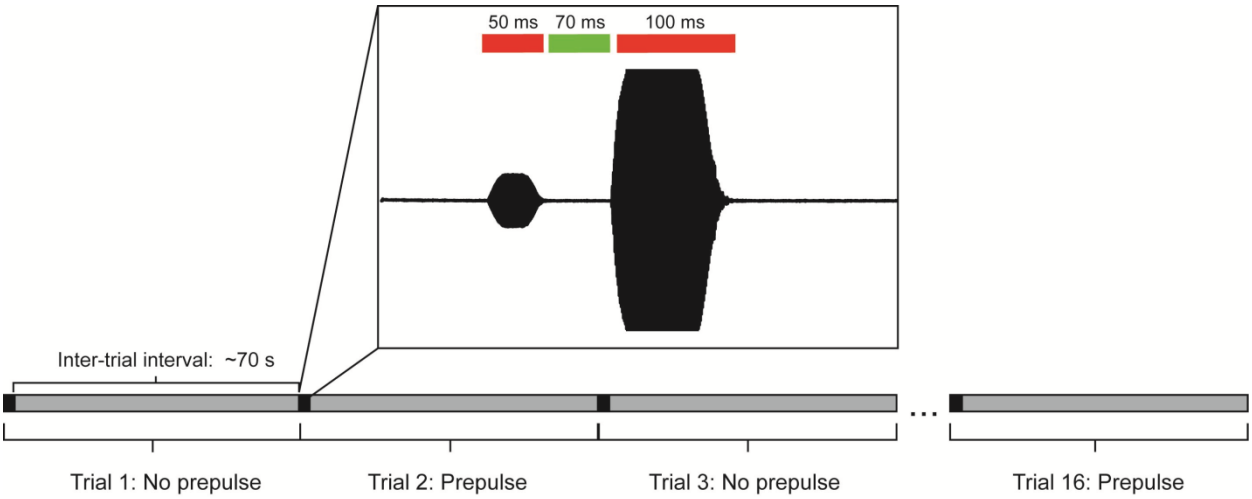

Figure 2.4. **Diagram of PPI protocol**. Prepulse trials and no prepulse catch trials are interleaved together with an inter-trial interval of ~70 s for a total of 16 trials per group. Prepulse frequency and level are randomized between trials, but all trials contain the same catch stimulus. [Inset] Example of a prepulse trial. A 50 ms prepulse stimulus is separated from the 100 ms catch stimulus by an empirically determined 70 ms inter-stimulus interval.

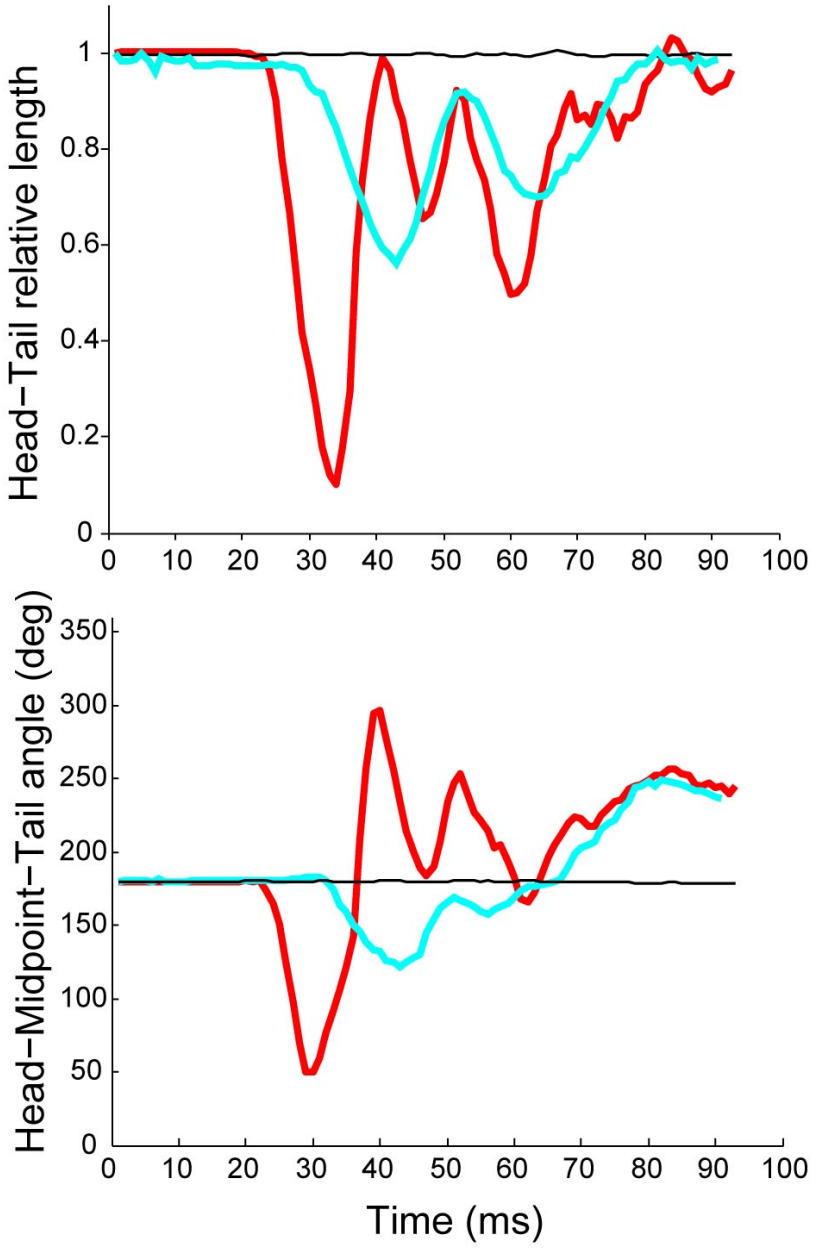

Figure 2.5. **Example of the acoustic startle response characterization**. A. Euclidean length from head-tail throughout response, relative to initial length. Time 0 denotes the onset of the stimulus. A positive startle response (red) shows a marked decrease in head-tail distance at the apex of the initial c-bend, followed by smaller refractory bends. Non-startle response (cyan) shows some decrease, but is smaller in magnitude than the startle. No response (black is also shown). B. Head-midpoint-tail angle (degrees) throughout response. Initial position is 180°. Length and angle measurements are highly correlated (r = 0.83, N=18 responses).

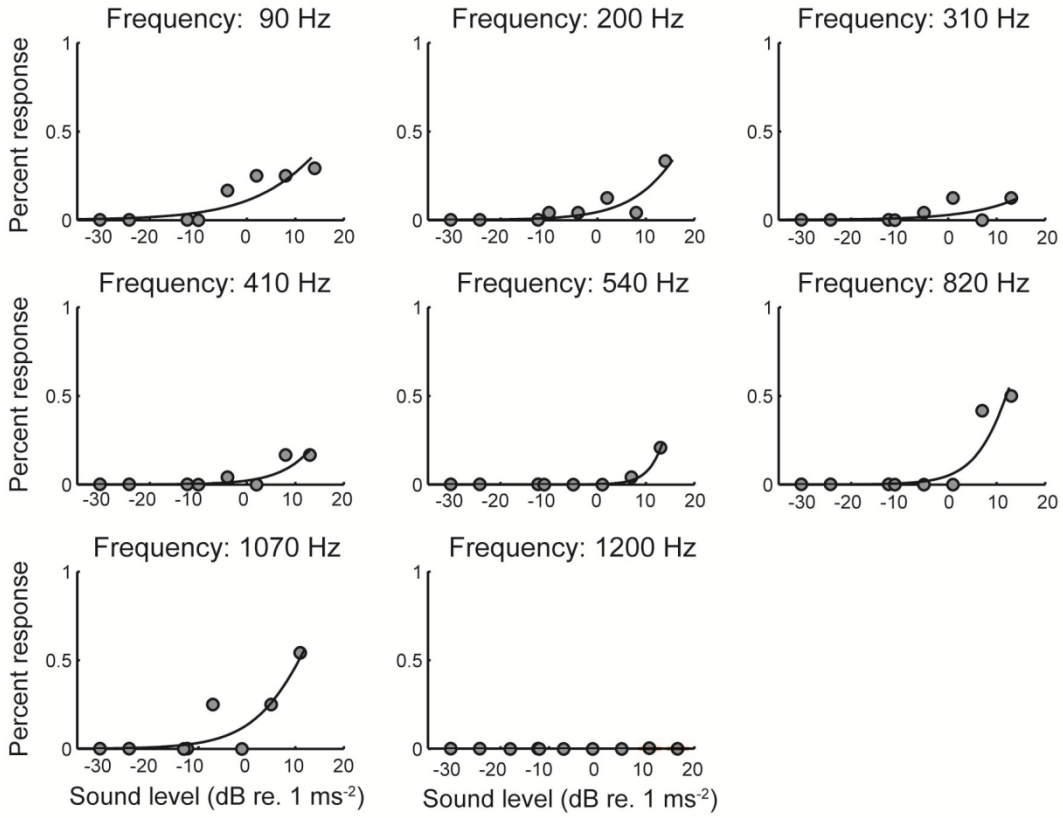

Figure 2.6**. Curve fitting to startle response data**. Representative examples of curve-fitting the acoustic startle response probability at a given frequency at the various stimulus levels tested for 5-6 dpf zebrafish. For each frequency, observed response percentages are shown in red. The Weibull curve is fitted using an MLE criterion and threshold is characterized as the sound level (dB re: 1g; x-axis) at which the model predicts a response probability of 5% (y-axis).

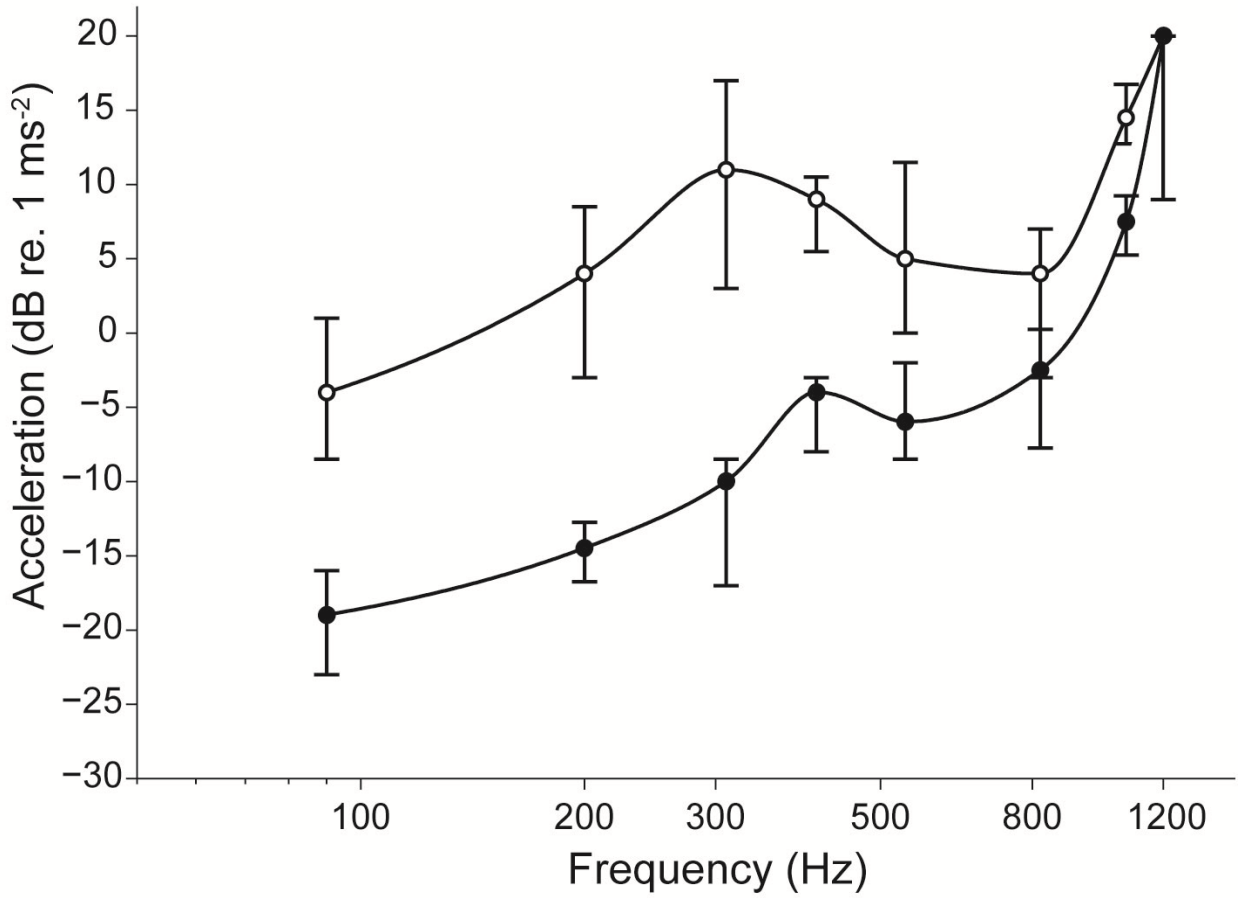

Figure 2.7. **Behavioral audiogram of the acoustic startle response of 5-6 dpf zebrafish** (n = 10 plates of 24 fish) to particle motion stimuli (open circles) and audiogram of hearing sensitivity using the PPI assay (n = 10 plates; closed circles) to sub-threshold particle motion stimuli. Thresholds were defined as a 5% probability of startle for startle response assay or 5% inhibition of startle from the paired catch trials in the PPI assay. Data presented as median $\pm$ 1 quartile. Lower numbers indicate higher sensitivities. Note that all prepulse thresholds are significantly lower than startle response thresholds

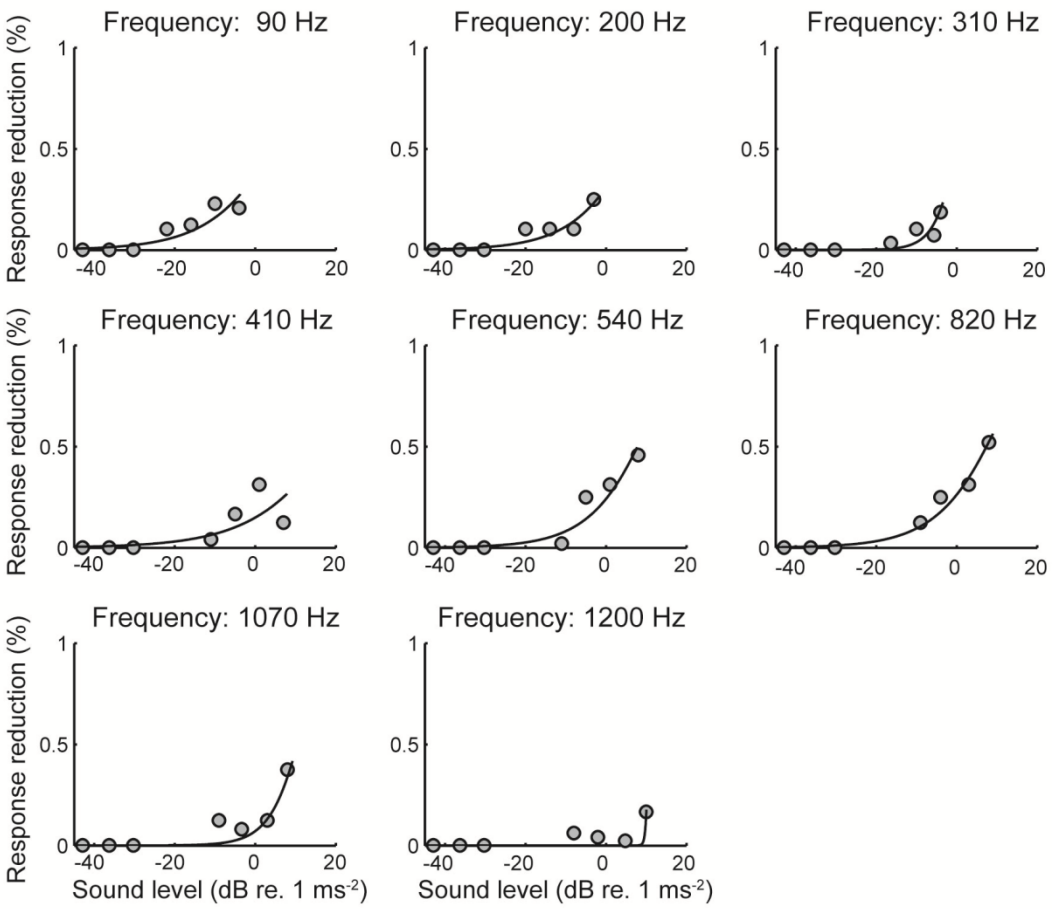

Figure 2.8. **Example of curve-fitting to prepulse response data**. Each point shown is the mean difference between response percentage at that frequency and sound level (dB re: 1g; x-axis) and paired no-prepulse catch trials preceding and following the prepulse stimulus. Similar to the startle response data, a Weibull curve is fitted using an MLE criterion and threshold is characterized as the sound level at which the model predicts a response reduction of 5% (y-axis).

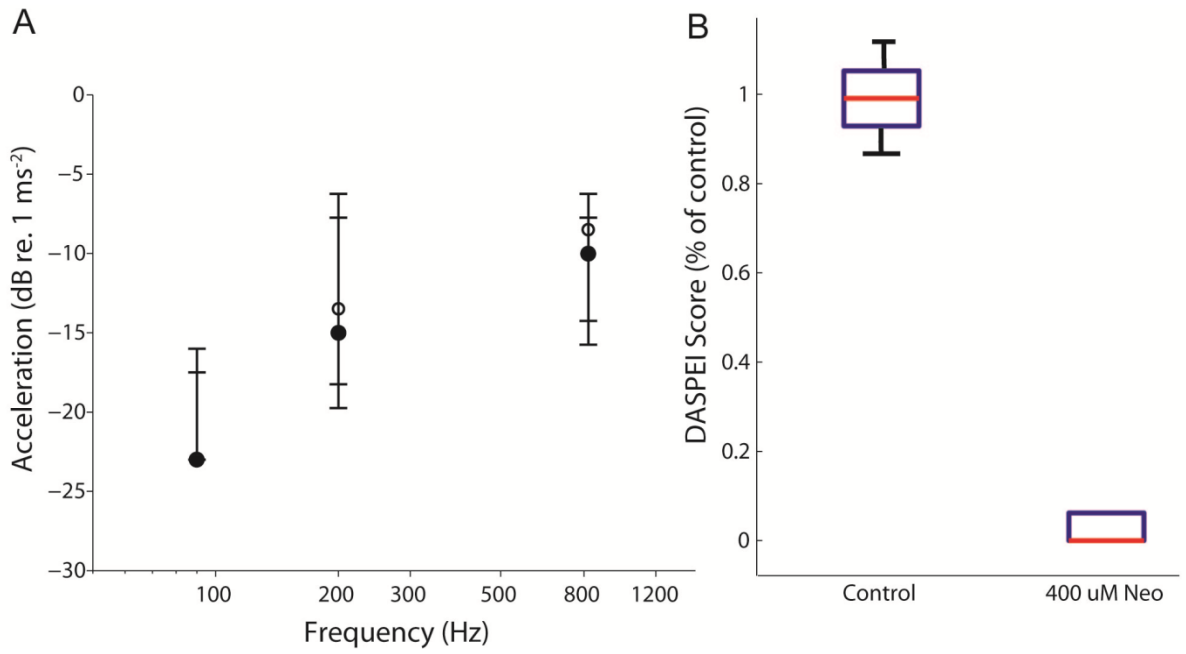

Figure 2.9. **Prepulse inhibition is not affected by lateral line ablation.** A. Prepulse inhibition thresholds of 5-6 dpf larval zebrafish (n = 10 plates) treated with 400 µM neomycin at 90, 210, and 820 Hz. There is no difference between neomycin treated (open circles) and control (filled circles) plates. All data are presented as median $\pm$ 1 quartile. Note that at 90 Hz, medians for treated and control data overlap and lower quartile bars may be slightly obscured. B. Box plots of DASPEI scores for control and neomycin treated fish, normalized to percentage relative to DASPEI scores for controls. 400 µM neomycin treatment had a high rate of superficial neuromast hair cell ablation. Note that the 95% CI bars are obscured for the 400 µM neomycin treatment.

Chapter 3. NOISE-INDUCED HYPERSENSITIZATION OF THE ACOUSTIC STARTLE RESPONSE IN LARVAL ZEBRAFISH

3.1 SUMMARY

Exposure to loud noise is known to lead to deficits in auditory sensitivity and perception. One of these changes in auditory perception is hyperacusis, a condition characterized by an abnormal oversensitivity to environmental sound. Here, we studied the effects of noise exposure on the auditory-evoked startle response in larval (5-7 dpf) zebrafish (*Danio rerio*). We observed a temporary 10-15 dB decrease in startle response threshold after 18 hours of flat-spectrum noise at 10 dB m/s^2. This noise-induced sensitization was not due to changes in absolute threshold and was specific to the auditory evoked escape responses. Noise exposure also resulted in a decrease in habituation to startle inducing stimuli. 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.

3.2 INTRODUCTION

Overexposure to loud noise can cause temporary or permanent noise-induced hearing loss (Wang et al. 2002, Park et al. 2013). Previous work has shown that overexposure to intense noise can cause serious damage to the peripheral auditory system, including hair cell damage and death (Liberman and Dodds 1987, Pickles et al 1987) and physical damage to the organ of Corti (Patterson and Hamernik 1997, Wolf et al 2009) leading to permanent hearing loss and auditory neuropathy. These detrimental effects of acoustic overexposure on the peripheral auditory system can often lead to changes in physiology and central auditory processing in the inferior colliculus (Szcepaniak and Moller 1996, Salvi et al. 1992, Wang et al. 2002) and auditory cortex (Syka et al. 1994, Seki and Eggermont 2002).

Acoustic overexposure can also lead to noise-induced perceptual abnormalities, such as temporary threshold shifts (TTS), where auditory sensitivity is reduced temporarily, but gradually recovers over time to pre-exposure levels. The severity of the TTS depends on the loudness and exposure time to noise (Miller et al 1963, Mills et al 1979). Long duration (8-48 hr) noise exposure is known to produce a TTS effect that rapidly increases toward an asymptote and then decays with an exponential function. The severity of TTS is asymptotic, reaching a maximum level after ~8-10 hours of constant exposure. Noise-induced TTS is known to occur in humans (Melnick 1991), rodents (Carder and Miller 1972, Chen et al 2013), marine mammals (Finneran 2012), and fishes (Smith 2012), which suggests that this effect is conserved across vertebrates. Although there is no hair cell death associated with TTS, primary afferent nerve terminals become swollen at the hair cell synapse, presumably as a result of glutamate

excitotoxicity (Spoendlin 1971, Robertson 1983). In rodents, application of AMPA receptor blockers such as NBQX coupled with noise exposure can prevent synaptic swelling (Hickox and Liberman 2014) and application of glutamate agonists in the absence of sound can induce synaptic swelling, mimicking the effect of noise overexposure. Previous work has shown that although TTS is temporary, this short-term condition can have long-term deleterious effects on spiral ganglion neurons leading to cochlear nerve degeneration and progressive hearing loss (Kujawa and Liberman 2006, 2009) and premature aging of the cochlear neurons (Fernandez et al 2015).

Another common perceptual disorder closely associated with acoustic overexposure is hyperacusis, a condition characterized by an exaggerated or inappropriate response to loud sounds (Baguley 2003). It is estimated that 2-15% of the general human population suffers from hyperacusis (Sammeth et al. 2000, Fabijanska et al. 1999). This condition is described most commonly as an intolerance toward loud, non-specific sounds and is co-morbid with tinnitus, as 90% of hyperacusic patients also show symptoms of tinnitus. In rodents, hyperacusis-like effects are commonly observed after overexposure to noise. Mice overexposed for 2 hrs to high noise levels (94-100 dB SPL) show marked increases in acoustic startle responses, prepulse inhibition, auditory CNS activation and behavioral hyperactivity (Hickox and Liberman, 2014).

Hyperacusis has been assessed behaviorally in animal models by measuring changes in the startle response after acoustic or pharmacological insult (Hickox and Liberman 2014, Turner and Parrish 2008). Similar to rodents, zebrafish (*Danio rerio*) possess an acoustic startle response that is easy to measure and quantify. In addition, the acoustic startle response pathway in

zebrafish is well-studied and perhaps 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 (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 response (Tabor et al. 2014). Furthermore, zebrafish at 5 days post-fertilization have a fully functioning auditory system with only ~80 hair cells in the saccule, the main organ of hearing (Inoue et al. 2013), providing a tractable auditory system that can be readily used to examine the effects of noise on the inner ear and the behavioral response pathway.

Here, we investigate the effects of long-term noise exposure on loudness perception in zebrafish. We hypothesize that overexposure to loud noise will affect loudness perception and induce a hyperacusis-like sensitization to startle-inducing stimuli. We describe a perceptual condition in larval zebrafish where startle sensitivity increases temporarily after noise exposure. We also explore physiological changes in response to noise and the role of AMPA in mediating this hypersensitive state.

3.3 METHODS

3.3.1 *Animal care*

Larval zebrafish (5-7 dpf) were used for all experiments. 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 in order 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.

3.3.2 *Behavioral testing*

Startle response and PPI experiments were performed as described in Chapter 2 (Figure 2.1). 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 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 (i.e., particle motion in the x or y axes), providing a reliable and repeatable stimulus signal. Acoustic stimuli were generated using MATLAB software and relayed to the shaker via a TDT System III. 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 $\pm$ 1°C) and lighting conditions for 15 mins before each test. A single test consisted of 24 fish in the central 6x4 wells, which were presented with randomized pure-tone stimuli of the frequencies listed above at sound levels ranging from 14 dB to -16 dB re. 1 m/s2 in 6 dB steps (startle) or -34 to -16 dB re. 1 m/s2 in 6 dB steps (prepulse). Because the dB 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/sec. Inter-trial 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 probability of response reached 0.05 (see Chapter 2).

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 one hour 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.

3.3.3 *Noise exposure*

Noise exposure was conducted in the same apparatus as described for the startle response experiments (Figure 2.1). 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" (1-10000 Hz) stimulus at 20 dB re. 1 m/s^2 for 18 hours. Due to instrumental constraints, the noise stimulus was a looped 1 second sound with a 20 ms cosine gate. Sound level was measured using the accelerometer and was calibrated for the 1 second period. During the sound exposure protocol, fish were monitored and fed every 4-6 hours and

embryo medium was added where necessary. Control fish were siblings from the same mating and were placed in the sound-isolation chamber, but were not exposed to noise.

In order to test for the time course of noise exposure onset, startle response thresholds were measured before and after noise exposure using a repeated measures design. Cohorts of 24 fish were tested at 1, 8, 12, and 24-36 hours of noise exposure. After noise exposure, all fish were removed from the shaker system and allowed to reacclimatize for ~15 minutes before further testing.

In order to test for recovery from noise exposure, fish were exposed to 18-24 hours of 20 dB (re. m/s^2) noise and allowed to rest in a quiet environment (average amplitude of -55 dB re. 1 m/s^2) for 1, 8, or 12 hours after noise exposure. After rest, fish were tested using the startle response assay.

3.3.4 *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 3 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 measured 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 hours of 20 dB (re. m/s^2) noise, fish were tested again using the same stimuli presented in the same randomized order as the pre-noise condition. The effect of noise was calculated as the difference in response percentage to EF pulses using a repeated measures design.

3.3.5 *Pharmacology*

The excitatory pathway of the startle response is mediated through glutamatergic pathways; therefore, all pharmacological treatments were conducted before behavioral experiments in order to control for this confound. 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. 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 hour 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 m/s^2) for the 18 hour 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.

3.3.6 *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 (see Chapter 2). Thresholds were interpolated from the curve at each frequency and were defined as the stimulus level at which the startle response could be elicited at a 0.05 probability. 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 non-parametric 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 particular frequencies were carried out using *post hoc* pairwise Wilcoxon rank sum tests. All 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.

3.4 RESULTS

3.4.1 *Behavioral thresholds to acoustic stimuli: control vs. noise-exposed*

Moderate exposure to flat-spectrum noise at 20 dB (re. 1 m/s^2) for 18 hours led to profound decreases in startle response threshold (Figure 3.1). Startle response thresholds decreased by 8-14 dB (re. 1 m/s^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 m/s^2) and noise-exposed fish (Mdn = -15 dB, IQR: - 9 to -21 dB re. m/s^2).

Noise induced sensitization also had a time-dependent effect. In fish tested before and after noise exposure, significant decreases in overall threshold were observed after 12 hours of 20 dB re. m/s^2 noise exposure ($X^2(1) = 8.35$, $p = 0.003$, n = 8 groups of 24 fish; Figure 3.2). There were no differences between noise exposures of 12 and 24-36 hours, 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 hour of noise ($X^2(1) = 1.21$, $p = 0.27$, n = 8 groups of 24 fish) or 8 hours of noise ($X^2(1) = 1.8$, $p = 0.18$, n = 8 groups of 24 fish). However, at these exposure times, thresholds were not significantly higher, even at 30 Hz (5.5 dB re. m/s^2), the frequency of highest startle sensitivity.

Recovery from noise exposure was also time-dependent (Figure 3.3). After 18-24 hours 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 one hour of recovery in an environment with mean sound levels < -60 dB (re. 1 m/s^2), startle thresholds were the same as startle thresholds measured immediately

after noise exposure (data not shown in figure). However, after 8 hours 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 hours) led to a significant increase in startle thresholds compared to controls ($X^2(1) = 7.53$, $p = 0.006$, $n = 7$). However, this increase was driven primarily by a difference in startle threshold between noise-exposed (Mdn = 12 dB, IQR: 14 to 8 dB re m/s^2) and control fish (Mdn = -12 dB, IQR: -14 to -7 dB re m/s^2) at 30 Hz ($U = 29$, $p = 0.001$, $n = 7$).

In order to test whether decreases in startle threshold after noise exposure were due to generalized increase in locomotor activity in these fish, noise-exposed (18 hours at 20 dB re. m/s^2) and control fish were tracked for 30 min within one hour of noise-exposure cessation and locomotor activity was recorded. No differences in locomotor activity were observed between noise-exposed (546 $\pm$ 38 seconds SEM) and control fish (647 $\pm$ 50 s SEM) ($t(58) = -2.16$, $p = 0.035$, $\alpha=0.025$, $n = 30$; Figure 3.4). However, the total distance moved was significantly reduced in noise-exposed fish (1030 $\pm$ 68 mm SEM) compared to the control group (1379 $\pm$ 66 mm SEM) ($t(58) = -3.7$, $p < 0.001$, $n = 30$). Together, these data show that noise-exposed fish were slightly less active than the control group in normal locomotor activity.

Our previous work showed that the PPI assay was a more sensitive measure than the startle response assay for measuring absolute auditory thresholds (see Chapter 2), which was the motivation to conduct PPI experiments in this study. Prepulse inhibition thresholds were not significantly different between noise-exposed and control fish ($X^2(1) = 0.08$, $p = 0.77$, $n = 8$ groups; Figure 3.5). However, post-noise prepulse inhibition thresholds were also not

significantly different from post-noise startle thresholds at both 30 Hz (Mdn prepulse: -25 dB, IQR: -30 to -21 dB re. m/s^2, Mdn startle: -19 dB, IQR: -20 to -17 dB re. m/s^2) and 90 Hz (Mdn prepulse: -24 dB, IQR: -27 to -18 dB re. m/s^2, Mdn startle: -13 dB, IQR: -18 to -7 dB re. m/s^2). Visual observations confirmed that prepulse stimuli would occasionally result in a startle response (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' trial in order to estimate the prepulse effect. Analysis of the responses of the 'no prepulse' trials throughout the experiment showed a decrease in response percentage from the initial trial (84 $\pm$ 5%, Mean $\pm$ SEM) to the last trial (53 $\pm$ 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; Figure 3.6). Furthermore, the initial stimulus presentations resulted in higher response percentage in noise-exposed fish (98 $\pm$ 1%) compared with controls (84 $\pm$ 5% ($U = 139.5$, $p < 0.001$).

Prepulse inhibition has been shown to vary with the inter-stimulus interval (ISI) between the prepulse and the startle-inducing stimulus (Burgess & Granato 2007, Bergeron et al. 2015). In order 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 m/s^2) and startle stimulus were kept constant, but the ISIs were varied between 10-290 ms. The PPI effect was measured 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; Figure

3.7). However, at 10 ms ISI, there were no differences in prepulse inhibition effect between noise exposed (Mdn: 0.26, IQR: 0.17 to .29, n=6 groups) and control fish (Mdn: 0.1, IQR: 0.17 to 0.08, n=6 groups) ($U = 49$, $p = 0.13$).

3.4.2 *Electrical stimulation*

Direct stimulation of the Mauthner cells using electrical pulses showed a positive linear relationship between increasing stimulus voltage and response probability in both pre-noise and post-noise conditions ($\beta = 0.54$, $p < 0.001$; Figure 3.8). 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).

3.4.3 *Pharmacology*

Studies in mice have shown that startle hyper-excitability is mediated by AMPA receptors (Hickox et al. 2014). Similarly, NMDA receptors have been implicated in changes in startle excitability (Burgess and Granato 2007, Best et al. 2008, Bergeron et al. 2013) and habituation (Roberts et al. 2011) in zebrafish. 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; Figure 3.9). However, 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 hypersensitization of the startle response. Similarly, we 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), and were not significantly different from startle thresholds in control (vehicle, quiet) fish ($X^2(1) = 0.27$, $p = 0.61$, n = 6 groups of 24 fish; Figure 3.10).

3.5 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 induce sensitization to startle-inducing stimuli. We demonstrate that exposure to 18 hours of flat spectrum loud noise induces a temporary condition in larval zebrafish in which acoustic startle sensitivity increases by 10-15 dB. Our experiments also demonstrate that the changes leading to this hypersensitization are not due to overall changes in locomotor behavior (i.e. increased overall activity levels) or excitability of Mauthner cells. Interestingly, auditory sensitivity measured by the PPI thresholds was not significantly changed after noise exposure, which suggests that overall hearing sensitivity of 5-7 dpf zebrafish was not affected by this noise exposure paradigm.

Larval zebrafish show increased responsiveness to startle-inducing stimuli after noise exposure similar to rodent studies (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, our data are consistent with these previous findings that the startle response levels are higher near the startle threshold. A previous study in adult three-spined stickleback fish (*Gasterosteus aculeatus*) 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 exaggeration may be common motif found in both fish and mammals.

Startle thresholds decreased significantly after 12 hours of 20 dB (re. m/s^2) noise and returned to pre-noise levels within 8 hours of recovery, indicating a temporary effect of noise exposure. Studies in human subjects (Melnick 1991) and chinchillas (Carder and Miller 1972) have shown that temporary threshold shifts are exposure-dependent and rapidly increase toward an asymptotic threshold shift which is reached within 8-10 hours of exposure. Similarly, recovery from asymptotic threshold shifts of ~10 dB can require at least 48 hours of recovery to return to pre-noise levels in human subjects (Melnick 1976). Our data indicate 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 (Figures 3.2 and 3.3). Interestingly, the recovery data shows a subsequent increase in startle threshold after 12 hours at 30 Hz, the lowest frequency tested. This suggests a potential desensitization to startle-inducing stimuli at low frequencies after the 12 hour recovery period.

In order to examine whether the observed changes in startle sensitivity after noise exposure were specific to the acoustic startle response pathway, we measured startle responses to electrical 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 excitability. 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 minute period and no significant difference in the time spent moving compared to control fish (Figure 3.4). These data are consistent with previous observations in 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 are not likely due to generalized hyperactivity.

Locomotor activity and stimulus responsiveness are both aspects of arousal (Pfaff et al. 2008), and a decrease in overall locomotion could result in a greater probability of startle response to auditory stimuli. However, Burgess and Granato (2007) demonstrated in that startle responses are more likely to occur in actively moving 5 dpf zebrafish compared to a stationary fish. This would result in a decreased probability of response in noise-exposed animals compared to control animals and therefore, it is unlikely that a decrease locomotor activity would lead to a concomitant increase in startle sensitivity.

In contrast to the observed increase in startle sensitivity, auditory thresholds measured using a PPI assay showed no differences between noise-exposed and control fish, suggesting that overall hearing sensitivity is not significantly different in noise-exposed animals compared with non-exposed animals. These data are in contrast with 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), which show 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, Smith et al. 2006). These apparent discrepancies could be due to a few reasons. First, adult zebrafish, goldfish, and minnows can encode the pressure component of sound via hearing specializations, which may

result saccular damage following noise exposure. 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 on the effects of noise exposure and ontogeny 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 inter-pulse intervals (Fig. 3.7), suggesting a decrease in the prepulse inhibition effect above threshold. These data are in contrast with rodent studies, which 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. 2010, 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 a set of glycinergic interneurons in the hindbrain including the PHP cell, which 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. 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.

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 PnC (Krase et al. 1993, Miserendino and Davis 1993) and is involved in noise-induced cochlear synaptopathy (Liberman et al. 2011) and subsequent increase in startle responses (Hickox and Liberman 2013). Studies in adult goldfish show that AMPA receptor blockade leads to a reduction in the M-cell EPSP at club ending mixed synapse, indicating that AMPA receptors play a major role in signal transduction at the M-cell lateral dendrite (Mirjany and Faber 2011). This would suggest that hypersensitization could also be mediated through changes in synaptic strength at the lateral dendrite. It should be noted that DNQX can also bind to the kainate receptor with similar affinity (Honore et al. 1988) and therefore some of these effects could also potentially be kainate-mediated, as previously shown in guinea pigs and rats (Pujol et al. 1985). Why would AMPA blockade and not NMDA blockade reduce startle-induced hypersensitization? One explanation could be due to silent synapses at the saccule and lateral dendrite (Korn and Faber 2005). During noise exposure LTP, NMDA activity upregulates AMPA receptor number at the synapse, which increases activation (Malinow and Malenka, 2002). Application of DNQX may have directly interfered with this activation compared with indirect activation through NMDA receptor blockade by APV.

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 that noise exposure results in a decrease in habituation to repeated stimulus presentations (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 1981), 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 and is mediated by NMDA receptors (Roberts et al. 2011, Wolman et al. 2011) and through glycinergic feedforward inhibition through interneurons (Marsden and Granato 2015). 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).

Non-auditory effects, such as stress and fear-potentiation, have been suggested as a mediator for changes in the startle response of mammals (Davis et al 2006) and in zebrafish (Griffiths et al 2012). Noise exposure has been shown to transiently increase plasma cortisol levels in adult goldfish within 10 minutes of exposure (Smith et al. 2004). Therefore, the observed changes in startle response may also be modulated by central processes. Future research in modulating stress-related and auditory-related effects 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 could provide a new and tractable model to investigate novel treatments for noise-induced perceptual changes, the mechanisms of which may be conserved across vertebrate taxa.

3.6 FIGURES

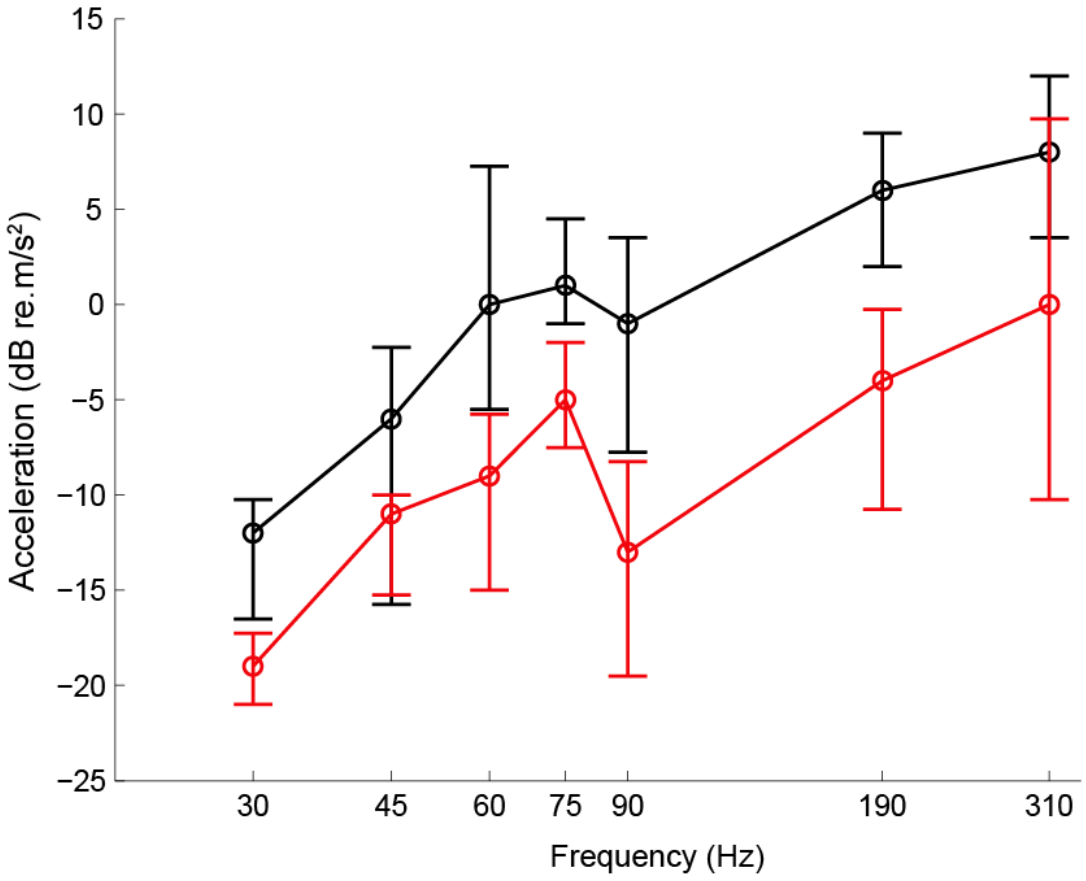

Figure 3.1. **Noise exposure results in decreased startle thresholds**. Startle response thresholds to particle motion stimuli in 18 hour white noise-exposed (red) and control (black) conditions (n = 10 groups of 24 fish). Thresholds were defined as a 5% probability of startle. Data presented as (median $\pm$ 1 quartile) and numbers indicate higher sensitivities.

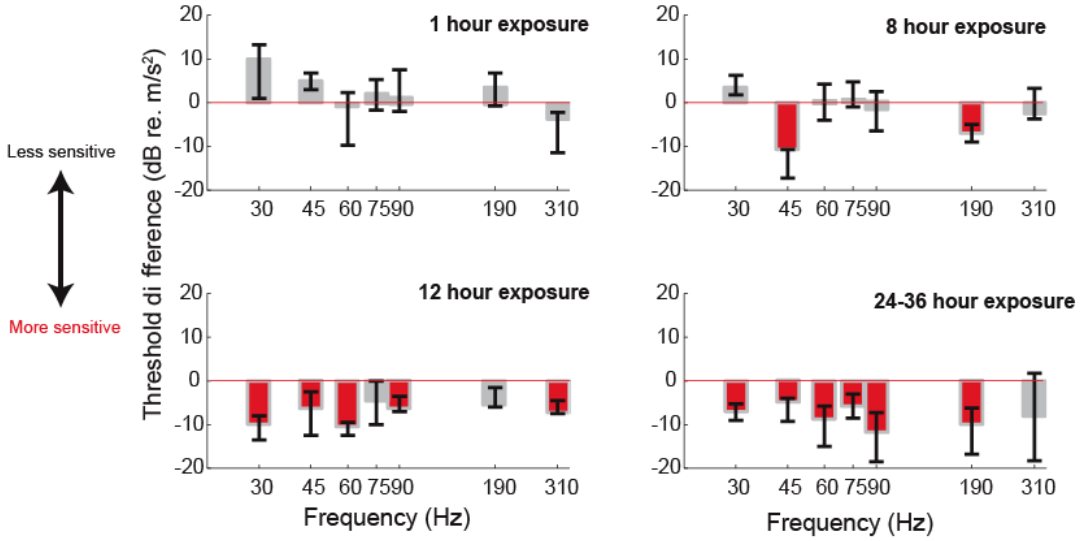

Figure 3.2. **Noise-induced sensitization is time-dependent.** Differences between pre-noise exposure and post-noise exposure startle thresholds at 1 hour noise exposure (top left, n = 8 groups of 24 fish), 8 hour noise exposure (top right, n = 8 groups of 24 fish), 12 hour noise exposure (bottom left, n = 8 groups of 24 fish), and 24-36 noise exposure (bottom right, n = 8 groups of 24 fish) tested at 7 frequencies. Bars in red denote statistical significance. Note that no change is equal to a threshold difference of 0.

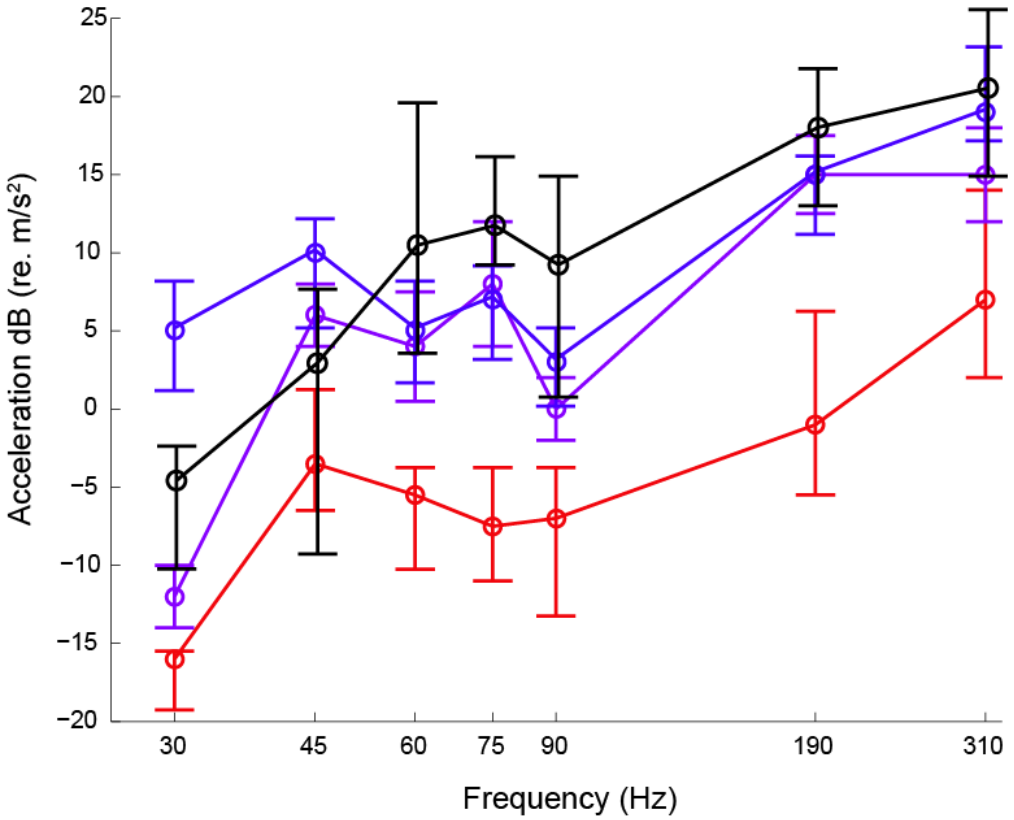

Figure 3.3. **Recovery from noise-exposure is time-dependent**. Startle thresholds to pure-tone stimuli after noise exposure immediately after 18 hour noise exposure (red, n = 10), 4 hour recovery (purple, n = 7 groups of 24 fish), and 8 hour recovery (blue, n = 7 groups of 24 fish). Control (no noise exposure) startle thresholds are plotted in black (n = 8 groups of 24 fish). Thresholds are reported as medians $\pm$ 1 quartile.

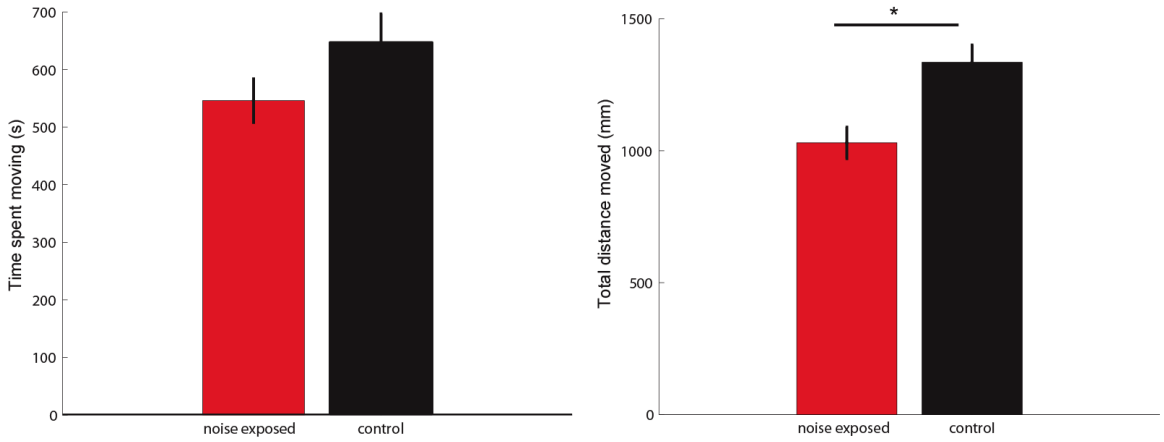

Figure 3.4. **Overall locomotor activity is not higher in noise-exposed fish**. Movement in noise-exposed (red, n = 30) and control (black, n = 30) fish. Animals were motion-tracked for 30 minutes in the absence of auditory stimuli and total time spent moving (top) and total distance moved (bottom) were measured. Total distance moved was significantly lower in noise-exposed fish compared to controls ($p < 0.001$), whereas total time spent moving was not significant overall.

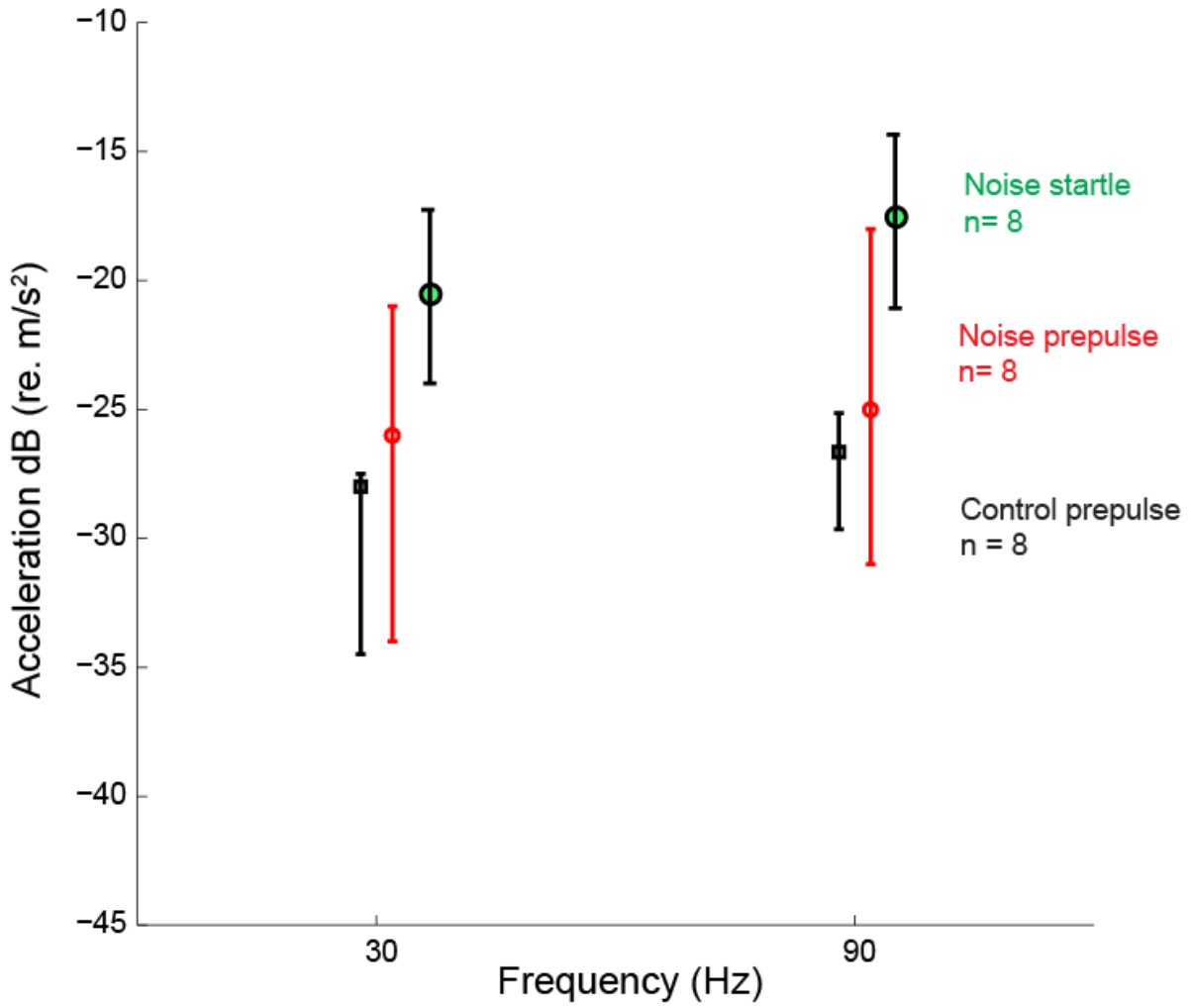

Figure 3.5. **Prepulse inhibition thresholds are not changed after noise exposure**. Auditory thresholds measured using a PPI assay showed no differences between control (black squares, n = 8 groups of 24 fish) and noise-exposed (red circles, n = 8) fish. PPI thresholds in noise-exposed fish were also not significantly different from startle thresholds in noise-exposed fish (green circles, n = 8).

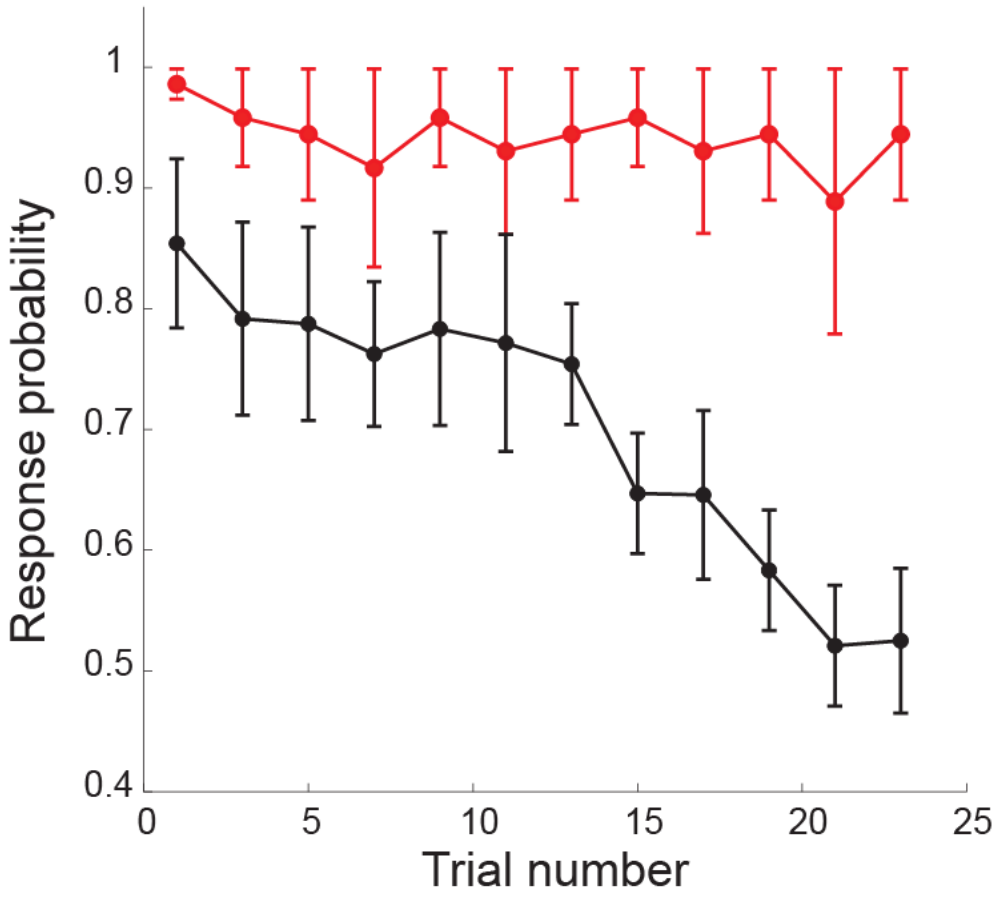

Figure 3.6. **Habituation to startle-inducing stimuli is reduced after noise exposure**. Response probability to the startle-inducing "catch" stimuli used in the PPI experiments are plotted by trial number in noise exposed (red, n= 8 groups of 24 fish) and controls (black, n = 8). Noise-exposed fish have significantly lower response probability at the first stimulus presentation and have a steeper decline than noise-exposed fish.

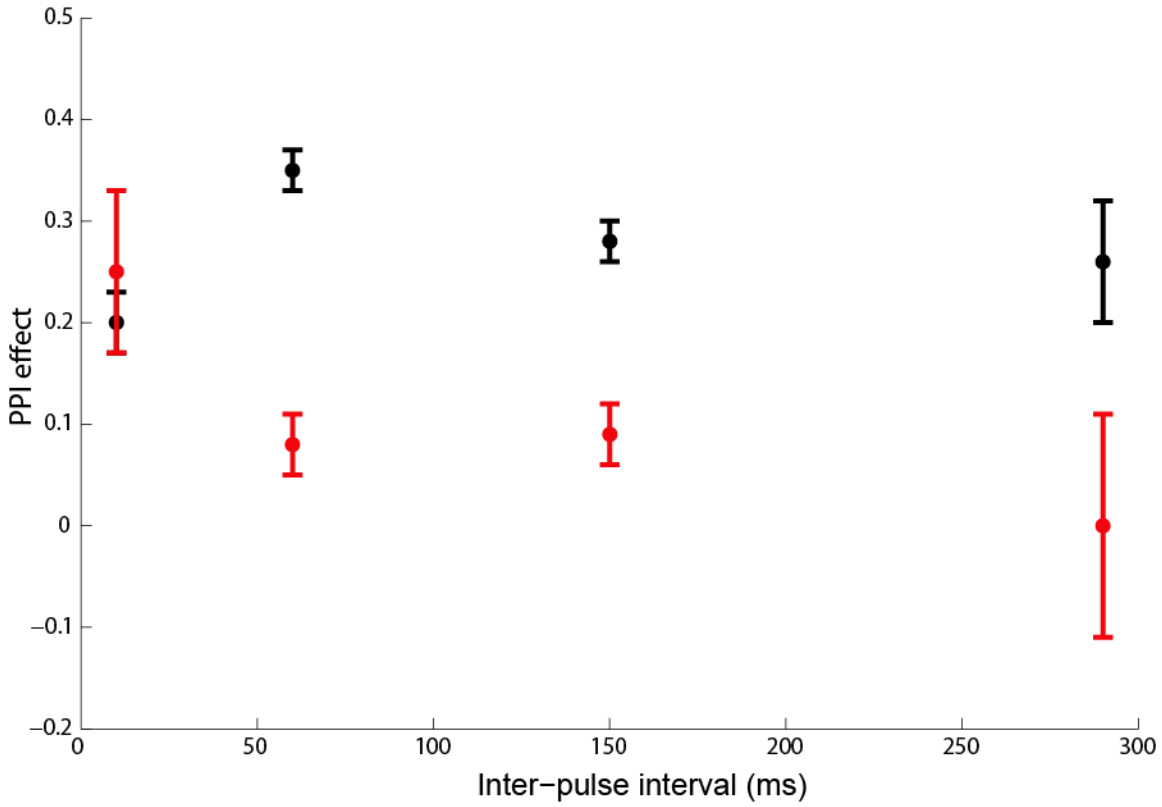

Figure 3.7**. Prepulse inhibition effect is reduced at longer inter-stimulus intervals**. Prepulse inhibition effect, measured as the total decrease in response probability to a 90 Hz, -16 dB re. 1 m/s^2 prepulse, plotted against increasing inter-stimulus intervals for noise-exposed (red, n = 6 groups of 24 fish) and control (black, n = 6) conditions. Noise-exposed fish had significantly lower prepulse inhibition at all inter-stimulus intervals except 10 ms. Inter-stimulus intervals were defined as the time between the end of the prepulse stimulus and the beginning of the startle-inducing "catch" stimulus.

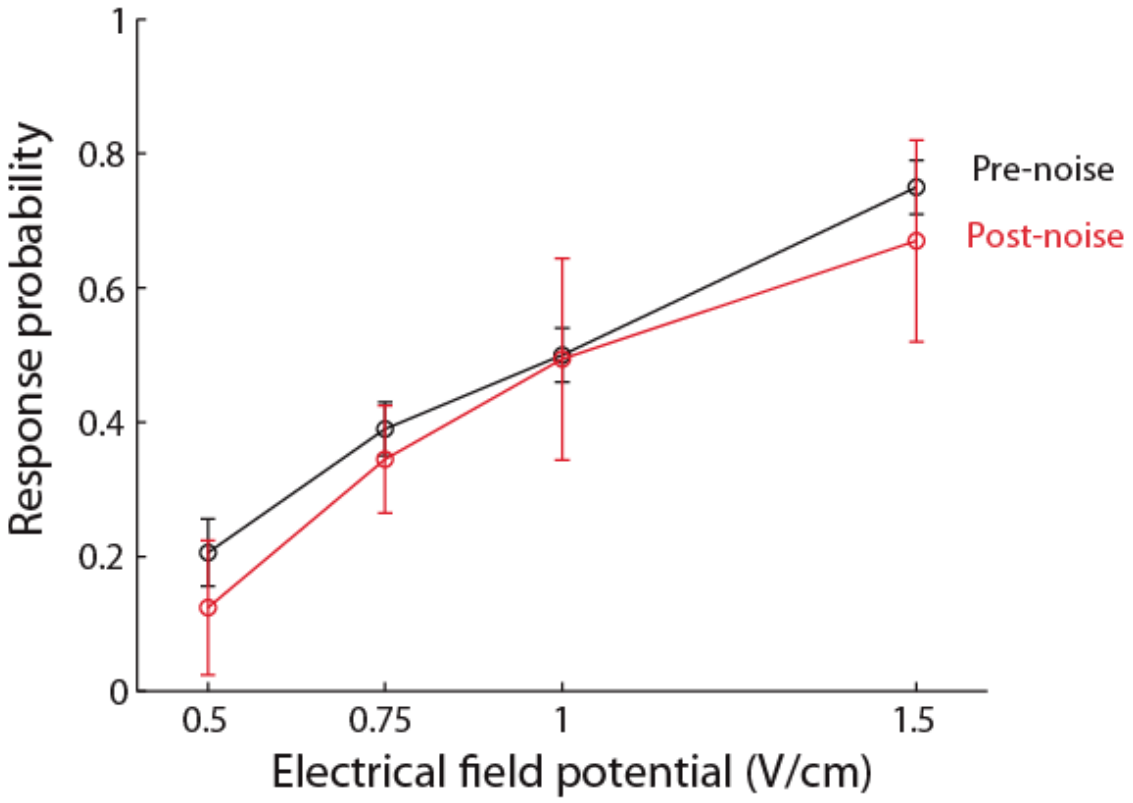

Figure 3.8. **Mauthner cell response probability is not affected by noise exposure**. Response probabilities to direct electrical field pulses in pre-noise (n = 8 groups of 3 fish) and post-noise (n = 8 groups of 3 fish) conditions. Responses were defined as M-cell mediated startle responses in animals aligned within 30° of the anode-cathode axis. There were no significant differences in responsivity between pre-noise and post-noise conditions.

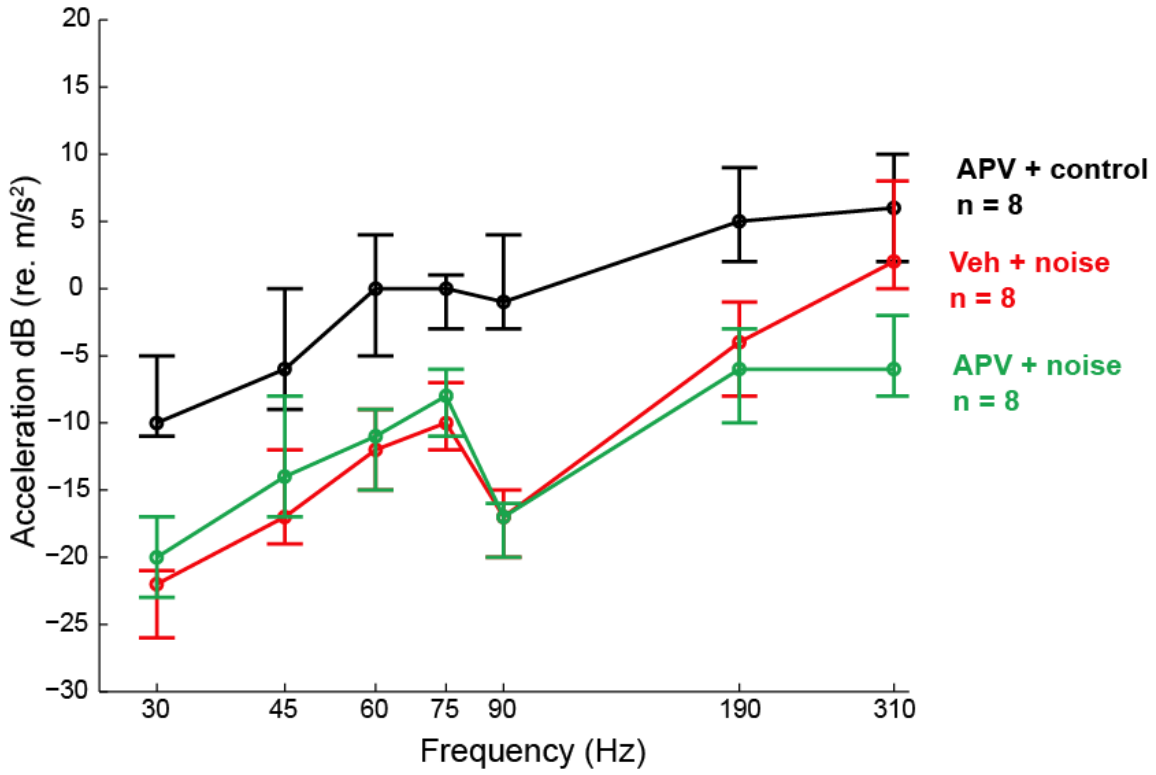

Figure 3.9. **NMDA receptor blockade does not disrupt noise-induced startle sensitization**. Startle response thresholds in groups of animals treated with a bath-applied NMDA receptor antagonist, APV. Animals treated with 25 uM APV during noise exposure (green) had significant decreases in startle threshold compared with animals treated with 25 uM APV kept in quiet (black). Groups treated with 25 uM APV did not have significantly different thresholds from animals treated with the vehicle, DMSO, and exposed to noise (red).

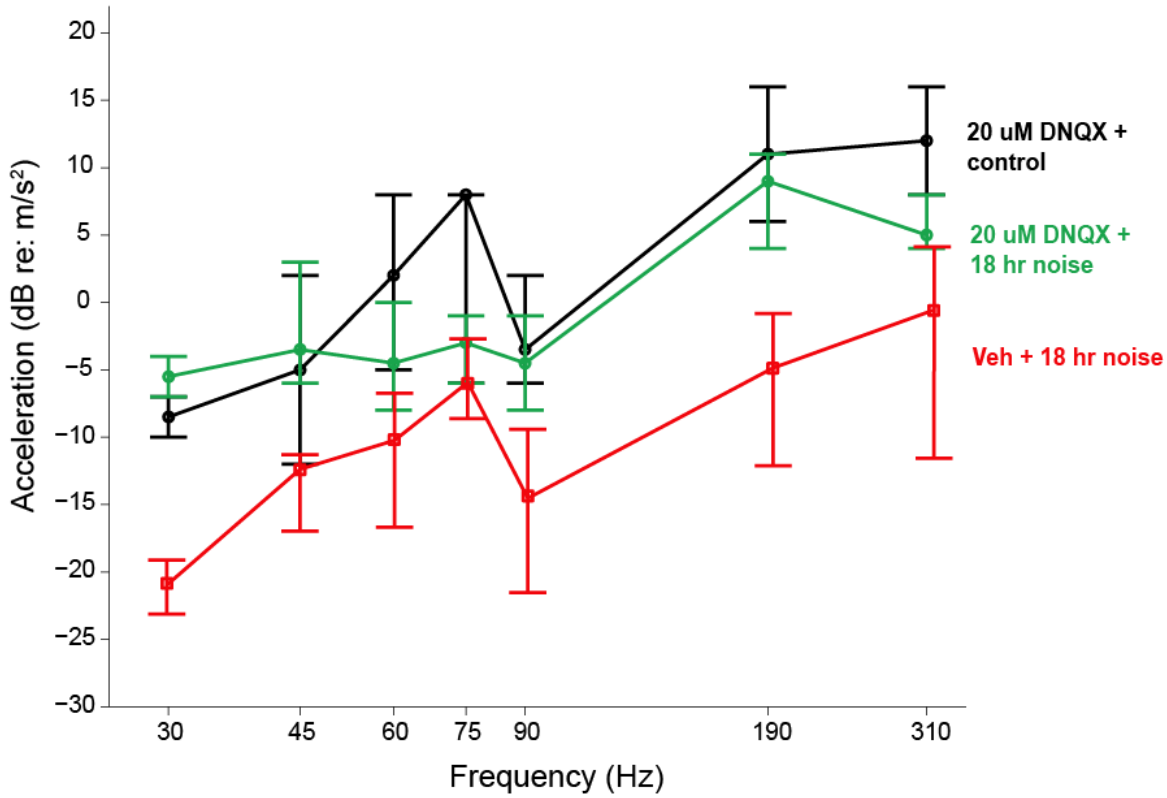

Figure 3.10. **AMPA receptor blockade results in disruption of noise-induced startle sensitization**. Startle response thresholds in groups of animals treated with a bath-applied AMPA receptor antagonist, DNQX. Animals treated with 20 uM DNQX during noise exposure (green) were not significantly different compared with animals treated with 20 uM DNQX and kept in quiet (black). However, animals treated with the vehicle, DMSO (red), had significant decreases in startle threshold compared to both DNQX groups.

Chapter 4. STARTLE RESPONSE VARIATION AND SUSCEPTIBILITY TO NOISE-INDUCED HYPERSENSITIVITY IN MULTIPLE ZEBRAFISH LINES

4.1 SUMMARY

Genetic and environmental effects on auditory-evoked behaviors have been demonstrated across vertebrates. In the zebrafish (*Danio rerio*), a powerful genetic model for auditory research, mutations that confer profound hearing and vestibular defects have been well-studied. Less is known about variability and the influence of genetic background on auditory-evoked behaviors and auditory perception in larval zebrafish. In this study, we measured startle response thresholds in three wild-type zebrafish lines: AB, Tubingen (TU), and WIK. In these experiments, AB fish had significantly lower startle thresholds than TU and WIK. However, the AB/WIK hybrid line had startle and prepulse inhibition thresholds that were more similar to AB than to WIK, suggesting that the AB-like phenotype may be dominant. Furthermore, AB/WIK fish showed less noise-induced hypersensitization compared to AB alone. Finally, studies within genetically similar AB fish showed that noise-induced hypersensitization differed across individuals. These inter-strain and individual differences in auditory startle behavior could lead to better understanding of how gene-environment interactions can shape sensorimotor behavior.

4.2 INTRODUCTION

There is a large degree of variation in auditory sensitivity and perception in mammals, including humans. The most extreme cases of variation arise from mutations that result in congenital hearing loss (Gorlin et al. 1995, Nicolson 2005, Dror and Avraham 2009). However, healthy individuals can also show a high variability in susceptibility to noise-induced hearing loss (NIHL; Carlsson et al. 2005, Konings et al. 2009) and in age-related hearing loss (Tremblay et al. 2003, Liu and Yan 2007). Several studies in mice and rats have also shown strain-specific differences in auditory-evoked behavioral responses and in ABR thresholds in healthy animals (Borg 1982, Zhou et al. 2006, Jones et al. 2006, Glowa and Hansen 1993). Therefore, understanding how genes and environment interact to influence hearing is crucial within and across individuals in the study of auditory plasticity.

Genetic studies of hearing loss in humans have found over 46 genes associated with some form of hearing loss as well as 100 loci of some effect (reviewed in Hilgert et al. 2009). These genes are heterogeneous in their expression and effect, and a fundamental question in hearing is to identify the mechanisms by which these genes effect hearing. Mouse models have shown over 200 genes associated with hearing loss, with 44 genes in common between mouse and human (Friedman et al. 2007). Many of the genes of strong effect, such as the Pou4f3$^{-/-}$ and Cdh23$^{-/-}$, also result in severe behavioral deficits in not only auditory, but vestibular function.

A major goal in hearing research is to determine candidate genes that influence susceptibility to NIHL. Although animal studies have suggested that some subjects are more resilient to noise-induced hearing loss than others, the mechanisms underlying this resiliency is not well-

understood. In a study on the guinea pig (*Cavia porcellus*), loud noise exposure led to a large variance in damage to stereocilia and subsequent effects on the compound action potential of the cochlear nerve and microphonic potentials (Cody and Robertson, 1983). Similarly, strain-specific differences in noise-exposure susceptibility are well characterized in the mouse. Some lines, such as the C57BL/6 mouse, are highly susceptible to noise-induced hearing loss (Li et al. 1992, Erway et al. 1996, Davis et al. 1999), whereas other lines, such as 129/SvEv, are highly resilient against noise-related auditory injury (Yoshida et al. 2000). More recently, a genome-wide association study in mice discovered that a mutation in the Nox3 gene lead to a greater increase in ABR threshold and a decrease in the ABR wave-1 amplitude compared to wild-type siblings, suggesting a genetic difference in the effect of noise on spiral ganglion neurons (Lavinsky et al. 2015).

The acoustic startle response (ASR) is one of the most common behavioral methods to investigate genetic influences on hearing. The ASR is a reflex response to a loud perceived acoustic stimulus and has a well-described mechanism in both mammals (Davis 1984) and non-mammalian vertebrates (Cioni et al. 1989, Korn and Faber 2005). Studies in rats and mice have shown large inter-strain variability in ASR (Willott et al. 2003, Glowa and Hansen 1993) and in the prepulse inhibition of the ASR (Paylor and Crawley 1997). This variability across lines has led to genetic mapping studies to determine candidate loci in the studies of hereditary, age-related, and noise-induced hearing loss.

The zebrafish (*Danio rerio)* is a powerful model species to study the genetics of hearing and auditory-related behaviors. There are more than 10 known genetic mutations that affect inner ear

and lateral line development and can lead to total hearing and vestibular loss (Nicolson 2005). The mechanism of ASR is well-studied in zebrafish, and mutations that lead to loss of function in the ASR have also been described (Granato et al. 1996, Burgess et al. 2009). Less is known about genetic loci associated with mild or moderate loss of function in zebrafish and susceptibility to noise-induced changes in ASR. There are at least 10 wild-type zebrafish lines used in auditory research, and little is known about auditory sensitivity and ASR in these wild-type lines. Thus, it is important to understand how genetic background can influence hearing in these fish.

Here, we investigated the effects of genetic background and noise exposure on startle sensitivity in larval zebrafish. To test the hypothesis that genetic background influences auditory-evoked startle responses in these fish, we used a behavioral acoustic startle assay to determine startle threshold in three inbred wild-type zebrafish lines (AB, Tubingen (TU), WIK) and one hybrid wild-type line (AB/WIK) at 5-7 dpf. We also investigated the inter-individual variability in startle threshold and the effects of initial threshold on susceptibility to noise-induced startle hypersensitization within the inbred AB wild-type line. We report large differences in startle threshold between wild-type zebrafish lines and significant differences in susceptibility to noise-induced startle hypersensitization. Together, these data suggest a strong effect of genetic background and gene-environment interactions in shaping auditory behaviors.

4.3 MATERIALS AND METHODS

4.3.1 *Animal care*

Zebrafish larvae (*Danio rerio*) were obtained from an adult zebrafish colony housed at the University of Washington. For these experiments, four wild-type lines were used: AB, TU, and WIK, with one AB/WIK hybrid line. AB/WIK wild-type fish were a kind gift from Dr. John Clark. It should be noted that the AB/WIK fish used were not F0 hybrids between AB and WIK but rather from a stable hybrid line.

Mating and egg collection were performed according to Westerfield (2000). Embryos were raised in petri dishes (densities $\leq$ 50 larvae/dish) housed in incubators at 28.5 ˚C with a 12:12 hour light-dark cycle. All fish were transferred between containers and to the experimental apparatus using wide-bore pipettes in order to minimize damage of the lateral line neuromasts. Fish were tested between 5-7 dpf in all lines, and were fed rotifers on the day before testing. Larvae were allowed to acclimate to the experimental lighting and temperature (28 $\pm$ 1 °C) conditions for 30 minutes before the experiments were conducted. Animal rearing and experimental procedures were approved by the University of Washington Animal Care and Use Committee.

4.3.2 *Startle response experiments*

Behavioral measurement of startle sensitivity has been described previously in this dissertation (Chapters 2 and 3). Briefly, groups of 24 wild-type fish were placed in the experimental apparatus consisting of a 96-well plate attached to an acrylic platform and mounted on top of a Bruel-Kjaer Type 4810 shaker (Figure 2.1). The apparatus was placed on a vibration isolation

table in a sound-attenuation chamber in order to reduce background noise. Measured sound levels in the chamber did not exceed -40 dB re. 1 m/s^2. The shaker delivered particle motion stimuli in the vertical axis and a PCB model 355B04 accelerometer was attached to the acrylic platform in order to measure the stimulus amplitude. Fish were placed individually in the central 24 wells of the 96 well plate and each well was filled with ~400 µL of embryo medium.

Stimuli consisted of pure tones of 30, 45, 60, 74, 90, 190, and 310 Hz each presented at 14 dB to -16 dB re. 1 m/s^2 in 6 dB steps using a method of constant stimulus experimental design. Stimuli were presented in random order with a 70 $\pm$ 10 second inter-stimulus interval in order to minimize habituation (Zeddies and Fay 2005). These frequencies were empirically determined to minimize resonance of the system and off-axis particle motion components (Chapter 1). Stimuli were presented with a 24 ms cosine-squared gated ramp time in order to further reduce resonance frequencies. All stimuli were generated using MATLAB 2009B (Mathworks Inc, Natick, MA) and relayed to the shaker using a TDT System III processor.

Auditory-evoked startle responses were observed using a Photron FastCam 1024PCI high speed camera at a frame rate of 1000 frames/sec. Kinematic analysis and categorization of the behavioral response to startle-inducing stimuli were done using the same methods described in Chapter 2. Positive responses were defined as a startle response that was initiated less than 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°. If these two criteria were met, responses were coded as a positive startle. Responses were coded as a binary variable (1 for response and 0 for no response). For each frequency, the proportion of fish that exhibited a positive response at

each amplitude tested 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 probability of response reached 0.05. Because we do not observe ASRs in the absence of a loud stimulus, this threshold allowed for a good estimate of the minimum sound level that led to a robust, repeatable response.

For all comparison experiments, the wild-type AB line was always tested along with one or more other lines. This allowed for comparison to existing datasets obtained in Chapters 2 and 3 and served as a *de facto* control. If ASR thresholds for the tested AB fish were found to be outside the 95% confidence interval from previous datasets, both AB and the paired wild-type startle threshold data was not used in the analysis.

4.3.3 *Prepulse inhibition experiments*

In order to test for differences in absolute auditory threshold in AB/WIK fish compared to the inbred AB line, we used the PPI assay described in Chapter 2. Using the same apparatus described in Section 3.3.2, a known startle-inducing stimulus (820 Hz at 20 dB re. 1 m/s^2) was delivered a cohort of 24 fish. A PPI trial consisted of a 100 ms randomized prepulse stimulus with a 24 ms ramp time followed by the startle-inducing stimulus. Groups of 24 fish were tested using prepulse stimulus frequencies of 30, 60, 90, 190, and 310 Hz at four sound levels. These sound levels were empirically determined to be below the startle threshold at each of the frequencies in order to minimize the startle responses to the prepulse alone. Each PPI-startle stimulus presentation (trial) was paired with a no-prepulse trial to measure the effect of the prepulse. The PPI effect was calculated as the difference in response between a no-prepulse and prepulse condition. For all prepulse experiments, plates of fish were presented with no more than

20 total stimuli. After 20 stimuli, these fish were replaced with naïve fish from the same cohort. Therefore, each replicate used 48 fish to estimate PPI thresholds.

4.3.4 *Individual startle response experiments*

In order to test whether the differences in startle thresholds were biased by changes in threshold of a relatively small number of individuals, we used a startle response protocol where a group of 24 fish was tested using only 90 Hz (8 to -10 dB re. 1 m/s^2), but each stimulus was repeated 15 times. This frequency was used because of the high sensitivity of control fish to 90 Hz stimuli and because we found the largest effect of noise-exposure on startle hypersensitivity to this frequency in Chapter 3. Fish were tested before and after 18 hour noise exposure in order to minimize variability between fish. Non-responding fish in either the pre-noise or post-noise condition were excluded from analysis. This allowed for a better estimate of individual startle thresholds to 90 Hz stimuli in control and noise-exposed fish.

4.3.5 *Noise exposure*

The noise exposure protocol to determine susceptibility has been described previously in Chapter 3. Noise exposure was conducted in the same apparatus used for startle response testing. Cohorts of 24-48 fish were presented with a flat spectrum "white noise" (1-10000 Hz) stimulus at 10 dB re. 1 m/s^2. Due to instrumental constraints, the noise stimulus was a looped 1 second sound with a 20 ms cosine gate. Sound level was measured using the accelerometer and was calibrated for the 1 second period. During the sound exposure protocol, fish were monitored and fed every 4-6 hours and embryo medium was added where necessary. Control fish were siblings from the same clutch and were placed in the sound-isolation chamber, but were not exposed to noise. After

noise, fish were removed from the experimental apparatus and allowed to rest for 15 minutes before testing. All fish were tested for ASR responsivity within one hour of noise exposure.

4.3.6 *Data analysis*

Startle response data was fit to a cumulative Weibull distribution function using a maximum likelihood method with two degrees of freedom. Thresholds were calculated by interpolating from the best-fit function and were set at a response level of 5%. If there was no best-fit function (ie. No responses to any of the sound levels tested), threshold was coded at 20 dB re. 1 m/s^2. This allowed for a valid comparison between groups of fish tested. If a group of fish did not respond to any of the stimuli used in this experiment, it was removed from analysis.

For the individual startle response experiments, a similar Weibull fitting method was used to determine threshold. In addition, a classifier was developed to determine threshold for hypersensitization across individuals. Threshold calculations calculated for pre-noise and post-noise conditions were analyzed for reliability using a receiver operating curve (ROC) curve analysis. Area under the curve (AUC) was calculated as a measure of discriminability between pre-noise and post-noise conditions using a trapezoidal approximation method (Cortes and Mohri 2003). This statistic is equivalent to a Mann-Whitney U statistic or Wilcoxon Rank-Sum statistic and can provide an estimate for test validity as well as provide information about the optimum threshold for classifying a startle response threshold as ‘control-like’ or ‘noise-exposed’.

Differences in startle response thresholds between wild-type strains were analyzed using non-parametric statistical tests. Because the threshold value is an extreme (ie. The descriptive statistic is not about the mean, but rather at one tail, and therefore heteroscedastic), all comparisons

between wild-type strains were conducted using Friedman tests. Tests for differences at particular frequencies were carried out using *post hoc* pairwise Wilcoxon rank sum tests, adjusted using Bonferroni comparisons when appropriate. For individual fish, a Wilcoxon rank sum test was used to test the effect of noise exposure.

All statistical analyses were conducted using MATLAB 2009B software and all figures were created in MATLAB and Adobe Illustrator.

4.4 RESULTS

4.4.1 *Differences in startle response threshold between wild-type lines*

All wild-type lines responded to startle-inducing stimuli at at least one frequency. There were no significant differences in response latency ($F(2,27) = 1.08$, $p = 0.35$) or maximum bend angle ($F(2,27) = 1.88$, $p = 0.17$) of the ASR across all lines, suggesting that there were no differences in kinematics of startle responses between the different wild-type zebrafish lines. Startle response percentages also increased as a function of sound level for all lines tested (Figure 4.1). However, due to the low response rates of WIK and TU fish, estimated startle thresholds were more variable than for AB and AB/WIK wild-type fish.

All startle thresholds were compared against AB fish, due to a larger existing dataset from Chapters 2 and 3. Startle thresholds for AB fish were similar to those obtained in previous experiments ($X^2(1) = 0.009$, $p = 0.92$). Furthermore, there were no significant differences between AB fish tested across days. Therefore, all AB data was pooled together for analysis.

We observed large differences in startle threshold between AB and the other two wild-type lines tested. WIK startle thresholds were significantly higher than AB thresholds at all frequencies tested ($X^2(1) = 84.86$, $p < 0.001$, $n = 9$; Figure 4.2). Differences in threshold were greater than 10 dB at all frequencies. The minimum difference in startle threshold between WIK (Median (Mdn): 1 dB, Interquartile interval (IQR): -14 to -21 dB re. 1 m/s^2) and AB (Mdn: -11 dB, IQR: -6 to -14 dB re. 1 m/s^2) was at 30 Hz ($U = 124.5$, $p < 0.001$, $n = 9$), and the largest difference in startle threshold between WIK (Mdn: 13 dB, IQR: 9 to 19 dB re. 1 m/s^2) and AB (Mdn: -4 dB, IQR: 4 to -5 dB re. 1 m/s^2) was at 90 Hz ($U = 126$, $p < 0.001$, $n = 9$).

Startle threshold differences between AB and TU lines were smaller in magnitude, but showed overall significant differences ($X^2(1) = 62.7$, $p < 0.001$, $n = 9$)(Figure 4.2). These differences were largely driven by pairwise threshold differences between TU (Mdn: -4 dB, IQR: 4 to -5 dB re. 1 m/s^2) and AB lines (Mdn: 12 dB, IQR: 10 to 19 dB re. 1 m/s^2) at 90 Hz ($U = 126$, $p < 0.001$) and differences between AB (Mdn: 5 dB, IQR: 2 to 10 dB re. 1 m/s^2) and TU (Mdn: 14 dB, IQR: 12 to 16 dB re. 1 m/s^2) at 190 Hz ($U = 116$, $p < 0.001$). Startle response thresholds at 60 Hz and 310 Hz were not significant overall.

There were no overall significant differences in startle threshold between TU and WIK ($X^2(1) = 3.79$, $p = 0.06$, $n = 9$) and between AB and AB/WIK lines ($X^2(1) = 0.02$, $p = 0.99$, $n = 9$; Figure 4.2). Interestingly, at all frequencies tested, AB/WIK startle thresholds were equivalent to or lower than AB thresholds, although they did not show statistical significance. Moreover, the variability of startle response threshold was much lower in the AB/WIK line at all frequencies

(Mean IQR: 4.71 dB $\pm$ 0.72 dB re. 1 m/s^2 SEM) compared to the AB line alone (Mean IQR: 10.42 $\pm$ 1.12 dB re. 1 m/s^2 SEM) and WIK line alone (Mean IQR: 7 $\pm$ 0.57 dB re. 1 m/s^2 SEM).

We used a PPI assay to further investigate the auditory sensitivity of the AB/WIK strain. Auditory thresholds determined using the PPI method were not significantly different between AB and AB/WIK fish (X2(1) = 0.08p = 0.77, n = 6) at the five frequencies tested (Figure 4.3). The largest pairwise difference between AB (Mdn: -26 dB, IQR: -25 dB to -28 dB re. 1 m/s^2) and AB/WIK (Mdn: -32 dB, IQR: -30 dB to -35 dB re. 1 m/s^2) lines was at 90 Hz, but this difference was not significant after Bonferroni correction (U = 26.5, p = 0.04, n = 6).

Surprisingly, AB/WIK fish did not show noise-induced hypersensitization of the startle response. After 18 hours of white noise exposure, overall startle thresholds were not different between noise-exposed and control fish (X^2(1) = 3.53, p = 0.06, n = 8) (Figure 4.4). Startle thresholds between noise-exposed AB/WIK and noise-exposed AB fish were not significantly different (X^2(1) = 1.65, p = 0.2, n = 8)

4.4.2 *Individual startle response experiments*

Analysis of individual thresholds showed that individual startle thresholds (Mdn = -1 dB, IQR: 7 to -3 dB re. m/s^2) not significantly different from group-level startle thresholds (Mdn = 3 dB, IQR: 6 to -4 dB re. m/s^2) (U = 1.1, p = 0.94) at 90 Hz, the only frequency tested (Figure 4.5). Therefore, our underlying assumption that group-level percent response is equivalent to individual probability of response holds. Startle thresholds decreased after noise exposure (Median change = -9 dB, IQR: -12 to -2 dB re. m/s^2) in individual responses similar observations in group-level data (U = 131, p < 0.001) (Figure 4.6). Furthermore, analysis of startle thresholds

before and after noise exposure in individual fish showed that initial thresholds and post-noise exposure thresholds were negatively related ($\beta = -0.63$, $p = 0.005$, $r^2 = 0.89$, n=63 fish) (Figure 4.7). That is, fish with higher initial thresholds had greater sensitization compared to fish with lower initial thresholds.

In order to determine whether individual fish could be classified as being from a 'pre-noise' or 'post-noise' condition by their startle thresholds alone, an ROC analysis was conducted on the threshold data (Figure 4.8). Pre-noise and post-noise conditions were discriminable (AUC = 0.76 $\pm$ 0.04). An AUC measure of 1 denotes perfect discriminability and an AUC of 0.5 is no different from chance (Figure 4.8, dashed line). Although there are many analyses to determine 'goodness of fit' for a classification system, by convention, an AUC of 0.75 or greater is considered to have good discriminability.

4.5 DISCUSSION

The goal of these experiments was to observe the effects of genetic background on startle thresholds in larval zebrafish. We found that inbred wild-type WIK and TU lines had higher startle response thresholds compared to the AB line. However, the hybrid AB/WIK line showed similar startle thresholds compared to the AB line with a lower response variability. Startle thresholds for AB/WIK were similar to AB, but AB/WIK fish did not show a similar magnitude of noise-induced hypersensitization when exposed to 18 hours of 10 dB (re. $1/ms^2$) noise. Individual variation within the AB line was similar to variation across groups (Figure 4.5). Furthermore, pre-noise and post-noise startle threshold were largely uncorrelated, suggesting that individual changes in startle response threshold were not dependent on initial sensitivity.

Together, these results demonstrate a large influence on genetic background and gene-environment interaction on startle sensitivity in larval zebrafish.

We found large differences in startle response thresholds between wild-type lines in larval zebrafish. Startle thresholds were more similar between AB and TU when compared to WIK. These results are somewhat unsurprising, as genetic mapping using over 2000 SNPs across multiple wild-type lines has shown that AB and TU lines are more closely related than AB and WIK (Guryev et al. 2006). However, it should be noted that these similarities in startle response may be due to differences in the Mauthner cell and not necessarily due to differences in hearing sensitivity. Previous studies have shown significant differences in startle between AB and TU fish (Burgess and Granato 2008) and between TU and Tubingen longfin (TL) (Burgess and Granato 2007). These differences are presumed to be due to differences in the startle pathway, with AB fish showing proportionally more M-cell mediated short latency startle responses and TU and TL showing a greater proportion of long-latency startle responses to identical stimuli. Because our analysis only considers the short-latency startle response as a 'true' response to the auditory stimulus, it is possible that subtler differences across lines could exist in sensorimotor circuits that mediate auditory-evoked startle behaviors. It should be noted that even though we did not find significant differences in startle response latency across wild-type zebrafish lines, our use of a ramped stimulus may contribute to an increase in variance. Future experiments using non-ramped stimuli are required to determine whether this non-result holds across multiple wild-type lines.

Startle threshold differences across multiple zebrafish lines are similar to previously described differences across rodent strains. Studies in 40 strains of mice (Willott et al. 2003) and 46 strains of rats (Glowa and Hansen 1993) have shown significant inter-strain variability in startle response to a broadband stimulus. In both species, the highest responding strains have startle amplitudes 9-10 times greater than the lowest responding strains. In comparison, our data show a maximum of 17 dB (7 times) difference in startle probability between AB and WIK lines at 90 Hz.

The mechanisms underlying these differences across multiple lines remain unexplored. Studies in multiple mouse strains show similar magnitude differences in both ABR threshold and ABR Wave I amplitude (Zheng et al. 1999), suggesting that these changes could be due to differences in peripheral auditory function. However, it should be noted that many of these strains tested undergo early-onset permanent hearing loss, which is not observed in zebrafish. Similarly, differences in central auditory processing, including spiral ganglion neuron and inhibitory interneuron function (Liu and Fetcho 1999, Lorent et al. 2001), and altered transmission at the mixed synapse at the lateral dendrite of the Mauthner cell (Marsden and Granato 2015, Pereda et al. 2003) all could play a role in modulating the startle response across multiple lines. Finally, differences in neuromodulation through dopaminergic and/or serotonergic processes are known to influence startle sensitivity and could play a role in regulating startle response thresholds (Korn and Faber 2005, Burgess and Granato 2007, Fetcho et al. 2008).

Hybridization of the startle-responsive AB line with the relatively non-responsive WIK line resulted in AB/WIK fish that not only had similar startle thresholds to AB fish, but showed a

smaller magnitude of hypersensitization after noise exposure. This difference could be due to a few reasons. First, our findings that there were no differences between noise exposed AB fish and noise-exposed AB/WIK fish suggests that there is a ceiling effect of noise exposure. Because AB/WIK fish had slightly lower startle thresholds in the control condition, it is possible that the maximum level of hypersensitization is similar across wild-type lines.

However, an alternate explanation could be that AB/WIK fish were more resistant to noise-induced startle sensitization. Studies in mice have shown that the 129/SvEv strain has greater resistance to acoustic injury compared to other commonly used strains (Yoshida et al. 2000). Furthermore, Erway et al. (1996) showed that hybridization of CBA/H-T6J, a mouse line resistant to age-related hearing loss, with non-sensitive lines resulted in F1 hybrids that exhibited greater resiliency against age-related hearing loss compared to all inbred lines (including the parental CBA/H-T6J), suggesting a protective effect of hybridization.

We also found no differences in PPI thresholds between AB and AB/WIK fish. This finding suggests that central auditory processing is similar in the two lines. Because AB and AB/WIK had similar startle and PPI thresholds and were significantly different from WIK startle thresholds, it would seem that the AB phenotype is haplosufficient. However, it should be noted that the AB/WIK line is a stable line and that more careful experiments need to be conducted with F0 hybrids between AB and WIK strains to determine the contribution of AB and WIK alleles to auditory-related processes. Measurement of PPI thresholds in WIK (and TU) fish was not possible due to their low responsivity to the startle-inducing (‘catch’) stimulus using our protocol. Thus, it is possible, though unlikely, that PPI thresholds for WIK and AB/WIK fish are

similar. Previous studies in rodent models have shown that most F1 hybrids between sensitive and non-sensitive strains show startle and PPI phenotypes similar to the sensitive strain (Logue et al. 1997). However, this is not a general rule and the effect of hybridization on startle responsivity and PPI is heterogeneous and should be empirically measured.

Individual variability in startle sensitivity within AB fish was similar to the variability between groups. This is expected and supports our assumption that the probability of response for a single individual is approximated by group-level data (Chapter 1). It is generally assumed that auditory sensitivity between individuals within a strain is similar, due to genetic homogeneity (Yoshida et al. 2000). However, we found high variability in noise-induced startle hypersensitization across individuals within the inbred AB line. Furthermore, we found that initial startle threshold and susceptibility to noise-induced hypersensitization were negatively related. Naively, we would expect that individuals with greater initial startle sensitivity would have larger sensitization effect. However, our data suggests that the ceiling effect of noise exposure reduces the variability between individual responses after noise exposure. Future experiments should be conducted to investigate whether the time course of noise-induced hypersensitization differs between high threshold and low threshold individuals. This could provide information about whether susceptibility to noise-induced hypersensitization of the startle response is correlated with initial sensitivity.

We were also able to show that we could categorize noise-exposed AB fish from controls with relative precision using an ROC analysis. This allows us to determine whether an animal is susceptible to noise-induced hypersensitization based on the threshold alone. The

discriminability (measured using AUC) was 0.76, indicating moderate discriminability. The use of AUC for measuring discriminability has been recently criticized in favor of the d' statistic (Pencina et al. 2008). However, the AUC criterion used is robust to heteroscedastic data, whereas the d' statistic works best for normally distributed data. Nevertheless, this classification method allows for efficient categorization of noise-exposed fish and could be used in future studies to analyze individuals with higher susceptibility to noise-induced hypersensitization.

Auditory sensitivity in zebrafish increases throughout development (Bang et al. 2001, Higgs et al. 2003), and differences in startle response between strains would be expected to increase concomitantly. Future experiments in adult zebrafish should seek to understand whether these differences in startle arise from differences in peripheral auditory sensitivity (eg. differences in AEP threshold) or whether these changes are due to auditory processing in the startle circuitry. Previous studies in rodents have also shown within-strain differences in PPI and startle response between males and females (Lehmann et al. 1999), and these differences are thought to be through regulation of dopaminergic neurons mediated by estrogen (Koch 1998). Although larval zebrafish are not sexually dimorphic and therefore do not show sex-specific differences, studies in adult zebrafish could further elucidate sex differences in startle response and PPI across wild-type lines.

Zebrafish have become a powerful model species in the study of auditory function. This study shows that wild-type lines have a large effect on auditory evoked behaviors and should be considered when comparing across groups. Future startle response experiments on F2 hybrids using a similar individual identification classifier could help uncover novel loci that influence

how genes and gene-environment interactions can influence auditory sensitivity and auditory-evoked behaviors.

4.6 FIGURES

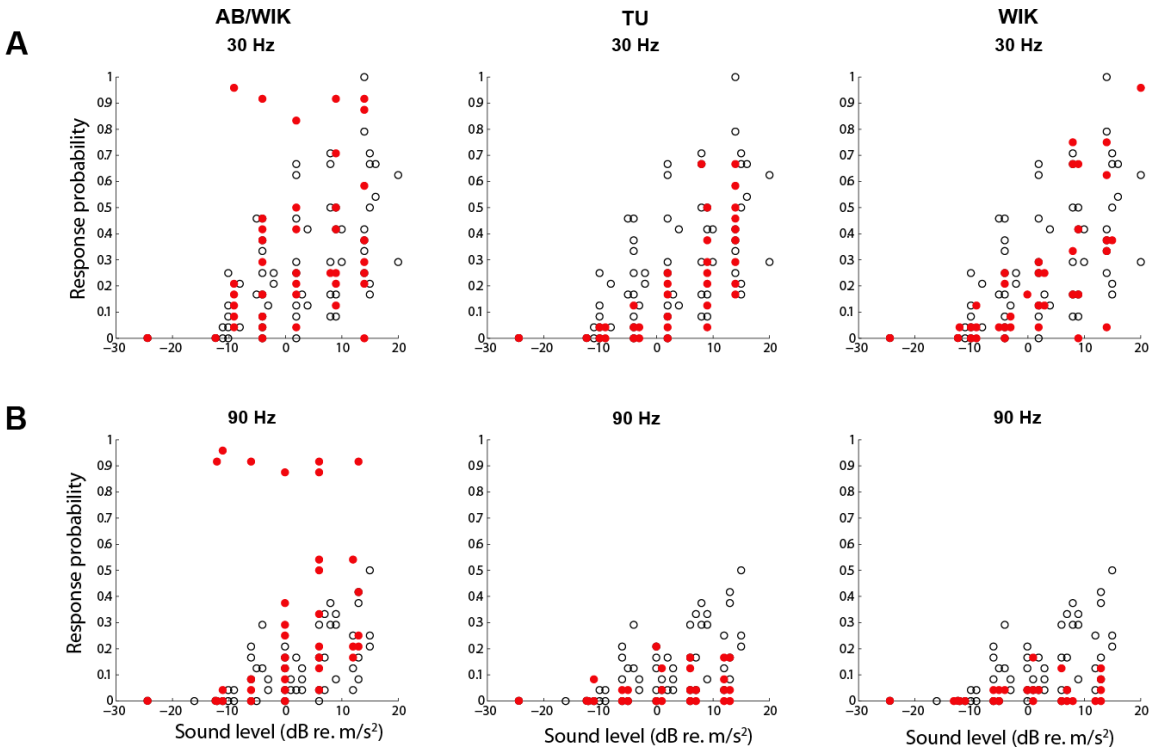

Figure 4.1. **Group-level startle probabilities across multiple wild-type zebrafish lines**. A. Startle response probabilities for AB/WIK (left), TU (middle), and WIK (right) at 30 Hz (red circles) compared with startle response probabilities for AB fish (open circles). Data are plotted as response probabilities for groups of 24 fish against sound levels. B. Startle response probabilities for AB/WIK (left), TU (middle), and WIK (right) at 90 Hz (red circles) compared with startle response probabilities for AB fish (open circles). Data are plotted as response probabilities for groups of 24 fish against sound levels. In both (A) and (B), AB/WIK fish are more responsive near threshold and above threshold compared to AB, TU, and WIK.

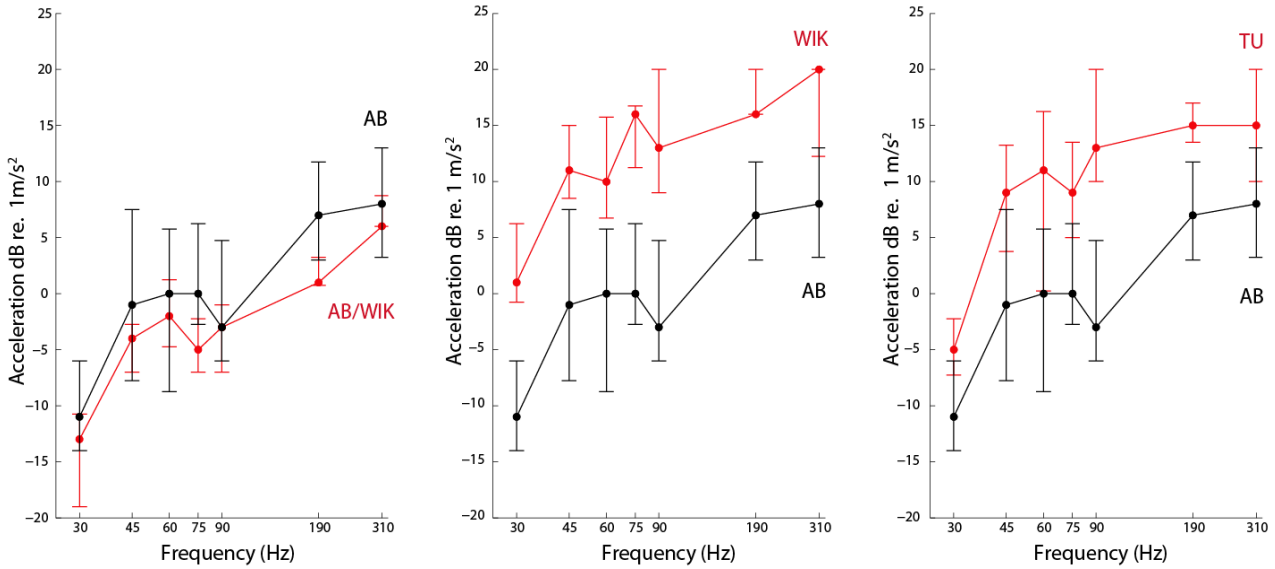

Figure 4.2. **Differences in startle threshold between AB, WIK, TU, and AB/WIK lines**. Startle response thresholds to pure tone stimuli compared between AB and AB/WIK (left), AB and WIK (middle), and AB and TU (right) lines. Startle thresholds for AB (black, n = 10 groups of 24 fish) are the same for all three figures and are for direct comparison. Differences between AB and WIK (middle, red, n = 10) and AB and TU (right, red, n = 10) are significantly different. AB and AB/WIK (right, red, n = 10) startle thresholds were not significantly different at any of the tested frequencies. Threshold data are reported as medians $\pm$ 1 quartile.

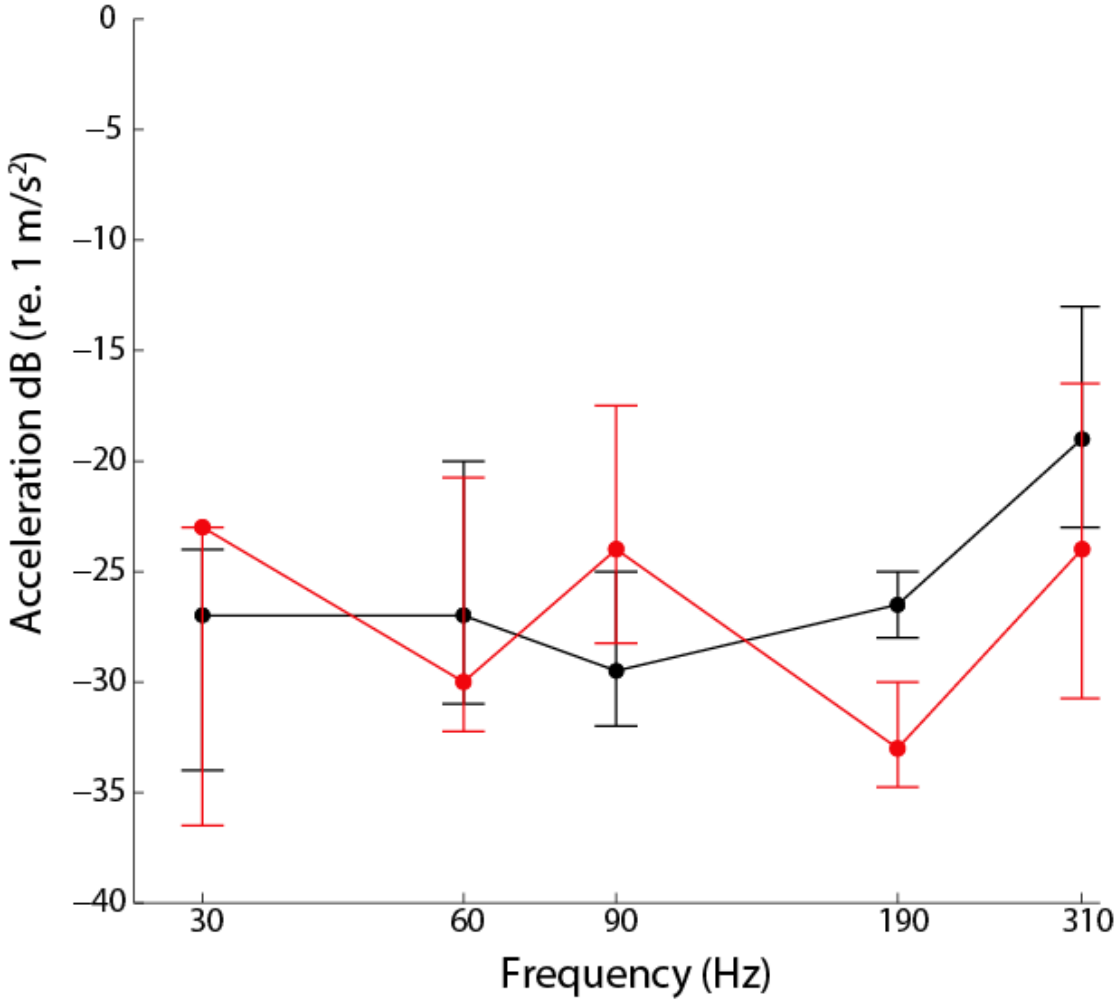

Figure 4.3. **PPI thresholds between AB and AB/WIK are not significantly different**. Auditory thresholds determined using a PPI assay for AB (black, n = 7 groups of 24 fish) and AB/WIK lines (red, n = 7 groups of 24 fish) at 5 tested frequencies. Thresholds are defined as the prepulse sound level that results in a 5% reduction of the startle response to a 'catch' stimulus. Data are reported as medians ± 1 quartile.

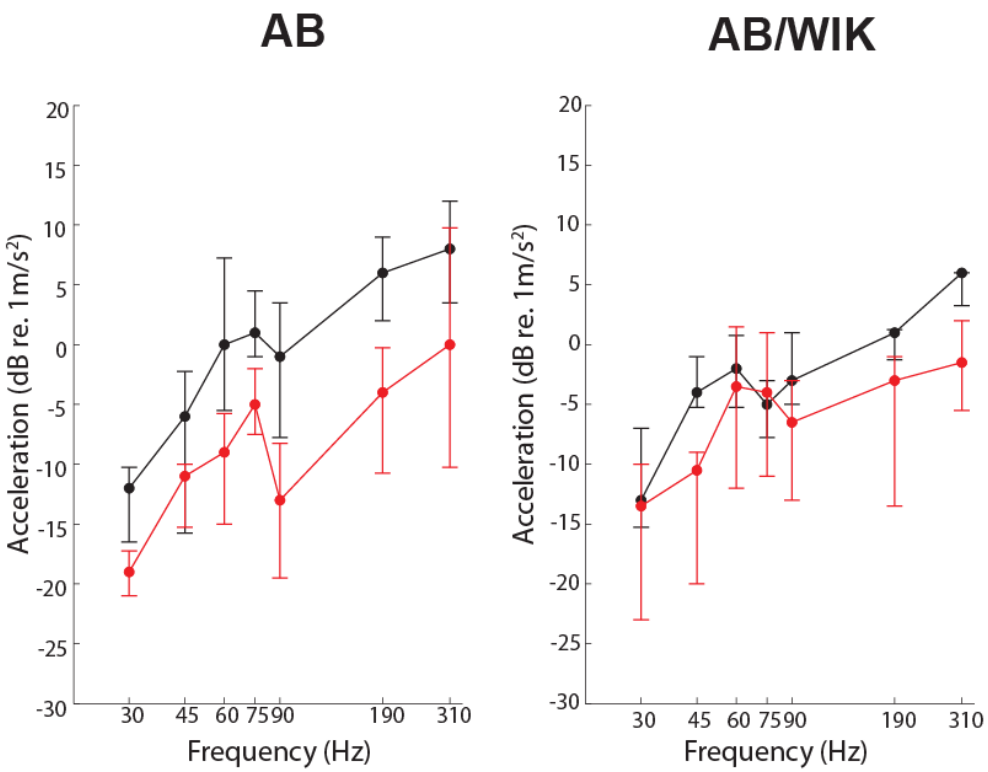

Figure 4.4. **Differences in noise-induced startle sensitization between AB and AB/WIK fish**. Comparison of startle threshold in noise exposed (red, n = 10 groups of 24 fish) and control (black, n = 10 groups of 24 fish) in AB (left) and AB/WIK (right) lines. AB data are reproduced from Figure 3.1 for comparisons with AB/WIK. Startle thresholds are not significantly different between noise-exposed AB and noise-exposed AB/WIK groups.

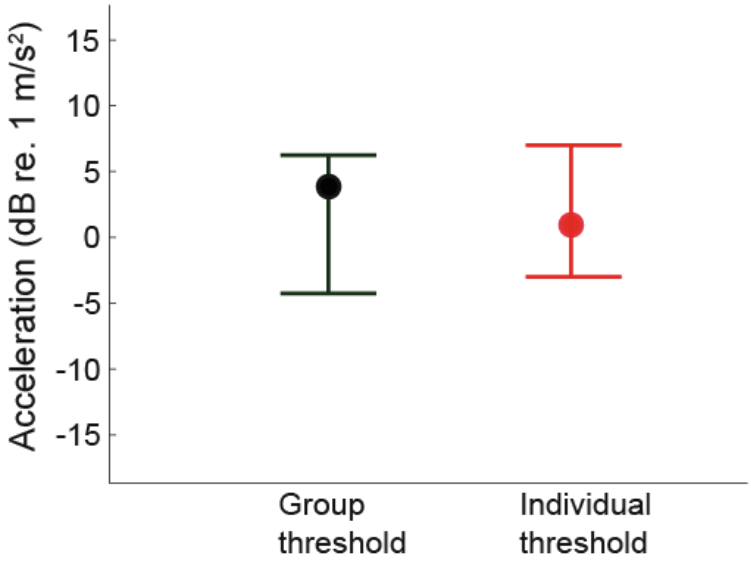

Figure 4.5. **Comparison of group-level and individual startle thresholds in AB fish at 90 Hz**. Startle threshold comparisons to pure-tone stimuli between group-level data (Chapter 2, n = 10 groups of 24 fish) and individual startle thresholds (n = 24 fish). Group-level and individual-level data are not significantly different at the p = 0.05 criterion.

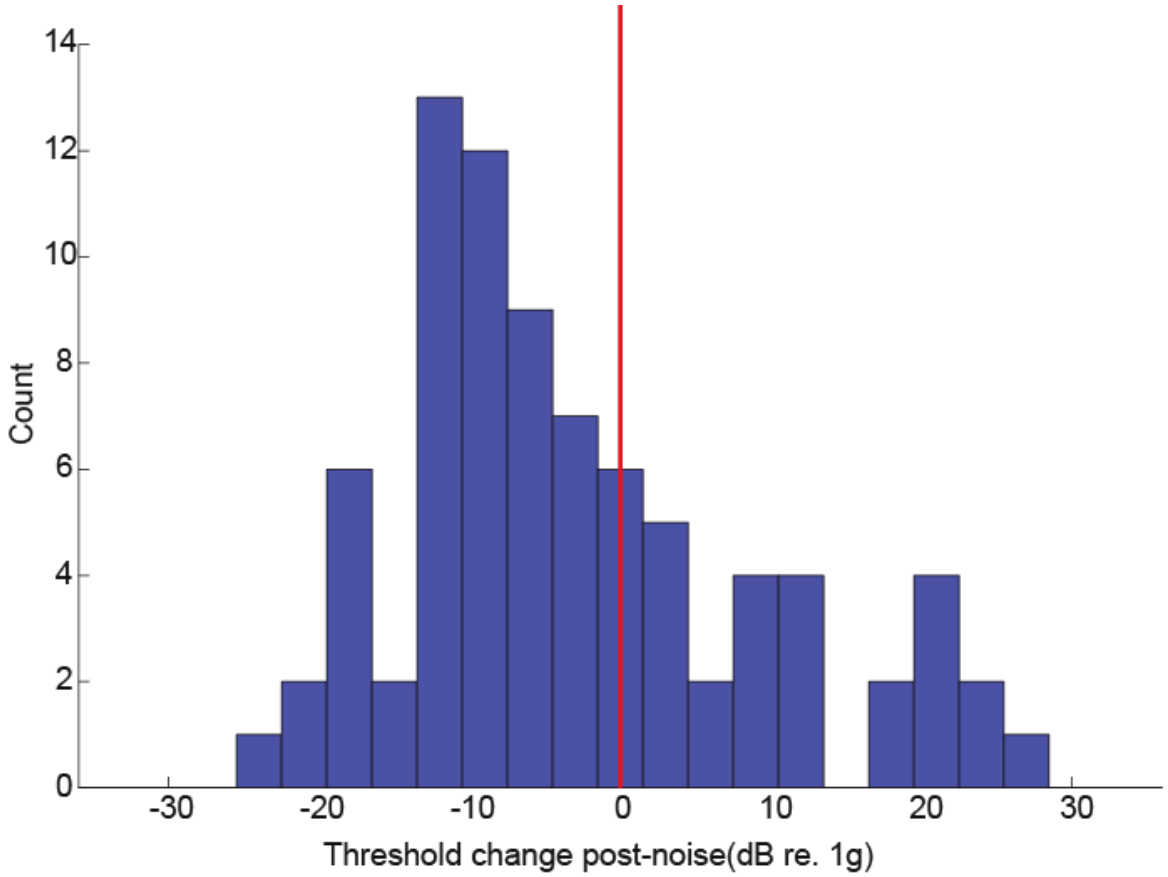

Figure 4.6. **Distribution of threshold changes in individual fish after noise exposure**. Histogram of individual threshold shifts to 90 Hz pure tone stimuli after noise exposure in n = 81 AB zebrafish. Individual threshold shifts were calculated as the threshold difference between pre-noise and post-noise conditions, and negative numbers indicate sensitization. The red line indicates no change between pre- and post-noise thresholds.

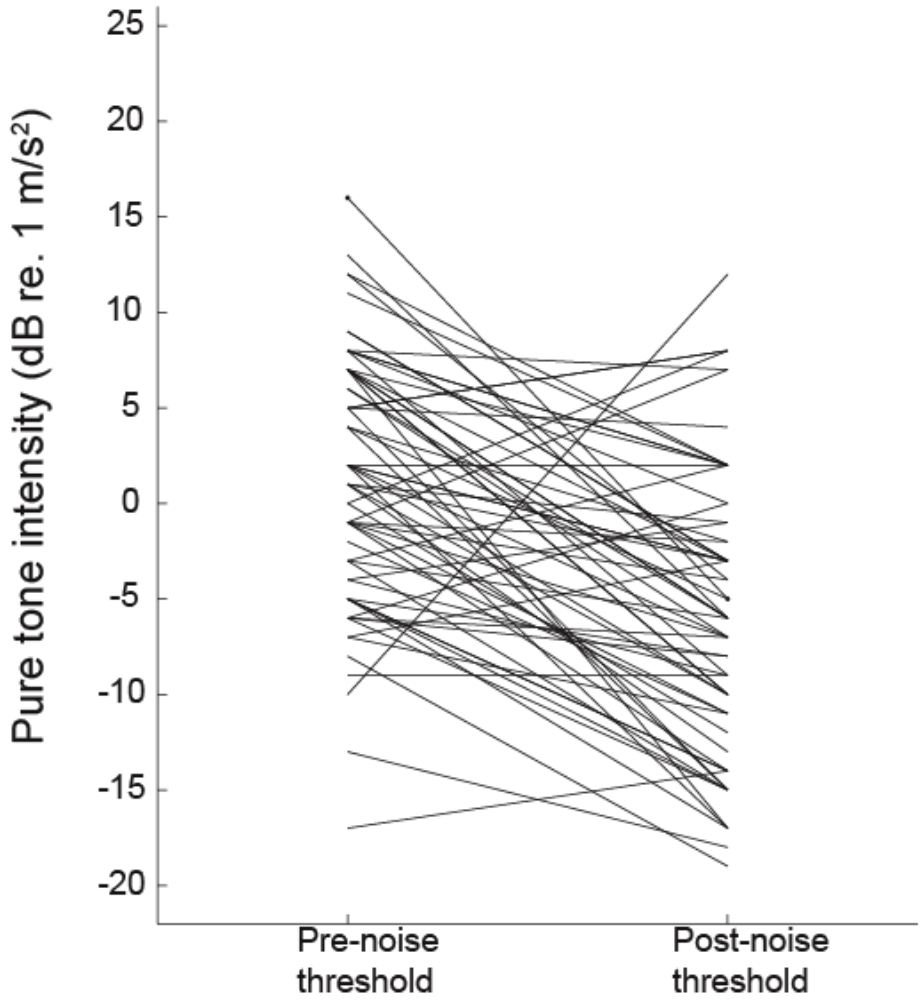

Figure 4.7. **Comparison of pre-noise and post-noise startle threshold in AB zebrafish**. Startle response thresholds to 90 Hz pure tone stimuli for individual fish (n = 63) in pre-noise and post-noise conditions. Pre-noise thresholds and post-noise thresholds were negatively correlated ($\beta = -0.63$, $p = 0.005$, $r^2 = 0.89$).

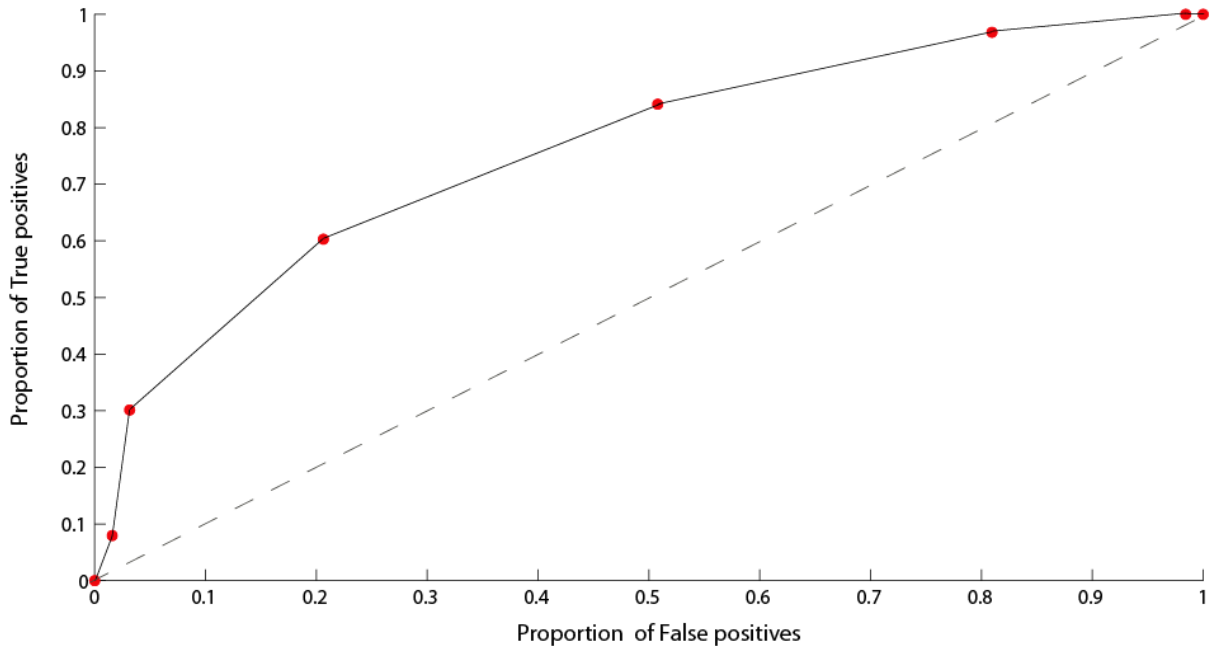

Figure 4.8. **ROC curve for classification of noise-exposed and control fish**. ROC analysis was conducted for pre-noise exposure and post-noise exposure fish (n = 63) for classification. Area under the curve (AUC) is 0.76 $\pm$ 0.04 and the dashed line indicates chance level (AUC = 0.5).

Chapter 5. SUMMARY AND FUTURE DIRECTIONS

This dissertation explored the role of extrinsic and intrinsic factors on auditory sensitivity and auditory-evoked behaviors in larval zebrafish. I developed a method using PPI of the ASR to determine the limit of auditory sensitivity in larval zebrafish and used that method to explore the effects of noise-exposure, genetic background, and inter-individual variation on startle sensitivity. In this chapter, I will summarize these results in greater detail and present questions about future studies. Finally, I will explore future avenues of research using these techniques in zebrafish.

5.1 THE USE OF ASR AND PPI TO MEASURE AUDITORY FUNCTION

In Chapter 2, I described the use of pure tones to determine startle threshold and a novel prepulse inhibition method to determine absolute thresholds in larval zebrafish. Using the PPI method, I showed that auditory threshold was 10-15 dB greater than previously predicted. I also demonstrated, by aminoglycoside ablation of the lateral line neuromasts, that the lateral line did not have a significant input into PPI of the startle response at the frequencies tested.

The latter finding is interesting for a number of reasons. Prepulse inhibition of the startle response has been shown to be cross-modal and visual, tactile, and auditory prepulse stimuli have all been shown to modulate the ASR in larval zebrafish (Korn and Faber 2005). Furthermore, studies in adult goldfish have shown that the M-cell is innervated by both the anterior and posterior lateral line (Zottoli and Van Horne 1983, Mirjany and Faber 2011). *What is the contribution of the lateral line to auditory-evoked startle responses in larval zebrafish?* One possibility is that lateral line information would enhance fidelity of the startle response.

Because both auditory and lateral line inputs are mechanosensory, one would presume that these would provide complementary information about the sound source to the animal. The lateral line has been implicated in providing directional information about sound sources and thus determines the initial startle response direction (Mirjany et al. 2011). Careful control of particle motion stimuli using a three-dimensional shaker system and use of targeted lateral line knockouts could be used with the startle response and PPI assays to measure lateral line sensitivity as well as auditory sensitivity.

5.2 Interaction of habituation and sensitization of the startle response in zebrafish

In Chapter 3, I demonstrated noise-induced hypersensitization of the startle response in 5-7 dpf zebrafish. I showed that 18 hours of noise exposure led to a 10-15 dB decrease in startle threshold. Using glutamatergic antagonists, I determined that this hypersensitization effect is likely mediated by AMPA-type receptors. Finally, I also showed that noise exposure led to a decrease in habituation to startle.

One immediate question that arises from these results is: *What is the role and relationship of habituation and sensitization as a result of noise exposure in the startle pathway in zebrafish?* Classically, the dual process theory of response plasticity states that habituation is a function of the stimulus-response pathway and sensitization is a function of the 'state', or the neural circuits underlying overall responsiveness of an organism (Groves and Thompson 1970). These two pathways are independent, but interact together to determine the overall behavioral output of the animal. Our data shows that noise exposure increases sensitivity and decreases habituation to subsequent stimulation. It is imperative to understand whether this result is due to an increase in

the synaptic strength of the startle-response pathway (ie. potentiation at the M-cell lateral dendrite), changes in 'state', or both. Understanding the interaction of these two processes could provide insight onto fundamental mechanisms of learning and memory in this sensorimotor circuit.

5.3 Effects of genetic background on startle responses

In Chapter 4, I used the startle response and PPI measured to investigate the effects of genetic background and the contribution of individual variation on auditory-evoked behaviors. Using multiple zebrafish lines, I was able to show that startle response thresholds were different by up to 15 dB in different lines. I also demonstrated that the AB/WIK hybrids not only had low startle and PPI thresholds, but were also less susceptible to noise exposure.

How can selective breeding between these lines lead to such dramatic differences within a single species? How does hybridization of multiple lines lead to a decrease in both thresholds and noise-induced sensitization? Further testing of outbred lines (such as TL) and testing true wild-type fish, as well as closely related congeners could help provide vital information about the extent of variability in auditory-evoked behaviors in larval zebrafish. Comparisons between wild-type and hybrid startle thresholds could also determine whether the effect of hybridization is due to group-level differences (due to increased genetic diversity) or due to a specific interaction between genetic loci found in two inbred lines. Finally, analysis of F2 hybrids could help provide vital information about novel loci that influence auditory sensitivity and auditory evoked behaviors in larval zebrafish.

5.4 FUTURE DIRECTIONS

Together, these studies suggest a dynamic interaction between the neural circuits involved in startle response and PPI, environmental influences, and genetic background. Below, I discuss potential new avenues of research.

5.4.1 *How does ontogeny affect auditory function?*

Changes in auditory sensitivity throughout development are well-established in the zebrafish (Higgs et al. 2003, Yao et al. 2016). After 8-10 dpf, zebrafish begin to increase saccular hair cell number rapidly (Bever and Fekete 2002). Furthermore, by 15 dpf, a third auditory endorgan, the lagena, emerges from the posterior saccule (Bever and Fekete 1999). Finally, the Weberian ossicles, which transmit pressure information to the inner ear, develop around 30-50 dpf (Bang et al. 2001, Higgs et al. 2003). Each of these developments is thought to increase auditory sensitivity. However, little is known about how these developments affect auditory evoked behaviors.

The PPI assay of auditory sensitivity is an ideal measure to track how these developments affect absolute limits of hearing. Using a combination of longitudinal studies in fish from 5 dpf to adulthood, the effects of each of these developmental milestones can be better understood. Finally, although Weberian ossicle development and its concomitant effects on auditory sensitivity are well-studied, these studies have been conducted primarily using speakers, which present both particle motion and pressure stimuli (Radford et al. 2012). Using a shaker system and the PPI assay, it would be possible to determine changes in particle motion sensitivity after the development of Weberian ossicles *independent of* changes due to pressure sensitivity onset.

5.4.2 *What are the functions of catacholaminergic neuromodulation in noise-induced startle hypersensitivity?*

Monoaminergic influences on auditory function have been reported across the auditory systems of vertebrates, from dopaminergic inhibition of hair cell activity in the zebrafish lateral line (Toro et al. 2015) to increased auditory discrimination learning in the auditory cortex in mammals (Schiknick et al. 2008). Monoaminergic modulation of the startle response and PPI pathways has also been well-described using a combination of histological (McLean and Fetcho 2004) and pharmacological techniques (Burgess and Granato 2007, Mintz et al. 1989). How is dopaminergic and serotonergic modulation of startle and PPI influenced by noise exposure? Careful manipulations of dopamine and serotonin at the M-cell lateral dendrite and in the inner ear could provide insights on the effects of noise on top-down auditory processing, potentially revealing how these small vertebrates process their auditory world.

BIBLIOGRAPHY

Amoser, S., & Ladich, F. (2003). Diversity in noise-induced temporary hearing loss in otophysine fishes. *The Journal of the Acoustical Society of America*, *113*(4), 2170-2179.

Alario, P., Gamallo, A., Beato, M. J., & Trancho, G. (1987). Body weight gain, food intake and adrenal development in chronic noise stressed rats. *Physiology & behavior*, *40*(1), 29-32.

Baguley DM (2003). Hyperacusis. *Journal of the Royal Society of Medicine*, *96*(12), 582-585.

Bang PI, Sewell WF, Malicki JJ (2000) Behavioral screen for dominant mutations affecting zebrafish auditory system. *Abst. Assn. Res. Otolaryngol.* **23**, 177-187.

Bang PI, Sewell WF, Malicki JJ (2001) Morphology and cell type heterogeneities of the inner ear epithelia in adult and juvenile zebrafish (*Danio rerio*). *J. Comp. Neurol.* **438**,173-190.

Bang PI, Yelick PC, Malicki JJ, Sewell WF (2002) High-throughput behavioral screening method for detecting auditory response defects in zebrafish. *J. Neurosci. Met.* **118**, 177-87.

Barnes, J. (Ed.). (2014). *Complete Works of Aristotle, Volume 1: The Revised Oxford Translation* (Vol. 1). Princeton University Press.

Bergeron, S. A., Carrier, N., Li, G. H., Ahn, S., & Burgess, H. A. (2015). Gsx1 expression defines neurons required for prepulse inhibition. *Molecular psychiatry*, *20*(8), 974-985.

Bergström, B., & Nyström, B. (1986). Development of Hearing Loss During Long-Term Exposure to Occupational Noise A 20-year Follow-up Study. *Scandinavian audiology*, *15*(4), 227-234.

Best JD, Berghmans S, Hunt JJ, Clarke SC, Fleming A, Goldsmith P, Roach AG (2008). Non-associative learning in larval zebrafish. *Neuropsychopharmacology*, *33*(5), 1206-1215.

Bever, M. M., & Fekete, D. M. (2002). Atlas of the developing inner ear in zebrafish. Developmental dynamics, 223(4), 536-543.

Bodnar, D. A., & Bass, A. H. (1997). Temporal coding of concurrent acoustic signals in auditory midbrain. *The Journal of neuroscience*, *17*(19), 7553-7564.

Borg, E. (1982). Auditory thresholds in rats of different age and strain. A behavioral and electrophysiological study. *Hearing research*, *8*(2), 101-115.

Buck LMJ, Winter MJ, Redfern WS, Whitfield (2012) Ototoxin-induced cellular damage in neuromasts disrupts lateral line function in larval zebrafish. *Hear. Res.* **284**, 67–81.

Buonomano, D. V., & Merzenich, M. M. (1998). Cortical plasticity: from synapses to maps. *Annual review of neuroscience*, *21*(1), 149-186.

Burgess HA, Granato M (2007) Sensorimotor gating in larval zebrafish. *J. Neurosci.* **27**, 4984–4994

Burgess, H. A., & Granato, M. (2008). The neurogenetic frontier—lessons from misbehaving zebrafish. *Briefings in functional genomics & proteomics*,*7*(6), 474-482.

Burgess, H. A., Johnson, S. L., & Granato, M. (2009). Unidirectional startle responses and disrupted left–right co-ordination of motor behaviors in robo3 mutant zebrafish. *Genes, Brain and Behavior*, *8*(5), 500-511.

Busch-Nentwich E, Sollner C, Roehl H, Nicolson (2004) The deafness gene dfna5 is crucial for ugdh expression and HA production in the developing ear in zebrafish. *Development* **131**, 943–51.

Carder, H. M., Miller, J. D. (1972). Temporary threshold shifts from prolonged exposure to noise. *Journal of Speech, Language, and Hearing Research*, *15*(3), 603-623.

Cai, S., Ma, W. L. D., & Young, E. D. (2009). Encoding intensity in ventral cochlear nucleus following acoustic trauma: implications for loudness recruitment. *Journal of the Association for Research in Otolaryngology*, *10*(1), 5-22.

Carlsson, P. I., Van Laer, L., Borg, E., Bondeson, M. L., Thys, M., Fransen, E., & Van Camp, G. (2005). The influence of genetic variation in oxidative stress genes on human noise susceptibility. *Hearing research*, *202*(1), 87-96.

Carr, C. E. (1986). Time coding in electric fish and barn owls. *Brain, behavior and evolution*, *28*(1-3), 122-133.

Cervi AL, Poling KR, Higgs DM (2012) Behavioral measure of frequency detection and discrimination in the zebrafish, *Danio rerio*. *Zebrafish* **9**, 1-7.

Chang, E. F., & Merzenich, M. M. (2003). Environmental noise retards auditory cortical development. *Science*, *300*(5618), 498-502.

Chen G, Lee C, Sandridge SA, Butler HM, Manzoor NF, Kaltenbach JA (2013). Behavioral evidence for possible simultaneous induction of hyperacusis and tinnitus following intense sound exposure. *Journal of the Association for Research in Otolaryngology*, *14*(3), 413-424.

Chen YC, Li X, Liu L, Wang J, Lu CQ, Yang M… Sun W (2015). Tinnitus and hyperacusis involve hyperactivity and enhanced connectivity in auditory-limbic-arousal-cerebellar network. *Elife*, *4*, e06576.

Cioni, C., De Palma, F., & Stefanelli, A. (1989). Morphology of afferent synapses in the Mauthner cell of larval Xenopus laevis. *Journal of Comparative Neurology*, *284*(2), 205-214.

Clark, J. A., & Pickles, J. O. (1996). The effects of moderate and low levels of acoustic overstimulation on stereocilia and their tip links in the guinea pig. *Hearing research*, *99*(1), 119-128.

Cody, A. R., & Robertson, D. (1983). Variability of noise-induced damage in the guinea pig cochlea: electrophysiological and morphological correlates after strictly controlled exposures. *Hearing research*, *9*(1), 55-70.

Coffin A, Kelley M, Manley GA, Popper AN (2004) Evolution of sensory hair cells. In: *Evolution of the Vertebrate Auditory System* (Manley GA, Popper AN, Fay RR, eds), pp. 55-94. New York: Springer-Verlag.

Cortes, C., & Mohri, M. (2004). AUC optimization vs. error rate minimization. *Advances in neural information processing systems*, *16*(16), 313-320.

Davis, M. (1984). The mammalian startle response. In *Neural mechanisms of startle behavior* (pp. 287-351). Springer US.

Davis, R. R., Cheever, M. L., Krieg, E. F., & Erway, L. C. (1999). Quantitative measure of genetic differences in susceptibility to noise-induced hearing loss in two strains of mice. *Hearing research*, *134*(1), 9-15.

De Vries, H. L. (1950). The mechanics of the labyrinth otoliths. *Acta oto-laryngologica*, *38*(3), 262-273.

DePalma, R. G., Burris, D. G., Champion, H. R., & Hodgson, M. J. (2005). Blast injuries. *New England Journal of Medicine*, *352*(13), 1335-1342.

Dror, A. A., & Avraham, K. B. (2009). Hearing loss: mechanisms revealed by genetics and cell biology. *Annual review of genetics*, *43*, 411-437.

Easter SS, Nicola GN (1996) The development of vision in the zebrafish (*Danio rerio*). *Dev. Biol.* **15**, 646-663.

Eaton RC, Farley RD, Kimmel CB, Schabtach **E** (1977) Functional development in the Mauthner cell system of embryos and larvae of the zebrafish. *J. Neurobiol.* **8**, 151–172.

Eaton TC, Lee RKK, Foreman MB (2001). The Mauthner cell and other identified neurons of the brainstem escape network of fish. *Progress in neurobiology*, **63**, 467-485.

Edds-Walton, P. L., & Fay, R. R. (2003). Directional selectivity and frequency tuning of midbrain cells in the oyster toadfish, Opsanus tau. *Journal of Comparative Physiology A*, *189*(7), 527-543.

Eggermont, J. J. (2013). Hearing loss, hyperacusis, or tinnitus: what is modeled in animal research? *Hearing research*, *295*, 140-149.

Enger, P. S. (1963). Single unit activity in peripheral auditory system of a teleost fish. *Acta Physiologica Scandinavica*, *59*, 9.

Enger, P. S., Hawkins, A. D., Sand, O., & Chapman, C. J. (1973). Directional sensitivity of saccular microphonic potentials in the haddock. *Journal of Experimental Biology*, *59*(2), 425-433.

Engström, B., Flock, Å., & Borg, E. (1983). Ultrastructural studies of stereocilia in noise-exposed rabbits. *Hearing research*, *12*(2), 251-264.

Erway, L. C., Shiau, Y. W., Davis, R. R., & Krieg, E. F. (1996). Genetics of age-related hearing loss in mice. III. Susceptibility of inbred and F1 hybrid strains to noise-induced hearing loss. *Hearing research*, *93*(1), 181-187.

Evans, G. W., Hygge, S., & Bullinger, M. (1995). Chronic noise and psychological stress. *Psychological Science*, 333-338.

Fabijanska, A., Rogowski, M., Bartnik, G., & Skarzynski, H. (1999, September). Epidemiology of tinnitus and hyperacusis in Poland. In*Proceedings of the sixth international tinnitus seminar* (pp. 569-571). Tinnitus and Hyperacusis Center London.

Fay RR (1981) Coding of acoustic information in the eighth nerve. In: *Hearing and sound communication in fishes* (WN Tavolga, AN Popper, RR Fay, eds), pp. 189-221. New York: Springer.

Fay RR (1984) The goldfish ear codes the axis of acoustic particle motion in three dimensions. *Science* **225**, 951-954.

Fay RR (1995) Perception of spectrally and temporally complex sounds by the goldfish (*Carassius auratus*). Hear Res 89(1): 146-154.

Fay RR, Edds-Walton PL (1997) Diversity in frequency response properties of saccular afferents of the toadfish, *Opsanus tau.* Hear Res 113(1): 235-246.

Fay RR, Edds-Walton PL (2000) Directional encoding by fish auditory systems. Phil Trans Roy Soc Lon B 355(1401): 1281-1284.

Fay RR, Edds-Walton PL, Highstein SL (1994) Directional sensitivity of saccular afferents of the toadfish to linear acceleration at audio frequencies. *Biol. Bull.* **187**, 258-259.

Fay RR, Olsho LW (1979) Discharge patterns of lagenar and saccular neurones of the goldfish eighth nerve: displacement sensitivity and directional characteristics. *Comp. Biochem. Phys. A.* **62**, 377-386.

Fay RR, Popper AN (1974) Acoustic stimulation of the ear of the goldfish (*Carassius auratus*). *J. Exp. Biol.* **61**, 243-260.

Fay, R. R. (1977). Auditory temporal modulation transfer function for the goldfish. *The Journal of the Acoustical Society of America*, *62*(S1), S88-S88.

Fay, R. R. (1978). Coding of information in single auditory-nerve fibers of the goldfish. *The Journal of the Acoustical Society of America*, *63*(1), 136-146.

Fay, R. R. (1979). Physiological and psychophysical studies of amplitude modulation detection by the goldfish. *The Journal of the Acoustical Society of America*, *65*(S1), S82-S82.

Fay, R. R., & Coombs, S. (1983). Neural mechanisms in sound detection and temporal summation. *Hearing research*, *10*(1), 69-92.

Fernandez, K. A., Jeffers, P. W., Lall, K., Liberman, M. C., & Kujawa, S. G. (2015). Aging after Noise Exposure: Acceleration of Cochlear Synaptopathy in "Recovered" Ears. *The Journal of Neuroscience*, *35*(19), 7509-7520.

Fetcho, J. R., Higashijima, S. I., & McLean, D. L. (2008). Zebrafish and motor control over the last decade. *Brain research reviews*, *57*(1), 86-93.

Finneran, J. J. (2012). Auditory effects of underwater noise in odontocetes. In *The Effects of Noise on Aquatic Life* (pp. 197-202). Springer New York.

Foreman MB, Eaton RC (1993) The direction change concept for reticulospinal control of goldfish escape. *J. Neurosci.* **3**, 4101–4113

Friedman, L. M., Dror, A. A., & Avraham, K. B. (2007). Mouse models to study inner ear development and hereditary hearing loss. *International Journal of Developmental Biology*, *51*(6/7), 609.

Frisch, K von (1938) The sense of hearing in fish. *Nature* **141**, 8-11.

Frisina, R. D. (2009). Age-related hearing loss. *Annals of the New york Academy of Sciences*, *1170*(1), 708-717.

Fritz, J. B., Elhilali, M., David, S. V., & Shamma, S. A. (2007). Auditory attention—focusing the searchlight on sound. *Current opinion in neurobiology*, *17*(4), 437-455.

Furukawa, T., & Ishii, Y. (1967). Neurophysiological studies on hearing in goldfish. *Journal of Neurophysiology*, *30*(6), 1377-1403.

Gao, E., & Suga, N. (2000). Experience-dependent plasticity in the auditory cortex and the inferior colliculus of bats: role of the corticofugal system.*Proceedings of the National Academy of Sciences*, *97*(14), 8081-8086.

Glowa, J. R., & Hansen, C. T. (1994). Differences in response to an acoustic startle stimulus among forty-six rat strains. *Behavior genetics*, *24*(1), 79-84.

Gorlin, R. J., Toriello, H. V., & Cohen, M. M. (1995). *Hereditary hearing loss and its syndromes* (No. 28). Oxford University Press, USA.

Gotow, T., Triller, A., & Korn, H. (1990). Differential distribution of serotoninergic inputs on the goldfish Mauthner cell. *Journal of Comparative Neurology*, *292*(2), 255-268.

Granato M, van Eeden FJM, Schach U, Trowe T, et al (1996) Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**, 399-413.

Granato, M., Van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., ... & Kane, D. A. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development*, *123*(1), 399-413.

Grande T, Young B (2004) The ontogeny and homology of the Weberian apparatus in the zebrafish *Danio rerio* (Ostariophysi: Cypriniformes). *Zool. J. Linnean Soc.* **140**, 241–254

Gray L, Rubel EW (1985) Development of absolute thresholds in chickens. *J. Acoust. Soc. Am.* **77**, 1162-1172.

Groves, P. M., & Thompson, R. F. (1970). Habituation: a dual-process theory. *Psychological review*, *77*(5), 419.

Guryev, V., Koudijs, M. J., Berezikov, E., Johnson, S. L., Plasterk, R. H., Van Eeden, F. J., & Cuppen, E. (2006). Genetic variation in the zebrafish.*Genome research*, *16*(4), 491-497.

Haddon, C., & Lewis, J. (1996). Early ear development in the embryo of the zebrafish, Danio rerio. *Journal of Comparative Neurology*, *365*(1), 113-128.

Hamernik, R. P., Henderson, D., Crossley, J. J., & Salvi, R. J. (1974). Interaction of continuous and impulse noise: audiometric and histological effects. *The Journal of the Acoustical Society of America*, *55*(1), 117-121.

Hamernik, R. P., Turrentine, G., Roberto, M., Salvi, R., & Henderson, D. (1984). Anatomical correlates of impulse noise-induced mechanical damage in the cochlea. *Hearing research*, *13*(3), 229-247.

Hammond, K. L., Loynes, H. E., Folarin, A. A., Smith, J., & Whitfield, T. T. (2003). Hedgehog signalling is required for correct anteroposterior patterning of the zebrafish otic vesicle. *Development*, *130*(7), 1403-1417.

Hanley JA, McNeil BJ (1982) The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 143(1): 29-36.

Harris JA, Cheng AG, Cunningham LL, MacDonald G et al (2003) Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). *J. Assoc. Res. Otolaryngol.* **4**, 219–234.

Hawkins AD (1993) Underwater sound and fish behaviour. In: *Behaviour of teleost fishes* (Pitcher TJ, ed), pp. 129-169. New York: Springer.

Hawkins, E. (1973). Ototoxic mechanisms: a working hypothesis. *Audiology*,*12*(5-6), 383-393.

Hawkins, J. E., Johnsson, L. G., Stebbins, W. C., Moody, D. B., & Coombs, S. L. (1976). Hearing loss and cochlear pathology in monkeys after noise exposure. *Acta oto-laryngologica*, *81*(3-6), 337-343.

Hazell, J. W., & Jastreboff, P. J. (1990). Tinnitus. I: Auditory mechanisms: a model for tinnitus and hearing impairment. *The Journal of otolaryngology*,*19*(1), 1-5.

Hedrick TL (2008) Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspiration & Biomimetics* **3**, 034001.

Hickox, A. E., & Liberman, M. C. (2014). Is noise-induced cochlear neuropathy key to the generation of hyperacusis or tinnitus?. *Journal of Neurophysiology*, *111*(3), 552-564.

Higgs DM, Radford CR (2013) The contribution of the lateral line to 'hearing' in fish. *J. Exp. Biol.* **216**, 1484-1490.

Higgs DM, Rollo AK, Souza MJ, Popper AN (2003) Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *J. Acoust. Soc. Am.* **113**, 1145-1154.

Hilgert, N., Smith, R. J., & Van Camp, G. (2009). Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics?. *Mutation Research/Reviews in Mutation Research*, *681*(2), 189-196.

Hoffman HS, Ison JR (1980) Principles of reflex modification in the domain of startle: I. Some empirical findings and their implications for the interpretation of how the nervous system processes sensory input. *Psychological Review* **87**, 175-189.

Honore, T., Davies, S. N., Drejer, J., Fletcher, E. J., Jacobsen, P., Lodge, D., & Nielsen, F. E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science*, *241*(4866), 701-703.

Husbands, J. M., Steinberg, S. A., Kurian, R., & Saunders, J. C. (1999). Tip-link integrity on chick tall hair cell stereocilia following intense sound exposure. *Hearing research*, *135*(1), 135-145.

Inoue, M., Tanimoto, M., & Oda, Y. (2013). The role of ear stone size in hair cell acoustic sensory transduction. *Scientific reports*, *3*

Ison JR (1982). Temporal acuity in auditory function in the rat: Reflex inhibition by brief gaps in noise. *J. Comp. Phys. Psych.* **96**, 945-954.

Ison JR, Pinckney LA (1983). Reflex inhibition in humans: Sensitivity to brief silent periods in white noise. *Perception & Psychophysics* **34**, 84-88.

Jastreboff, P. J., & Jastreboff, M. M. (2015). Decreased sound tolerance: hyperacusis, misophonia, diplacousis, and polyacousis. *Handb Clin Neurol*,*129*, 375-387.

Jones, S. M., Jones, T. A., Johnson, K. R., Yu, H., Erway, L. C., & Zheng, Q. Y. (2006). A comparison of vestibular and auditory phenotypes in inbred mouse strains. *Brain research*, *1091*(1), 40-46.

Kalmijn AJ 1988) Hydrodynamic and acoustic field detection. *Sensory biology of aquatic animals*, 83-130.

Kappler JA, Starr CJ, Chan DK, Kollman R, Hudspeth AJ (2004) A nonsense mutation in the gene encoding a zebrafish myosin VI isoform causes defects in hair-cell mechanotransduction. *Proc. Natl. Acad. Sci.* **101**, 13056–61.

Katzenell, U., & Segal, S. (2001). Hyperacusis: review and clinical guidelines.*Otology & Neurotology*, *22*(3), 321-327.

Kawasaki, M., & Guo, Y. X. (1998). Parallel projection of amplitude and phase information from the hindbrain to the midbrain of the African electric fish Gymnarchus niloticus. *The Journal of neuroscience*, *18*(18), 7599-7611.

Kerr, A. G. (1980). The effects of blast on the ear. *The Journal of Laryngology & Otology*, *94*(01), 107-110.

Kiang, N. Y., Liberman, M. C., & Levine, R. A. (1976). Auditory-nerve activity in cats exposed to ototoxic drugs and high-intensity sounds. *Annals of Otology, Rhinology & Laryngology*, *85*(6), 752-768.

Kilgard, M. P., & Merzenich, M. M. (1998). Plasticity of temporal information processing in the primary auditory cortex. *Nature neuroscience*, *1*(8), 727-731.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Developmental dynamics* **203**, 253–310.

Kimmel, C. B., Patterson, J., & Kimmel, R. O. (1974). The development and behavioral characteristics of the startle response in the zebra fish.*Developmental psychobiology*, *7*(1), 47-60.

Kimmel, C. B., Sessions, S. K., & Kimmel, R. J. (1978). Radiosensitivity and time of origin of Mauthner neuron in the zebra fish. *Developmental biology*,*62*(2), 526-529.

Knudsen, E. I. (1999). Mechanisms of experience-dependent plasticity in the auditory localization pathway of the barn owl. *Journal of Comparative Physiology A*, *185*(4), 305-321.

Koch, M. (1998). Sensorimotor gating changes across the estrous cycle in female rats. *Physiology & behavior*, *64*(5), 625-628.

Kohashi T, Oda Y (2008) Initiation of Mauthner or non-Mauthner-mediated fast escape evoked by different modes of sensory input. *J. Neurosci.* **28**, 10641–10653.

Konings, A., Van Laer, L., & Van Camp, G. (2009). Genetic studies on noise-induced hearing loss: a review. *Ear and hearing*, *30*(2), 151-159.

Korn, H., & Faber, D. S. (2005). The Mauthner cell half a century later: a neurobiological model for decision-making?. *Neuron*, *47*(1), 13-28.

Kozlowski DJ, Whitfield TT, Hukriede NA, Lam WK, Weinberg ES (2005) The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line. *Dev. Biol.* **277**, 27–41.

Kujawa, S. G. (2015). Noise-induced cochlear synaptopathy: Extending effects of noise on man?. *The Journal of the Acoustical Society of America*,*137*(4), 2247-2248.

Kujawa, S. G., & Liberman, M. C. (2006). Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. *The Journal of Neuroscience*, *26*(7), 2115-2123.

Kujawa, S. G., & Liberman, M. C. (2009). Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *The Journal of Neuroscience*, *29*(45), 14077-14085.

Ladich, F., & Fay, R. R. (2013). Auditory evoked potential audiometry in fish.*Reviews in fish biology and fisheries*, *23*(3), 317-364.

Lavinsky, J., Crow, A. L., Pan, C., Wang, J., Aaron, K. A., Ho, M. K., ... & Eskin, E. (2015). Genome-wide association study identifies Nox3 as a critical gene for susceptibility to noise-induced hearing loss. *PLoS Genet*, *11*(4), e1005094.

Le Prell, C. G., Yamashita, D., Minami, S. B., Yamasoba, T., & Miller, J. M. (2007). Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. *Hearing research*, *226*(1), 22-43.

Lehmann, J., Pryce, C. R., & Feldon, J. (1999). Sex differences in the acoustic startle response and prepulse inhibition in Wistar rats. *Behavioural brain research*, *104*(1), 113-117.

Li, H. S., Johnson, A. C., Borg, E., & Höglund, G. (1992). Auditory degeneration after exposure to toluene in two genotypes of mice. *Archives of toxicology*, *66*(6), 382-386.

Li, L., Du, Y., Li, N., Wu, X., & Wu, Y. (2009). Top–down modulation of prepulse inhibition of the startle reflex in humans and rats. *Neuroscience & Biobehavioral Reviews*, *33*(8), 1157-1167.

Liao JC, Ballo O, Akanyeti O (2012) Frequency response of afferent neurons in the posterior lateral line system of larval zebrafish (*Danio rerio*). Program No. 460.01. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

Liberman, M. C. (1987). Chronic ultrastructural changes in acoustic trauma: serial-section reconstruction of stereocilia and cuticular plates. *Hearing research*, *26*(1), 65-88.

Liberman, M. C., & Dodds, L. W. (1984). Single-neuron labeling and chronic cochlear pathology. III. Stereocilia damage and alterations of threshold tuning curves. *Hearing research*, *16*(1), 55-74.

Liberman, M. C., & Dodds, L. W. (1987). Acute ultrastructural changes in acoustic trauma: serial-section reconstruction of stereocilia and cuticular plates. *Hearing research*, *26*(1), 45-64.

Liberman, M. C., & Kiang, N. Y. (1978). Acoustic trauma in cats: cochlear pathology and auditory-nerve activity. *Acta oto-laryngologica*.

Liberman, M.C., Mulroy, M.J., 1982. Acute and chronic effects of acoustic trauma: Cochlear pathology and auditory nerve pathophysiology. In: Hamernik, R.P., Henderson, D., Salvi, R.J. (Eds.), New Perspectives on Noise-Induced Hearing Loss, Raven Press, New York, pp. 105–135.

Lin, J. W., & Faber, D. S. (1988). Synaptic transmission mediated by single club endings on the goldfish Mauthner cell. II. Plasticity of excitatory postsynaptic potentials. *The Journal of neuroscience*, *8*(4), 1313-1325.

Liu, K. S., & Fetcho, J. R. (1999). Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. *Neuron*, *23*(2), 325-335.

Liu, X. Z., & Yan, D. (2007). Ageing and hearing loss. *The Journal of pathology*, *211*(2), 188-197.

Logue, S. F., Owen, E. H., Rasmussen, D. L., & Wehner, J. M. (1997). Assessment of locomotor activity, acoustic and tactile startle, and prepulse inhibition of startle in inbred mouse strains and F1 hybrids: implications of genetic background for single gene and quantitative trait loci analyses. *Neuroscience*, *80*(4), 1075-1086.

Lorent, K., Liu, K. S., Fetcho, J. R., & Granato, M. (2001). The zebrafish space cadet gene controls axonal pathfinding of neurons that modulate fast turning movements. *Development*, *128*(11), 2131-2142.

Lu Z, DeSmidt AA (2013). Early development of hearing in zebrafish. J Assoc Res Otolaryngol 14(4): 509-521.

Lu Z, Xu Z, Buscher WJ (2004) Coding of acoustic particle motion by utricular fibers in the sleeper goby, *Dormitator latifrons*. *J. Comp. Phys. A.* **190**, 923-938.

Lu, Z., Popper, A. N., & Fay, R. R. (1996). Behavioral detection of acoustic particle motion by a teleost fish (Astronotus ocellatus): sensitivity and directionality. *Journal of Comparative Physiology A*, *179*(2), 227-233.

Ma EY, Rubel EW, Raible DE (2008) Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *J. Neurosci.* **28**, 2261-2273.

Malinow, R., & Malenka, R. C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annual review of neuroscience*, *25*(1), 103-126.

Manikandan, S., Padma, M. K., Srikumar, R., Parthasarathy, N. J., Muthuvel, A., & Devi, R. S. (2006). Effects of chronic noise stress on spatial memory of rats in relation to neuronal dendritic alteration and free radical-imbalance in hippocampus and medial prefrontal cortex. *Neuroscience letters*, *399*(1), 17-22.

Mann, D. A., Lu, Z., & Popper, A. N. (1997). Ultrasound detection by a teleost fish. *Nature*, *389*, 341.

Marini, M., Trevisan, P., Dondi, M. A., & Stefanelli, A. (1991). The mauthner neurons in salamandra. *Italian Journal of Zoology*, *58*(1), 43-48.

Marsden, K. C., & Granato, M. (2015). In Vivo Ca 2+ Imaging Reveals that Decreased Dendritic Excitability Drives Startle Habituation. *Cell reports*,*13*(9), 1733-1740.

Maruska, K. P., & Tricas, T. C. (2009). Encoding properties of auditory neurons in the brain of a soniferous damselfish: response to simple tones and complex conspecific signals. *Journal of Comparative Physiology A*,*195*(11), 1071-1088.

McKibben, J. R., & Bass, A. H. (1999). Peripheral encoding of behaviorally relevant acoustic signals in a vocal fish: single tones. *Journal of Comparative Physiology A*, *184*(6), 563-576.

McLean, D. L., & Fetcho, J. R. (2004). Relationship of tyrosine hydroxylase and serotonin immunoreactivity to sensorimotor circuitry in larval zebrafish. *Journal of Comparative Neurology*, *480*(1), 57-71.

Melnick, W. (1991). Human temporary threshold shift (TTS) and damage risk.*The Journal of the Acoustical Society of America*, *90*(1), 147-154.

Meltser, I., Tahera, Y., Simpson, E., Hultcrantz, M., Charitidi, K., Gustafsson, J. Å., & Canlon, B. (2008). Estrogen receptor β protects against acoustic trauma in mice. *The Journal of clinical investigation*, *118*(4), 1563-1570.

Meyer M, Fay RR, Popper AN (2010) Frequency tuning and intensity coding of sound in the auditory periphery of the lake sturgeon, *Acipenser fulvescens*. *J. Exp. Biol.* **213**, 1567-1578.

Miller, J. M., Watson, C. S., & Covell, W. P. (1963). Deafening Effects of Noise on the Cat. *Journal of Occupational and Environmental Medicine*,*5*(11), 555.

Mills, J. H., Gilbert, R. M., & Adkins, W. Y. (1979). Temporary threshold shifts in humans exposed to octave bands of noise for 16 to 24 hours. *The Journal of the Acoustical Society of America*, *65*(5), 1238-1248.

Mintz, I., Gotow, T., Triller, A., & Korn, H. (1989). Effect of serotonergic afferents on quantal release at central inhibitory synapses. *Science*,*245*(4914), 190-192.

Mirjany, M., & Faber, D. S. (2011). Characteristics of the anterior lateral line nerve input to the Mauthner cell. *The Journal of experimental biology*,*214*(20), 3368-3377.

Mirjany, M., Preuss, T., & Faber, D. S. (2011). Role of the lateral line mechanosensory system in directionality of goldfish auditory evoked escape response. *Journal of Experimental Biology*, *214*(20), 3358-3367.

Montgomery JC, Coombs S, Halstead M (1995) Biology of the mechanosensory lateral line in fishes. *Reviews in Fish Biology and Fisheries* **5**, 399-416

Moore BCJ (1998). Cochlear hearing loss: physiological, psychological and technical issues. John Wiley & Sons.

Moorman SJ, Buirress C, Cordova R, Slater J (1999) Stimulus dependence on the development of the zebrafish (*Danio rerio*) vestibular system. *J. Neurobiol.* **38**, 247-258.

Murakami SL, Cunningham LL, Werner LA, Bauer E, Pujol R, Raible DW, Rubel EW (2003) Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*). *Hear. Res.* **186**, 47–56.

Namdaran P, Reinhart KE, Owens KN, Raible DW, Rubel EW (2012) Identification of modulators of hair cell regeneration in the zebrafish lateral line. *J. Neurosci.* **32**, 3516–3528.

Neumeister, H., Ripley, B., Preuss, T., & Gilly, W. F. (2000). Effects of temperature on escape jetting in the squid Loligo opalescens. *Journal of Experimental Biology*, *203*(3), 547-557.

Nicolson T (2005) The genetics of hearing and balance in zebrafish. *Ann. Rev. Genet.* **39**, 9-22.

Nicolson T, Rusch A, Friedrich RW, Granato M, Ruppersberg JP, Nusslein-Volhard C (1998) Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron* **20**, 271-283.

Nicolson, T., Rüsch, A., Friedrich, R. W., Granato, M., Ruppersberg, J. P., & Nüsslein-Volhard, C. (1998). Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron*, *20*(2), 271-283.

Owens KN, Coffin AB, Hong LS, Bennett KO, Rubel EW, Raible DW (2009) Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. *Hear. Res.* **253**, 32–41.

Parham, K., & Willott, J. F. (1988). Acoustic startle response in young and aging C57BL/6J and CBA/J mice. *Behavioral neuroscience*, *102*(6), 881.

Park, S. N., Back, S. A., Park, K. H., Seo, J. H., Noh, H. I., Akil, O., ... & Yeo, S. W. (2013). Comparison of functional and morphologic characteristics of mice models of noise-induced hearing loss. *Auris Nasus Larynx*, *40*(1), 11-17.

Parker GH (1903) The sense of hearing in fishes. Am Nat 37: 185-204.

Patterson, J. H., & Hamernik, R. P. (1997). Blast overpressure induced structural and functional changes in the auditory system. *Toxicology*, *121*(1), 29-40.

Paylor, R., & Crawley, J. N. (1997). Inbred strain differences in prepulse inhibition of the mouse startle response. *Psychopharmacology*, *132*(2), 169-180.

Pencina, M. J., D'Agostino, R. B., & Vasan, R. S. (2008). Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Statistics in medicine*, *27*(2), 157-172.

Pereda, A., O'Brien, J., Nagy, J. I., Bukauskas, F., Davidson, K. G. V., Kamasawa, N., ... & Rash, J. E. (2003). Connexin35 mediates electrical transmission at mixed synapses on Mauthner cells. *The Journal of neuroscience*, *23*(20), 7489-7503.

Pereda, A., Triller, A., Korn, H., & Faber, D. S. (1992). Dopamine enhances both electrotonic coupling and chemical excitatory postsynaptic potentials at mixed synapses. *Proceedings of the National Academy of Sciences*, *89*(24), 12088-12092.

Pickles, J. O., Osborne, M. P., & Comis, S. D. (1987). Vulnerability of tip links between stereocilia to acoustic trauma in the guinea pig. *Hearing research*, *25*(2), 173-183.

Picton, T. W., & Hillyard, S. A. (1974). Human auditory evoked potentials. II: Effects of attention. *Electroencephalography and clinical neurophysiology*,*36*, 191-200.

Platt, C., & Popper, A. N. (1981). Fine structure and function of the ear. In*Hearing and sound communication in fishes* (pp. 3-38). Springer New York.

Popper AN, Fay RR (1993) Sound detection and processing by fish: critical review and major research questions. Brain Behav Evol 41(1): 14-25.

Popper AN, Fay RR (2011) Rethinking sound detection by fishes. *Hear. Res.* **273**, 25–36.

Popper AN, Fay RR (2011) Rethinking sound detection by fishes. Hear Res 273(1): 25-36.

Popper, A. N., & Hastings, M. C. (2009). The effects of human-generated sound on fish. *Integrative Zoology*, *4*(1), 43-52.

Popper, A. N., Fay, R. R., Platt, C., & Sand, O. (2003). Sound detection mechanisms and capabilities of teleost fishes. In *Sensory processing in aquatic environments* (pp. 3-38). Springer New York.

Pujol, R., Lenoir, M., Robertson, D., Eybalin, M., & Johnstone, B. M. (1985). Kainic acid selectively alters auditory dendrites connected with cochlear inner hair cells. *Hearing research*, *18*(2), 145-151.

Pujol, R., Puel, J. L., D'aldin, C. G., & Eybalin, M. (1993). Pathophysiology of the glutamatergic synapses in the cochlea. *Acta oto-laryngologica*, *113*(3), 330-334.

Purser, J., & Radford, A. N. (2011). Acoustic noise induces attention shifts and reduces foraging performance in three-spined sticklebacks (Gasterosteus aculeatus). *PLoS One*, *6*(2), e17478.

Qiu, C., Salvi, R., Ding, D., & Burkard, R. (2000). Inner hair cell loss leads to enhanced response amplitudes in auditory cortex of unanesthetized chinchillas: evidence for increased system gain. *Hearing research*, *139*(1), 153-171.

Radford CA, Montgomery JC, Caiger P, Higgs DM (2012) Pressure and particle motion detection thresholds in fish: a re-examination of salient auditory cues in teleosts. *J. Exp. Biol.* **215**, 3429-3435.

Radford, C. A., Montgomery, J. C., Caiger, P., & Higgs, D. M. (2012). Pressure and particle motion detection thresholds in fish: a re-examination of salient auditory cues in teleosts. *Journal of Experimental Biology*, *215*(19), 3429-3435.

Raible DW, Kruse GJ (2000) Organization of the lateral line system in embryonic zebrafish. *J. Comp. Neurol.* **421**, 189–198.

Retzius, G. (1881). Das Gehororgan der Wirbeltheire. Vol. I (Samson and Wallin: Stockholm).

Riley BB, Moorman SJ **(**2000) Development of utricular otoliths, but not saccular otoliths, is necessary for vestibular function and survival in zebrafish. *J. Neurobiol.* **43**, 329-337.

Riley BB, Phillips BT (2003) Ringing in the new ear: resolution of cell interactions in otic development. *Dev. Biol.* **261**, 289–312.

Roberto, M., Hamernik, R. P., & Turrentine, G. A. (1989). Damage of the auditory system associated with acute blast trauma. *Annals of Otology, Rhinology & Laryngology*, *98*(5 suppl), 23-34.

Roberts, A. C., Reichl, J., Song, M. Y., Dearinger, A. D., Moridzadeh, N., Lu, E. D., ... & Glanzman, D. L. (2011). Habituation of the C-start response in larval zebrafish exhibits several distinct phases and sensitivity to NMDA receptor blockade. *PLOS one*, *6*(12), e29132.

Robertson, D. (1983). Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. *Hearing research*, *9*(3), 263-278.

Rubel, E. W. (1978). Ontogeny of structure and function in the vertebrate auditory system. In *Development of sensory systems* (pp. 135-237). Springer Berlin Heidelberg.

Ruben, R. J., & Rapin, I. (1980). Plasticity of the developing auditory system.*Annals of Otology, Rhinology & Laryngology*, *89*(4), 303-311.

Salvi, R. J., Hamernik, R. P., & Henderson, D. (1979). Auditory nerve activity and cochlear morphology after noise exposure. *Archives of oto-rhino-laryngology*, *224*(1-2), 111-116.

Salvi, R. J., Saunders, S. S., Gratton, M. A., Arehole, S., & Powers, N. (1990). Enhanced evoked response amplitudes in the inferior colliculus of the chinchilla following acoustic trauma. *Hearing research*, *50*(1), 245-257.

Salvi, R. J., Wang, J., & Ding, D. (2000). Auditory plasticity and hyperactivity following cochlear damage. *Hearing research*, *147*(1), 261-274.

Sammeth, C. A., Preves, D. A., & Brandy, W. T. (2000). Hyperacusis: Case studies and evaluation of electronic loudness suppression devices as a treatment approach. *Scandinavian Audiology*, *29*(1), 28-36.

Schicknick, H., Schott, B. H., Budinger, E., Smalla, K. H., Riedel, A., Seidenbecher, C. I., ... & Tischmeyer, W. (2008). Dopaminergic modulation of auditory cortex-dependent memory consolidation through mTOR. *Cerebral Cortex*, *18*(11), 2646-2658.

Scholik, A. R., & Yan, H. Y. (2001). Effects of underwater noise on auditory sensitivity of a cyprinid fish. *Hearing research*, *152*(1), 17-24.

Siegel S, Castellan NJ (1988) Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill.

Sisneros, J. A. (2009). Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, Porichthys notatus. *Journal of neurophysiology*, *102*(2), 1121-1131.

Sisneros, J. A., & Bass, A. H. (2003). Seasonal plasticity of peripheral auditory frequency sensitivity. *The Journal of neuroscience*, *23*(3), 1049-1058.

Sisneros, J. A., Popper, A. N., Hawkins, A. D., & Fay, R. R. (2016). Auditory Evoked Potential Audiograms Compared with Behavioral Audiograms in Aquatic Animals. In *The Effects of Noise on Aquatic Life II* (pp. 1049-1056). Springer New York.

Smith, M. E., Kane, A. S., & Popper, A. N. (2004). Noise-induced stress response and hearing loss in goldfish (Carassius auratus). *Journal of Experimental Biology*, *207*(3), 427-435.

Smith, M. E., Coffin, A. B., Miller, D. L., & Popper, A. N. (2006). Anatomical and functional recovery of the goldfish (Carassius auratus) ear following noise exposure. *Journal of Experimental Biology*, *209*(21), 4193-4202.

Smith, M. E., Schuck, J. B., Gilley, R. R., & Rogers, B. D. (2011). Structural and functional effects of acoustic exposure in goldfish: evidence for tonotopy in the teleost saccule. *BMC neuroscience*, *12*(1), 19.

Smith, M. E. (2012). Predicting hearing loss in fishes. In *The effects of noise on aquatic life* (pp. 259-262). Springer New York.

Spoendlin, H. (1971). Primary structural changes in the organ of Corti after acoustic overstimulation. *Acta oto-laryngologica*, *71*(1-6), 166-176.

Starr CJ, Kappler JA, Chan DK, Kollman R, Hudspeth AJ (2004) Mutation of the zebrafish choroideremia gene encoding Rab escort protein 1 devastates hair cells. *Proc. Nat. Acad. Sci. USA.* **24,** 2572-2577.

Sturm, J. J., & Weisz, C. J. (2015). Hyperactivity in the medial olivocochlear efferent system is a common feature of tinnitus and hyperacusis in humans.*Journal of neurophysiology*, *114*(5), 2551-2554.

Sun, W., Deng, A., Jayaram, A., & Gibson, B. (2012). Noise exposure enhances auditory cortex responses related to hyperacusis behavior. *Brain research*, *1485*, 108-116.

Syka, J., Rybalko, N., & Popelář, J. (1994). Enhancement of the auditory cortex evoked responses in awake guinea pigs after noise exposure. *Hearing research*, *78*(2), 158-168.

Szczepaniak, W. S., & Møller, A. R. (1996). Evidence of neuronal plasticity within the inferior colliculus after noise exposure: a study of evoked potentials in the rat. *Electroencephalography and Clinical Neurophysiology/Evoked Potentials Section*, *100*(2), 158-164.

Tabor, K. M., Bergeron, S. A., Horstick, E. J., Jordan, D. C., Aho, V., Porkka-Heiskanen, T., ... & Burgess, H. A. (2014). Direct activation of the Mauthner cell by electric field pulses drives ultrarapid escape responses.*Journal of neurophysiology*, *112*(4), 834-844.

Tarter, S. K., & Robins, T. G. (1990). Chronic noise exposure, high-frequency hearing loss, and hypertension among automotive assembly workers. *Journal of Occupational and Environmental Medicine*, *32*(8), 685-689.

Thiel, C. M., Friston, K. J., & Dolan, R. J. (2002). Cholinergic modulation of experience-dependent plasticity in human auditory cortex. *Neuron*, *35*(3), 567-574.

Tilney, L. G., Saunders, J. C., Egelman, E., & DeRosier, D. J. (1982). Changes in the organization of actin filaments in the stereocilia of noise-damaged lizard cochleae. *Hearing research*, *7*(2), 181-197.

Toro, C., Trapani, J. G., Pacentine, I., Maeda, R., Sheets, L., Mo, W., & Nicolson, T. (2015). Dopamine Modulates the Activity of Sensory Hair Cells.*The Journal of Neuroscience*, *35*(50), 16494-16503.

Tremblay, K. L., Piskosz, M., & Souza, P. (2003). Effects of age and age-related hearing loss on the neural representation of speech cues. *Clinical Neurophysiology*, *114*(7), 1332-1343.

Treutwein B (1995) Adaptive psychophysical procedures. *Vis. Res.* **35**, 2503-2522.

Walter M, Tziridis K, Ahlf S, Schulze H (2012) Context dependent auditory thresholds determined by brainstem audiometry and prepulse inhibition in Mongolian gerbils. *Open J. Acoust.* **2**, 34-49.

Wang, J., Ding, D., & Salvi, R. J. (2002). Functional reorganization in chinchilla inferior colliculus associated with chronic and acute cochlear damage. *Hearing research*, *168*(1), 238-249.

Webb JF, Shirey JE (2003) Postembryonic development of the cranial lateral line canals and neuromasts in zebrafish. *Dev. Dyn.* **228**, 370-385.

Weber EH. 1820. *De Aure et Auditu Hominis et Animalium, Pars I. De Aure Animalium Aquatium*. Leipzig: Gerhardum Fleischerum.

Weeg, M., Fay, R., & Bass, A. (2002). Directionality and frequency tuning of primary saccular afferents of a vocal fish, the plainfin midshipman (Porichthys notatus). *Journal of Comparative Physiology A*, *188*(8), 631-641.

Weiss SA, Zottoli SJ, Do SC, Faber DS, Preuss T (2006) Correlation of C-start behaviors with neural activity recorded from the hindbrain in free-swimming goldfish (*Carassius auratus*). *J. Exp. Biol.*, **209**, 4788-4801.

Weiss, S. A., Preuss, T., & Faber, D. S. (2008). A role of electrical inhibition in sensorimotor integration. *Proceedings of the National Academy of Sciences*,*105*(46), 18047-18052.

Westerfield M (2000) The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed, Eugene, OR: Univ. of Oregon Press.

Whitfield TT (2002) Zebrafish as a model for hearing and deafness. *J. Neurobiol.* **53**, 157-171.

Whitfield TT, Granato M, van Eeden JM, Schach U, Brand M, Futurani-Seiki M et al (1996) Mutations affecting development of the zebrafish inner ear and lateral line. *Development* **123**, 241-254.

Whitfield TT, Riley BB, Chiang MY, Phillips B (2002) Development of the zebrafish inner ear. *Dev. Dyn.* **223**, 427–58.

Wichmann FA, Hill NJ (2001) The psychometric function: I. Fitting, sampling, and goodness of fit. *Perception & Psychophysics* **63**, 1314-1329.

Willott, J. F., & Turner, J. G. (2000). Neural plasticity in the mouse inferior colliculus: relationship to hearing loss, augmented acoustic stimulation, and prepulse inhibition. *Hearing research*, *147*(1), 275-281.

Willott, J. F., Aitkin, L. M., & McFadden, S. L. (1993). Plasticity of auditory cortex associated with sensorineural hearing loss in adult C57BL/6J mice.*Journal of comparative neurology*, *329*(3), 402-411.

Willott, J. F., Tanner, L., O'Steen, J., Johnson, K. R., Bogue, M. A., & Gagnon, L. (2003). Acoustic startle and prepulse inhibition in 40 inbred strains of mice. *Behavioral neuroscience*, *117*(4), 716.

Wilson DM (1959) Function of giant Mauthner's neurons in the lungfish. Science 129(3352): 841-842.

Wolf, S. J., Bebarta, V. S., Bonnett, C. J., Pons, P. T., & Cantrill, S. V. (2009). Blast injuries. *The Lancet*, *374*(9687), 405-415.

Wolman M, Granato M (2012) Behavioral genetics in larval zebrafish: learning from the young. *Dev. Neurobiol.* **72**, 366–372

Yang, G., Lobarinas, E., Zhang, L., Turner, J., Stolzberg, D., Salvi, R., & Sun, W. (2007). Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. *Hearing research*, *226*(1), 244-253.

Yang, X. D., Korn, H., & Faber, D. S. (1990). Long-term potentiation of electrotonic coupling at mixed synapses.

Yao, C., Vanderpool, K. G., Delfiner, M., Eddy, V., Lucaci, A. G., Soto-Riveros, C., ... & Pereda, A. E. (2014). Electrical synaptic transmission in developing zebrafish: properties and molecular composition of gap junctions at a central auditory synapse. *Journal of neurophysiology*, *112*(9), 2102-2113.

Yao, Q., DeSmidt, A. A., Tekin, M., Liu, X., & Lu, Z. (2016). Hearing Assessment in Zebrafish During the First Week Postfertilization. zebrafish, 13(2), 79-86.

Yelverton, J. T., Richmond, D. R., Hicks, W., Saunders, H., & Fletcher, E. R. (1975). *The relationship between fish size and their response to underwater blast*. Lovelace Foundation for Medical Education and Research. Albuquerque, NM.

Yerkes RM (1905). The sense of hearing in frogs. *J. Comp. Neurol. Psych.* **15**, 279-304.

Yokogawa, T., Hannan, M. C., & Burgess, H. A. (2012). The dorsal raphe modulates sensory responsiveness during arousal in zebrafish. *The Journal of Neuroscience*, *32*(43), 15205-15215.

Yoshida, N., Hequembourg, S. J., Atencio, C. A., Rosowski, J. J., & Liberman, M. C. (2000). Acoustic injury in mice: 129/SvEv is exceptionally resistant to noise-induced hearing loss. *Hearing research*, *141*(1), 97-106.

Young JS, Fechter LD (1983) Reflex inhibition procedures for animal audiometry: A technique to assess ototoxicity. *J. Acoust. Soc. Am.* **73**, 1686-1693.

Zar JH (1999) Biostatistical Analysis. 4th ed. New Jersey: Prentice Hall.

Zeddies DG, Fay RR (2005) Development of the acoustic startle response of zebrafish to tones. *J. Exp. Biol.* **208**, 1363-1372.

Zheng, Q. Y., Johnson, K. R., & Erway, L. C. (1999). Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hearing research*,*130*(1), 94-107.

Zhou, X., Jen, P. H. S., Seburn, K. L., Frankel, W. N., & Zheng, Q. Y. (2006). Auditory brainstem responses in 10 inbred strains of mice. *Brain research*,*1091*(1), 16-26.

Zottoli, S. J., & Faber, D. S. (2000). The Mauthner Cell: What Has it Taught us? *The Neuroscientist*, *6*(1), 26-38.

Zottoli, S. J., & Van Horne, C. (1983). Posterior lateral line afferent and efferent pathways within the central nervous system of the goldfish with special reference to the Mauthner cell. *Journal of comparative neurology*,*219*(1), 100-111.

Zottoli, S. J., Bentley, A. P., Prendergast, B. J., & Rieff, H. I. (1995). Comparative studies on the Mauthner cell of teleost fish in relation to sensory input. *Brain, behavior and evolution*, *46*(3), 151-164.

APPENDIX A

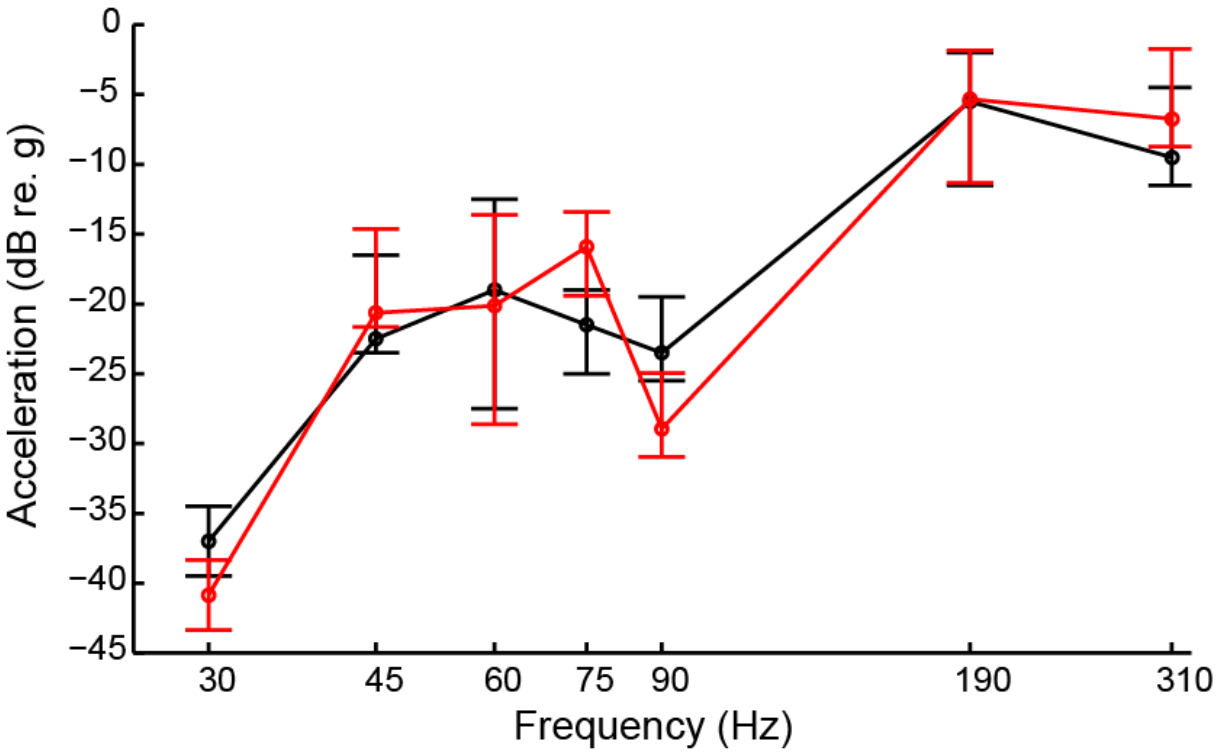

Figure A.1. **Re-test validation of the startle response assay on group-level data**. Startle response thresholds are reported for groups of 24 wild-type (AB) zebrafish tested at 5 dpf (black, n = 7) and retested at 6 dpf (red, n = 7).

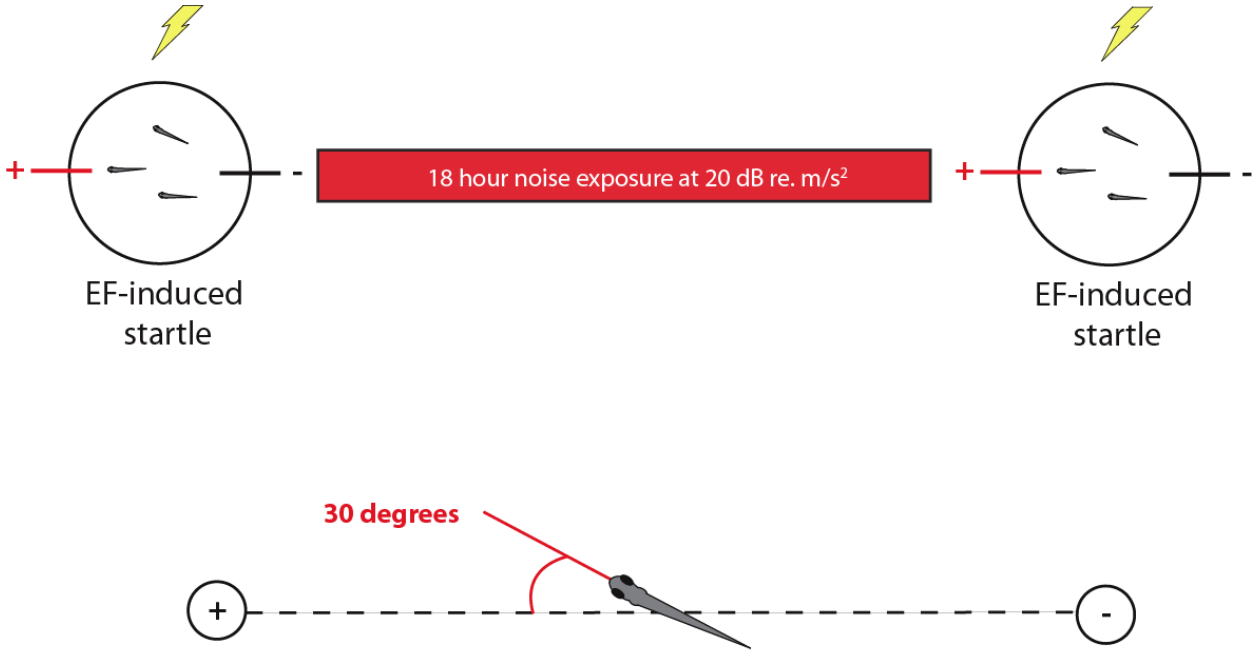

Figure A.2. **Diagram of experimental design for EFP experiments**. Groups of 3 fish were placed in a 3 cm diameter arena and startle responses were induced using EF pulses at 0.25 – 1.5 V/cm 10 times, for a total of 40 stimulus presentations. Only animals that were within 30° off-axis from the anode-cathode axis were used for analysis. After 18 hours of noise exposure at 20 dB re. 1 m/s^2, fish were tested again using the same EF stimuli.

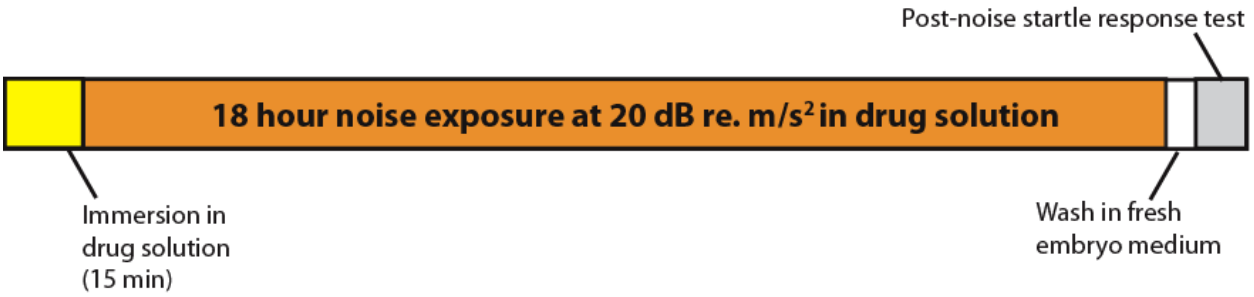

Figure A.3. **Diagram of experimental design for pharmacological experiments**. Cohorts of 24 zebrafish (5-7 dpf) were immersed in solutions containing DNQX, APV, or vehicle and allowed to acclimate for 15 minutes. Groups of fish were then exposed to 18-24 hours of 20 dB re. m/s^2 white noise in the drug solutions (orange). After cessation of the noise exposure, fish were washed in embryo medium three times (white), allowed to acclimate in fresh embryo medium, and tested for startle responsivity to pure tone stimuli. Note that the bars are not to scale.

VITA

Ashwin Bhandiwad was born to Anirudha and Vidya Bhandiwad in August, 1985, in Mysore, Karnataka, India. The Bhandiwad family moved to Bay City, MI, in 1995, and Ashwin graduated from Bay City Central High School in June 2003. He attended Johns Hopkins University, graduating with a Bachelor of Arts in Biophysics in 2007. He then enrolled in Northeastern University's Three Seas/East West program in 2008, graduating with a Master's of Science in Marine Biology and Ecology in 2009. During his Master's program, he worked with Dr. Sonke Johnsen at Duke University investigating the effects of salinity and temperature on tissue transparency in the grass shrimp, *Palaemonetes pugio*. Ashwin has been at the University of Washington since August 2010 working in the Sisneros lab on questions related to hearing in fishes, in particular the midshipman fish (*Porichthys notatus*), three-spined stickleback (*Gasterosteus aculeatus*), and the zebrafish (*Danio rerio*).