Research Article

Auditory Evoked Potential Bioassay of Function and Dysfunction in Zebrafish

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Abstract

For several years, zebrafish (*Danio rerio*) have been important toward the development of an animal model to explore sensory neural hearing function. Zebrafish present with several advantages over rodent models, including a relatively small size, ease of maintenance, efficient delivery of water soluble chemicals and drugs, homologous inner ear structures as mammals, and hearing dysfunction that can be readily established. Our goal in this investigation was to optimize experimental conditions for using the zebrafish as a laboratory model to evaluate the auditory sensory neural axis as a biological assay utilizing the auditory evoked potential (AEP). Taking into consideration previous experimental protocols developed by others, and several refinements employed by us, we examined in the adult zebrafish the far-field AEP using three stimulus parameters (intensity, frequency, repetition rate), three ototoxic drugs (Pentylenetetrazol, Cisplatin, and Gentamicin), and after noise overstimulation (1- day, 3 - days, 5 — days exposure). The AEP assay provided indices of sensory neural functioning reflected as down-regulation of the amplitude of key wave components and a prolongation of latency. In aggregate, the results lend further support to previous investigations of the utility of using the AEP of the zebrafish as an effective bioassay of the function and dysfunction of the auditory sensory neural axis.

ABBREVIATIONS

AEP: Auditory Evoked Potential; dB SPL; decibel Sound Pressure Level; Hz: Hertz or cycles per second; MS-222: Tricaine Methyl Sulfonic; PTZ: Pentylenetetrazol

INTRODUCTION

An ideal *in vivo* model biological system would be welcomed as one to test interventions that alleviate or correct conditions that create deficits in sensory neural hearing. The zebrafish (*Danio rerio*) [1] appears to meet the basic primary requirements of such a system since it has a long-standing history of successful use for recordings in auditory research, as reflected in numerous studies of fish in which the auditory evoked potential (AEP) has in general been explored in several fish species [2-14], including specifically the zebrafish [7-9,11,13]. Furthermore, the zebrafish has been the subject of translational research strategies, used as effective disease models analogous to mammals [15-21].

Although rodent models are available for a number of hearing dysfunctions, the use of the zebrafish offers rapid phenotypic testing of genetic-, chemical- or noise-induced pathologies. Using available but refined tools might lead to a better workable roadmap for the discovery and development of therapeutic agents for hearing loss prevention and treatment in humans [22], as with animals [23,24], or re-purposing of already FDA approved drugs that are already available on the market for treating other medical conditions. In this research, we used the adult zebrafish as a biological model to induce deficits in hearing by testing various drugs and using noise over-exposure as tools to down-regulate electrophysiologic function [6,7,26].

Hearing in fish

When the AEP is recorded in humans and other mammals such as laboratory rodents, a sufficiently loud acoustic signal such as a click, gated noise or a short tone burst (e.g., 80 – 90 dB SPL) will elicit 4 – 7 vertex positive-negative peaks that occur at an interval of milliseconds, modulated on a slow wave of approximately 1.0 kHz [27-29], and we designated the positive peaks as waves I, II, III, IV, and V. The waveform peaks in mammals are electrophysiologic indicants of various anatomical structures along the afferent auditory pathway, and can be readily identified based on their morphology, latency and amplitude - due in part to various recording parameters and disease states [30,31]. In fact, the changes in waveform parameters can be used to diagnose

normal or abnormal conditions of the underlying generators of the various peaks. Thus, the waveforms seen in other mammalian species are reflected in the zebrafish [2,4,7-10,32-34], although the elaboration of the afferent auditory pathway in mammals is not analogous to pathway in the zebrafish. As the sound impulse impinges on the fish detection system via the swim bladder, Weberian ossicles, Baudelot ligament, and the lateral line system, the receptor hair cells detect the vibrations of the surrounding water and thus generate a receptor potential. We propose that this is reflected in the far field peak shown as wave I. Wave II is perhaps indicative of the first order neurons of the bipolar auditory nerve via chemical and/or electrical transmission. Second order neurons are thought by us to be reflected in the electrical activity generated by the Mauthner cell (wave III) with its output synapsing at the first motor neuron of the spinal tract. The motor neuron innervates muscle cells of the spinal tract that plays a critical role in the escape response [35], seen as a "C-bend" in the trunk of the fish as voluntary escape is initiated. The remaining far field peaks (IV, V) have not been well characterized in zebrafish, nor is the slow negative-going wave of approximately 1.0 kHz on which the individual peaks both positive- and negative - going are superimposed.

Using the zebrafish as a model system to record auditory electrophysiologic responses in the form of AEPs, we show results similar to other investigators that the response is an effective sensory neural assay for the evaluation of inner ear and motor - brain function. We used the technique for untreated and treated animals and the results were sensitive to stimulus parameters, drug treatment, and over - exposure to noise. Here we demonstrate that the AEP can be used to assess the sensory neural auditory pathway of the zebrafish, and suggest that its use can be helpful in understanding fundamental and clinical aspects of auditory abnormalities in humans. Our results indicate that AEP morphology, amplitude and latency of responses serve as a vital adjunct to differential assay of the correlates of sensory neural hearing loss in humans.

MATERIALS AND METHODS

Animals

Animal procedures were approved by the UNT-IACUC (Approval number: 14001) and were performed in accordance with regulations for the care and use of laboratory animals. Adult zebrafish were obtained from a local fish store and kept in aquaria water that were maintained at 25°C, filtrated, aerated, pH balanced 7.0 – 8.0, with frequent monitoring of excess contaminants such as nitrate, nitrite, ammonia, chloramines, and chloride. A 12h day/12h night cycle was maintained in the room housing the fish. Exchange of conditioned or treated tap water occurred at regular intervals. Two bottom feeder fish (Bristle nose catfish, *Ancistrus temmincki*) were kept in the aquaria to help reduce the accumulation of waste. Animals were fed twice daily from an automated controlled feeder (EHEIM 3581090, Deizisau, Germany) using Freshwater Flakes (Omega One, Omega Sea, Sitka, AK) that was sterilized overnight using UV illumination.

Drugs and solutions

Tricaine Methylsulfonic (MS-222) (E10521) ($C_9H_{11}O_2N+CH_3SO_3H$, Molar mass = 261.3), Pentylenetetra-

zol (P6500) ($C_6H_{10}N_4$, Molar mass = 138.171 g/mol), Cisplatin (C2210000) ($Cl_2H_6N_2Pt$, Molar mass = 300.01 g/mol), and Gentamicin sulfate (G1397) ($C_{21}H_{43}N_5O_7$, Molar mass = 477.596 g/mol) were obtained in powder form from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in autoclaved water (except Cisplatin, which was dissolved in a saline solution of 0.9%, $NaCl_2$) to produce an appropriate stock solution (mM range, except MS-222) from which various titrations were calculated according to the drug, and purpose of the experiments. Fresh solutions of the drugs were made daily from the refrigerated stock solutions.

Drug administration

The concentration of the PTZ was 100 μM, the Gentamicin was 500 μM, and the Cisplatin was 100 μM. Fish were exposed to a 10 ml solution for 10 min, applied from a syringe using droplets of drugs over the surface of the head. Following a 10-min interval after the application of drugs, the AEP testing began. Electrodes remained in place during the entire duration of an experiment.

Auditory Evoked Potential (AEP) Assays

Adult zebrafish of 3 - 4 cm in length were used to record the AEP. Fish were sedated using 0.075g/L of Ethyl-3-aminobenzate, methylsulfonic acid salt (MS-222). After sedation, the fish was placed in a mesh sling (Figure 1) and suspended in a solution of fish water (36), which contained MS-222 in order to maintain a quiescent level of sedation during the experimental test sessions, which lasted about 30 min (3). The head of the fish was slightly elevated above the water line, but was submerged sufficiently to maintain snout and gill respiration of water, at a temperature of 25°C (76 mm Immersion thermometer, Curtin Matheson Scientific, Morris Plains, NJ). An epidermal stainless steel double Ag/Ag - chlorided (~1.0 mm exposed tip, laid bare by scalpel) needle electrode (Grass Instruments, W. Warwick, RI) was placed at the anterior surface of the head (Active), a second electrode was placed at the posterior surface of the head (Reference), with
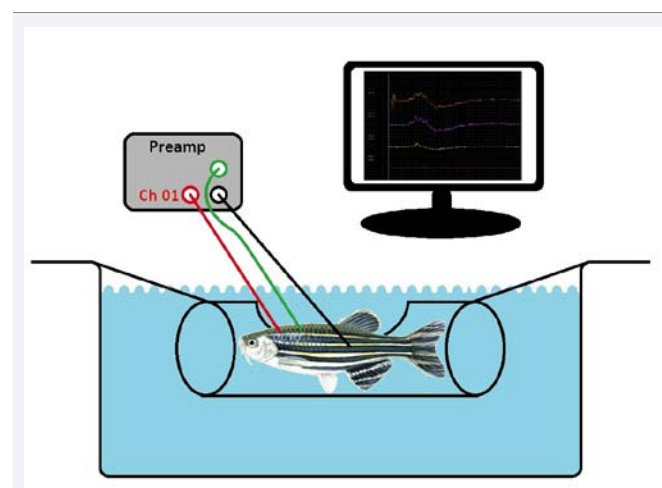
Figure 1 Highly simplified cartoon of the experimental setup used for recording auditory evoked potentials from zebrafish. The fish is suspended in a mesh sling inside of a cylinder submerged in a bath containing fish water. Three needle electrodes are placed just beneath the epidermis as shown. The electrophysiologic activity is routed to appropriate recording instrumentation. An audio speaker (not shown) is located 90o directly above the head of the fish.

a third electrode inserted into the dorsal body (Ground) near the posterior fin. The impedance of the electrodes at initial testing was typically measured at or below 5.0 kOhms. Impedances were systematically checked before and at the end of an experimental run to monitor consistency. Real time visual monitoring of the fish was accomplished using a CCD camera (Panasonic HCV-110, Osaka, Japan) and monitor (JVC cm31720, Yokohama, Japan).

The AEP was obtained using an alternating 0.1 ms square wave electrical pulse presented at 9.3/s (ISI = 107.5 ms), except where noted. The pass band of the amplifier was from 10 Hz – 5.0 kHz. A typical AEP was based on a minimum of 1024 epochs, superimposing at least two replicate runs at the same experimental condition. Threshold was estimated at a level in which waveform peaks were not visually detectable. At this level, the residual noise was measured to be within the nanovolts range. The analysis time was 10 ms using 512 data points over the entire analysis period. Thus, the dwell time for the data points was about 1.95 μs. The click used was generated by a 0.1ms electrical pulse that produced an acoustic transient of 3 – 5 ms from an audio speaker (LG 6400ETJI3, Seoul, South Korea) suspended 40 cm/90° superior to the head of the fish. Tone burst stimuli consisted of full-cycle 3.0 - 5.0 ms short sine waves (e.g., at 1.0 kHz, with five complete waves, and so forth) using a sigmoidal ramp, generated by a multifunction processor (RX6, Tucker Davis Technologies, Alachua, FL). The stimulus intensity was controlled by a programmable electronic attenuator (RX6, TDT, Alachua, FL) in 5 or 10 dB steps, especially near threshold. The electrophysiologic signal was amplified 1 x 10⁴, filtered from 10–5 kHz, and routed to the input of the data processor (Multifunction Processor RX6, and SigGen RP 4.4, TDT, Alachua, FL). The lowest SPL to produce reproducible responses 2x was defined as threshold. Hearing was tested using either a click, or short tone bursts at frequencies of 500, 1000, 2000, and 4000 Hz (10). The sound pressure limit of the equipment was calculated to be approximately 50 - 60 dB SPL (Audio amplifier MS-25B, McMartin, Omaha, NE) above the normal adult fish hearing threshold. All equipment was calibrated before and after each experiment using a Function Generator (Beckman FG2A, Brea, CA), Universal Counter (HP5334A, Palo Alto, CA), Multimeter (HP 3435A, Palo Alto, CA), and an Oscilloscope (Tektronix 2205, Beaverton, OR), with signals routed through a multipurpose Patch Panel (HP353A, Palo Alto, CA). Each experiment used 3 - 4 animals to obtain consistent trends for data analyses.

Noise over-exposure

The noise over-exposure experiments were conducted in two phases. The initial phase consisted of obtaining an AEP (baseline) to an intensity series of 110, 90, 70, 50 dB SPL - replicated twice. The fish was then allowed to recover from the sedation over a 1.0 hr. period. The fish was then placed in a custom built "noise exposure chamber" consisting of a bucket of fish water (36, 37) over which was suspended a loud speaker 25 cm above the container (TOA Corp, Model F-121CM, Indianapolis, IN), and was driven by an oscillator (HP 200CD, Palo Alto, CA), with voltage or current levels monitored by a multimeter (Fluke 8000A, Wilmington, NC). The water chamber was enclosed inside of a custom built noise excluding custom-made box that contained noise silencing materials. The frequency of a continuous noise

exposure tone was 800 Hz, and at a level of 115 dB SPL at the water surface. A protective net was placed in the water to ensure swimming at a shallow depth of approximately 10 cm. It should be noted that if the protective net was not used, the fish would dive toward the bottom of the tank to perhaps escape the noise signal. The noise exposure total time was 1-day, 3-days or 5-days. The fish were then removed from the noise exposure chamber and immediately sedated (see above). A second phase of AEP testing (AEP after noise exposure) was initiated. Thus, fish were tested using the AEP after 1.0, 3.0 or 5.0 days of exposure to test for changes in various parameters of the AEP such as morphology, amplitude and latency. A control condition consisted of a sham experiment in which noise was withheld for 1.0 day that was conducted under similar conditions. The AEP under these conditions did not differ significantly for pre- and post-testing.

RESULTS AND DISCUSSION

Effects of stimulus intensity

Figure (2) shows the AEP as a function of stimulus intensity, in which the sound pressure level (SPL) of clicks were presented at 110, 90, 70, and 50 dB SPL (Re. 1.0 μPa). The repeatability of the electrophysiologic traces is close to 100% identical for the two separate replications at each intensity. That is, the superimposition of the replicates is seen to be highly repeatable. The waveforms at 110 dB SPL and 90 dB SPL show the five prominent peaks labeled: I, II, III, IV, and V, following the nomenclature for these waves recorded in humans [28]. A more robust or largest wave V peak, readily identifiable in humans, however, was not the most robust response in the zebrafish. This reflects a difference in the anatomical substrates responsible for generating the various peaks. We should note that the exact anatomical loci in the fish, however, has not been completely verified or compared to human and, thus, our waveform designations are at best - a first approximation with no precedence of the results of the literature [32].

The various waves or peaks can be seen to be superimposed on a negative - positive going slow wave that tends to swing back toward baseline at about 5.0 ms, and remains essentially steady-state for the remainder of the electrophysiologic trace of 10 ms. The same waveform peaks are present also at 90 dB SPL, albeit, there is a sharp decline of the amplitude of the peaks, and a prolongation of the latency for various peaks. At 70 dB SPL, only one – two, or perhaps three of the peaks can be visualized. At this intensity, peak II shows a prominent prolongation in latency. Thus, as the intensity of the stimulus was decreased, the latency of each peak increased (e.g., follow the vertical hashed line), and the amplitude of the waves decreased. At a level of 50 dB SPL, there were no visually detectable peaks. Increasing the sensitivity of the display failed to reveal any remnants of any of the peaks. Thus, input-output amplitude or latency functions could be derived for each of the waves as a function of intensity. The calibration bar for amplitude (μV) and latency (ms) are displayed under the 50 dB SPL traces.

We have determined using 10 or 5 dB steps that the threshold (data not shown) for the fish as determined by these peaks occurs between approximately 50 - 60 dB SPL. The sharp negative-positive transient at the beginning of the trace at 110

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dB SPL is from the radiation of the stimulus artifact (perhaps a combination of electromagnetic/electrostatic coupling) picked up by the electrode/amplifier interface at this high intensity level of stimulation, as its amplitude could be linked to the input impedance of the electrodes. That is, a higher input impedance would yield a comparable increase in the artifact. In earlier experiments, we attempted to suppress the artifact but found later its utility as an indicator of the electrostatic/electromagnetic coupling between the electrode/amplifier interface and the intensity of the input traveling wave. It is noted that the transient is not present at the lower intensities of the input stimulus, an indicant of less radiated voltage/current from the speaker. Intra-impedance of the three electrodes ranged from approximately 3 – 10 kOhms across the various conditions. When these AEP waveforms were recorded to different polarities of the stimulus, i.e., rarefaction phase vs. condensation phase, the averaged waveforms did not cancel out each other, thus, highly indicative of neural substrates underlying the various peaks, and not sensory or mechanical factors. At lower intensities, the waves tended to lose some of the sharp "peakness."

Effects of stimulus frequency

The AEP to various audio frequencies are depicted in Figure (3). The AEP traces were obtained at frequencies of 500, 1000, 2000, and 4000 Hz. Frequency tuning of the fish auditory system is evident, with underlying generators presumed to be unique to these test frequencies. The sigmoidal rise/fall times of each frequency was 1.0 ms with a peak duration of 3.0 ms. Control conditions to eliminate frequency and phase artifacts were routinely conducted. The entire audiogram derivation for each individual fish, however, was not attempted. This was done in a few fish, however, to test the frequency limits of our experimental system. Two replicates of the responses were obtained at each frequency tested. The intensity of the stimulus was held constant at 110 dB SPL, the repetition rate was held constant at 9.3/s, and the number of epochs averaged was 1024.

As can be seen (Figure 3), the most robust responses were obtained at 1000 and 500 Hz. At frequencies greater than 2000 Hz, the AEP is severely diminished in amplitude, although the latency of the responses occurs earlier in time. In words, as frequency is increased, the amplitude of the responses decreases, but there is a corresponding shift as an earlier occurrence of latency or onset of the frequency response. AEP responses to a frequency of 500 Hz is seen as a carrier frequency (stimulus-locked waveforms) and would appear to be multiple replicates of the individual responses to clicks. The first harmonics of the stimulus were down far enough (25 – 35 dB SPL) (power spectral data not shown) so that distortional products were not recorded. Thus, there was no evidence of frequency "splatter" at any of the frequencies tested (re.: 110 dB SPL). The familiar 4 – 5 peaks recorded to clicks are not present at the various frequencies tested. This is a reflection of the slower rise time (1.0 ms) of the various sinusoids. Psychophysical tuning of the responses was not measured but was presumed to be present from the electrophysiological recordings - zebrafish hearing show frequency selectivity from about 100 – 4000 Hz [10].

Effects of stimulus repetition rate

Given that we held the stimulus constant at a repetition rate

of 9.3/s while varying the intensity (Figure 2), we, likewise, held the intensity of the click stimulus constant at 110 dB SPL, and varied the repetition rate (Figure 4), using 3.1/s, 9.3/s, 19.3/s, 39.1/s, 67.1/s, and 91.1/s. The increasing repetition rates were chosen based on their periodicity lacking to appear as multiple integrals of the 60-cycle mains frequency. It is noted that the inter-stimulus interval has a corresponding decrease in latency to increasing repetition rates/decreasing inter-stimulus interval (e.g., 3.2/ 312 ms, 9.3/107 ms, etc.). At the lower repetition rates, the morphology of the responses is very similar to those of the intensity series. The 4 - 5 major peaks (i.e., I, II, III and IV) are present at the lower repetition rates, but the morphology of the peaks tend to alter their appearance at higher repetition rates (e.g., see at 91.1/s). As the repetition rate increased, the latency of the peaks increased (e.g., follow the dashed line intersecting initially wave II at 3.1/s up to the fastest rate tested of 91.1/s).

It is noted also (Figure 4) that the magnitude of the peaks decreased to a corresponding increase in repetition rate. The slow negative excursion appearing at about 4.0 ms (see horizontal line) is present, with the familiar swing toward positivity at about 5.0 ms. However, the slow negativity excursion shows a diminution of amplitude as a function of the increase in repetition rate, e.g., at 39.1/s or greater. Since various responses to various repetition rates show the dynamics of recovery of sensory neural elements, repetition rate is simply a corollary of inter-stimulus interval, provided the duration of the stimulus remains constant across the various repetition rates, evident for our data.

Effects of pentylenetetrazol (PTZ)

PTZ is used to create epileptic-type seizure activity in an animal model of epilepsy [38]; we have used it to create a tinnitus-like activity (increased brain or ear electrical activity) in an *in vitro* model of tinnitus [39]. Figure (5) displays the effects of the GABA antagonist and pro-convulsant, Pentylenetetrazol (PTZ), as a function of stimulus intensity.

The baseline AEP traces (green) were obtained to an intensity series at 110, 90, 70, and 50 dB SPL. The effects of the PTZ administrated at 100 M on the AEP are depicted by the red traces obtained at each of the intensities. A vertical line has been drawn to intersect wave I at the highest intensity level of 110 dB SPL, as a shift in the latency of the peak to the right of the vertical line is indicative of a delayed onset under the drug condition at various intensities. At 110 and 90 dB SPL, the reduction in amplitude and a delay in latency are readily apparent by the action of the drug (red traces). The effects of the drug even at 70 dB SPL is also readily apparent. Likewise, there is a decrease in amplitude and an increase in latency of the respective peaks due to the effects of the drug on the neural system of the fish. The slow negative wave of at approximately 4.0 ms is affected also by the PTZ, displaying a shallower trough, or reduced amplitude, for the drug-treated condition.

Effects of cisplatin

The effects of the cancer treatment drug Cisplatin were tested as an assay of neurotoxicity (Figure 6). Trials in which the drug was not present are displayed in green, while those obtained after exposure to a 300 μM concentration of Cisplatin are rendered in red. In the presence of Cisplatin, the latency of the peaks is shifted

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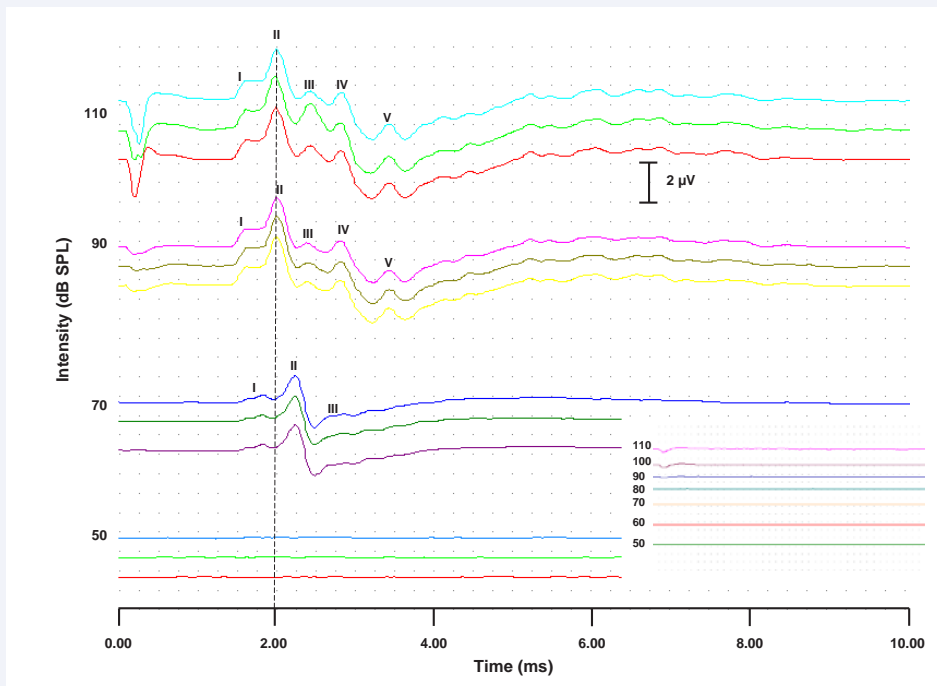
Figure 2 Auditory evoked potential (AEP) waveforms to intensity, recorded from the adult zebrafish (*Danio rerio*). The three traces at each intensity level represent three consecutive recordings to display the repeatability of the AEPs. Peaks of the response are labeled as waves I, II, III, IV, and V. Responses were obtained at intensity levels of 110, 90, 70, and 50 dB SPL. Peak II can be visualized down to 70 dB SPL. None of the peaks can be detected at 50 dB SPL. The inset displays traces obtained from a dead fish, confirming the electrophysiologic origin of the various waveform peaks.
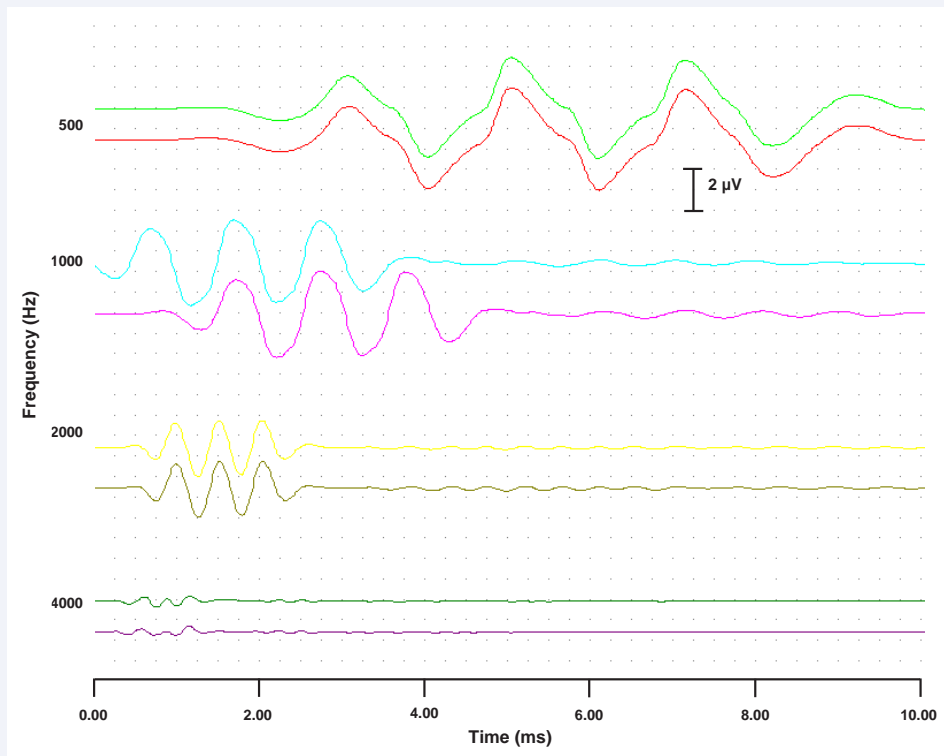
Figure 3 Auditory evoked potential (AEP) waveforms as a to frequency in the adult zebrafish (*Danio rerio*). Peaks in the waveform tend to phase lock as a function of the center frequency. The two traces at each frequency represent consecutive recordings and thus display the repeatability of the AEP. A total of 1024 individual averages constitute the composite response at each frequency. The repetition rate was 9.3/s. The filters were from 10 Hz - 5 x 10^3 kHz (-3 dB). The analysis time was 10 ms using 512 data points. A 0.1 ms square wave generated the acoustical signal of ~3.0 ms.
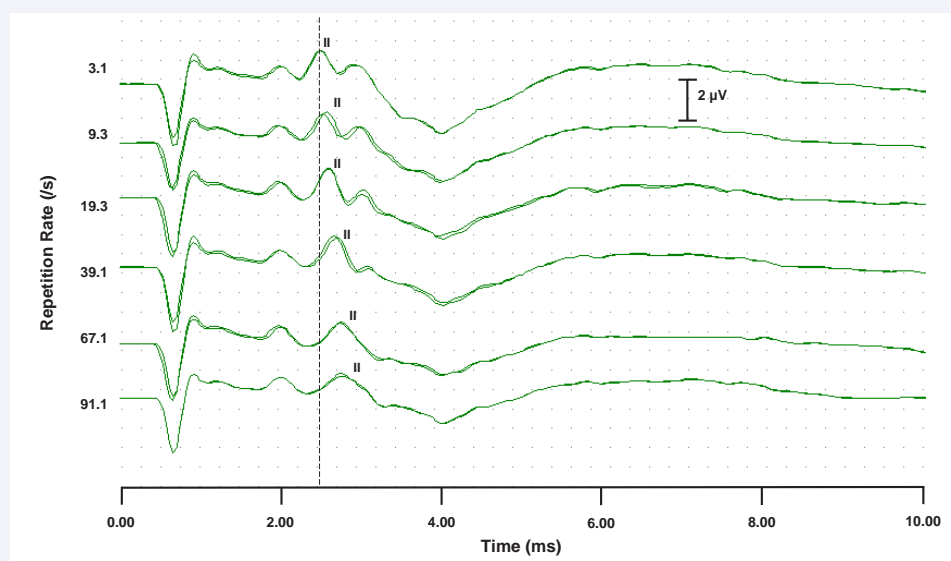
Figure 4 Auditory evoked potentials (AEP) as a function of repetition rate, recorded from the adult zebrafish (*Danio rerio*). Wave II is labeled. Averages were obtained at a repetition rate of 3.1/s, 9.3/s, 19.3/s, 39.1/s, 67.1/s, and 91.1/s. Peaks I, II and III can be visualized down to the fastest repetition rate of 91.1/s. A total of 1024 individual averages constitute the composite response at each repetition rate. The band Pass of the filters was from 10 Hz - 5 x 10^3 kHz (-3 dB). The analysis time was 10 ms using 512 data points.
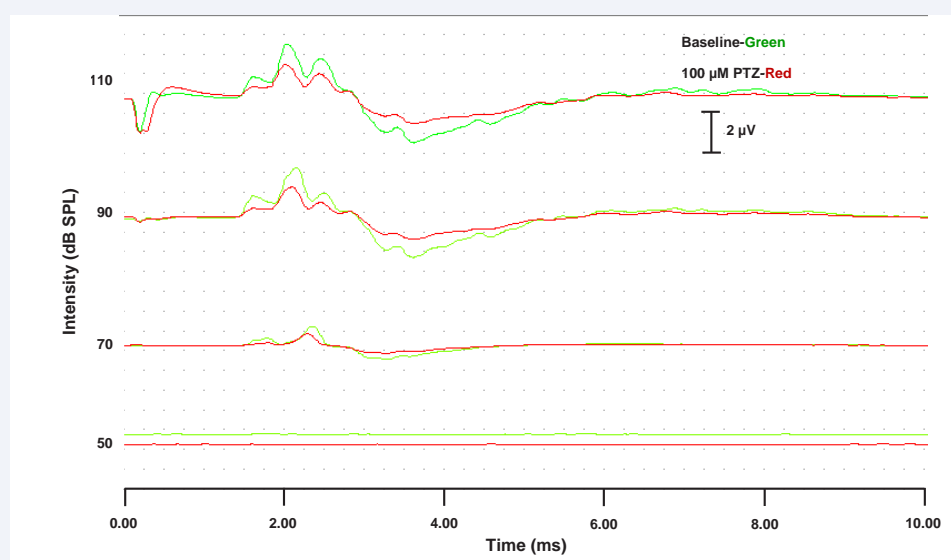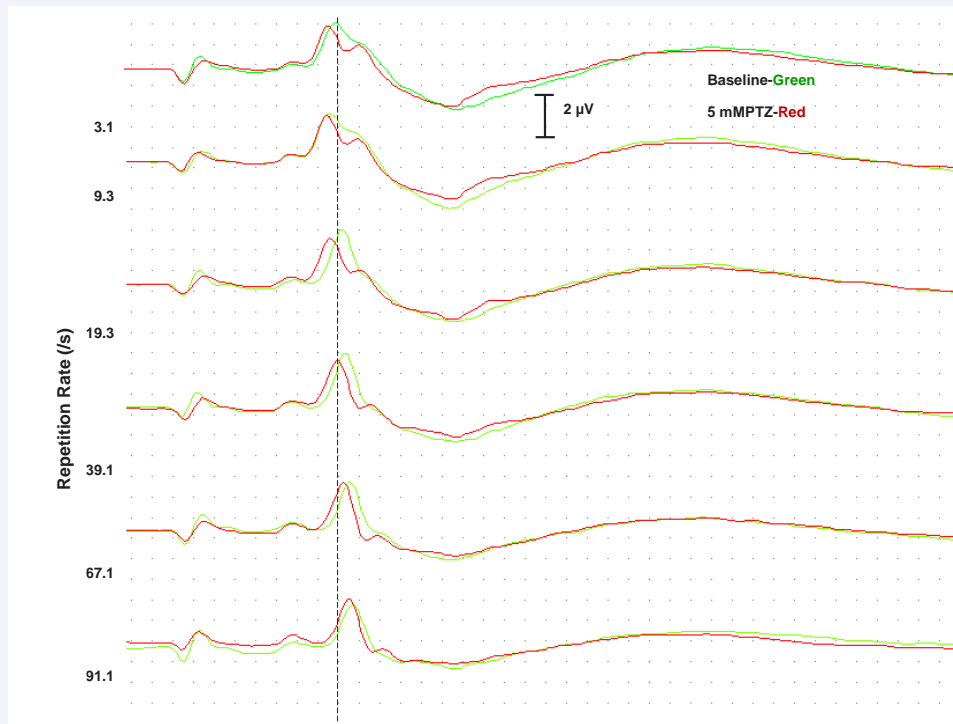

Figure 5 Auditory evoked potentials (AEP) to the application of 100 µM Pentylenetetrazol (PTZ), recorded from the adult zebrafish (*Danio rerio*). The green trace represents baseline, the red trace is post PTZ treatment. AEP averages were obtained at a repetition rate of 9.3/s. Peaks I, II and III can be visualized down to an intensity level of 70 dB SPL. A total of 1024 individual averages constitute the composite responses at each intensity level. The band Pass of the filters was from 10 Hz - 5 x 10^3 kHz (-3 dB).

slightly to the right in the form of a delayed response. There is a detectable decrease in the amplitude of the response so that at 70 dB SPL, a small almost undetectable wave I is present, while wave II is shifted to the right of the vertical interrupted line. Responses were not detectable at control and drug treated condition at 50 dB SPL. The slow negative-positive wave was affected also showing a decrease in amplitude in the presence of the drug.

Effects of gentamicin

For comparison to other published work we also tested the aminoglycoside antibiotic Gentamicin that is primarily used to treat gram positive bacterial infection. Typical results are displayed in Figure (7). During the control or baseline recordings (green), the complex of waves I, II, III and IV are readily apparent at the highest intensities of 110 dB SPL, and I, II, and III at 90 dB SPL. Under these conditions, only a wave I remnant could be detected at 70 dB SPL. When the animal is exposed to the 500 µM concentration of Gentamicin (red traces) the latency is prolonged and the amplitude is decreased, especially at 110 dB SPL. Unlike the responses to PTZ or Cisplatin, the four major peaks of the AEP

Figure 6 Auditory evoked potentials (AEP) as a function of 300 µM Cisplatin, recorded from the adult zebrafish. Waves are labeled using the nomenclature of I, II, II, and IV. The green trace represents baseline; red trace is post Cisplatin treatment. AEP averages were obtained at intensity levels of 110, 90, 70, and 50 dB SPL. Repetition rate was 9.3/s. Peak II can be visualized down to an intensity level of 70 dB SPL. 1024 individual averages constitute the composite responses for baseline or treatment. The band Pass of the filters was from 10 Hz - 5 x 10^3 kHz (-3 dB).
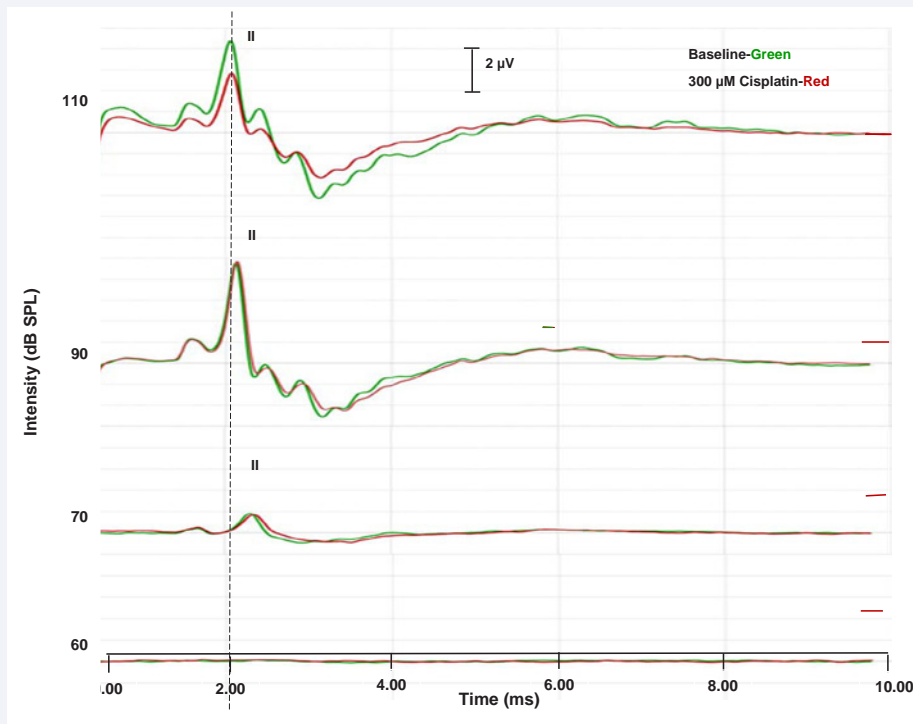
Figure 7 Auditory evoked potentials (AEP) to 500 µM Gentamicin, recorded from the adult zebrafish. The green trace represents baseline, while he red trace is post Gentamicin treatment. AEP averages were obtained at intensity levels of 110, 90, 70, and 50 dB SPL. Repetition rate was 9.3/s. Peak II can be visualized down to an intensity level of 70 dB SPL. A total of 1024 individual averages constitute the composite responses at baseline and treatment. The band pass of the filters was from 10 Hz - 5 x 10^3 kHz (-3 dB). The analysis time was 10 ms using 512 data points.

were not detectable at 90 dB SPL. Thus, none of the AEP peaks were detectable at or below 70 dB SPL. The slow negative wave at about 4.0 ms is severely diminished under the drug condition at 110 dB SPL. These modes of action on the amplitude and latency of the various peaks of the AEP at high intensity levels were perhaps mediated by the prevention of the generation and conduction of the depolarized nerve impulses within the zebrafish neuraxis that are responsible for the appearance of the various peaks.

Effects of noise over-exposure

The typical pattern of AEP waves I – V were recorded prior to the exposure of the fish to noise. Upon completion of baseline collection of data, the fish was placed in the noise exposure chamber and exposed to a continuous 400 Hz pure tone of 115 dB SPL. AEPs were recorded at baseline, 1.0 - day post - exposure, at 3.0 - days post - exposure, and 5.0 - days post - exposure. All post-exposure recordings consisted of AEP responses at an intensity series of 110, 95, 80, and 65 dB SPL. These data are displayed in Figure (8).

A second phase of AEP testing (AEP > noise exposure) was commenced to determine the temporary threshold shift exhibited by latency and amplitude. Since the AEPs were similar over the various periods of exposure showing synchronous activity at all intensity levels, it was assumed that the noise and time of exposure did not diminish the ability of underlying elements responsible for synchrony. However, it is noted that the robustness of the various peaks tended to diminish more so when

exposed over a 5-day period of exposure as compared to, e.g., the 1- day of exposure. Since the AEP is surmised to occur from a large population of synchronous neurons, the reduction in amplitude after 5-day exposure perhaps posits that the full complement of neurons is not available or incapable of responding after the longer times of exposure [40].

The effects of various stimulus parameters, effects of various drugs, and a noise over exposure regimen on the AEP of fish were quite remarkable and predictable. This overall high reliability of the recordings made it advantageous to use the AEP responses as indicants of auditory sensory neural processing or encoding within the fish auditory sensory neural system. Thus, we were able to interpret the data based on the effects of stimulus parameters such as intensity, frequency, and repetition rate, effects of drugs, as well as the effects of noise over exposure to a noxious stimulus. Testing under these conditions caused a down-regulation of the amplitude of the AEP and reflected as a decrease in latency.

We characterized the AEP from the zebrafish while varying stimulus parameters. We then treated the fish with various ototoxic or neurotoxic agents. These tests individually and in combination resulted in changes in the morphology, latency and amplitude of the AEP responses. That is, the ototoxic drugs reduced the amplitude and prolonged the latency of the responses. Finally, we demonstrated that the fish is sensitive to noise over-exposure that was presented continuously over various periods of time. Thus, there is a direct relationship between the amount of time of the exposure to a decrement in the amplitude of the
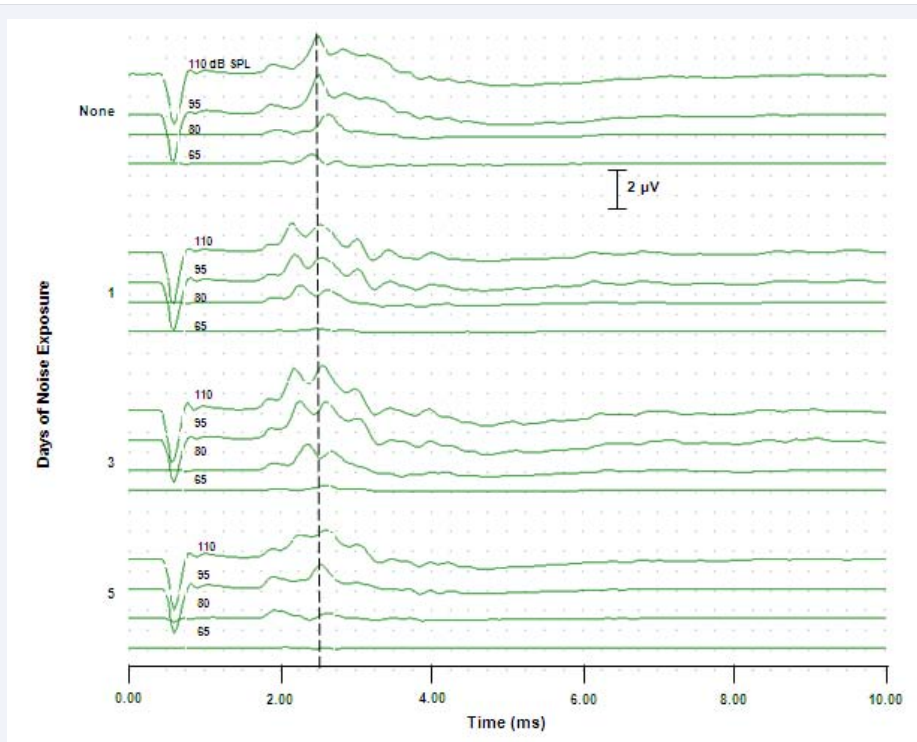
Figure 8 Auditory evoked potentials (AEP) waveforms to noise exposure from the adult zebrafish (*Danio rerio*). AEPs were obtained at intensity levels of 110, 95, 80, and 65 dB SPL, with no noise exposure, 1 - day noise exposure, 3 - days noise exposure, and 5 - days noise exposure. The noise exposure was a continuous tone of 800 Hz, at 115 dB SPL. Repetition rate was 9.3/s. Peak II can be visualized down to an intensity level of 70 dB SPL. A total of 1024 individual averages contribute to the composite responses.

response, as well as an alteration in the latency or onset of the various peaks of the AEP. We thus demonstrated that the AEP can serve as a sensitive assay of the function/dysfunction of the sensory neural auditory system of which the fish uses for monitoring the surrounding environs of its water milieu. These data are highly consistent with previous reports that have used the zebrafish AEP as a test of auditory function [4,7,8,10,13,32]. Moreover, our results demonstrate that the click stimulated AEP can increase the sensitivity of the electrophysiological response.

As previously found in humans, as well as in zebrafish, waves I – V of the AEP are perhaps a series of successive depolarization (summation of EPSPs, APs, far-field potentials, or a combination of all) of inner ear and auditory/motor structures. It should be noted, however, that the zebrafish does not possess a brainstem, thus, designation of the response as an AEP, rather than an auditory brainstem response seemed appropriate. We have no direct experimental proof, and only speculate that Wave I is thought to arise at the level of the statoacoustic nerve innervating the saccules and utricle [20]. Some investigators have reported that a wave I' can be recorded in mammals that precedes wave I with appropriate experimental manipulations to control stimulus artifact [41-47]. Whether the wave I seen in fish recordings is perhaps wave I' is unsupported. It has been speculated that I' is derived from the distal auditory nerve dendrites, thus, indicative of an EPSP or summating potential [42,46]. We were able to rule out the origin of wave I as a microphonic response (nee "cochlear microphonic" in mammals) response since it is readily present and robust to alternating stimuli, thus, there was no phase reversal of our responses to different phases of the click stimuli.

Wave II is surmised to be generated by the Mauthner cell (or M-cell) that serve as an input to the CNS via the statoacoustic nerve. Wave III and IV are perhaps generated by brain stem and motor nuclei since it is not unusual to elicit the C-bend reflex to an intense stimulus such as the maximum signal used in this investigation [2]. The stimulus parameters of intensity, frequency and repetition rate (the latter, the corollary of inter-stimulus interval, or ISI) proved to be a critical independent variable of which the zebrafish depicts AEP dependence [4]. Waveform morphology, latency of the peaks, and the amplitude of the peaks exhibit well – defined changes that can be quantified. For example, the morphology or appearance of the AEP to various experimental manipulations can be seen to undergo an alteration in appearance. There is a change in latency to reflect shorter or longer latency depending on stimulus amplitude, i.e., as the intensity of the stimulus is increased, the latency will decrease, and vice versa, while the amplitude will increase. Frequency of the stimulus exerts its influence by showing the tendency to "frequency follow", or Phase-lock especially to a low frequency of, e.g., 500 Hz, up to 4000 Hz, although amplitude is severely diminished beginning at approximately 2000 Hz. The rise time of the various frequencies can be seen to be reflected in a shorter latency as the stimulus is increased in frequency. Likewise, the repetition rate or inter-stimulus interval, also reflects changes in latency and amplitude. As repetition rate increased or inter-stimulus interval decreased, the latency of the AEP responses increased, with a corresponding decrease in amplitude. Showing a reliable trend in the stimulus dependence of the vertex positive AEP peaks of three stimulus parameters in untreated zebrafish,

led us to explore the effects of three highly water-soluble drugs – Pentylenetetrazol (PTZ), Cisplatin, and Gentamicin – using the stimulus same stimulus parameters while recording the AEP.

At 100 µM, PTZ decreased the amplitude of the AEP waves *in vivo*. This suggests that the drug is acting as an antagonist. There was a noticeable increase in latency after PTZ drug application. This is consistent with inhibition of GABAergic components, as it binds to the picrotoxin site of the GABA-A receptor complex. Another mode of mechanism to consider may be the PTZ influence on neuronal ion channels via calcium and sodium influx, modulating effects that would tend to depolarize available neurons. Of interest also to these inferences is that PTZ modulates also calcium channels, causing them to lose their calcium selectivity, and are replaced by a sodium conductance [48].

Cisplatin (a platinum-based chemotherapy drug) exhibited a similar effect on the potentials as that of PTZ. Its mechanism of action is aquation in cells in which chloride ligands eventually displace water, and allows the platinum atom to bind to bases, such as guanine, but also purine [49]. Its action on the AEP was to decrease the amplitude and prolong the latency of the AEP. These relationships are more than likely causing Gentamicin to act in this instance as an antagonist [50].

We were tempted to use the nomenclature of I' for the first peak designated as wave I, as the I' potential in humans and other animals is presumed to be generated by the dendrites of the auditory nerve. Thus, a possible reduction in the amplitude of I' is perhaps indicative of an effect of the drug on the neural projection of the peripheral auditory nerve prior to its myelinated part. Given that the pathway is more than likely wired in series, a reduction of the input from the hair cells to the Mauthner cell (M-Cell) would be reflected in the subsequent waves II – V, thus, being perhaps a reflection of various levels of nuclei of the sensory and motor pathway leading from the M-cell to the spinal tract. Generator potentials, both spontaneous and evoked, have been recorded from VIIIth nerve saccular fibers of the goldfish [5], as well as patch clamp lateral line neuromast hair cells [51].

CONCLUSIONS

Sensory neural processes underlying three physical parameters of the auditory signal were used to characterize the AEP in zebrafish. The intensity of the stimulus was surmised to be related to the spatial configuration of sensory neural activity in active neurons and fiber tracts. The frequency of stimulation was surmised to be related to loci of stimulation along the utricle and saccules as tonotopicity is present in these structures, with subsequent tuning further along the afferent tract sub-serving sensory neural and motor tracts. The variable of time in the form of the repetition rate or its intrinsic counterpart, inter-stimulus interval, is related to the integration of auditory energy and the relative distribution of excitatory, as well as perhaps inhibitory nets [52]. Three drugs were tested (PTZ, Cisplatin, Gentamicin) to chemically perturb the auditory system (in other words, force it from a presumed "state of equilibrium"). Furthermore, a noise paradigm that exposed fish for various periods of time, resulted in a diminution in functioning [53]. Thus, the basic assumptions posited herein for these fundamental observations must give

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credit to basic concepts of electrophysiology, dependent upon the recordings in zebrafish, as it serves as a robust biological model [21] to investigate auditory sensory neural functioning using the AEP components.

Authors Contributions

Conceived idea for research (EM, TL), designed research (EM, TL, DL), performed experiments (MB, DL, DP, CP, GR), data analysis (MB, DL, CP, DP, GR, EM), wrote the paper (EM, TL).

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