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Evaluation of Auditory Brainstem Response in Chicken Hatchlings

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Abstract

The auditory brainstem response (ABR) is an invaluable assay in clinical audiology, non-human animals, and human research. Despite the widespread use of ABRs in measuring auditory neural synchrony and estimating hearing sensitivity in other vertebrate model systems, methods for recording ABRs in the chicken have not been reported in nearly four decades. Chickens provide a robust animal research model because their auditory system is near functional maturation during late embryonic and early hatchling stages. We have demonstrated methods used to elicit one or two-channel ABR recordings using subdermal needle electrode arrays in chicken hatchlings. Regardless of electrode recording configuration (i.e., montage), ABR recordings included 3–4 positive-going peak waveforms within the first 6 ms of a suprathreshold click stimulus. Peak-to-trough waveform amplitudes ranged from 2–11 μV at high-intensity levels, with positive peaks exhibiting expected latency-intensity functions (i.e., increase in latency as a function of decreased intensity). Standardized earphone position was critical for optimal recordings as loose skin can occlude the ear canal, and animal movement can dislodge the stimulus transducer. Peak amplitudes were smaller, and latencies were longer as animal body temperature lowered, supporting the need for maintaining physiological body temperature. For young hatchlings (<3 h post-hatch day 1), thresholds were elevated by ~5 dB, peak latencies increased ~1–2 ms, and peak to trough amplitudes were decreased ~1 μV compared to older hatchlings. This suggests a potential conductive-related issue (i.e., fluid in the middle ear cavity) and should be considered for young hatchlings. Overall, the ABR methods outlined here permit accurate and reproducible recording of *in-vivo* auditory function in chicken hatchlings that could be applied to different stages of development. Such findings are easily compared to human and mammalian models of hearing loss, aging, or other auditory-related manipulations.

SUMMARY:

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DISCLOSURES:
The authors have nothing to disclose.

We have used standard auditory brainstem response (ABR) techniques and applied them to hatchling chickens, a precocious avian model for auditory function. The protocol outlines animal preparation and ABR acquisition techniques in detail, with steps that could translate to other avian or rodent models.

Keywords

auditory brainstem response; ABR; hearing threshold; central auditory processing; electrophysiology; auditory pathway; chicken

INTRODUCTION:

The study of evoked neural responses to sound stimuli dates back over half a century[1]. The auditory brainstem response (ABR) is an evoked potential that has been utilized as a measure of auditory function in both non-human animals and humans for decades. The human ABR presents with five to seven waveform peaks conventionally labeled by Roman numerals (I-VII)[2]. These peaks are analyzed based on their latency (time of occurrence in milliseconds) and amplitude (peak-to-trough size in microvolts) of the neural responses. The ABR is instrumental in evaluating the function and integrity of the auditory nerve and brainstem, as well as hearing threshold sensitivity. Deficits in the auditory system result in absent, reduced, prolonged, or abnormal ABR latencies and amplitudes. Remarkably, these parameters are nearly identical in humans and other animals, making it a consistent objective test of auditory function across vertebrate models[3].

One such model system is the chicken, and it is especially useful for a variety of reasons. Birds can be classified as altricial or precocial[4]. Altricial birds hatch with senses still developing; for example, barn owls do not show a consistent ABR until four days post hatch[5]. Precocious animals like the chicken hatch with near mature senses. The onset of hearing occurs in embryonic development, such that days before hatch (embryonic day 21), the auditory system is near functional maturation[6–8]. Altricial birds and most mammalian models are susceptible to extrinsic factors that influence the development and require animal husbandry until the hearing is mature. Chicken ABRs can be performed the same day as the hatch, forgoing the need for feeding or an enriched environment.

The embryonic chicken has been a well-studied model for physiology and development, especially in the auditory brainstem. Specific structures include the chicken cochlear nucleus, divided into nucleus magnocellularis (NM) and nucleus angularis (NA), and the avian correlate of the medial superior olive known as nucleus laminaris (NL)[6,7]. The ABR is ideal for focusing on central auditory function before the level of the forebrain and cortex. Translation between *in-vivo* ABR measurements and *in-vitro* neuronal studies of development[8], physiology[9], tonotopy[10], and genetics[11,12] provides ideal research opportunities that support studies of overall auditory function.

Although the ABR has been extensively studied in mammalian models, there has been less focus for avians. Previous avian ABR studies include characterizations of the budgerigar[13], woodpecker[14], seagull[15], diving birds[16], zebra finch[17], diurnal raptors[18], canary[19], three

species of owl[5,20–22] and chicken[23]. Given the nearly four decades since the last thorough characterization of the chicken ABR, many of the equipment and techniques previously used have changed. Insights from studies in other avian models can help develop modern chicken ABR methodology while also serving as a comparison to the chicken ABR. This paper will outline the experimental setup and design to allow for ABR recording in hatchling chickens that could also be applied to embryonic stages of development and other small rodent and avian models. Additionally, given the precocious development of the chicken, developmental manipulations can be performed without any extensive animal husbandry. Manipulations to a developing embryo can be evaluated just a few hours after the animal hatches with near mature hearing capabilities.

PROTOCOL:

The experiments described here were approved by Northwestern University's Institutional Animal Care and Use Committees (IACUC) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1. Chicken husbandry

1.1 Acquire fertilized white leghorn chicken eggs.

NOTE: There are several chicken breeds used in scientific research, but the results shown here are from white leghorn chicken (*Gallus gallus domesticus*). While ABR variability between breeds is unknown, some differences have been found when comparing adult egg-laying chickens to meat-producing broiler chickens[24,25].

1.2 Incubate eggs at 38 °C, humidity at 50%, for 21 days before the desired testing date.

NOTE: If eggs are not immediately incubated at 38 °C, they can be stored at 14 °C, humidity at 40%. However, the longer eggs are kept at 14 °C, the less likely they are to develop into viable hatchlings. Egg viability can drop as low as 50% depending on how long eggs are kept at 14 °C. Egg viability will also drop in the winter months.

1.3 Periodically turn the eggs 2–3 times a day. Most incubators have a mechanism to perform this automatically.

1.4 If using a Styrofoam incubator or an incubator holding more than 6 eggs, transfer eggs to a small 38 °C incubator the day before hatch, embryonic day 20 (E20). Eggs should hatch 21 days (E21) after being set in the incubator.

NOTE: In the hatching process, the animal will begin "pipping" out of the egg, making a small hole that eventually goes around the entire egg. If conditions are too dry, the egg can dry up, and the animal will be unable to hatch. The humidity should be kept around 50%, based on previous studies on egg hatching viability[26–29].

1.5 Determine the animal's age. If the hatch is not witnessed in person, the only indication of age is the 2–3 h it takes for the amniotic fluid to dry.

NOTE: The hatchling incubator should be thoroughly cleaned daily based on how many hatchlings are processed. Chicken hatchlings often leave excrement, feathers, and amniotic fluid in the incubator, which can contaminate conditions and air quality.

2. Drug preparation

2.1 Weigh the animal by placing it in a large weighing boat. With a gentle enough placement, the animal should not move.

NOTE: Mass can range from 30–45 g. Younger animals are often heavier because of yolk reserves and not yet excreting waste. Older animals approaching 24 h of age and P2 usually weigh less.

2.2 Prepare an anesthetic cocktail of Ketamine (100 mg/mL) and Xylazine (20 mg/mL) such that the dosage is 50 mg/kg Ketamine and 16.68 mg/kg Xylazine based on animal weight.

NOTE: This drug cocktail can be made with 1 mL of Ketamine (100 mg/mL), 1.5 mL of Xylazine (20 mg/mL), and 2.5 mL of H_2O. Anesthetic cocktail injections will range from 0.05–0.1 mL based on the 30–45 g range in animal weight.

3. Drug injection and animal prep

3.1 Hold the animal in one hand, making sure to hold the legs down.

3.2 Feel for the breastbone of the animal, the keel. On either side of the keel will be breast muscle.

3.3 Use a 29-G needle and syringe to penetrate 5 mm into the skin and inject the Ketamine/Xylazine cocktail into the breast muscle. Inject between 0.05–0.1 mL based on animal weight.

3.4 Place the animal back in the incubator after injection. Maintain animal body temperature for a few minutes as the anesthetic takes effect.

3.4.1 Use forceps to pinch the toe of the animal and check if the neck is limp. If there is no reflex and a limp neck, the animal is unconscious.

3.5 Determine the sex of the chicken using its wing feathers. If the feathers are all the same length, the animal is male. If the feathers vary in length, the animal is female[30].

NOTE: Another method of sexing the animal is venting. The male genitalia can be seen in the cloaca[31]. This method is very difficult and can harm the animal if not done correctly. It is recommended to use the wing feather method.

3.6 Apply depilatory cream with a cotton tip applicator to the head and neck area, especially near the ear opening for the bird.

3.7 Use 70% isopropyl alcohol wipes to wipe off feathers, any remaining depilatory cream, and the skin on the head and neck.

3.8 Use a 70% isopropyl alcohol wipe to sterilize the subdermal electrodes and rectal probe.

3.9 Place the animal in a sound isolation and electrically shielded chamber. Ensure that the environment has minimal electrical and acoustical noise for the best recordings.

NOTE: The experiments here were made in a custom sound isolated enclosure measuring 24 × 24 × 25 inches. Any chamber or room that eliminates acoustical noise, as well as electrical noise from alternating electric current (60 Hz in the United States), is sufficient.

3.10 Use a heating pad or temperature control system to maintain animal body temperature.

3.11 Insert the rectal probe to ensure the animal's temperature is maintained between 37–41 °C (98.6–105 °F)[32,33].

NOTE: If the probe is an incorrect size, the animal can lay on top of the temperature probe.

3.12 Fix the animal's head in place or rest the beak against an object to avoid unwanted movement. This can be done with modeling clay if breathing is not obstructed.

3.13 Administer a supplementary injection of anesthetic cocktail that is one-half the original dosage if the animal begins to regain consciousness during testing.

NOTE: Any body movement or vocalization is a sign that a supplement dosage must be administered. Miniscule beak movements indicate breathing and are acceptable.

4. Electrode placement

4.1 Use three stainless steel, silver chloride needle electrodes with the following designations: the reference electrode, the active electrode, and the common ground electrode.

NOTE: The reference electrode is also referred to as inverting or "-". The active electrode is also referred to as noninverting or "+".

4.2 Place each electrode sub-dermally 2–3 mm into the head, but not deep enough to penetrate the skull.

4.3 Poke the electrode out of the skin, exposing the tip. This helps to minimize contact with the skin and ensure consistent insertion depth across animals[34].

NOTE: The electrode wire should have sufficient slack such that after placing the electrode, there is no tension that will pull it out or pull the skin taut.

4.4 For single-channel recording, place the active electrode above the skull at the midline, as far caudal as the ear canal.

4.4.1 Place the reference electrode behind the ear where the stimulus will be delivered, and place the ground electrode behind the contralateral ear in the neck.

NOTE: If performing surgery at the skull or ear canal of the animal, place the reference electrode in the neck at the midline of the animal. Both this and step 4.4.1 are considered horizontal electrode recording montages.

4.5 For two-channel recording, use two negative electrodes and a combined positive electrode that requires an adapter cable. Place the ground electrode subdermally in the neck and one reference electrode behind each ear canal.

4.6 Check the electrode impedance. Ensure that the overall electrode impedance does not exceed 5.0 kΩ. Maintain the interelectrode impedance below 3.0 kΩ.

5. ABR recording

5.1 Depending on acquisition hardware and software, be sure to perform calibration for correct sound levels across stimulus frequencies used.

NOTE: Calibration techniques will vary based on equipment (see discussion). For some programs, sound attenuation can be edited within the software. Calibration procedures performed here involved using a 1/8-inch B&K 4138 condenser microphone to record frequency stimuli within a closed coupler system that approximated the chick ear canal (~5 mm). A chicken hatchling calibration table is provided as a Supplemental Table.

5.2 Move the sound transducer apparatus toward the active ear of the animal. Place the sound transducer at a shallow depth of 2 mm in the ear canal.

NOTE: Depending on the sound transducer, a plastic speculum can be attached and inserted into the ear canal. The speculum placement is critical. If the sound is blocked by the canal wall or the ear canal is pinched shut, ABRs will be absent or resemble a ~40 dB shift in the threshold.

5.3 Check on the animal during testing if results look abnormal or absent. If they are, reposition the sound transducer in the ear canal.

NOTE: Since the skin is loose and animal movement is possible, the speculum placement may shift during recording. However, with correct anesthetic injection and the animal fully unconscious, recording can go uninterrupted for 30–45 min.

6. Data acquisition

6.1 Utilize sufficient equipment/software to generate sound stimuli and record/acquire ABR recordings.

NOTE: There are many commercially available or custom systems for ABR acquisition. For these experiments, the commercially available Intelligent Hearing Systems (IHS) SmartEP USB platform was used. The ability to manipulate recording parameters is critical; these include, but are not limited to stimulus intensity, stimulus length, stimulus frequency, stimulus presentation rate, high pass and low pass filter, artifact rejection, number of sweeps, sampling rate, envelope shape, and stimulus polarization.

6.2 Set the artifact rejection (AR) upper and lower limits to ±25 μV, such that animal movement or noise during a sweep will exclude that sweep from the analysis. Across the population tested, less than 1% of total sweeps were rejected due to artifacts.

6.3 Collect at least 1024 sweeps to obtain a grand averaged response. This can be done in two recordings of 512 sweeps each. This also ensures that the response is stimulus-evoked and repeatable.

6.4 Set the gain to 100,000, the low pass filter to 100 Hz, and the high pass filter to 3000 Hz.

NOTE: The low and high pass filter settings were optimal for recordings using the IHS system. Therefore, these parameters are recommendations. ABR recordings in other avian species using the BIOSIG software filtered the signal between 30 and 3000 Hz [5,13,14,16,22].

6.5 Set the stimulus presentation rate between 10 and 20 stimuli per second. High presentation rates will shift ABR peak latency, especially for later peaks[13]. Low presentation rates will increase the time required to acquire the ABR.

6.6 Set the time duration of the click stimulus to 100 μs.

6.6.1 If using a tone burst stimulus, edit the frequency and duration of the stimulus based on the desired effect. A range of 100–4000 Hz was used for tone burst stimuli, although the range of behavioral hearing in adult chickens ranges from 2–9000 Hz[35].

NOTE: In the IHS system, the rise and fall time of a tone burst stimulus can only be modified if the spectral envelope shape is a trapezoid. However, the cosine squared and Blackman envelopes provide a preset rise and fall time that is commonly used in animal ABR experiments. The IHS system can display the spectral envelope of a tone burst to ensure appropriate rise and fall times. The rise and fall time of a click stimulus cannot be edited in IHS.

6.7 Set the sampling rate to the highest value allowable (usually 40 kHz) for the best resolution data.

NOTE: Some systems, including IHS, use a limited number of sampling points and will alter the length of the recording window. A 40 kHz (25 μs period) sampling rate may only allow for a 12 ms recording window, so to capture a tone burst ABR, a 20 kHz sampling rate (50 μs period) was used to allow for a 24 ms recording window. If directly comparing click and tone burst ABRs, keep the sampling rate constant to maintain the same resolution.

6.8 Set the stimulus polarization to alternating. This is done to eliminate the visualization of the cochlear microphonic from ABR recordings. To visualize the cochlear microphonic, use rarefaction or condensation for stimulus polarity.

NOTE: Many settings can be changed when selecting stimuli. The gain and filter settings provided may not be optimal for other equipment setups. Factory defaults on most ABR machines are not set for recording in hatchling chicken.

6.9 If recording 512 sweeps, combine two separate tests to create a 1024 sweep average.

6.10 For a click or tone burst stimulus, acquire an ABR at a suprathreshold intensity.

6.11 Continue recording at lower and lower intensities until the evoked response can no longer be identified.

6.12 Define ABR threshold as the lowest stimulus intensity that elicits a detectable evoked response. Lower the stimulus intensity by steps of 5 dBSPL to find the lowest stimulus intensity that elicits a detectable peak.

7. Euthanasia and experiment end

7.1 Once ABRs are acquired, prepare an overdose (0.1 mL) of euthanasia solution (Pentobarbital Sodium 390 mg/mL Phenytoin Sodium 50 mg/mL).

7.2 Inject the euthanasia solution in the breast muscle with a 29-G needle at a 5 mm depth. The injection technique is the same as the anesthetic injection.

NOTE: The animal will expire after a few minutes. Do not manipulate or decapitate the animal until no movement is detected.

7.3 As soon as the animal is not reflexive, rapidly decapitate with sharp scissors or shears.

7.4 Clean the heating pad, rectal probe, and silver chloride electrodes with 70% isopropyl alcohol wipes.

7.5 Make sure all acquired traces have been saved. For further analysis, export files as .txt files which can be viewed in notepad or imported into a spreadsheet.

REPRESENTATIVE RESULTS:

Representative ABR recordings for hatchling chicks

The following representative and population results come from ABR recordings made in 43 animals. In response to a suprathreshold click stimulus (75 dBSPL), three positive-going peaks were consistently observed across all hatchlings. These peaks occurred within 6 ms after stimulus onset. Infrequently, a fourth peak was also observed at ~6 ms. While the identification of ABR peaks in birds varied among animals (see discussion), peaks were labeled and identified as Roman numeral Waves I–IV. A representative ABR waveform with labeled peaks is shown in Figure 1A (top trace). Figure 1B shows the latency-intensity function for Waves I and III labeled in the representative trace. Wave I peak latency increased by ~0.3 ms for each 20 dB decrease in stimulus intensity. On average, Waves I–III occurred at 1.50 ms (±0.02 ms), 3.00 ms (±0.06 ms), and 4.13 ms (±0.09 ms) at 75 dBSPL, respectively (Figure 1C). Wave I and Wave III always presented as a singular peak. Occasionally for Wave II, multiple small peaks were seen between 2.5–3.2 ms. Each peak had a corresponding trough, and the peak-to-trough amplitude of Wave I – the largest of all the peaks – averaged 7 μV and approached a maximum amplitude of 11 μV at 75 dB SPL.

In addition to the largest amplitude, Wave I of the chick ABR presented with the least variability in peak latency among animals. Therefore, this peak was used to estimate hearing threshold sensitivity. ABR thresholds were defined as the lowest stimulus intensity that elicited an identifiable and repeatable waveform peak. This was subjectively determined by the experimenter and cross-checked by a second experimenter for threshold agreement. Peaks were better defined and easier to identify when using click stimuli, but tone bursts also generated defined and identifiable peaks that varied depending on stimulus frequency and its parameters (Figure 1D, n = 4 chicks). The click-evoked ABR threshold was lower than the tone burst evoked threshold, with the exception of 1000 Hz. Thresholds varied between 10–30 dBSPL for click stimuli. Click-evoked ABRs that did not show identifiable peaks >30 dBSPL were often the result of the speculum becoming dislodged from the ear canal due to animal movement.

Decreased body temperature increases ABR latencies

The speed of neural activity – as measured by the peak occurrence of a waveform amplitude (i.e., latency) – is known to decrease at lower body temperatures[36,37]. This phenomenon was observed in hatchling chicken ABRs using a 75 dBSPL click stimulus. A representative trace is shown in Figure 2A. As body temperature decreased from 39 °C, the latency of ABR peaks occurred later in time, despite the same stimulus intensity level. Figure 2B shows the latency of Waves I and III as a function of lower body temperatures for the representative trace. There was a strong correlation ($R^2 = 0.89$) between lower body temperatures and the occurrence of Wave I peak latency (Figure 2C, n = 5 chicks). These results demonstrate the need for maintaining a near-normal body temperature during ABR recordings. If near-normal body temperature is not maintained, latency-intensity functions and amplitude measurements of the ABR are highly variable and often inaccurate.

Latency and amplitude differences in early hatchlings

Research has shown that neural activity related to the onset of hearing for the chick is near maturation at late embryonic ages[8]. However, for a subset of very early hatchlings (<3 h post-hatch), we observed a peak latency shift of ABR waveforms (n = 4) in response to a 75 dB SPL click stimulus or evoked potentials were not identifiable (n = 2 chicks). In 2 young hatchlings, no tone burst ABR could be elicited, and click thresholds were elevated by 50 dBSPL. This could be due to a conductive issue where there is still fluid in the ear canal/middle ear cavity of the animal, or an underdeveloped neural component. Mammalian studies have reported threshold shifts of 50 dB in newborns[38,39]. Representative animals used here were >3 h old, which also coincided with the length of time it takes for the feathers to dry. Figure 3A shows ABRs recorded from young (P1, <3 h old) and older hatchlings (P2). For analysis, only 3 young hatchlings presented with all three ABR peaks. Peak waveform latencies were significantly prolonged, and waveform amplitudes were slightly reduced when compared to older hatchlings (Figure 3B–C, respectively).

Reference electrode placement and two-channel ABR recordings

In Figure 4, the reference electrode placement was modified between 2 different locations but still resulted in comparable ABR recordings. A comparison between 75 dBSPL click traces in the same animal with the two reference electrode placements showed minimal

differences in peak-to-trough waveform amplitudes and peak waveform latencies (Figure 4A). The mastoid placement was methodologically like mammalian ABR experiments with the reference electrode placed on the mastoid or pinna. Using a neck placement for the reference electrode would be beneficial if manipulation or surgery was performed on either ear. Interestingly, Wave II peak amplitude for the mastoid placement (red trace) occurred 1 ms after the Wave II peak for the neck placement (black trace). This time difference likely reflects the site(s) of ABR neural generation relative to the electrode placement.

Using a two-channel setup, one active recording electrode (top of head placement) and two reference electrodes (mastoid placements) were used to obtain ABRs for both the left and right ears (Figure 4B). The responses between the two ears were similar, with minor changes in peak amplitudes likely due to earphone positioning. The latency of both the left and right ear being equivalent supported the equally healthy function of both ears and brainstem hemispheres in the hatchling chicken. The two-channel recording montage could be used for binaural ABRs as well, but there would be additional considerations necessary for those recordings.

DISCUSSION:

The auditory brainstem of birds is well studied, and many structures are analogous to the mammalian auditory pathway. The auditory nerve provides excitatory inputs onto the two first-order central nuclei, the cochlear nucleus magnocellularis (NM) and angularis (NA). NM sends an excitatory projection bilaterally to its auditory target, nucleus laminaris (NL)[7]. NL projects to the nucleus mesencephalicus lateralis, pars dorsalis (MLd)[40,41]. NL also projects to the superior olivary nucleus (SON), which provides feedback inhibition to NM, NA, and NL[42]. This lower auditory brainstem microcircuit is exquisitely conserved for the function it subserves, sound localization, and binaural hearing[33]. The upper auditory brainstem regions of the bird also have nuclei analogous to the mammalian lateral lemniscus and inferior colliculus in the midbrain. Given these similarities, the composition of the avian ABR up to the auditory midbrain is comparable across all vertebrates.

While multiple avian species show three positive peaks within 6 ms following stimulus onset, the correlation of ABR peaks with central auditory structures does has some variability. Wave I can be reasonably assumed to be the first neural response from the peripheral basilar papilla and auditory nerve and displays little variability among individuals (Figure 1C). Subsequent Wave identification is less certain and may differ between species. Kuokkanen et al.[17] recently determined that Wave III of the barn owl's ABR is generated by NL; thus, it is reasonable to argue that Wave II originates from NM and NA of the cochlear nucleus[20]. However, the owl Wave III was defined as the positive peak generated 3 ms after stimulus onset. This corresponds to Wave II as defined in the hatchling chicken ABR. In the barn owl ABR, waves I and II were combined.

While the hatchling chicken usually presented with three peaks within 6 ms, a fourth peak was occasionally observed (e.g., see Figure 1A). Population data, larger sample size, and additional experimental paradigms would be needed to support a fourth wave, and in

some cases, a five-wave chicken ABR. The most consistent finding was the three peak representations shown here.

Since the ABR is defined as a measure of neural synchrony, the major nuclei in the auditory pathway could represent each positive-going peak in the ABR. The signal passing from the auditory nerve to NM/NA and then to NL may define Waves I, II, and III in the hatchling chicken ABR, respectively. Additionally, the later occurring fourth peak of the chicken ABR could represent an upper brainstem or midbrain auditory structure. The characterization of avian ABRs should also consider the difference between precocial and altricial birds. The maturation of auditory responses will vary among species and is also affected by other critical traits like predator behavior and/or vocal learning[4]. Regardless, the methods and techniques described are easily applied to a variety of avian and vertebrate species.

The importance of maintaining animal body temperature is illustrated in Figure 2. As the internal body temperature decreased, the latency of ABR responses increased for the same stimulus intensity level. This is more pronounced when body temperature drops below 32 °C[36,37]. The roughly 1 ms latency increase in the ABR is less than previously reported in the chicken[23]. However, Katayama[23] used a 12-day old hatchling that was cooled and subsequently warmed over a 4 h period. The data in Figure 2 was recorded during the cooling process over a 20-min period. To acquire the best quality and most consistent recordings, the animal's body temperature must be maintained, and all recordings should be done at the same physiological temperature among animals.

The effect of age on the ABR is slight but important to consider. While only the latency of Waves I and II of the ABR was significantly different, this is in part because only three young hatchlings were used in Figure 3; the other three did not present with three identifiable ABR peaks. ABR amplitude and threshold shifts may also be evident if using large sample sizes or comparing frequency-specific ABRs. This age-related effect could be caused by fluid in the middle ear of the chicken. Such conductive changes lead to a marked increase in ABR thresholds for both human and other mammalian models[38,39].

Using two different recording montages, similar responses were observed (Figure 4A). While the most common montage places the reference electrode behind the stimulus receiving ear, having the reference electrode in the neck tissue can be useful if there is surgical intervention accompanying the ABR. However, if two-channel ABR recordings are used, the reference electrodes should be separately and symmetrically placed, which is difficult if placing the reference electrode in the neck. The mastoid position for the reference electrode is recommended to standardize as many aspects of recording as possible. Two-channel ABR recording is an effective tool requiring little extra preparation and results in similar responses between the ears. Minor amplitude differences were likely due to the positioning of the earphone. Two-channel recording allows for easy comparison between an experimentally manipulated ear or brain hemisphere versus a control. This setup would also be required for testing binaural ABRs. Future experiments using the chicken ABR can refer to previous literature on recording configurations and montages[34].

This methodology does come with several limitations. As mentioned in step 5.1, poor speculum placement can lead to a 40 dBSPL shift in response. This could cause an incorrect interpretation of a manipulated or modified animal. The following precautions are recommended: acquire a large sample of control data before acquiring the ABRs of manipulated or mutant models. Do not decrease stimulus intensity by more than 20 dBSPL between recordings. If the amplitude or latency shifts more than expected, check on the animal and speculum position. Repeat that ABR stimulus to observe changes. If the speculum has moved, reacquire previous tests. Another limitation is the calibration of ABRs. Without proper calibration to record the sound pressure level, the intensity presented to the animal is unknown. When measuring sound output, use the same speculum as in experimental recording and a small microphone inside a cavity that approximates the animal's ear canal length (~5 mm). Measure the same tone frequencies used in experiments, as calibrations are frequency specific. The manual for both hardware and software systems may come with directions for calibration. There are also additional filters such as linear phase and minimum phase filters, which can improve click and tone burst ABRs[43]. These filters were not used in the present study. Additional considerations, like the rise and fall time of a tone burst spectral envelope changing as a function of frequency or changing the rise and fall time of the click stimuli was not examined either. These are good future investigations once reliable and consistent ABRs can be acquired.

The comparison of the hatchling chicken to other avian models is promising. Budgerigars and eastern screech-owls also display three positive microvolt peaks within the first 6 ms of the ABR[13,22]. In different species of woodpeckers, three peaks are seen as well, but their latency is later in time. Additionally, the range of best frequency sensitivity in woodpeckers is between 1500 and 4000 Hz, which is somewhat higher than the chicken's best threshold at 1000 Hz. In the adult chicken, the best sensitivity is at 2000 Hz[35], so there may be improved hearing of high frequencies as chicken hatchlings develop into adults. That development will differ among bird species, taking into account the altricial or precocious development of the animal[4].

The experimental methods outlined here can help determine what factors lead to detriments or changes in auditory responses and thresholds, as well as studies at different stages of embryonic development. Genetic manipulation, aging, and noise exposure are all known manipulations in animals and other avian models[24,25,44,45]. These methods should be extended to the chicken model now that techniques like *in-ovo* electroporation allow for the expression of proteins that are focally and temporally controlled at one side of the auditory brainstem[12,46]. This permits the direct comparison of ABRs from the genetically manipulated ear to the contralateral control ear using a two-channel recording paradigm.

Overall, the ABR of hatchling chickens is a useful research method, nearly identical to measures of hearing function in human and other mammalian models. It is also a non-invasive, *in-vivo* methodology. Apart from anesthetic injection and subdermal electrode placement of a few millimeters, no other physical manipulation is required. A hatchling could theoretically be tested multiple times over a developmental time course of days or weeks if kept in an appropriate environment. This protocol not only lays out the necessary steps and recording parameters for the hatchling chicken ABR, but it proposes

characteristics of an avian ABR that can inform further testing into auditory brainstem function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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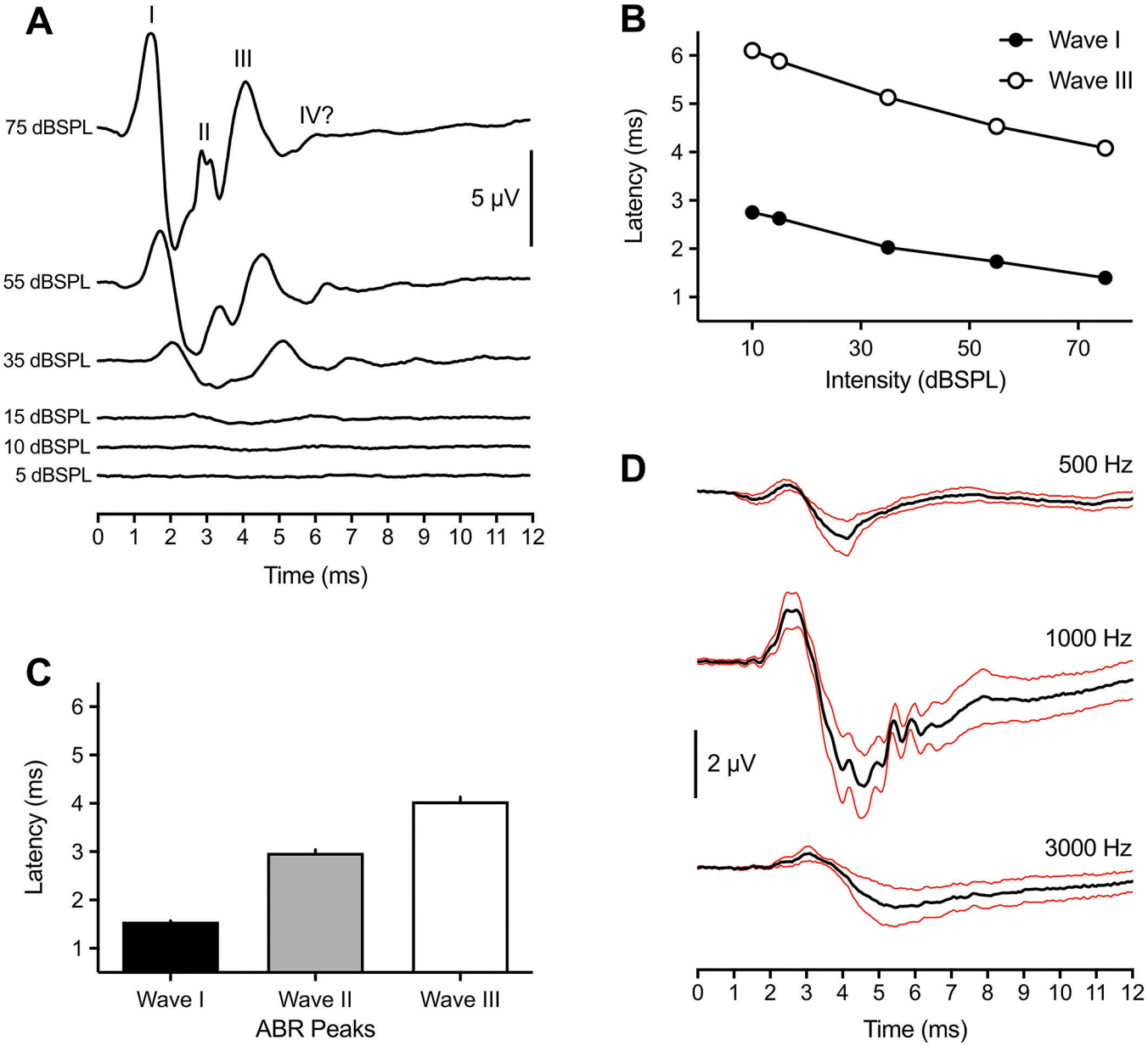

Figure 1: Representative recordings of hatchling chicks to click- and tone-evoked stimuli.

(**A**) Representative ABR recordings from a hatchling chick (P2) as a function of different stimulus intensity levels. Three to four positive peaks in microvolts (μV) can be identified within 6 ms post-stimulus onset (time = 0 ms). Waves were identified using Roman numerals. Peak-to-trough amplitudes decrease at lower stimulus intensity levels. (**B**) Latency-intensity functions of Waves I and III for the representative trace shown in (**A**). Only these peaks were analyzed, as Wave II was typically not observed at intensities <45 dBSPL. (**C**) Latency of click-evoked ABR peak waveforms (n = 43 chicks). Error bars denote the standard error of the mean (SEM). (**D**) Averaged tone-evoked ABRs (black traces) for four hatchling chicks at three different frequencies. Red traces = standard error of the mean (SEM) Stimuli = 75 dBSPL. In this and subsequent figures, error bars denote

SEM, and the right ear was the stimulus ear. (exception for Figure 4B where both ears were stimulated).

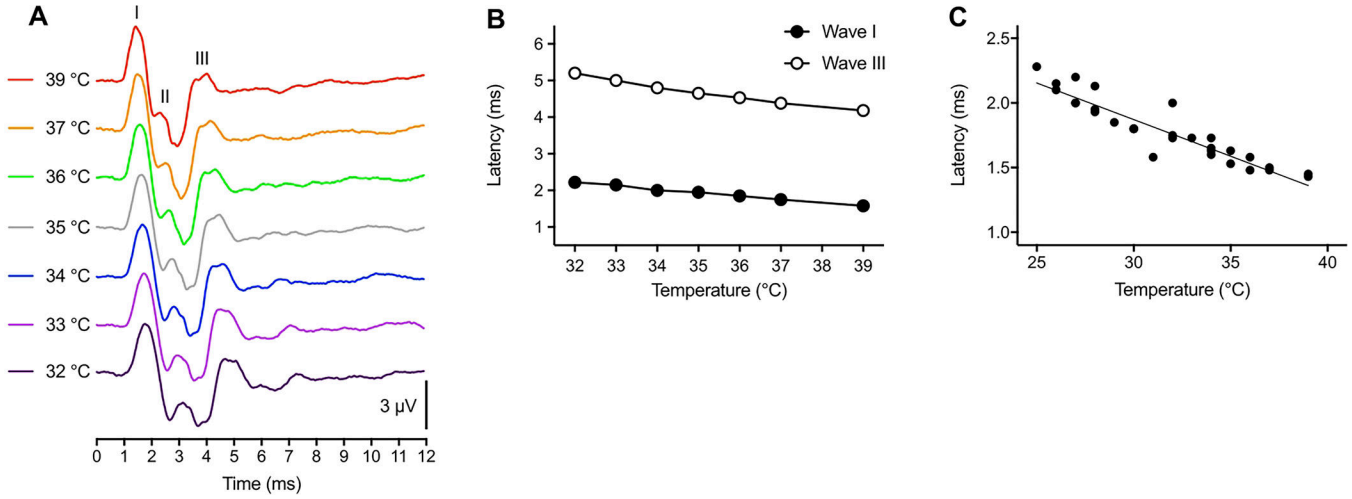

Figure 2: Effect of body temperature on ABR recordings.

(**A**) Representative ABR recordings from a hatchling chick (P2) as a function of body temperature. For lower body temperatures, peak waveform latencies increased while peak-to-trough amplitudes remained relatively unchanged. (**B**) Latency-temperature function of Waves I and III for the representative traces shown in (**A**). (**C**) Population data showing the relationship between latency and temperature changes for 5 chicks ($p < 0.01$, $R^2 = 0.89$). A similar trend was observed for Waves II and III (data not shown).

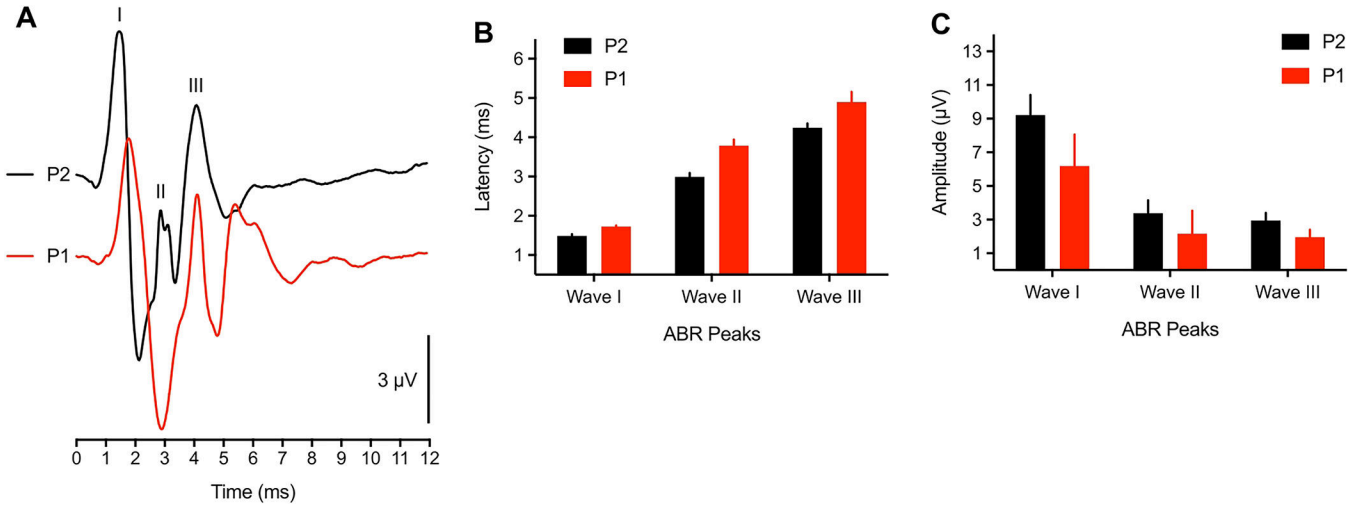

Figure 3: Age-related differences on ABR recordings.

(**A**) Representative ABR recordings (overlapped) of a representative hatchling chick at P2 (black trace) and P1 (<3 h post-hatch, red trace). (**B**) Peak waveform latencies for Waves I, II, and III as a function of age. The latencies for Waves I-III were significantly different between ages ($P < 0.05$, $n = 6$ chicks). (**C**) Peak-to-trough waveform amplitudes of Waves I, II, and III as a function of age.

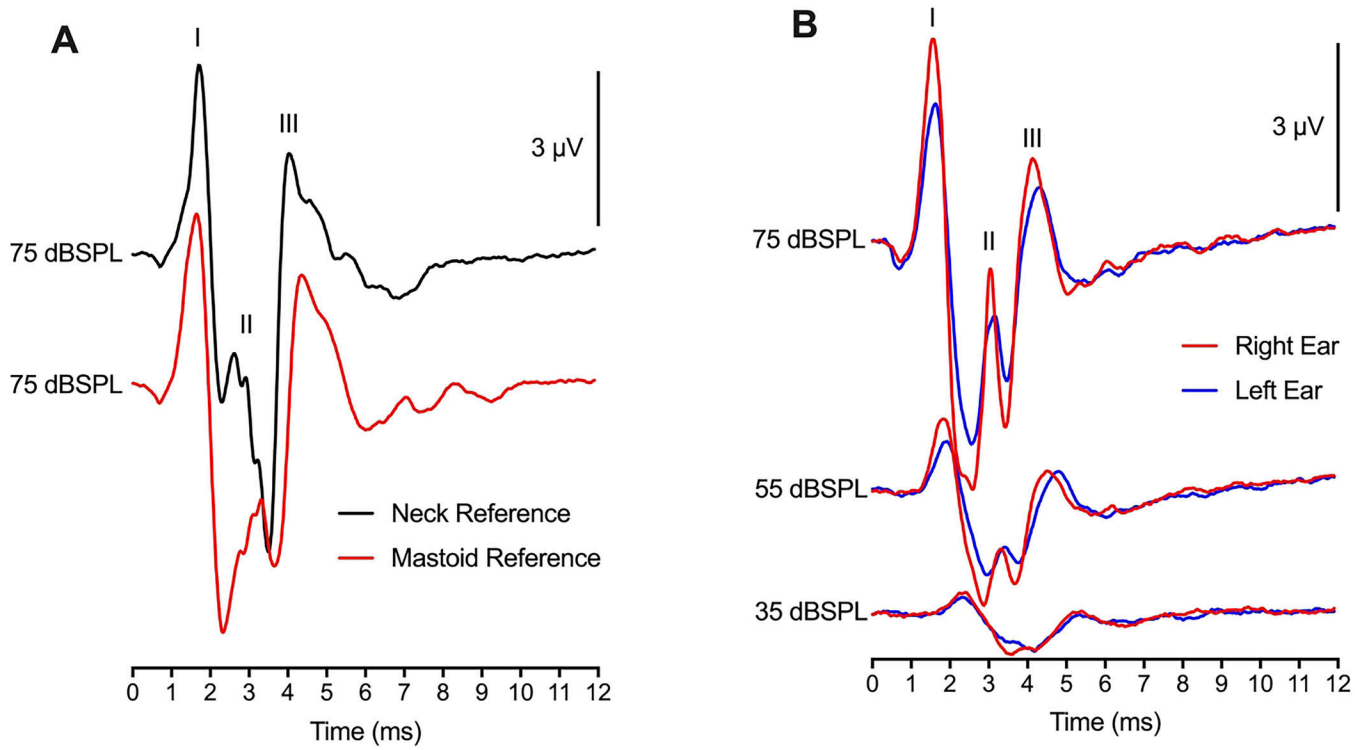

Figure 4: Electrode placement and two-channel ABR recordings:

(**A**) Representative ABR recordings (overlapped) from the same hatchling chick (P2) with the reference electrode placed in the neck (black trace) or mastoid (red trace). The active electrode was placed at the midline of the skull for both electrode recording montages. The latency of Waves I and III, and the amplitude of Waves I and III are nearly identical in both conditions. The latency of Wave II is earlier, and the amplitude is larger for the electrode placed in the neck tissue. (**B**) Two-channel recording while sequentially stimulating the right and left ears. Representative ABR recordings (overlapped) from the same hatchling chick (P2) with the reference electrodes placed in the mastoid of the left ear (blue traces) and right ear (red traces) at three different intensity levels.