Abstract—Electrophysiological responses to pulsed linear acceleration stimuli were recorded in chicken embryos incubated for 19 or 20 days (E19/E20). Responses occurred within the first 16 ms following the stimulus onset. The evoked potentials disappeared following bilateral labyrinthectomy, but persisted following cochlear destruction alone, thus demonstrating that the responses were vestibular. Approximately 8 to 10 response peaks could be identified. The first 4 positive and corresponding negative components (early peaks with latencies < 6.0 ms) were scored and latencies and amplitudes quantified. Vestibular response latencies were significantly longer ($P < 0.01$) and amplitudes significantly smaller ($P < 0.001$) than those observed in 2-week-old birds. Mean response threshold for anesthetized embryos was $-15.9\text{dB}re 1.0\text{g/ms}$, which was significantly higher ($P < 0.03$) than those observed in 2-week-old birds ($-23.0\text{dB}re 1.0\text{g/ms}$). Latency/intensity functions (that is, slopes) were not significantly different between embryos and 2-week-old animals, but amplitude/intensity functions for embryos were significantly shallower than those for 2-week-old birds ($P < 0.061$). We presume that these differences reflect the refinement of sensory function that occurs following 19 to 20 days of incubation. The recording of vestibular evoked potentials provides an objective, direct and noninvasive measure of peripheral vestibular function in the embryo and, as such, the method shows promise as an investigative tool. The results of the present study form the definitive basis for using vestibular evoked potentials in the detailed study of avian vestibular ontogeny and factors that may influence it.

Keywords—vestibular ontogeny; vestibular evoked potentials; linear acceleration; birds; linear jerk.

Introduction

Vestibular evoked potentials have been reported in several species, including chickens (1,2), rats (3–5), chinchillas (6), cats (7), and humans (8), using linear or angular acceleration stimuli. In post-hatch chickens, noninvasively recorded responses to linear acceleration transients have been described as consisting of 6 to 8 peaks occurring within 8 to 10 milliseconds of stimulation (1,2). Jones and coworkers have shown that these responses are not masked by cochlear extirpation, but disappear following cochlear destruction alone, thus demonstrating that the responses were vestibular. Approximately 8 to 10 response peaks could be identified. The first 4 positive and corresponding negative components (early peaks with latencies < 6.0 ms) were scored and latencies and amplitudes quantified. Vestibular response latencies were significantly longer ($P < 0.01$) and amplitudes significantly smaller ($P < 0.001$) than those observed in 2-week-old birds. Mean response threshold for anesthetized embryos was $-15.9\text{dB}re 1.0\text{g/ms}$, which was significantly higher ($P < 0.03$) than those observed in 2-week-old birds ($-23.0\text{dB}re 1.0\text{g/ms}$). Latency/intensity functions (that is, slopes) were not significantly different between embryos and 2-week-old animals, but amplitude/intensity functions for embryos were significantly shallower than those for 2-week-old birds ($P < 0.061$). We presume that these differences reflect the refinement of sensory function that occurs following 19 to 20 days of incubation. The recording of vestibular evoked potentials provides an objective, direct and noninvasive measure of peripheral vestibular function in the embryo and, as such, the method shows promise as an investigative tool. The results of the present study form the definitive basis for using vestibular evoked potentials in the detailed study of avian vestibular ontogeny and factors that may influence it.

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Introduction

Vestibular evoked potentials have been reported in several species, including chickens (1,2), rats (3–5), chinchillas (6), cats (7), and humans (8), using linear or angular acceleration stimuli. In post-hatch chickens, noninvasively recorded responses to linear acceleration transients have been described as consisting of 6 to 8 peaks occurring within 8 to 10 milliseconds of stimulation (1,2). Jones and coworkers have shown that these responses are not affected significantly by auditory maskers or cochlear extirpation, but disappear following labyrinthectomy or pharmacological blockade of the labyrinth. These studies have provided definitive evidence that responses to linear acceleration transients in the post-hatch chicken are in fact vestibular. In addition, studies have distinguished that early response peaks (P1 and P2) are dependent upon peripheral generators, whereas response peaks beyond P2 are produced by central generator sites (9,10).
The bird has long been recognized and widely used as a valuable model for studies of development. However, little information is available concerning functional development of the avian vestibular system. Vestibular evoked potentials could be an important non-invasive measurement technique in studying the development of gravity receptors and the influence of gravity on ontogeny. Only one cursory description of responses to linear acceleration transients in the chicken embryo has been offered (11). Roll and colleagues described embryonic waveforms that consisted of response peaks having latencies between 6 and 11 ms with peak-to-peak amplitudes between 0.5 and 5 μV. When these embryonic responses are compared to responses published elsewhere for the post-hatch bird, waveform morphology appears less well defined overall, response peaks are fewer, and peak latencies are considerably longer. Unfortunately, the embryonic responses described by Roll and colleagues (11) were obtained to single polarity stimuli which makes comparisons difficult. In addition, these embryonic responses were not shown explicitly to have vestibular origins. Hence, the objectives of the present study were 1) to provide a more complete description of embryonic responses to pulsed linear acceleration, and 2) to test the hypothesis that embryonic responses depend critically upon eighth nerve function, but are independent of the cochlea.

Methods

Animals and Animal Preparation

The present study used 32 chicken embryos (Gallus domesticus) and 6 2-week-old hatchlings. The embryos completed 19 or 20 full days of incubation and, as such, were designated as E19 or E20, respectively (the normal incubation time is 21 days). Embryonic ages corresponded to stages 44–45 according to Hamburger and Hamilton (12). These ages were chosen because responses to pulsed linear acceleration stimuli could not be resolved in substantially younger embryos. For comparison, post-hatch birds were included to provide responses from relatively mature animals under similar conditions. It was not our intention to undertake a detailed developmental study at many different ages. Such studies are underway in our laboratories.

Animals were anesthetized with an intramuscular injection of EquiThesin (0.005 mL/g, hatchlings) or EquiThesin:saline (ratio 1:4, 0.10 mL, embryos). [EquiThesin is a mixture of Na-pentobarbital (0.972 g), chloral hydrate (4.251 g), magnesium sulfate (2.125 g), ethanol (12.5 mL), and propylene glycol (42.6 mL) in distilled water to a total volume of 100 mL.] Anesthesia was administered at the beginning of each experiment in all but 7 animals. In the latter case, 7 embryos were prepared noninvasively with surface electrodes, and responses were recorded before and after the administration of anesthetic (EquiThesin, 0.10 mL). In both the present and previous studies this strategy and dose were used to evaluate the effects of anesthesia (2).

In anesthetized hatchlings, a tracheotomy was performed and the beak secured in plaster to the stimulus platform as described in detail elsewhere (2). Each embryo’s head was removed from the eggshell through a small opening and secured to the mechanical shaker platform in a similar manner. Tracheotomies were unnecessary in embryos since they did not exhibit pulmonary ventilation. For all animals, the head was oriented with the naso-occipital axis in the vertical plane parallel to the Earth G axis. Subcutaneous electrodes (that is, stainless steel wires, 0.2-mm diameter) were placed at the vertex (noninverting), behind the left and right external auditory meati (inverting) and at the neck (ground). Egg temperature was monitored with a thermistor inserted into the extraembryonic fluid. A brain thermistor inserted into the neostriatum on the right side was used to monitor and maintain brain temperature at 39.0 ± 0.1 °C in embryos. Rectal (cloacal) temperature was monitored in hatchlings and maintained within 39.0 ± 0.1 °C. The care and use of animals reported on in this study were approved by the
Embryonic Vestibular Responses

University of Missouri (IACUC #2334) Institutional Animal Care and Use Committee.

Recording Responses

Responses to auditory and vestibular stimuli were recorded in 5 embryos using 2-channel signal averaging. Single-channel recordings of vestibular responses were made in the 2-week-old birds and in the remaining 27 embryos. Auditory stimuli were clicks produced by applying 1.0 V, 0.10 ms rectangular pulses, shaped by a graphic equalizer, to an Etymotic ER-2 insert earphone sealed at the left external auditory meatus (EAM). Clicks were presented at a rate of 2/s. Stimulus intensity at the output of the earphone was 100 dB peak equivalent SPL (dBpeSPL, re: 20 p.Pa) measured with a calibrated 0.25-inch Bruel and Kjaer microphone in a 0.1 cc coupler. Peak equivalent SPL (peSPL) was derived by comparing the peak-to-peak (p-p) microphone output voltage produced by the click waveform to the p-p output voltage produced by a sine wave tone of known rms SPL. Click level was then expressed as $20 \log \left( \frac{V_{cp-p}}{V_{tp-p}} \right) + X$ dB SPL, where $V_{cp-p}$ is the p-p voltage of the click, $V_{tp-p}$ is the p-p voltage of the test tone, and $X$ is the SPL level of the test tone in dB SPL, re: 20 µPa.

Vestibular stimuli were linear rectangular jerk pulses (jerk is the first derivative of linear acceleration, $da/dt$) of 2.0 ms duration presented to the bird's head via a mechanical shaker platform. Stimulus coupling in both embryos and hatchlings was accomplished by seating the tip of the beak on the platform and embedding it in plaster as described elsewhere (2, 11). A calibrated accelerometer was mounted on the platform and used to monitor acceleration. Jerk amplitude was determined by electronically differentiating the accelerometer output. The differentiator was calibrated using known jerk stimuli and was linear over the range of amplitudes used in the present study. The maximum stimulus amplitude used in the present study was 1.0 g/ms. Stimulus amplitude was attenuated in 3-dB steps (re: 1.0 g/ms) to obtain response thresholds. A stimulus rate of approximately 2/s was used for studies of embryos, whereas 9/s was used for hatchlings. The slower rate was used for the embryos because our experience has shown that faster stimulus rates may significantly reduce response amplitudes in embryos, but not in 2-week-old hatchlings. This rate effect was not examined here, but is the focus of ongoing study. The stimulus was turned off for at least 2 min between all trace recordings in embryos.

Two stimulus polarities, normal and inverted, were used, where normal polarity was defined as an initial upward jerk and inverted as an initial downward jerk. Final individual response traces were produced by summing one averaged response to each stimulus polarity and dividing the result by 2. This served to reduce mechanically induced electrode wire artifacts in traces and was an alternative to using stimuli of alternating polarity during signal averaging (2). Electrode wire artifacts occasionally do not cancel precisely, and in these cases they are seen in final recording traces. Generally, wire artifacts can be distinguished from and bear little resemblance to physiological responses. When present, they remain after bilateral labyrinthectomy and after death. These artifacts have been described previously (2) and are found at all ages.

Signal averaging was used to extract auditory and vestibular responses from the amplified (100,000 times) and filtered (300 to 3000 Hz) electroencephalographic (EEG) activity. The noninverting electrode was placed at the vertex of the skull. For single channel averaging, the reference electrode was placed behind the left EAM. For two channel averaging, reference electrodes were placed behind both the left (channel 1) and the right (channel 2) EAM. This enabled simultaneous recording from both sides of the head and provided one means of assessing the effectiveness of the initial unilateral right labyrinthectomies where they were performed (see below). The EEG activity was digitized (1024 points, 20 or 16 µs/point for embryos; 16 µs/point for hatchlings) beginning at the onset of the acoustic or jerk
stimulus. To produce one averaged response waveform, 128 primary responses were averaged. Responses were replicated for all conditions in each animal.

Overview of Measurements

Vestibular response thresholds were determined by systematically increasing stimulus intensity from approximately 0.045 to 1.0 g/ms in 3-dB steps (1.0 g/ms = 9.81 m/s²/s). Two final response traces were recorded at each level. This was completed for the 6 hatchlings and 27 embryos. In 7 of the 27 embryos, vestibular response thresholds were recorded before and after anesthesia, as outlined above. Threshold was defined as the stimulus intensity midway between the minimum intensity producing a response and the maximum intensity failing to produce any response (that is, no discernible replicating response peaks present). However, in 7 embryos, remnants of response peaks persisted at the lowest stimulus intensities used. In these animals, the following convention was used: Threshold was defined as 1.5 dB below the lowest stimulus intensity presented.

This first 4 positive and negative response peaks were scored and labeled for the embryos and hatchlings according to the convention of Jones and coworkers (1,2,4,9). Peak latencies were defined as the time delay, in milliseconds (ms), from the onset of the stimulus to the occurrence of each respective peak (P1 to N4). Four peak-to-peak response amplitudes were calculated by subtracting the amplitudes of negative peaks (N1 to N4) from the respective positive peaks (P1 to P4). The resulting amplitudes were labeled as P1/N1, P2/N2, P3/N3, and P4/N4 and expressed in microvolts (μV).

The latencies and amplitudes of response components were expressed as a function of stimulus intensity. Latency/intensity (L/I) and amplitude/intensity (A/I) functions were quantitatively described for each animal, using linear regression analysis. Regression slopes were determined first for each animal, and these values were used to calculate group means and standard deviations across animals. Group means generated in this fashion were used in statistical comparisons. In figures, raw latency and amplitude data were also pooled and displayed as a function of intensity for each age group. Multivariate analysis of variance (MANOVA) was used for statistical inferences about the effects of age on response parameters. A Mann–Whitney U–Wilcoxon rank sum W test (MWUWRS) was used where the assumption of equal sample variances was in question.

Surgical Strategies for Isolating Peripheral Generators

Five embryos were anesthetized and prepared as described above, and vestibular responses were recorded prior to surgery to verify that a viable response was present. The right labyrinth was then surgically destroyed to isolate the intact left labyrinth for study, and vestibular responses were recorded again. The left ear was then surgically accessed using a posterolateral approach. Neck muscles were removed from the left side to expose the exoccipital bone. The skull was opened in this area allowing entrance to the middle ear space and visualization of the bony plate overlying the recessus scala tympani. Fluid in the middle ear cavity was removed with absorbent pledgets to allow stimulation by air borne sound. Vestibular and auditory responses were recorded at this point to verify the presence of responses. The left cochlea was subsequently opened by removing the bony plate over the scala tympani and incising the membranous labyrinth. Responses to auditory and vestibular stimuli were recorded following surgery to verify normal function, hence each animal served as its own control for effects of the surgical access. The left cochlea was surgically destroyed (mechanical compression) or aspirated (or both), and responses were recorded after each manipulation to evaluate whether responses were dependent upon an intact cochlea. A labyrinthectomy was completed on the left side by surgical aspiration or by applying a specific neural blocking agent tetrodotoxin (TTX (13), Sigma, 0.05 mg in 16%
polyvinyl alcohol) to the opened vestibule (2), and responses were recorded again. In this case, any response remnants that relied on an intact labyrinth would be eliminated. Finally, recordings were made after the death of the embryo (decapitation) to help identify wire motion artifacts.

**Results**

Figure 1 displays representative responses to pulsed linear acceleration stimuli (that is, jerk) at 1.0 g/ms for one embryo and one hatchling. Response morphology was more variable across embryos, and responses were not well formed compared to those of 2-week-old chicks. In 3 of the 32 embryos studied, no discernible responses could be obtained to 1.0 g/ms stimuli. In most responses, there were up to 10 response peaks, each having a positive (P) and negative (N) component. The first 4 of these have been labeled in Figure 1, and they occurred with latencies shorter than 6.0 ms at stimulus intensities of 1.0 g/ms. One or more late dominant peaks (latency > 6.0 ms) were also seen in 10 of the 29 embryos evidencing responses. These late dominant peaks appeared to be anesthesia labile. This was suggested by results in 7 animals where responses were recorded before and after drug administration. Anesthesia in these cases reduced or eliminated late dominant components (Figure 2). The magnitude of the effect was quite

\[ E_{19} - E_{20} \]

\[ P_1 \rightarrow P_4 \]

\[ 1.0 \text{ g/ms} \]

\[ \text{Two-Week-Old} \]

\[ 2 \mu V \]

\[ P_2 \]

\[ 0 \]

\[ 2 \text{ ms} \]

\[ -6 \]

\[ -12 \]

\[ -15 \]

\[ -18 \]

\[ -21 \]

\[ -24 \]

Figure 1. Representative response waveforms recorded from the left side of one anesthetized E19/E20 chicken embryo and one 2-week-old hatchling. Six to eight response peaks can be found. The first 4 positive peaks are labeled as shown (early components, P1 to P4, with latencies < 6.0 ms). Note the large dominant late components for the embryo. Late components are not explicitly labeled, but they can be observed at latencies greater than 6.0 ms and are found in both embryos and hatchlings. In this particular case, anesthesia virtually eliminated the late components in the post-hatch traces. These responses were obtained with linear jerk stimuli from \(-27 \text{ dB}_{re} \, 1.0 \text{ g/ms} \) to \(0 \text{ dB}_{re} \, 1.0 \text{ g/ms} \). Early components of embryonic vestibular responses (that is, P1 to P4) showed longer latencies and smaller amplitudes than did those of 2-week-old responses. Threshold for this embryo was scored at \(-19.5 \text{ dB}_{re} \, 1.0 \text{ g/ms} \), whereas the 2-week-old threshold was scored at \(-25.5 \text{ dB}_{re} \, 1.0 \text{ g/ms} \). Amplitude calibrations are 2 \( \mu V \), and time calibrations are 2 ms. The entire trace duration is 16 ms.
variable across animals, and in many cases the late potentials, although reduced, were well resolved after the anesthetic dose. In Figure 2, early response components remain following anesthesia, whereas late potentials virtually disappear. Graded doses of anesthesia were not given. Therefore, a more complete description of the effect of anesthesia on vestibular responses was not possible. The earliest embryonic response peaks \( P_1 \) through \( N_4 \) (latency \(< 5.0\) ms) were considerably smaller and less distinct in comparison to those of 2-week-old animals. Positive peaks \( P_1 \) to \( P_3 \) and negative peaks \( N_1 \) to \( N_3 \) were discerned in most animals, whereas \( P_4 \) was observed less consistently. Furthermore, due to the small amplitudes of these earliest response components, latency scoring was often difficult.

Table 1 summarizes the mean latencies for embryos and hatchlings at stimulus intensities of 1.0 g/ms. Latencies of response components \( P_1 \) through \( N_4 \) were between approximately 1.20 and 5.90 ms. Latencies for both positive (\( P_1, P_2, P_3 \)) and negative (\( N_1, N_2 \)) peaks were significantly longer for embryos than for 2-week-old birds (\( P < 0.01 \), MANOVA and MWUWRS). Response peaks \( P_4, N_3, \) and \( N_4 \) were absent in more than 50% of the animals, and were not included in the analysis for that reason. The difference between latencies

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**Table 1. Mean Latencies (μs) and Standard Deviations (±SD) for 1.0 g/ms Stimuli**

<table>
<thead>
<tr>
<th>Peak</th>
<th>E19/E20</th>
<th>PH</th>
<th>E19/E20</th>
<th>PH</th>
<th>E19/E20</th>
<th>PH</th>
<th>E19/E20</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_1 )</td>
<td>Mean (ms)</td>
<td>1.540</td>
<td>1.267</td>
<td>2.463</td>
<td>1.902</td>
<td>3.565</td>
<td>2.736</td>
<td>4.496</td>
</tr>
<tr>
<td>( N_1 )</td>
<td>Mean (ms)</td>
<td>2.096</td>
<td>1.584</td>
<td>3.213</td>
<td>2.404</td>
<td>4.024</td>
<td>3.184</td>
<td>5.132</td>
</tr>
<tr>
<td>( P_2 )</td>
<td>Mean (ms)</td>
<td>1.267</td>
<td>0.982</td>
<td>1.449</td>
<td>1.140</td>
<td>0.357</td>
<td>0.012</td>
<td>0.431</td>
</tr>
<tr>
<td>( N_2 )</td>
<td>Mean (ms)</td>
<td>1.584</td>
<td>0.919</td>
<td>0.440</td>
<td>0.176</td>
<td>0.350</td>
<td>0.170</td>
<td>0.588</td>
</tr>
<tr>
<td>( P_3 )</td>
<td>Mean (ms)</td>
<td>2.463</td>
<td>1.402</td>
<td>6.024</td>
<td>6.176</td>
<td>8.350</td>
<td>8.170</td>
<td>6.588</td>
</tr>
<tr>
<td>( N_3 )</td>
<td>Mean (ms)</td>
<td>3.565</td>
<td>2.736</td>
<td>4.024</td>
<td>3.184</td>
<td>5.132</td>
<td>3.908</td>
<td></td>
</tr>
<tr>
<td>( P_4 )</td>
<td>Mean (ms)</td>
<td>4.496</td>
<td>3.515</td>
<td>5.132</td>
<td>3.908</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( E19/E20 = \) embryos at 19 or 20 days of incubation. 
\( PH = \) post-hatch birds 2 weeks old. 
\( n = \) number of observations.
of embryos and 2-week-old animals increased progressively from earlier to later peaks, as can be seen in Figure 3. Moreover, at a stimulus intensity of 1.0 g/ms, embryonic peak-to-peak amplitudes were significantly smaller than were 2-week-old peak-to-peak amplitudes (Table 2, P1/N1, P2/N2, P < 0.001, MANOVA and MWUWRS). Mean response threshold in dB (re: 1.0 g/ms), which is a general indicator of the end organ's sensitivity, was significantly higher for embryos than for 2-week-old animals (Table 3, P < 0.03, t-test). Embryonic vestibular response thresholds were not affected significantly by anesthesia (Table 4).

In general, group mean latencies systematically decreased with increasing stimulus intensity for both ages (Figure 4), especially at intensities above -20 dB. Below -20 dB, sample sizes changed for the 2 ages, thus variability increased and the group latency progression was modified. For clarity, Figure 4 summarizes findings for P1 and P2 only, however, all response peaks showed similar functions within their respective latency ranges. Table 5 summarizes the mean L/I slopes for P1, P2, P3, N1, and N2 based upon the regression slopes calculated for individual animals. These results show that there was a strong trend for the slope of P1 to be shallower in 2-week-old animals, but MANOVA results revealed no significant effects of age on the L/I slopes for response peaks overall.

Mean group amplitudes plotted as a function of stimulus intensity revealed some important differences between embryos and hatchlings (Figure 5). The most striking differences evident in Figure 5 are the response amplitudes obtained at the highest stimulus intensities, where 2-week-old birds exhibited substantially larger amplitudes as noted above.

In general, it is clear that group amplitudes increase with increasing stimulus intensities. For P2/N2, the change in group amplitudes with a given increase in stimulus intensity is much greater for 2-week-old birds in comparison to embryos. This relationship also holds for P1/N1, however, this is not clear based on Figure 5 alone. The sample size (n) for P1/N1 decreased at intensities below -15 dB, thus distorting the progression. A/I regression slopes determined for each animal can be used

Table 2. Mean Amplitudes (μV) and Standard Deviations (±SD) for 1.0 g/ms Stimuli

<table>
<thead>
<tr>
<th>Peak to Peak</th>
<th>P1/N1</th>
<th>P2/N2</th>
<th>P3/N3</th>
<th>P4/N4</th>
</tr>
</thead>
<tbody>
<tr>
<td>E19/20 PH</td>
<td>E19/20 PH</td>
<td>E19/20 PH</td>
<td>E19/20 PH</td>
<td>E19/20 PH</td>
</tr>
<tr>
<td>Mean (μV)</td>
<td>0.323 0.959</td>
<td>0.372 1.866</td>
<td>0.345 1.527</td>
<td>0.677 1.124</td>
</tr>
<tr>
<td>(±SD)</td>
<td>0.224 0.408</td>
<td>0.332 1.015</td>
<td>0.138 0.754</td>
<td>0.502 0.438</td>
</tr>
<tr>
<td>n</td>
<td>17 6</td>
<td>12 6</td>
<td>7 6</td>
<td>6 5</td>
</tr>
</tbody>
</table>

E19/20 = embryos at 19 or 20 days of incubation.
PH = post-hatch birds 2 weeks old.
n = number of observations.
Table 3. Thresholds for Embryonic and Post-hatch Vestibular Responses to Jerk (g/ms) Stimuli

<table>
<thead>
<tr>
<th></th>
<th>Threshold dB (re: 1.0 g/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>E19/E20</td>
<td>-15.94</td>
</tr>
<tr>
<td>Post-hatch</td>
<td>-23.00</td>
</tr>
</tbody>
</table>

All animals anesthetized.

$ t = 2.048; \ p < 0.025. $

to resolve this issue for both peaks. Table 5 summarizes the mean A/I regression slopes for animals of each age. Mean values of individual linear regression slopes for P1/N1 and P2/N2 across embryos were relatively flat (approximately 0.01 μV/dB), whereas hatchlings demonstrated significantly steeper slopes (up to 0.08 μV/dB, Table 5, $ P < 0.001 $, MANOVA and MWUWRS).

Figures 6, 7, and 8 illustrate the results of strategic surgical manipulations for 3 representative animals. All 5 animals studied in this manner demonstrated qualitatively similar results. Responses to auditory and vestibular stimuli are shown as recorded from the left side of the head. The top pair of traces in each figure (6, 7, and 8) were recorded after a right labyrinthectomy, which eliminated any contributions from that ear. In addition, the left middle ear was surgically accessed and cleared of fluid, and an opening in the cochlear wall and membranous labyrinth was made at the basal end of the left cochlea (Before). Robust responses to auditory and jerk stimuli were obtained under these conditions. Under direct microscopic observation, a wire stylette (having a diameter between 500 μm and 750 μm of the cross-sectional diameter of the osseous cochlear duct) was inserted through the opening in the cochlea at the base and advanced to the apical end of the cochlear duct (3 to 4 mm dis-

Table 4. Thresholds for Unanesthetized and Anesthetized Embryonic Vestibular Evoked Responses to Jerk (g/ms) Stimuli

<table>
<thead>
<tr>
<th></th>
<th>Threshold dB (re: 1.0 g/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>E19 unanesthetized</td>
<td>-13.07</td>
</tr>
<tr>
<td>E19 anesthetized</td>
<td>-11.57</td>
</tr>
</tbody>
</table>
Table 5. Linear Regression Slopes, Amplitudes and Latencies Versus dB re: 1.0 g/ms

<table>
<thead>
<tr>
<th>E19/E20 embryos</th>
<th>Post-hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplitude</strong></td>
<td></td>
</tr>
<tr>
<td>Slope (µV/dB)</td>
<td>±SD</td>
</tr>
<tr>
<td>P1/N1</td>
<td>0.013</td>
</tr>
<tr>
<td>P2/N2</td>
<td>0.010</td>
</tr>
</tbody>
</table>

| **Latency**     |            |
| Slope (µV/dB)   | ±SD        | N  |
| P1              | -58.49     | 31.94 | 16  | -24.53 | 3.15  | 4  |
| P2              | -62.64     | 20.53 | 11  | -52.20 | 8.98  | 6  |
| P3              | -58.78     | 30.15 | 8   | -50.13 | 9.66  | 6  |
| N1              | -54.87     | 26.38 | 15  | -36.43 | 6.53  | 6  |
| N2              | -48.34     | 20.70 | 10  | -58.22 | 23.77 | 6  |

Figure 6. Vestibular (left column) and auditory (right column) responses for one representative animal at the various stages of surgical manipulation. Early vestibular response peaks are marked for orientation. Robust evoked potentials are present in response to both vestibular and auditory stimuli after right labyrinthectomy and left cochlear fenestration (Before). Both early (<6.0 ms) and late (>6.0 ms) vestibular components are present. Vestibular responses persist under all conditions, including cochlear aspiration. Vestibular evoked potential latencies and amplitudes are essentially unchanged as the cochlea is crushed (After). In contrast, only remnants of cochlear responses to click stimuli appear after cochlear compression, thus providing functional verification of the destructive effect of the surgery (After). All cochlear potentials disappear following its aspiration, whereas only minor changes occur in vestibular responses (Cochlear Aspiration). Responses to jerk stimuli virtually disappear after labyrinthectomy. The remaining activity is likely wire artifact. Amplitude calibration is 2 µV, time calibration is 2 ms, and the entire trace duration is 20 ms. Auditory stimuli were clicks at 100 dBpeSPL. Vestibular stimuli were 1.0 g/ms cranial jerks.
gradients across fluid compartments in the labyrinth. The procedure therefore carried with it a high risk of damaging vestibular structures due to hydraulic forces acting at some distance from the tip of the aspiration probe. It is not surprising, therefore, that some changes were observed in responses to jerk following aspiration. The degree of change was generally small, but it varied across animals, as can be seen in Figure 6, 7, and 8. Both early and late response peaks remained in all cases. Only after destruction of the entire left labyrinth or with the application of TTX did responses to jerk stimuli vanish (Left Labyrinthectomy). Electrode wire artifacts remain after labyrinthectomy in Figure 6 and 7.

**Discussion**

The present study provides the first detailed description of embryonic responses to pulsed linear acceleration as well as convincing evidence that these responses are vestibular. The responses are quite complex and include both early (latencies < 6.0 ms, anesthesia resistant) and late (latencies > 6.0 ms, anesthesia labile) components, as is the case in post-hatch birds. Clearly, responses to jerks do not depend critically on an intact auditory end organ. However, they are strictly dependent upon an intact labyrinth. We conclude therefore, that the evoked potentials, including early and late components, depend upon the activation of vestibular neurons and are therefore vestibular responses.

Embryonic responses to pulsed linear acceleration are more variable across animals, and early components are not as well formed as those in 2-week-old animals. Generally, one or more late dominant peaks are present in at least one-third of anesthetized embryos. These peaks appear to be anesthesia labile and can be orders of magnitude larger than early peaks in unanesthetized embryos. In unanesthetized

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**Figure 7.** The effects of the surgical manipulations described in Figure 6 are illustrated here for another animal. Qualitatively similar results were obtained Before and After cochlear compression and aspiration. Vestibular responses disappear only after labyrinthectomy. Wire artifacts persist after labyrinthectomy. Stimuli and calibration marks are the same as in Figure 6.
embryos, therefore, late dominant peaks may be more frequently identified. The late peaks are clearly dependent upon vestibular activation and are therefore considered to be an integral part of the vestibular response to jerk. The fact that they have long latencies and are influenced by anesthesia suggests that they may arise from higher order elements of the response circuitry, including myogenic sources. Anesthesia-labile response components have also been reported by Jones in posthatch animals (2), where similar arguments were made. Regardless of the precise level of origin, the late dominant peaks are dependent upon an intact labyrinth and clearly depend on the activation of the peripheral vestibular system and not the cochlea.

In embryos, the earliest response peaks are significantly smaller in amplitude and have significantly longer latencies when compared to 2-week-old chicks. In addition, response thresholds are higher (by approximately 7 dB) for embryos than for 2-week-old birds, and it is important to note that, in 3 of the 32 embryos studied, vestibular responses could not be resolved at 1.0 g/ms (maximum stimulus intensity used). Embryonic peak latencies decrease as a function of intensity in a manner similar to hatchlings; however, embryonic amplitudes do not increase in proportion to stimulus intensity at a rate equivalent to hatchlings. In general, these differences likely reflect the immature status of the embryonic vestibular system in the E19/E20 bird. The basic structural elements of vestibular sensory end organs are in place by at least E16 (14-17). However, the final refinements of sensory elements, including myelination of afferents,
represent obligate neuroanatomical events that occur during development beyond E19 (14-16). Other maturational processes occurring after E19 may include dimensional changes in the labyrinth during growth (C. Fermin, unpublished data) as well as changes in otocochlear membranes and mechanical coupling between hair cells and overlying structures (18). Elements of structural development such as these may contribute to the conditions predisposing functional differences noted in the present study for E19/E20 embryos.

Embryonic response thresholds presented here are comparable to E19 response thresholds reported by Roll and colleagues (11). Early response components with latencies less than approximately 6.0 ms and labeled here as P1 to N4 were not identified by Roll and colleagues. Instead, Roll and coworkers evaluated only response components having latencies of 6.0 ms and longer, which correspond to the late dominant peaks discussed here. Latencies and amplitudes of early peaks therefore cannot be compared. The present study nonetheless confirms the presence of the late vestibular components in embryos and suggests further that these rather dominant late components are sensitive to anesthesia. Early peaks, P1 to N4, appear to be resistant to anesthesia effects and thus correspond more closely to the early peaks P1 to N4 reported for hatchlings here and elsewhere (2).

Embryonic responses to jerk stimuli clearly are not dependent upon the cochlea, and they disappear following the destruction of the labyrinth. We have argued, therefore, that embryonic responses to jerk (and hence responses to pulsed linear acceleration) are vestibular. Moreover, responses are presumably neural because all response components disappear after TTX application. The generator sites for the individual response components (for example, peripheral versus central sites) remain to be established for the embryo. Late components beyond N4 appear to be sensitive to anesthesia. As discussed, this may reflect myogenic origins, although this hypothesis has not been explicitly tested. The precise origins of the earliest peaks (P1 to P4) similarly must remain hypothetical. Nonetheless, peripheral (P1/N1) and central (beyond N2) response components have been distinguished in post-hatch birds (9,10). By analogy one can propose that a similar distinction holds for embryonic response peaks P1/N1 and those beyond N2. This will be an important hypothesis to test in future studies, because it may provide a means of distinguishing between central and peripheral developmental patterns.

Studies of single primary afferent and second-order vestibular neurons in newborn rats have revealed a number of important functional immaturities (19,20). These investigators reported highly variable single unit responses, decreased input-output sensitivities, and gain as well as delayed timing and phase lags for neural activation in newborn animals. Comparable measurements of single vestibular neurons in the bird embryo have not been reported. However, the reduced amplitudes, elevated thresholds, and delayed latencies of vestibular compound action potentials reported here may be indicative of similar immaturities in avian embryonic vestibular neurons. Determining the actual basis for embryonic differences in the bird remains as an important focus for future research.

We are only meagerly informed about vestibular functional development in the bird. This contrasts with the widespread study of avian auditory ontogeny and the resulting depth of knowledge generated by the avian auditory model. We believe there is as much to gain in the study of the avian vestibular system. The recording of vestibular evoked potentials provides an objective, direct, and noninvasive measure of peripheral vestibular function in the embryo, and as such the method shows great promise as an investigative tool. The results of the present study form the definitive basis for using vestibular evoked potentials in the detailed study of avian vestibular ontogeny.

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