INNOVATIVE METHODOLOGY | Sensory Processing

Implementing the chick embryo model to study vestibular developmental disorders

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Submitted 9 July 2019; accepted in final form 30 September 2019

Seal HE, Lilian SJ, Popratiloff A, Hirsch JC, Peusner KD. Implementing the chick embryo model to study vestibular developmental disorders. J Neurophysiol 122: 2272–2283, 2019. First published October 2, 2019; doi:10.1152/jn.00434.2019.—Children with congenital vestibular disorders show delayed motor development and challenges in maintaining posture and balance. Computed tomography images reveal that these children have abnormal inner ears in the form of a sac, with the semicircular canals missing or truncated. Little is known about how this inner ear abnormality affects central vestibular development. At present, mice with the chromodomain helicase DNA-binding protein 7 mutation are the most common model for studying congenital vestibular disorders, despite forming multiple diverse inner ear phenotypes and inducing abnormal cerebellar and visual system development. To identify the effects of a sac-like inner ear on central vestibular development, we have designed and implemented a new model, the anterior-posterior axis rotated otocyst (ARO) chick, which forms a sac-like inner ear in 85% of cases. The ARO chick is produced by anterior-posterior rotation of the otocyst at embryonic day 2. Here, we describe for the first time the 15% of ARO chicks that form three small semicircular canals and rename the ARO chicks forming sacs (ARO/s chicks). The basic features of the vestibular sensory organs in ARO/s chicks are similar to those found in patients’ sacs, and ARO/s hatchlings experience balance and walking problems like patients. Thus, ARO/s chicks have a reproducible inner ear phenotype without abnormalities in vestibular-related structures, making the model a relatively simple one to evaluate the relationship between the sac-like inner ear pathology and formation of the central vestibular neural circuitry. Here, we describe unpublished details on the surgical approaches to produce ARO chicks, including pitfalls and difficulties to avoid.

NEW & NOTEWORTHY This paper describes simple techniques for chick otocyst rotation resulting in a sac-like inner ear (85%), the common phenotype in congenital vestibular disorders. We now describe anterior-posterior axis rotated otocyst chicks, which form three small canals (15%), and rename chicks forming a sac (ARO/s chicks). Basic protocols and potential complications of otocyst rotation are described. With the use of ARO/s chicks, it will be possible to determine how the vestibular neural circuit is modified by sac-like inner ear formation.

inner ear pathology; otocyst rotation; vestibular nuclei neurons

INTRODUCTION

Despite low incidence for individual congenital vestibular disorders in the overall United States school population, 5.3–10% of children collectively experience vestibular deficits (Elmaleh-Bergès et al. 2013; Layman et al. 2010; Li et al. 2016; Naficy et al. 1997; Rine et al. 2016). From computerized tomography (CT) images, children with the congenital vestibular disorder called “CHARGE syndrome” most commonly form a sac-like inner ear with the semicircular canals missing or truncated (Abadie et al. 2000). Because the chromodomain helicase DNA-binding protein 7 (CHD7) gene is often mutated in CHARGE syndrome patients, the peripheral vestibular system has been studied in Chd7 mutant mice and zebrafish mutant rock solo<sup>LNS</sup>. Both mutants form diverse inner ear phenotypes resulting from variable gene penetrance (Adams et al. 2007; Roberts et al. 2017). Because the vestibular inner ear defects in these mutant animal models are variable, it would be difficult, if not impossible, to perform systematic studies on the development of the central vestibular circuitry with mutant models.

The chick embryo is a classic animal model that has illuminated many basic concepts in inner ear and nervous system development. Technical advantages of the chick include accessibility of the inner ear precursor (otocyst) to surgical manipulation and relative ease of young operated embryos to continue developing after surgery. Indeed, otocyst rotation surgery was performed in the 1930s (Harrison 1935) and used in the 1990s to study factors involved in inner ear development (e.g., Hutson et al. 1999; for review, see Wu and Kelley 2012). If the otocyst of 2-day-old chick embryos (E2) is surgically rotated 180° in ovo in the anterior-posterior and dorsal-ventral axes, with the medial-lateral axis fixed, a sac-like inner ear is formed with all three semicircular canals missing or truncated in 85% of cases (Lilian et al. 2019). Because the protocol involves anterior-posterior axis rotation of the otocyst, we call the model the ARO chick. Here we describe for the first time the abnormalities in the other 15% of ARO chicks that form three small semicircular canals and an intact cochlea (ARO/sc chick) and we rename the ARO chicks that form a sac-like inner ear (ARO/s chick). A detailed description of the inner ear malformations and counts of certain brainstem vestibular nuclei neurons are available from Nissl-stained serial tissue sections of E13 ARO/s chicks, along with vestibular reflex testing on 5-day-old ARO/s hatchlings (H5; Lilian et al. 2019). An
intermediate gestational stage of E13 was selected for study so that interventions could be made to correct the abnormalities before their full impact materializes postnatally (chick incubation takes 21 days). E13 is also a prime time for experimentation because the basic pattern of the chick inner ear has emerged, including orthogonal positioning of the semicircular canals (E9–E10; Bissonnette and Fekete 1996), and certain vestibular nuclei neurons have differentiated to the extent that they are easy to identify (Peusner and Moster 1977a, 1977b, 1977c).

At E13, ARO/s chicks contain all three cristae, macula utriculi, and macula sacculi, although the superior crista and macula utriculi are shortened in anterior-posterior extent and disoriented (Lilian et al. 2019). Type I hair cells are enclosed by calycine endings in all the vestibular sensory organs on the normal and rotated sides. Finally, the number of principal cells in the tangential nucleus (TN), a major avian vestibular nucleus, decreases 66% on the rotated side of ARO/s chicks but remains normal on the nonrotated side (n = 300 neurons/TN). Studies of vestibular reflex behavior in ARO/s hatchlings reveal that the malformed inner ear adversely affects motor performance (Lilian et al. 2019). Although no difference is detected in righting reflex time, ARO/s hatchlings display a constant right head tilt, and, unlike normal chicks, they stumble and walk with a widened base after performing the righting reflex.

In patients with congenital vestibular disorders, certain features of the cristae and maculae have been characterized (Joshi et al. 2012), along with vestibular reflex testing, which shows that the otoliths function but the canals do not (Wiener-Vacher et al. 1999). Thus, findings from clinical testing of children indicate normal otolith function but dysfunction of the semicircular canals. From similarities in the sac-like inner ear gross anatomy, absence of the three semicircular canals, presence of certain abnormalities in the cristae and/or maculae, and abnormal vestibular reflex testing, we propose that the ARO/s chick offers a realistic model to study brain development in subjects missing the semicircular canals. We further propose that identifying the structure and function of abnormally developed brain regions in ARO/s chicks will help to identify the cause(s) of vestibular dysfunction encountered in children with congenital vestibular disorders and aid in the discovery of innovative treatments. This work will also add to our understanding of the basic mechanisms underlying the normal assembly of the central vestibular neural circuitry. For example, to what extent do missing semicircular canals affect vestibuloocular reflex performance and acquisition of spatial orientation (Yakushin et al. 2011)?

Because major findings on inner ear pathology and vestibular nuclei neuron development in ARO/s chicks have been published, here we focus on describing important unpublished details on the optimum methods to produce ARO/s chicks, including chick embryo egg incubation before and after otocyst rotation, surgical techniques for otocyst rotation, and a computer imaging program to improve visualization of the three-dimensional (3D) relationship of the sac-like inner ear with the vestibular nerves and brain. For the first time, we provide details on ARO chicks, which do not form a sac-like inner ear but instead produce an inner ear with three significantly smaller semicircular canals and an intact cochlea (ARO/sc chicks). Thus, we now subdivide ARO chicks into the major group that forms a sac-like inner ear (ARO/s chicks) and the smaller subset that forms an inner ear with three small semicircular canals and a small cochlea (ARO/sc chicks). We also present unpublished complications and pitfalls to avoid when producing ARO chicks.

**MATERIALS AND METHODS**

**Animals and Incubation Conditions**

All animal protocols were approved by the Institutional Animal Care and Use Committee of the George Washington University. The experiments also conformed to the International Guidelines for the Ethical Treatment of Animals. Efforts were made to minimize the number of embryos and hatchlings used in the experiments.

White Leghorn chick embryo eggs (*Gallus gallus*) were purchased as cold freshly fertile eggs from the University of Connecticut Poultry Unit (Storrs, CT). Eggs were stored at 55°F (60% humidity, Vinotemp refrigerator) for up to 5 days before incubation with little loss of viability. Cold eggs were allowed to warm to room temperature for about 2 h before being loaded in a laboratory incubator (GGF incubator, Sportsman model 1502) with temperature (100°F) and humidity (60%) controls and an automatic egg turner unit. Humidity was increased by adding wick pads to the incubator’s internal moisture pan.

**Otocyst Rotation**

Under laboratory conditions, eggs were incubated 64–69 h to reach E2, equivalent to stage (St) 15–St 17 of the Hamburger and Hamilton staging method (Hamburger and Hamilton 1951). A ½-in. oval area was sanded down on the bottom eggshell surface with a low-vibration Dremel drill (20,000 rpm, micro model 8050; Fig. 1A). This approach minimized damage to the embryo and extraembryonic blood vessels from drill vibration and drill heat because young embryos floated on top of the yolk sac near the top surface of the egg. After inverting the egg, the sanded-down shell and underlying shell membranes were removed with forceps (no. 5, RS-4955; Roboz) to view the embryo in ovo under a stereo dissecting microscope (Zeiss Discovery V8) with fiber optics illumination. To improve otocyst visibility, fast green FCF [0.1% in 0.1 M phosphate-buffered saline (PBS); Boulland et al. 2010] was injected from a glass pipette (1.5 mm OD, borosilicate glass; World Precision Instruments) with the tip placed directly next to the head of the embryo near the otocyst (Fig. 1, B and C). Briefly, pipettes were pulled on a Brown/Flaming horizontal pipet puller (P-87; Sutter Instruments) with a sharp tip broken down to ~50 μm by lightly pressing the tip against a smooth surface. The pipette was attached to an aspirator tubing assembly (Sigma- Aldrich) and then filled with fast green by placing the tip in a droplet of dye on a parafilm sheet and then gently inhaling through the aspirator to draw the dye in the pipette shaft (Fig. 1D).

In all experiments, the right otocyst was rotated because the right side of the embryo routinely faced upward in ovo (Fig. 1C). Chorion and amnion were opened discretely over the otocyst using short forceps (70 mm) with 40-μm tips [RS-4913 (Roboz); Fig. 1D2]. A tungsten wire (125 μm diameter; A-M Systems) was flame sharpened to a tapered tip sufficiently sharp to cut through the tissues surrounding and underneath the otocyst (<10 μm diameter; Fig. 1D3). The tip was curved using fine forceps. The opposite end of the wire was heat sealed to a 1.5-mm glass pipette, broken to a 50- to 60-mm length, which served as a short handle easily maneuvered within the restricted space of the eggshell window. Once free from the surrounding tissues, the otocyst was not lifted but was glued along the embryo’s surface, either dorsally, anteriorly, or posteriorly, before rotating the otocyst 180°. Because the otocyst was glued but not lifted in ovo, it was easy to retain the medial-lateral axis of the otocyst. Thus, otocyst rotation reversed the dorsal-ventral and anterior-posterior axes but not the
Fig. 1. Tools for otocyst rotation on embryonic day (E) 2 chick embryos in ovo. A: Dremel drill clamped firmly in a vertical position beneath a platform with an opening over which the egg was held briefly (20 s) to sand down a small oval area (¼ in.) on the bottom eggshell surface. The egg diagram shows where the egg was seated over the platform. Shortly after the shell was sanded down, the egg was inverted so that the sanded-down eggshell area and underlying shell membranes were removed with forceps to view the embryo. B: drawing of E2 chick embryo showing the location of the right otocyst (O) with its endolymphatic duct (ED) in its normal position on the dorsal surface of the otocyst before rotation. At stage (St) 16, the amnion (A) has descended to the region of the vitelline arteries (VA) while the neural tube (NT) is located medial to the otocyst. Red arrow, site where glass pipets containing fast green were placed to inject dye under the embryo. Ant, anterior; Dor, dorsal; CH, cerebral hemisphere; e, eye; h, heart; T, tail bud. C: E2 (St 16) chick embryo in ovo during otocyst rotation. Fast green was injected near the embryo’s head to improve otocyst visibility (red arrow, B). The otocyst was accessed after using fine forceps to make a discrete tear in the chorion (Ch) and amnion overlying the otocyst. Tissues underneath and surrounding the otocyst were cut with a tungsten needle so that the otocyst could be glided, in this case, posteriorly along the embryo’s epithelial surface before rotating it 180° in the anterior-posterior axis. The otocyst was not forced out of the epithelial slot because this could result in tearing the neural tube or failure to dissociate the otocyst from the adjacent vestibular ganglion. Outlined white oval shape indicates the epithelial slot where the otocyst was seated before otocyst removal. In this figure, the otocyst has been rotated, since the endolymphatic duct is located on the ventral surface of the otocyst rather than the dorsal surface as normally found. The otocyst was glided back to the epithelial slot (not shown). D: microtools used for otocyst rotation. D1: the tip of a sharp-tipped glass pipet was broken down to ~50 μm and filled with fast green dye, which was injected near the chick’s head before otocyst rotation to enhance the visibility of the otocyst (see red arrow in B). D2: microdissecting forceps of 70-mm length and 0.04-mm single tip width were optimum for opening the chorion and amnion in ovo. D3: tungsten wire of 125 μm diameter was flame sharpened to a tip of <10 μm to cut the tissues around and underneath the otocyst. D4: a sharp-tipped glass pipet was passed quickly through a Bunsen burner flame to obtain a round glass ball tip of 75–100 μm diameter that was used to move the extirpated otocyst along the embryo’s epithelial surface.
medial-lateral axis. The curved back of the tapered tungsten wire or a glass pipette with heat-flamed round ball tip (75–100 μm diameter) was used to glide the rotated otocyst along the embryo’s surface back to its original epithelial slot (Fig. 1, C and D4).

Otocyst rotation was performed on St 15–St 17 embryos. St 14 embryos were not used because of their high capacity to regenerate (Levi-Montalcini 1949). Because the anterior cardinal vein often surrounds the otocyst at St 17, making hemorrhage likely during otocyst rotation, those St 17 embryos with the otocyst enclosed by the anterior cardinal veins were not operated on. When the preliminary manipulations of eggshell, fast green dye injection, and opening the chorion and amnion were performed carefully, little bleeding resulted. Surgery was usually completed in <5 min, with prolonged surgeries of >10 min possibly leading to chilling or drying out of the embryo and possible death. Damaged embryos usually died within 1–3 days after surgery.

The endolympathic duct and amnion were helpful structures in staging chick embryos. At St 15, the otocyst had an oval shape, but no endolympathic duct had emerged. The endolympathic duct first appeared on the dorsal otocyst surface by St 16 and thereafter provided a distinctive landmark to check the degree of otocyst rotation. From St 15 to St 17, the amnion transformed into a nearly complete sac enclosing the chick embryo by descending from the embryo’s head region to the tail region. At St 15, the amnion was located halfway between the heart and vitelline arteries. By St 16, the amnion had grown to its original epithelial slot (Fig. 1B). At St 17, the amnion formed a small oval opening over the tail region (not shown).

At E3, 1 day after otocyst rotation, the chick embryo still rested on top of the yolk sac in ovo so it could be viewed under a dissecting microscope. Because wound healing had begun, the condition of the otocyst and viability of the embryo was checked at E3 to confirm that the otocyst was in place within the epithelial slot and had not floated away because of the embryo’s amniotic fluid environment. The location of the rotated otocyst was photographed using Zen blue software and a Zeiss Axiocam Erc 5s camera attached to the Zeiss dissecting microscope. After otocyst rotation (~1 day), the torn chorion and amnion repaired naturally (Fig. 2A). Sometimes, an opaque chorionic clump formed over the otocyst, blocking view of the otocyst (Fig. 2B). In these cases, to check the position of the otocyst, the chorionic clump was removed with forceps, avoiding damage to the underlying embryo. After E3, older embryos usually sank into the yolk sac away from the surface so that embryo viability was checked indirectly by looking through the eggshell window for small and large extraembryonic blood vessels on the yolk sac surface and/or embryonic movements. Both features were good indicators of healthy growing embryos. By E11, embryo viability was checked using a digital egg monitor to measure heart beats per minute (Buddy, Avitronic, England), with 200–300 beats/min indicative of healthy E11–E19 chick embryos (Höchel et al. 1998).

After otocyst rotation, the shell window was sealed with surgical tape (Blenderm; 3M), and the egg was reincubated at 100°F, 70% humidity, with the egg-turning unit off (Grubbach egg incubator model 8014; Lyon Instruments). ARO chicks that were alive 3 days after surgery were usually healthy and viable at E13 (41% survival rate at E13; n = 17/42) when most were euthanized (Fig. 3 and Lilian et al. 2019). In control chicks, a window was made in the eggshell at E2, followed by reincubation without egg turning until hatching (29% survival rate to hatching; n = 2/7). In ARO chicks that were allowed to hatch, viability decreased mainly because of introducing the eggshell window, which precluded egg turning (17% to hatching; n = 7/42). Finally, normal chick embryo eggs that were allowed to hatch in the laboratory showed reduced viability, likely because of natural stresses related to hatching, since only 73% hatched (n = 11/15 eggs; Fig. 3). To improve hatching survival rate for all chick categories, the incubator temperature was lowered to 97–98°F starting at E19, with the humidity kept at 70% (Reis et al. 1997; Yildirim and Yetisir 2004). After hatching, some chicks walked with spraddled legs, an abnormality that was avoided by placing shelf-lining paper with open grids on the incubator floor. Thus, ARO chicks could hatch after in ovo incubation, unlike ex ovo cultured chick embryos, which can grow only up to E14–E15 before dying (Cloney and Franz-Odendaal 2015).

Paint Fills of Inner Ear

The membranous labyrinths were visualized by injecting opaque paint (Liquid Paper; Newell Rubbermaid) dissolved in methyl salicylate (M-6752; Sigma-Aldrich) in fixed E13 chick embryos and H5 hatchlings. Chicks were decapitated, the lower jaw and skin were removed, and the cranium and meninges were opened dorsally, followed by immersion in Bodian’s fixative (Bissonnette and Pekete 1996; Kiernan 2006; Martin and Swanson 1993). After 2 days, the brain was dissected out and processed separately (see Imaging Vestibular Nuclei Neurons). The cranium was cut in half, separating the

Fig. 2. Natural repair of torn chorion in embryonic day (E) 3 chick embryo after otocyst rotation surgery at E2. A: the chorion (Ch) repaired itself naturally ~1 day after otocyst rotation surgery following discrete tearing to access the otocyst for rotation at E2. In this case, the embryo appeared green because of injecting a heavy dose of fast green just before otocyst rotation. B: during repair of the chorion, the chorion often formed an opaque clump (Ch). If the clump obstructed viewing the otocyst, it was removed with forceps without injury to the underlying embryo. In B, the otocyst (*) was visible without removing the chorionic clump. e, Eye; h, heart. In A and B, dorsal is to the top and anterior to the right. Scale bar in B refers to A and B.
left and right inner ears, which were dehydrated using an increasing series of 70–100% ethanol for 2–24 h each step (20–50 ml solution/specimen) before clearing in methyl salicylate in the dark. To prevent tissue hardening or discoloration, ethanol dehydration steps were shortened (Sigma-Aldrich Technical Support).

The half-cranium was pinned down to a Sylgard-coated petri dish with its medial surface facing upward and immersed in methyl salicylate before being viewed under a dissecting microscope with dual fiber optics lights placed horizontally at opposite ends of the dish. To improve visibility of inner ear structures, 2% charcoal was added before solidification. At E13 and H5, the membranous labyrinths appeared semitransparent while the otoliths were opaque white, so that the otoliths represented good targets for placing the pipette tip in the utricle in both normal and ARO chicks (Fig. 4A). At E13, the temporal bones had not ossified, so the membranous labyrinths were injected with 1% paint using a glass pipette (31 gauge) that pierced through the bone to the utricle. Second, a glass pipette was inserted in the bony opening to inject the paint in the utricle. Because after methyl salicylate clearing tissues surrounding the membranous labyrinths were yellow at H5, the paint concentration was increased to 4%. Briefly, using a Microfil syringe needle (MF34G; World Precision Instruments) attached to a syringe containing paint, paint was backfilled in a sharp-tipped glass pipette (40- to 50-μm tip). The pipette was attached with plastic tubing to a 1-ml Hamilton syringe containing glycerol and mounted on a micromanipulator. The pipette tip was advanced slowly using the micromanipulator until the tip punctured the medial surface of the utricle (Fig. 4B). Gentle pressure was applied to the syringe plunger to inject the paint. In some specimens, paint fill was improved by reinjection. With experience, the success rate for paint fills was 80%.

**Inner Ear Measurements**

Here, we present for the first time measurements of the three small semicircular canals formed in 15% of ARO chicks, the ARO/sc
chicks, and measurements of the hatching ARO sac-like inner ears (Fig. 5). The semicircular canals from normal E13 chicks and E13 ARO/s chick sacs are included for comparison. Briefly, the overall dimensions of the paint-filled labyrinths were measured from photographs of the lateral surface of the inner ear using Zen blue computer program. Image background was blackened using Adobe Photoshop (version CC 2017). Inner ears were oriented with the lateral canal in the horizontal plane. The anterior-posterior expanse of the superior canal was measured from anterior to posterior tip, whereas the dorsal-ventral expanse was measured from the dorsal tip to the surface of the utricle. Likewise, the anterior-posterior expanse of the lateral canal and dorsal-ventral expanse of the posterior canal were measured. The length of the cochlea was measured from apex to base. The diameters of the vertical canals were measured 50 μm from the ampullae, whereas the lateral canal was measured at the canal center because the ampullary connection was not visible from a lateral surface view. The anterior-posterior and dorsal-ventral dimensions of the sacs were measured by placing their vertical long axes in the vertical plane. Finally, the distance was measured between the posterior edge of the eyeball and superior canal ampulla in ARO/sc chicks and the anterior surface of the ARO/s hatching sac.

Imaging Vestibular Nuclei Neurons

After fixation in Bodian’s solution for 2 days, the whole head, or the brain dissected out of the cranium, was immersed in 10% neutral buffered formaldehyde for at least 2 days, followed by paraffin embedment and serial sectioning at 20 μm. Sections were stained using the Nissl method (Luna 1968). Tissue sections of the brainstem with/without the inner ears intact were photographed and assembled in virtual slides by stitching together adjacent fields from a single Nissl-stained tissue section using a Zeiss Cell Observer light microscope equipped with ×100 objective (NA, 1.46).

The TN was selected for study because its principal cells are readily identified by the orderly arrangement of their large oval cell bodies near the lateral surface of the medulla oblongata between horizontally coursing primary vestibular fibers. In preliminary studies performed at E10–E12, principal cells were distinguished in lateral parts of the TN but not in medial parts where the neurons were small and not fully differentiated (Fig. 6). Thus, at E10–E12, principal cell identification and counts could not be made reliably. Accordingly, neuron counts were made at E13, when all the principal cell bodies have acquired characteristic size and shape. Finally, in some chicks, the head containing the inner ears and brain intact were processed for Nissl staining and serial tissue sections (Lilian et al. 2019). In these sections, the overall configuration of the membranous and bony labyrinths was identified as sac like in ARO/s chicks or having three small canals in ARO/sc chicks by tracing inner ear structures through serial tissue sections.

Immunolabeling and Confocal Imaging Combined with Imaris 3D Imaging of Whole Mount Chick Embryos

Whole mounts of E4–E5 normal and ARO chick embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS overnight, followed by incubation for 1 h in blocking solution composed of 0.1 M PBS containing 1% Triton X-100 (PBS-T), 10% normal goat serum, and 1% Boehringer-Mannheim blocking (BMB) stock solution (Kapinski et al. 2014; Lilian et al. 2019). BMB stock solution consisted of 0.1 M PBS-T, 0.2% fraction V bovine serum albumin, and 10% BMB blocking powder (Roche). Next, embryos were incubated in primary antibody in blocking solution at 4°C on a shaker for 3–4 days, followed by incubation in secondary antibody in blocking solution at 4°C on a shaker for 1–2 days. Embryos were dehydrated in an increasing series of methanol/0.1 M PBS, followed by clearing in 1:2 benzyl alcohol-benzyl benzoate (BABB) before imaging on a Zeiss LSM 710 confocal microscope. Primary antibodies included purified anti-tubulin-β, (TUJ1, no. 801202; 1:500 or 1:1,000; Biolegend), phallodin conjugated to Alexa Fluor 647 [A22287, 1:1,000; Invitrogen (not used in the sample shown in Fig. 7C)], and DAPI (268298; Calbiochem). Secondary antibody for tubulin was Alexa Fluor goat anti-mouse 488 IgG (A11029, 1:1,000 or 1:500; Invitrogen). DAPI was added to the secondary antibody solution in a concentration of 0.4–1 μg/mL dissolved in water.

For confocal imaging, embryos were placed on their side in a depression well of a glass slide, immersed in BABB, and covered with a cover slip. The otocyst was imaged from lateral to medial, producing serial optical sections in a z-stack, which rendered three-dimensional (3D) images of the developing otocyst, vestibular nerves, and medulla oblongata. Confocal z-stacks were further manipulated using Imaris 3D image-processing software (Bitplane AG). Individual image planes were cropped manually to retain the area of interest. Z-stacks of the cropped images were further processed to show the 3D structure of the developing otocyst and vestibular nerves, which were displayed...
**Tangential Nucleus (TN) in Normal and ARO/s Chick Embryos**

![Image of Principal Cells](image)

Fig. 6. Principal cells of the tangential nucleus (TN). In A–D, dorsal is at the top. In A and C, lateral is to the left, and, in B and D, lateral is to the right. A: embryonic day (E) 10 anterior-posterior axis rotated otocyst (ARO) chick, left intact side. Arrows point to principal cell bodies with typical oval shapes. Note many small neuron cell bodies (*) medial to the principal cells that may be undifferentiated principal cells since the full number of principal cells was not counted in the TN at E10 (n = 259 ± 77 principal cells at E13; Lilian et al. 2019). Thus, accurate principal cell counts could not be made at E10. B: E13 normal chick, right side. Large oval principal cell bodies were present throughout the medial-lateral extent of the TN. Their distinctive morphology allowed accurate neuron counts at E13 not possible at E10. C: E13 anterior-posterior axis rotated otocyst chick forming sac-like inner ear (AROs), left intact side. The number of principal cells in the TN were the same as found in normal E13 TN. D: E13 ARO/s chick, right rotated side. Note that fewer principal cell bodies are found in the right TN after right otocyst rotation at E2 (34%; Lilian et al. 2019). Although principal cell number was reduced on the right rotated side of E13 ARO/s chicks, the size and shape of the principal cell bodies were indistinguishable from normal E13 principal cells and the left intact side of ARO/s chicks. The restiform body (RB) runs next to the lateral brain surface and lateral to the TN. In A–D, a broken line demarcates the border between the RB and TN. Scale bar in C refers to A–D.

as solid or semitransparent structures to enhance the visualization of structures and their relationships. Thus, Imaris processing avoided volumetric segmentation of large volumes that may obstruct viewing deeper objects in the section series.

**Statistical Analysis**

Data are presented as averages ± SE or the deviation of the measurements divided by the square root of the number of measurements (n). Significant differences between groups were determined using a one-way ANOVA test, followed by the Tukey HSD posttest (α = 0.05). Statistics were performed in R (version 3.5.1, http://www.r-project.org). The significance level was set at P < 0.05. Statistical significance is stated as the statistical value and degrees of freedom of the one-way ANOVA [F(k-1,n-k)] with P value from the posttest. For reported statistics, the following three groups were compared: 1) normal chick embryos, including the left and right side; 2) left side of ARO chicks; and 3) right rotated side of ARO chicks.

**RESULTS**

**Complications and Pitfalls of Otocyst Rotation**

**ARO/sc chicks.** In 85% of E13 ARO chicks, the major phenotype was an inner ear forming a sac with all three semicircular canals missing or truncated (n = 45/52; Lilian et al. 2019). We now distinguish chicks forming a sac as ARO/s chicks, since here we describe for the first time the remaining 15% of E13 ARO chicks that formed a small semicircular canal phenotype (the ARO/sc chicks, n = 17/52; Fig. 5D and Table 1). All canals and cochleae in ARO/sc chicks were statistically smaller in expanse than those found in normal E13 chicks (P < 0.05). However, canal diameters were not changed.

Despite shorted canal expanses, the anterior-posterior position in the head of the inner ear relative to the eyeball in E13 ARO/sc chicks (4,807 ± 322 μm; n = 5) did not shift significantly compared with normal E13 chick inner ears [5,558 ± 176 μm; n = 9; t(6) = 2.045; P = 0.0868]. It is interesting that here we found that the sac-like inner ear in ARO/s hatching chicks also retained the same anterior-posterior position in the head relative to the eyeball (5,629 ± 241 μm; n = 9) as found for normal H5 chick inner ears (5,561 ± 307 μm; n = 8) and the inner ear on the left side of the H5 ARO/sc chicks [6,269 ± 321 μm; n = 4; F(2,18) = 1.247; P = 0.108]. This could be expected since E13 sac-like inner ears of ARO/s chicks retained the same anterior-posterior position relative to the eyeball compared with the inner ear in normal E13 chicks (Lilian et al. 2019).

**Neural tube damage.** At E2, the otocyst was closely apposed to the neural tube, which resided medially (Fig. 7A). The neural tube could be pulled and torn if the tissues separating it from the otocyst were not cut completely before otocyst rotation. Adhesion of the neural tube and otocyst was more likely to occur if the tissues dried out during prolonged surgeries.

**Vestibular ganglion damage.** Another pitfall of otocyst rotation at E2 resulted from the proximity of the otocyst to the vestibular ganglion, which was located anterior and ventral to the otocyst (Fig. 7B). If the attachment was not cut completely before otocyst rotation, the vestibular ganglion adhered to the otocyst wall and moved along with the otocyst during rotation. At E4, the relocated vestibular ganglion developed posterior to the otocyst rather than anteriorly (Fig. 7, C and D).

**Difficulty viewing the relationship between the otocyst and vestibular nerves.** Confocal imaging was performed on E4–E5 normal (n = 9) and E4–E5 otocyst-rotated (n = 7) chick embryos from whole mount preparations that were immunolabeled with tubulin to view microtubules (green), phalloidin to label the actin filaments (not shown here), and DAPI to label the cell nuclei (blue) (Fig. 7C). The peripheral courses of the vestibular nerves were traced in series of confocal images from
medial to more lateral optical sections. Despite immunolabeling, it was sometimes difficult to trace the peripheral vestibular nerve pathways, especially that of the posterior branch, which deviated in its normal course after otocyst rotation (Lilian et al. 2019). To better view the relationship of the otocyst with the vestibular nerves and medulla oblongata in E4–E5 normal and ARO/s chicks, 3D representations of these structures were prepared from serial confocal images of immunolabeled whole mount specimens using Imaris 9.2 software (Fig. 7, B and D). Confocal images rendered using Imaris clearly revealed the

Fig. 7. Images of young chick embryos depicting the relationship of the otocyst, neural tube, medulla oblongata, vestibular ganglion, and vestibular nerves. A: embryonic day (E) 2 living chick embryo in ovo after otocyst rotation under the dissecting microscope [note ventral position of endolymphatic duct (ED)]. The neural tube was torn (*) accidently by pulling rather than cutting tissues around or underneath the otocyst before rotating the otocyst. e, Eye; h, heart. B: Imaris image of otocyst and neural tube on left intact side of E4 anterior-posterior axis rotated otocyst (ARO) chick, assembled from a stack of confocal images. The otocyst appeared normal after immunolabeling for tubulin, phalloidin, and DAPI. Anterior (Va) and posterior (Vp) vestibular nerve branches terminated on the anterior and posterior surfaces of the otocyst wall, respectively, as in normal E4 chicks (see Lilian et al. 2019). Me, medulla oblongata; V, vestibular nerve near entry in medulla oblongata; Vg, vestibular ganglion; F, facial nerve; Gl, glossopharyngeal nerve; Vag, vagus nerve; Ant, anterior; Post, posterior; Dor, dorsal; Ven, ventral. The Imaris image shows the otocyst in white transparency; medulla oblongata in opaque blue; vestibular ganglion, vestibular nerves, and facial nerve in opaque red; and glossopharyngeal and vagus nerves in opaque pink. C, cochlea; O, otocyst. C and D: confocal and Imaris images, respectively, of the otocyst on the right rotated side of an E4 ARO chick immunolabeled for tubulin and DAPI (blue/green channel). Phalloidin immunolabeling was not performed on this specimen. During otocyst rotation, the vestibular ganglion adhered to the anterior otocyst wall so that the ganglion was moved along with the otocyst. At E4, the vestibular ganglion was located posterior to its normal position near the glossopharyngeal and vagus nerves rather than more anteriorly, close to the facial nerve. Because the boundary between the vestibular and glossopharyngeal/vagus nerves was not distinguished, the nerves were indicated by the same color (pink). This Imaris images show the otocyst in white transparency; medulla oblongata in opaque blue; vestibular ganglion/vestibular nerve, glossopharyngeal nerve, and vagus nerves in opaque pink; and facial nerve in green. Compare C and D with B.
intimate relationship between the vestibular ganglion and vestibular nerves to the facial nerve, located anterior to the otocyst, and the glossopharyngeal and vagus nerves, located posterior to the otocyst, in normal chicks and the left side of ARO/s chicks at E4 (Fig. 7B). In addition, Imaris 3D renderings from cleared and immunolabeled tissue revealed the complete trajectory of the anterior and posterior branches of the vestibular nerves relative to the otocyst by observing different aspects of the volume rather than inspecting segments of each nerve in single sections and reconstructing the nerve pathways through serial sections to determine the nerve trajectories (compare with Fig. 9 in Lilian et al. 2019). Clearly, the anterior vestibular nerve followed a direct pathway running along the anterior surface of the otocyst wall to end on the anterior otocyst wall, whereas the posterior vestibular nerve ran from the vestibular ganglion medially and then coursed laterally before ending on the posterior wall of the otocyst (Fig. 7B). In chick embryos with the vestibular ganglion rotated along with the otocyst, Imaris-visualized volumes showed that the position of the vestibular ganglion relative to the otocyst was shifted to the posterior and medial otocyst surface near the glossopharyngeal and vagus nerves rather than at the anterior surface of the otocyst near the facial nerve (Fig. 7, C and D). In embryos with rotated vestibular ganglion, the courses of the anterior and posterior vestibular nerve branches could not be traced.

**DISCUSSION**

**Research on Congenital Vestibular Disorders**

The vertebrate inner ear is an intricate structure housing two sets of vital relatively independent sensory organs, the vestibular subset that detects head movements necessary for maintaining posture and balance and the auditory subset that detects sound waves involved in hearing. Despite the functional independence of these sensory organs, children with congenital disorders who experience hearing impairment often experience vestibular deficits affecting posture, locomotion, and gait (Kaga et al. 2008). The vestibular system plays a major role in daily living, with damage affecting standing, walking, reading, learning language, eye-hand coordination, and eye tracking (Blake et al. 2008). Thus, treatment of patients’ inner ear pathology is important to maintain quality of life for these patients. Accordingly, it is surprising that vestibular function in children has been afforded little attention in the clinic and research laboratory (Janky and Givens 2015).

Children with congenital disorders that produce profound sensorineural hearing loss may be treated by cochlear implants with variable results in improving speech and comprehension (Vincenti et al. 2018). Current treatment for congenital vestibular disorders involves physical therapy to relieve the symptoms. Vestibular testing may be performed to determine which vestibular components are dysfunctional. Utricular and saccular dysfunction is identified using ocular and cervical vestibular evoked myogenic potentials, whereas semicircular canal pathology is determined using bithermic caloric tests, earth vertical axis rotation, or head impulse testing (Janky and Givens 2015). At present, there are no diagnostic tests to identify dysfunction at the cellular level of the cristae, maculae, or vestibular neural circuitry in the brain. However, analysis at the cellular level can be achieved by applying invasive techniques to animal models.

The ARO chick offers a useful model for this work because of its reproducible inner ear sac pathology in 85% of cases. In addition, chick embryo eggs are readily available so that ARO chicks can be produced in large numbers for large-scale repetition of experiments required for analyzing data and statistical analysis.

**Is the ARO/s Chick Sac Similar to Sacs Found in Children with Congenital Vestibular Disorders?**

The ARO/s chick sac mimics the gross anatomy of the bony and membranous labyrinths and the histology of the vestibular sensory organs commonly seen in children with congenital vestibular disorders. Because at present there is no one imaging technique that can demonstrate all components of the peripheral and central vestibular system in living organisms, a composite image must be assembled from combining different approaches. High-resolution CT images show the bony labyrinths, whereas magnetic resonance imaging (MRI) demonstrates the membranous labyrinths, fluid-filled spaces of the inner ear, and vestibular nerves. Functional MRI reveals brain...
regions but not the cristae and maculae (Joshi et al. 2012). Future technical advances are needed to produce high-resolution images showing the cellular components of the cristae, maculae, and central vestibular neurons in living subjects. Presently, the most detailed images of inner ear structures are obtained from histological preparations of postmortem specimens (e.g., Haginomori et al. 2002).

Gross anatomy of the bony labyrinths. From CT images, the consistent pathology in human congenital vestibular disorders is semicircular canal hypoplasia (e.g., Sando et al. 2001). Studies of CHARGE syndrome patients indicate that the predominant inner ear defect is a sac missing all three semicircular canals (e.g., Abadie et al. 2000). Thus, it is propitious that a sac is produced by otocyst rotation in the anterior-posterior and dorsal-ventral axes of E2 chicks. Presumably, ARO/s chick sacs arise by halting otocyst morphogenesis at E4, when the otocyst normally forms a dorsal-ventrally elongated sac with the cochlea starting to emerge ventrally (Bissonnette and Fekete 1996). It is interesting that ARO/s chicks routinely lack a cochlea (n = 22/25; Lilian et al. 2019).

Histology of vestibular sensory organs. Which vestibular sensory organs are present in the human sac-like inner ear? Studies of the inner ear in CHARGE syndrome patients are often based on sampling one to four patients (e.g., Amiel et al. 2001; Guyot et al. 1987, Guyot and Vibert 1999; Haginomori et al. 2002; Martin et al. 2001; Wright et al. 1986), although some studies contain a larger cohort of patients [e.g., n = 50 (Morgan et al. 1993); n = 17 (Abadie et al. 2000)]. Despite differences in sample size and imaging approaches, common findings have emerged. 1) All three semicircular canals are routinely absent. 2) More severe abnormalities are detected in the vestibular sensory organs innervated by the anterior vestibular nerve. 3) The utricular macula is reduced in size. 4) The cochlea is reduced in size or missing. Indeed, the common inner ear pathologies found in these patients also characterize ARO/s chicks, including absence of the cochlea (Lilian et al. 2019).

Although the semicircular canals are often absent in human congenital vestibular disorders and in ARO/s chicks, the cristae and maculae develop with few defects. The ARO/s chicks contain all three cristae, with the superior crista disoriented and reduced in anterior-posterior extent (Lilian et al. 2019). The macula utriculi is also reduced in anterior-posterior extent. Thus, the cristae and maculae, which differentiate between E3 and E4.5, develop after otocyst rotation at E2. However, otocyst rotation at E2 primarily affects the outgrowth of the semicircular canals that occurs at E6. Why chick otocyst rotation at E2 disrupts inner ear morphogenesis at E6, but not from E2–E6, remains to be determined.

Chd7 mutant mice do not form a sac missing all three semicircular canals (Adams et al. 2007). Instead, canal formation is variable. The superior canal is present with normal structure. The lateral canal is variable, sometimes absent or present with diverse shapes or diameters. The posterior canal is usually present but may have reduced diameter. All three cristae, maculae utriculi, and maculae sacculi are present routinely in Chd7 mutant mice. Although the superior cristae are saddle shaped, the lateral cristae have smaller widths with decreased vestibular nerve input. The posterior cristae are variable, sometimes forming a flattened saddle-shaped sensory epithelium or a round patch epithelium. No calyce endings or primary vestibular fibers are detected in patches of the posterior cristae. Thus, the vestibular sensory organs in Chd7 mutant mice are highly variable and differ from the most common forms found in human congenital vestibular disorders.

What Roles Do Semicircular Canals Play on Development of the Central Vestibular System?

In children, peripheral vestibular structural abnormalities correlate closely with diminished performance on clinical vestibular tests (Abadie et al. 2000). Semicircular canal abnormalities are associated with delayed development of posture and lack of responses in canal function tests. At present, physical therapy is the only treatment available for these patients. Despite treatment, these children do not recover to the level of normal children but experience poor visual acuity and poor balance throughout life (Janky and Givens 2015). H5 ARO hatchlings show a constant head tilt toward the right side (Lilian et al. 2019). Whereas no difference is detected in righting reflex time between normal and ARO hatchlings, after performing the righting reflex, ARO chicks stumble and walk with a widened base, which is not observed in normal chicks. Mice, which are heterozygous for Chd7 gene mutation and form abnormal semicircular canals, are hyperactive with head-bobbing and circling movements. Presently, important gaps exist in our understanding of what produces the symptoms of developmental disorders. In DiGeorge syndrome, it is thought that cognitive deficits result from formation of fewer axonal connections between association areas of cerebral cortex (Fernandez et al. 2019). However, what happens in the cerebral cortex may not offer an accurate template for other brain regions. In larval Xenopus, rotatory head movements generate motor commands to stabilize gaze only after a minimum semicircular canal diameter is acquired during development (Lambert et al. 2008). It is interesting that other components of the vestibular circuitry are functional before minimal semicircular canal diameter is achieved. Specifically, functional activity is present in the extraocular eye muscles, central vestibular neural pathways, and hair cells of the cristae before functional canal diameters are acquired. Because vestibular semicircular canals are largely conserved in vertebrates, it seems likely that birds and mammals also must acquire minimal semicircular canal diameters to activate their vestibular neural circuitries. Taken together, ARO/s and ARO/sc chicks may be incapable of generating sufficient hair-cell stimulation on rotation to evoke normal signaling in hair cells of the cristae. However, canal function in ARO chicks remains to be shown.

How does the precise topographic pattern of the central vestibular circuitry form? The major vestibular pathology in ARO/s chicks is the absence of all three semicircular canals. Because transformation of vestibular signals from the sensory organs into spatially precise motor commands for gaze and posture stabilization requires convergence of semicircular canal and otolith inputs onto single vestibular nuclei neurons in a precise pattern, it will be important to determine whether the normal convergent pattern is formed and maintained or how it changes in patients with sac-like inner ears. Already, we know from ARO/s chicks that only one-third of TN principal cells survive at E13. Is the decreased number of vestibular nuclei neurons in ARO/s chicks the result of fewer vestibular gan-
glion cells that give rise to fewer primary vestibular fibers in the brainstem? Do surviving vestibular nuclei neurons receive convergent canal and otolith inputs according to the normal pattern (Cox and Peusner 1990b; Popratiloff and Peusner 2007)? Do axons of surviving vestibular nuclei neurons follow the normal pathways that contain normal axon numbers projecting to target regions, whereas other pathways fail to form (Cox and Peusner 1990a)? Alternatively, are all axon pathways present but contain fewer axons? How do fewer vestibular nuclei neurons affect neuron numbers in target regions? Currently little is known on the mechanisms that wire projection neurons into stereotypical circuits (for review, see Lodato and Arlotta 2015).

ACKNOWLEDGMENTS

We thank Lakshmi Kammili of the George Washington University Pathology Core Laboratory for processing the chick specimens for paraffin embedding, tissue sectioning, and Nissl staining. Present address of S. J. Lilian: Lewis Katz School of Medicine at Temple University, Philadelphia, PA.

GRANTS

This work was supported in part by Research Funds from the George Washington University (GWU) Department of Anatomy and Cell Biology (K. D. Peusner), GWU Luther Rice Undergraduate Fellowships (S. J. Lilian and H. E. Seal), and the District of Columbia Intellectual and Developmental Disabilities Research Center (National Institute of Child Health and Human Development Grant 1U54-HD-090257 to A. Popratiloff).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.E.S., S.J.L., A.P., and K.D.P. performed experiments; H.E.S., S.J.L., J.C.H., and K.D.P. analyzed data; H.E.S., A.P., J.C.H., and K.D.P. interpreted results of experiments; H.E.S., A.P., and K.D.P. prepared figures; H.E.S. and K.D.P. drafted manuscript; H.E.S., A.P., and K.D.P. edited and revised manuscript; S.J.L., A.P., J.C.H., and K.D.P. approved final version of manuscript; K.D.P. conceived and designed research.

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