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Inner Ear Formation during the Early Larval Development of *Xenopus Laevis*

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Abstract

The formation of the eight independent endorgan compartments (sacculus, utricle, horizontal canal, anterior canal, posterior canal, lagena, amphibian papilla, and basilar papilla) of the *Xenopus laevis* inner ear is illustrated as the otic vesicle develops into a complex labyrinthine structure. The morphology of transverse sections and whole mounts of the inner ear was assessed in seven developmental stages (28, 31, 37, 42, 45, 47, 50) using brightfield and laser scanning confocal microscopy. The presence of mechanosensory hair cells in the sensory epithelia was determined by identification of stereociliary bundles in cryosectioned tissue and whole mounts of the inner ear labeled with the fluorescent F-actin probe, Alexa-488 phalloidin. Between stages 28 and 45 the otic vesicle grows in size, stereociliary bundles appear and increase in number, and the pars inferior and pars superior become visible. The initial formation of vestibular compartments with their nascent stereociliary bundles is seen by larval stage 47, and all eight vestibular and auditory compartments with their characteristic sensory fields are present by larval stage 50. Thus in *Xenopus*, inner ear compartments are established between stages 45 and 50, a two week period during which the ear quadruples in length in the anteroposterior dimension. The anatomical images presented here demonstrate the morphological changes that occur as the otic vesicle forms the auditory and vestibular endorgans of the inner ear. These images provide a resource for investigations of gene expression patterns in *Xenopus* during inner ear compartmentalization and morphogenesis.

Keywords

hair cell; sacculus; utricle; amphibian papilla; basilar papilla; semicircular canal; lagena; confocal; sensory ganglion; stereocilia

INTRODUCTION

The anatomy and physiology of the amphibian inner ear are well characterized, especially during adult life (Capranica, 1978; Duellman and Trueb, 1986; Smotherman and Narins, 2000). In particular, the amphibian inner ear has proved useful for discovery of cellular and physiological mechanisms that underlie encoding of the senses of hearing and balance, such as the biophysical events that occur during mechanoreception by sensory hair cells (Duellman and Trueb, 1986; Hudspeth, 1989; Eatock, 2000). Much less is known about the morphogenesis of the inner ear between embryonic stages and metamorphosis (Lewis, 1907; Li and Lewis, 1979; Duellman and Trueb, 1986; Boatright-Horowitz and Simmons, 1997). Among amphibians the African clawed frog, *Xenopus laevis*, offers many advantages for investigation of inner ear morphogenesis. *Xenopus* is an established model organism for

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developmental studies, especially of embryogenesis, because animals are relatively easy to house and to breed, and thorough descriptions of developmental stages are available (Nieuwkoop and Faber, 1967; Kay and Peng, 1991).

The *Xenopus* inner ear resembles that of other anuran amphibians (Geisler et al., 1964). It comprises eight sensory endorgans specialized for vestibular (anterior, horizontal, posterior semicircular canals; utricle; lagena), auditory (amphibian papilla; basilar papilla), and acoustico-vestibular (sacculus) sensation (Paterson, 1948; Nieuwkoop and Faber, 1967; Haddon and Lewis, 1991; Lopez-Anaya et al., 1997; Bever et al., 2003). As in most vertebrates, inner ear development begins with the invagination of a thickened epithelium near the hindbrain, the otic placode, that gives rise to a spherically shaped otic vesicle (Noramly and Grainger, 2002). In *X. laevis*, induction of the otic placode and specification of the otic vesicle are complete by neural tube stages, establishing that the ear is one of the first organs to be determined during organogenesis (Gallagher et al., 1996). The cellular and molecular events that convert the otic vesicle into the membranous labyrinth of the inner ear with its multiple sensory endorgans are not well understood. The progressive differentiation and tissue patterning that characterize inner ear morphogenesis are under inquiry in many organisms including *Xenopus*, zebrafish, chick, and mouse, through multiple approaches such as fate mapping, cell lineage analysis, and gene expression studies (Fekete and Wu, 2002; Fritzsche et al., 2002; Kil and Collazo, 2002; Noramly and Grainger, 2002).

Although many investigations of the *Xenopus* inner ear have focused on processes that occur during embryonic stages (Gallagher et al., 1996; Kil and Collazo, 2002), there is a growing interest in studying events such as innervation (Hellmann and Fritzsche, 1996; Lopez-Anaya et al., 1997), three-dimensional morphogenesis (Bever et al. 2003), hair cell differentiation (Diaz et al., 1995; Kil and Collazo, 2001; Serrano et al., 2001), and gene expression (Kil and Collazo, 2001; Serrano et al., 2001) in postembryonic animals. In adult *Xenopus*, neuroethologists have been able to record responses to ambient sound (Elepfandt et al., 2000), as well as distortion product otoacoustic emissions (van Dijk et al., 2002). Recently the potential of *Xenopus* as an organism for cellular and molecular studies of the inner ear has been augmented by new methods that enable production of large numbers of transgenic animals per day (Amaya and Kroll, 1999), and the validation of *Xenopus tropicalis* as a useful organism for molecular genetics and large scale sequencing efforts (Hirsch et al., 2002; Gilchrist et al., 2004). Thus *Xenopus* is a viable model for multifaceted studies of the auditory and vestibular systems that combine genetics with behavior and physiology.

The objective of this study was to illustrate the morphogenesis of the auditory and vestibular sensory endorgans of the *X. laevis* inner ear, as well as the initial appearance of stereociliary bundles in sensory epithelia. The images presented here were acquired with brightfield and confocal microscopy and are intended to highlight landmark anatomical features that may be useful to other investigators who begin to navigate the intricacies of the inner ear in developing *Xenopus* larvae. The cellular detail provided in the images complements anatomical information shown in a paint-fill-based atlas that depicts the three-dimensional gross morphology of the *X. laevis* inner ear for similar stages (Bever et al., 2003). Together, these data sets provide a breadth of anatomical perspectives that will be useful for those investigators interested in cell and tissue specific gene expression patterns as well as inner ear development in general. Anatomical features of the development of the *X. laevis* inner ear are anticipated to be relevant for *X. tropicalis* because preliminary studies have shown that the postmetamorphic ears of these closely related species contain auditory and vestibular organs of comparable size and shape that are similarly organized with regard to stereociliary bundle distribution on the sensory epithelia (Serrano and Quick, 2001).

RESULTS AND DISCUSSION

The anatomical changes that characterize inner ear formation in *X. laevis* during early larval development were assessed from histological data collected from specimens classified at larval stages 28, 31, 37, 42, 45, 47, and 50 according to standard descriptions by Nieuwkoop and Faber (1967). These stages were selected because distinct morphological changes in the inner ear can be observed between stages throughout development in spite of the phenotypic variation within similarly staged amphibian populations, particularly with regard to animal length (Nieuwkoop and Faber, 1967; Bever et al., 2003). Images of representative transverse sections illustrate key anatomical changes during the developmental progression of the inner ear from a single compartment otic vesicle at stage 28 (Fig. 1A) into a multi-compartmentalized structure with vestibular and auditory endorgans at stage 50 (Fig. 2B). Videos constructed from images of transverse sections through the entire inner ear of animals at different developmental stages are provided online as supplemental data. The process of inner ear compartmentalization occurs by about 15 days postfertilization at 23°C but can vary between animals and clutches because in amphibians, developmental progression is dependent on a variety of environmental and genetic factors such as animal population density, maternal inheritance, and habitat temperature (Nieuwkoop and Faber, 1967; Berven, 1990; Parichy and Kaplan, 1992; Brunkow and Collins, 1996; Miaud et al., 1999).

The anteroposterior dimension of the inner ear increases from approximately $60 \pm 10 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) at stage 28, to $1080 \pm 90 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) at stage 50. This 18-fold enlargement is estimated from the number of paraffin embedded sections acquired in the anterior to posterior direction that contain ear tissue (Table 1). At stage 28, the largest dorsoventral and mediolateral dimensions are typically about $100 \mu\text{m}$ and $60 \mu\text{m}$, respectively. By stage 50 the dorsoventral dimension of the inner ear can be as large as $1000 \mu\text{m}$, while the largest mediolateral dimension is about $700 \mu\text{m}$, further emphasizing the vast amount of growth and development that occur between larval stages 28 and 50. Estimates for inner ear size and growth are in agreement with those obtained by Bever et al. (2003). Images from their paint-fill-based developmental atlas show similar dimensions for the *X. laevis* inner ear, as well as an approximate 18-fold anteroposterior size increase in inner ear size between stages 28 and 50.

Growth and Progressive Compartmentalization of the Otic Vesicle

Inner ear vestibular and auditory endorgans originate from an otic vesicle that is identifiable in stage 28 sections (Fig 1A), where it can be seen lateral to the central nervous system and separated from the epidermis. At this stage, the otic vesicle comprises a single compartment and is estimated to measure $60 \pm 10 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) in the anteroposterior dimension (Table 1). The otic vesicle remains similarly organized and grows in size to attain anteroposterior dimensions of $90 \pm 10 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) and $130 \pm 20 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) at stage 31 and stage 37 respectively. At stages 28, 31, and 37, the epithelium of the otic vesicle appears thickest in the medioventral region (Figs. 1A, B, C). Positioning of the sensory ganglia in the developing *X. laevis* inner ear is comparable to that observed in the embryos and larvae of frogs (Geisler et al., 1964), zebrafish (Haddon and Lewis, 1996) and rainbow trout (Salem and Omura, 1998). At stage 31, sensory ganglion cells can be identified adjacent to the otic vesicle (Fig. 1B). By stage 37 the ventromedial epithelium resembles the primordial sensory epithelium described in *Bufo marinus* by Corwin (1985), and as in stage 31, sensory ganglion cells can be seen closely apposed to the developing otic vesicle (Fig. 1C).

In stage 42 sections, the pars superior and pars inferior that will serve as anlagen for vestibular and auditory structures are easily identifiable (Fig. 1D1). Sensory ganglion cells

are visible between the central nervous system and the pars inferior of the otic vesicle, and the early stages of capsule formation are evident (Fig. 1D). The initial development of the endolymphatic duct is apparent (Fig. 1D3), as are small invaginations (Fig. 1D2) in the lateral region of the otic vesicle where horizontal canal formation initiates (Paterson, 1948; Anniko, 1983; Haddon and Lewis, 1991). The stage 42 otic vesicle occupies approximately $180 \pm 30 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) in the anteroposterior dimension (Table 1).

At stage 45, the extensive invaginations of the vesicle wall that characterize initial formation of the compartments of the semicircular canals are visible as illustrated for the horizontal canal (Fig. 1E2). Previous studies have demonstrated that the compartments of the semicircular canals are formed by the fusion of these lateral protrusions of the otic vesicle (Paterson, 1948; Haddon and Lewis, 1991). In sections from some specimens at this stage, the initial formation of anterior and posterior canal compartments also can be seen (data not shown). Morphological protrusions of this type corresponding to semicircular canal formation previously have been observed in the brown trout, *Salmo trutta fario* (Becerra and Anadon, 1993) and rainbow trout, *Oncorhynchus mykiss* (Salem and Omura, 1998). The developing endolymphatic duct is present in middle sections (Fig. 1E2), while sensory ganglion cells are concentrated in both middle and posterior sections of the inner ear, between the otic vesicle and the central nervous system (Fig. 1E2,3). The encapsulation of the otic vesicle by cartilage, which is seen in stage 42 (Fig. 1D), is more pronounced at stage 45 (Fig. 1E). The anteroposterior dimension of the stage 45 otic vesicle is $230 \pm 30 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$), a 30% increase from its size at stage 42.

The initial compartmentalization of the utricle, anterior canal, horizontal canal, and sacculus can be distinguished in anterior sections of the inner ear at stage 47 (Fig. 2A1-4). Anterior sections of the inner ear show that the cristae of the anterior canal (Fig. 2A1) and the horizontal canal (Fig. 2A2) are localized in the same compartment as the developing utricle, while the sacculus is forming an independent compartment (Figs. 2A2-4). In middle sections, the sacculus is the largest of the six compartments (Fig. 2A3). The lagena and posterior canal are visible in posterior sections of the inner ear (Figs. 2A5,6). At stage 47, otolithic crystals, or otoconia, overlie the macula of the developing utricle and sacculus (Fig. 2A2,3,4), and sensory ganglion cells are positioned adjacent to the utricular and saccular compartments (Figs. 2A3,4). The cartilage that will chondrify to protect the soft tissue of the inner ear is well defined and can be seen encapsulating the emerging compartments (Fig. 2A). With an anteroposterior dimension of $540 \pm 40 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$), the stage 47 otic vesicle is 135% larger than at stage 45 (Table 1).

By larval stage 50 the inner ear measures $1080 \pm 90 \mu\text{m}$ (mean $\pm$ S.D.; $n = 3$) in the anteroposterior dimension, and is twice its size at stage 47 (Table 1). All eight endorgans that comprise the membranous labyrinth of the mature inner ear, including the auditory organs, the basilar papilla and the amphibian papilla, can be identified at this stage as well as their thickened sensory epithelia (Fig. 2B1-6). Anterior sections of the stage 50 inner ear indicate that the utricle and anterior canal still share a compartment (Fig. 2B1). Posterior sections demonstrate the distinct emergence of the amphibian papilla and basilar papilla as well as the presence of the lagena (Figs. 2B4,5,6).

Stereociliary Bundles and Sensory Ganglia

The actin-rich stereociliary bundles of mechanosensory hair cells can be visualized in cryosectioned tissue (Fig. 3) and in inner ear whole mounts (Fig. 4) using Alexa 488-phalloidin as a fluorescent probe for F-actin. Stereociliary bundles are detected as bright triangular shaped structures in the epithelia of the otic vesicle as early as stage 31 in some specimens (Fig. 3A). The propidium iodide that was used as a counterstain binds to nucleic acids, permitting identification of nuclei (DNA) and to a lesser extent, the cytosol (RNA), as

seen in the confocal images (Figs. 3,4). By stages 42 and 45, stereociliary bundles are very distinct and approximately 5 μm in length (Fig. 3C,D). Stereociliary bundles in cryosections from early stages (31-45) are localized in ventromedial regions of the otic vesicle corresponding to the pars inferior, consistent with previous observations made with SEM of the primordial sensory epithelium of *Bufo marinus* (Corwin, 1985).

As the inner ear grows and begins to form compartments, it becomes much more awkward to process paraffin and resin embedded tissue for sections due to the size and irregular shape of the structures. Furthermore, the complexity of the individual endorgans, as well as slight variations in the orientation of the compartments relative to the brain and VIIIth nerve makes standardization of protocols for production of replicate sections from different specimens a technical challenge. However, at stages 47 and older, it is possible to remove, label, and mount whole inner ears in preparation for flexible optical sectioning using a laser scanning confocal microscope.

In stage 47 whole mounts, stereociliary bundles are seen in the epithelia of regions where the sacculus, utricle, anterior canal, horizontal canal, posterior canal and lagena are beginning to form compartments (Fig. 4A) as shown in transverse sections of embedded tissue (Fig. 2A). In whole mounts of the stage 50 inner ear, all eight individual endorgans are identifiable by their relative location to one another and the VIIIth nerve, and by the distinct patterns established on their epithelia by stereociliary bundles (Fig. 4B). Stereociliary bundles in the sacculus are observed as a circular centralized patch in the sensory epithelium, while utricular stereociliary bundles are detected within a narrowly oblong-shaped region in the utricular macular located adjacent to the sacculus (Fig. 4B1). In the horizontal, anterior, and posterior canals, stereociliary bundles as long as 10 μm can be seen projecting from the cristae (Figs. 4B2,3,5). Stereociliary bundles in the lagena form a crescent-shaped sensory epithelium (Fig. 4B4). Auditory organ stereociliary bundles are visible in the pod-shaped amphibian papilla and donut-shaped basilar papilla (Fig. 4B5). By Stage 50, the sensory fields and shapes of the young endorgans resemble the morphology that has been previously reported for endorgans from older larval and adult *X. laevis* (Diaz et al., 1995; Lopez-Anaya et al., 1997; Serrano et al., 2001). Videos constructed from confocal optical sections of stage 50 endorgans labeled with Alexa 488-phalloidin are provided online as supplemental data.

The morphology seen in cryosections of the inner ear with confocal microscopy (Fig. 3B,C,D) resembles that of brightfield images of resin-embedded sections (Fig. 1C,D, E), permitting identification of key structures such as the endolymphatic duct and sensory ganglia. In Fig. 3B, the endolymphatic duct can be seen along the dorsal wall of the stage 37 otic vesicle, adjacent to the sensory ganglion and the pars inferior with its stereociliary bundles. In sections from stages 37, 42, and 45, the sensory ganglia appear as spherical or ellipsoid structures containing many cell nuclei and situated close to the stereociliary bundles and thickened epithelia of the pars inferior of the otic vesicle (Figs. 3B-D). However, with the fluorophores and methods used here, sensory ganglia are not identifiable in stage 31 sections that contained phalloidin-labeled stereociliary bundles (Fig. 3A). This was so even though sensory ganglion cells can be seen adjacent to the pars inferior in tissue specimens at this stage that were prepared for brightfield microscopy (Fig. 1B).

The mechanisms and environmental cues responsible for mechanosensory hair cell differentiation and axon guidance to inner ear target epithelia have been the subject of intense investigation in recent years (Fritzsche et al., 2002). Specifically there is interest in determining whether the differentiation of mechanosensory hair cells is under the influence of diffusible factors from sensory ganglion cells, as well as whether hair cell tropic and trophic factors play a role in axon pathfinding during synaptogenesis (Rubel and Fritzsche,

2002). Developmental studies of inner ear formation have shown that in some species, innervation by sensory ganglion cells precedes the appearance of mechanosensory hair cells (Anniko, 1983; Sans and Dechesne, 1987). Recent evidence suggests that sensory ganglion neuron survival is influenced by neurotrophins such as BDNF and NT-3. In BDNF and NT-3 knockout mice there is an absence of innervation of target inner ear epithelia by sensory ganglion neurons. Nevertheless, hair cells are morphologically identifiable in these animals, lending support to the hypothesis that ganglion cells are not required for hair cells to achieve some of their differentiated properties (Rubel and Fritzsch, 2002). Data presented here are not conclusive with regard to whether hair cell differentiation (assessed by bundle formation) precedes the appearance of the sensory ganglion in *X. laevis*. However, the close apposition of the sensory epithelia, the pars inferior, and the sensory ganglia could potentially facilitate exchange of diffusible factors between these regions, whether or not innervation is present in early developmental stages (Fig. 1,3).

Sensory Endorgan Formation and Morphogenesis

It is well established from numerous developmental studies of vertebrates that the inner ear is derived from an otic placode that gives rise to a single compartment otic vesicle (Lewis, 1907; Torres and Giraldez, 1998; Normaly and Grainger, 2002; Barald and Kelley, 2004). Various investigations also have shown that during inner ear organogenesis, the vestibular apparatus of the inner ear generally begins to develop before the auditory structures in many species including mouse (Anniko, 1983), chick (Bissonnette and Fekete, 1996), opossum (Larsell et al., 1935), zebrafish (Haddon and Lewis, 1996) and the northern native cat, *Dasyurus hallucatus*, (Gemmell and Nelson, 1992). Furthermore, during vestibular system development, semicircular canal formation can precede the appearance of otolithic compartments such as the utricle and sacculus (Haddon and Lewis, 1991; Wu and Oh, 1996).

The anatomical development of the *X. laevis* inner ear parallels that of other species. The ear is limited to an otic vesicle during early embryonic stages 28 and 31. The otic vesicle grows and first gives rise to vestibular compartments as shown here in transverse histological sections (Figs. 1-3) and whole mounts (Fig. 4), and by others with different approaches (Paterson, 1948; Nieuwkoop and Faber, 1967; Haddon and Lewis, 1991; Bever et al., 2003). The thickened epithelia of the pars inferior and the pars superior of the otic vesicle are well formed by stage 42 (Fig 1D1). It is interesting to note that during the hatchling stages (S37-S42) where the pars inferior and pars superior become distinct, animals cease to lie sideways and begin to swim. During these stages the animals, in response to pineal-dependent cues, swim upwards until they encounter a surface from which to hang vertically, such as the sides of holding tanks and objects that cast shadows. Hatchlings attach to these objects or surfaces by mucus secreted from a specialized cement gland (Jamieson and Roberts, 2001).

The early compartmentalization of the horizontal canal, as determined by the presence of small invaginations in the lateral wall of the otic vesicle is seen in sections from stage 42 animals (Fig. 1D2). These become larger and easily identifiable in stage 45 sections where they begin to fuse to form the walls of the horizontal semicircular canal (Fig. 1E2). Haddon and Lewis (1991) have shown that in *X. laevis*, the linear carbohydrate polymer, hyaluronan, is found inside the core of the invaginations and is believed to drive the inward extension of the vesicle wall that is necessary for semicircular canal formation. Recently, hyaluronan synthase 3 has been localized to the inner ear and cement gland of *X. laevis* (Vigotti et al., 2003). An immature endolymphatic duct appears at stages 42 and 45, and cartilage can be seen surrounding the vesicle, further characterizing the progression of the developing membranous labyrinth. By stage 45, larvae have begun to feed on their own.

Distinct differences in the cellular organization of the inner ear can be observed during the period between stages 45 and 50, when compartmentalization of the individual auditory and vestibular endorgans accelerates, and the sensory epithelia with hair cell bundles emerge (Table 1). Vestibular components of the membranous labyrinth (utricle, anterior canal, posterior canal, lagena, horizontal canal, and sacculus) are all visible by stage 47 (Figs. 2A, 4A). Stereociliary bundles are seen on the epithelia of endorgans whose compartments have not closed. Thus, significant tissue differentiation occurs in the 2 day interval between stages 45 and 47 when the inner ear doubles in size in the anteroposterior dimension (Table 1). The inner ear doubles in size again during the 10 days between stage 47 and stage 50, and during this interval, the auditory organs emerge and compartments begin to separate.

By stage 50, the VIIIth cranial nerve can be seen branching to posterior and anterior endorgans of the *X. laevis* inner ear. Eight clearly identifiable auditory and vestibular compartments with sensory epithelia containing stereociliary bundles can be distinguished at this stage (Figs. 2B, 4B). Although the inner ears of premetamorphic stage 50 larvae appear to be smaller and similarly organized versions of the inner ear of postmetamorphic animals (Lopez-Anaya et al., 1997), they contain far fewer stereociliary bundles. Comparative anatomical studies of the sensory endorgans of the inner ears of larval and postmetamorphic *X. laevis* have shown that the numbers of hair cell bundles (Diaz et al., 1995; Serrano et al., 2001) and VIIIth nerve axons (Lopez-Anaya et al., 1997) increase dramatically during the late larval stages leading to metamorphosis. Other changes that occur during metamorphosis include the development of the perilymphatic system of the inner ear, the maturation and initial ossification of the otic capsule, and the formation of the plectrum, operculum, and tympanic membrane (Paterson, 1948; Nieuwkoop and Faber, 1967). Thus, although many components of the membranous labyrinth are present by stage 50, the period leading to metamorphosis in *X. laevis* is characterized by augmented innervation and differentiation of mechanosensory hair cells in the auditory and vestibular endorgans, as well as by extensive tissue growth and development of the otic capsule and the middle ear (Duellman and Trueb, 1986).

Bundle Production in Auditory Organs during *Xenopus* life

Like other anuran amphibians, *X. laevis* has a sacculus that serves a dual acoustico-vestibular function, and two organs specialized for auditory processing, the amphibian papilla and the basilar papilla (Duellman and Trueb, 1986; Smotherman and Narins, 2000). These three organs originate from the pars inferior and allow *X. laevis* to detect auditory frequencies between 200-4000 Hz (Elepfandt et al., 2000). The development of the sense of hearing requires that neural circuits be established between the central nervous system and the auditory hair cells that are the mechanoreceptors for acoustical stimuli in the inner ear. However, few studies have examined the appearance and innervation of auditory (or vestibular) hair cells during the compartmentalization and morphogenesis of the larval amphibian inner ear (Corwin, 1985; Haddon and Lewis, 1991). Although data regarding the development of hearing in larval *X. laevis* are lacking, studies have shown that sound elicits neural activity in the auditory midbrain of posthatch *Rana catesbeiana*, and that during metamorphic climax, animals experience a brief “deaf” period during which auditory activity cannot be recorded from the midbrain (Boatright-Horowitz and Simmons, 1997).

In *X. laevis*, previous SEM studies have established the presence of morphologically diverse stereociliary bundles on the sensory epithelia of the sacculus and the amphibian papilla in larvae as young as stage 52, where they number approximately 1100 and 100 respectively (Diaz et al., 1995; Serrano et al., 2001). SEM of the sensory epithelia has shown that in recently metamorphosed juvenile (1 gm) animals the sensory fields of the sacculus, amphibian papilla, and basilar papilla contain about 2000, 370, and 40 stereociliary bundles, respectively, while those of adult (40-60 gm) *X. laevis* contain approximately 2400, 430, and

50 stereociliary bundles, respectively (Diaz et al., 1995; Serrano et al., 2001). Thus *X. laevis*, like other amphibians (Corwin, 1985), appear capable of producing new stereociliary hair cell bundles throughout life. The very small size of the inner ear and its endorgans poses challenges to the application of SEM for identification of stereociliary bundles in animals at younger stages, especially during otic vesicle development and compartmentalization (S28-S50). However, confocal laser scanning microscopy facilitates observation of phalloidin-labeled stereociliary bundles at these animal stages and as shown here, bundles can be detected in the *X. laevis* otic vesicle at stages 31-50. Although the presence of stereociliary bundles in auditory organs alone is not sufficient to establish that hair cells are capable of mechanotransduction, their emergence on the epithelia of inner ear compartments is a strong indicator that hair cell differentiation is in progress during these stages. The physiological implications for the sense of hearing in larval animals remains to be determined (Boatright-Horowitz and Simmons, 1997).

EXPERIMENTAL PROCEDURES

Xenopus laevis used in this study represent seven developmental stages (S28, S31, S37, S42, S45, S47, S50) that span a period between 1 and 15 days post-fertilization at 23°C. *X. laevis* were purchased from a commercial supplier as newly fertilized eggs or larva (NASCO, Fort Atkinson, WI) and maintained in aerated aquaria at 22-24°C under full spectrum lights. Ten to fifteen larvae, each from a separate breeding, were used to acquire data for each stage. Morphological features, as viewed under a dissecting microscope, were used to determine the developmental stage of the specimens according to the descriptions of Neuwkoop and Faber (1967). Criteria used to stage animals included characteristics such as the amount of pigmentation in the ventral portion of the tail region, the extent of eye and tentacle formation, the number of intestinal revolutions, the relative development of the hindbud, and the length of the body and hindlimb. The stages identified by Neuwkoop and Faber (1967) can be grouped into categories that represent embryonic (S28, S31), hatchling (S37, S42), and early larval (S45, S47, S50) developmental periods (McDiarmid and Altig, 1999; Bever et al., 2003). After staging, larvae were euthanized in buffered (pH 7.4) 0.25% 3-aminobenzoic acid ethyl ester methanesulfonate salt (Sigma A-5040) for 30 minutes prior to further tissue processing. Experimental procedures were approved by the Institutional Animal Care and Use Committee of New Mexico State University. Following euthanasia, larvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 140 mM NaCl, 2 mM KCl, 10 mM NaHPO₄, 1 mM KH₂PO₄) for 2 hours at room temperature (22-26 °C) then rinsed in PBS (3 times, 5 minutes each). Specimens were placed in 25 mM EDTA for 30 minutes at room temperature, rinsed in PBS (3 times, 5 minutes each) and processed for resin or paraffin embedding.

Figures illustrating the anatomical changes that occur during development were prepared using images of resin embedded sections. Specimens for resin embedded sections were dehydrated in 70% EtOH for 15 minutes, followed by 95% EtOH (2 times, 10 minutes each), and 100% EtOH (2 times, 10 minutes each). Specimens were submerged in 100% propylene oxide (PPO) for 5 minutes prior to infiltration with 50% resin in PPO (v/v) for 1 hour, and 75% resin in PPO (v/v) for 2-3 hours. Tissue then was placed in 100% ARALDITE 502 resin (EMS 10900) for 6-8 hours and cured at 50 °C overnight (18-22 hrs). Transverse 3-5 µm sections (perpendicular to the plane of the animal's body anterior to posterior) were prepared with a LKB ultramicrotome. Sections were mounted on Superfrost Plus slides (MJ Research), stained with 1% toluidine blue/1% borax (w/v), rinsed in tap water, then coverslipped using Histomount mounting medium (National Diagnostics HS-103). Staining times varied with animal stage.

Paraffin sections were used to assess animal variation in morphology and for dimensional measurements of the developing *X. laevis* inner ear. Specimens for paraffin embedded sections were dehydrated in a graded EtOH/H₂O (v/v) series (25%, 50%, 75%, 100%) for 10-15 minutes in each solution, then infiltrated with a graded xylene/EtOH (v/v) series (25%, 50%, 75%, 100%) for 10 minutes in each solution. Specimens were infiltrated with a graded paraplast/xylene (v/v) mixture (25%, 50%, 75%, 100%) for 20 – 25 minutes each at 55°C and embedded in 100% Paraplast Plus (Tyco Healthcare/Kendall 8889-502004). Transverse 10 µm sections were prepared with a Zeiss microtome and mounted on Superfrost Plus slides, then placed in an oven overnight at 55°C prior to staining. Sections were deparaffinized in 100% xylene for 7-10 minutes, rehydrated through a series of decreasing EtOH alcohol solutions for 5 minutes each (100%, 75%, 50%, 25%), then placed in water. Sections were stained with Harris hematoxylin (Sigma HHS-16), rinsed in tap water, differentiated in an acid alcohol solution, blued in Scott's solution, and stained with alcoholic eosin Y (Sigma E4382) in 95% ethanol. Slides were dehydrated in 95% and 100% EtOH/H₂O (2 times, 3 minutes each), cleared in Histoclear-II (National Diagnostics HS-202) for 5 minutes, then coverslipped using Histomount mounting medium.

Specimens were prepared for fluorescence microscopy and imaging of stereociliary bundles from specimens that were staged as above, then euthanized and fixed in 4% paraformaldehyde/PBS for 2 hours. For cryosections, specimens were rinsed in PBS for 10 minutes (2 times), embedded in 5% sucrose/5% agar (Becton Dickinson, 214010), and cryoprotected overnight at 4°C in 30% sucrose/0.1% sodium azide in PBS. The tissue was sectioned to 20 µm using a Leica CM 3050 cryotome, then stored at -20°C. Slides were placed at 37°C for 30 minutes before labelling with fluorescent probes. With our labelling protocols, the stereociliary bundle morphology in cryosections was superior to that in paraffin sections. Whole mounts were prepared for fluorescence microscopy from the inner ears of euthanized stage 47 and stage 50 specimens. Otic capsules (S47) and dissected inner ears (S50) were fixed in 4% paraformaldehyde/PBS for 2 hours, then immersed in 25 mM EDTA overnight at 4°C. The tissue was rinsed twice for 10 minutes in PBS prior to fluorescent staining. Sections and whole mounts were permeabilized and exposed to the F-actin label, Alexa 488 phalloidin (Molecular Probes A-12379), and the nucleic acid label, propidium iodide (Molecular Probes P-3566), according to the manufacturer's protocols, and without RNAase treatment. Cryosectioned tissue and whole mounts were coverslipped using SlowFade Light Antifade mounting medium (Molecular Probes S-7461).

Digitized images from paraffin and resin embedded sections were captured using a Zeiss Axioplan light microscope and WinView 32 image acquisition system (Princeton Instruments). A BioRad 1024 laser scanning confocal microscope configured with the E1 and T2 filter blocks was used to acquire digitized images from cryosectioned tissue and inner ear wholemounts labelled with fluorescent probes. Alexa 488 fluorescence was detected using a 522DF35 emission filter and the 488 Argon/Krypton laser line. Propidium iodide fluorescence was detected using a E585LP emission filter and excitation with the 568 Argon/Krypton laser line. Gain, aperture, and laser intensity settings were adjusted to minimize photobleaching. Autofluorescence was not detected from unlabelled inner ear tissue at the settings used for image capture from fluorophore-labelled tissue. Images were processed using BioRad Lasersharp 2000 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ac	anterior canal
ap	amphibian papilla
bp	basilar papilla
cr	cartilage
D	dorsal
ed	endolymphatic duct
hz	horizontal canal
L	lateral
lag	lagena
nc	notochord
oc	otoconia
ov	otic vesicle
pc	posterior canal
pi	pars inferior
ps	pars superior
sac	sacculus
sg	sensory ganglion
utr	utricle
S28	stage 28
S31	stage 31
S37	stage 37
S42	stage 42
S45	stage 45
S47	stage 47
S50	stage 50

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Figure 1.

The *X. laevis* inner ear appears as an otic vesicle in resin-embedded sections from stages 28 (A), 31 (B), 37 (C), 42 (D), and 45 (E) stained with toluidine blue. **A:** S28 inner ear sections show an otic vesicle with a thickened epithelium. **B:** At S31, the sensory ganglion is present in middle sections of the inner ear. **C:** Sections from S37 specimens resemble those from S31 but the otic vesicle is larger. **D:** Serial transverse sections from S42 *X. laevis* inner ear (anterior to posterior; D1-D3) containing the sensory ganglion. D1: Anterior section of the S42 inner ear showing the pars inferior (pi) and pars superior (ps) of the otic vesicle, and early stages of otic capsule formation by cartilage (cr); D2: Middle section of the S42 inner ear shows a thickened epithelium, a small invagination in the lateral portion of the otic vesicle (hz) and cartilage; D3: Posterior section of the S42 inner ear shows the endolymphatic duct. **E:** Serial transverse sections from S45 *X. laevis* (anterior to posterior; E1-E3). Cartilage is visible in anterior sections. E1: Anterior section of the S45 inner ear with thickened epithelia; E2: Middle section of the S45 inner ear showing the endolymphatic duct and invaginations in the lateral portion of otic vesicle where the horizontal canal is forming (hz) E3: Posterior section of the S45 inner ear with sensory ganglion and thickened epithelium. Scale bar = 100 μ m.

Figure 2.

The compartmentalization of the *X. laevis* inner ear at stage 47 (A) and stage 50 (B) is apparent in resin-embedded sections stained with toluidine blue. **A:** Compartments are visible in serial transverse sections from the S47 *X. laevis* inner ear (anterior to posterior; A1-A6). A well developed otic capsule is seen surrounding the developing membranous labyrinth of the inner ear in all sections. A1: utricle, crista of the anterior canal; A2: utricle, horizontal canal, sacculus, otoconia; A3: utricle, horizontal canal, anterior canal, sensory ganglion, sacculus, otoconia; A4: sensory ganglion, sacculus, otoconia; A5: lagena; A6: crista of posterior canal. **B:** Auditory and vestibular epithelia and compartments are shown in sections of S50 *X. laevis* inner ear. B1: crista of anterior canal, utricle, otoconia; B2: sacculus; B3: crista of horizontal canal; B4: lagena; B5: basilar papilla; B6: amphibian papilla. Scale bar = 100 μ m.

Figure 3.

The F-actin cytoskeleton (green) of stereociliary bundles is detected with Alexa 488 phalloidin fluorescence in the *X. laevis* otic vesicle. Arrows point to stereociliary bundles in confocal images of cryosections from stages 31 (**A**), 37 (**B**), 42 (**C**), and 45 (**D**). Propidium iodide counterstain (red) labels RNA (cytosol) and DNA (nuclei). Scale bar = 50 μm .

Figure 4.

Alexa 488 phalloidin fluorescence marks the F-actin cytoskeleton (green) of stereociliary bundles in confocal sections from S47 (**A**) and S50 (**B**) inner ear wholemounts. Arrows point to stereociliary bundles. Propidium iodide counterstain (red) labels RNA (cytosol) and DNA (nuclei). **A**:. S47 endorgan compartments contain regions where stereociliary bundles are identifiable. A1: sacculus, utricle; A2: crista of the anterior canal; A3: lagena, cristae of posterior and horizontal canals. **B**:. Representative confocal sections show that stereociliary bundles are visible in the epithelia of all S50 vestibular and auditory compartments. B1: utricle, sacculus; B2: cristae of horizontal and anterior canals; B3: crista of horizontal canal (transverse view); B4: lagena, sensory ganglion; B5: basilar papilla, amphibian papilla, crista of posterior canal, sensory ganglion adjacent to amphibian papilla. Scale bar = 50 μ m.

TABLE 1Anatomical features of the *X. laevis* inner ear during development

Stage	Inner ear size ¹ μm	Landmark morphological characteristics [Key behaviors]
S28	60 ± 10	otic vesicle [embryos lie along horizontal axis; body twitches]
S31	90 ± 10	otic vesicle; pars inferior; sensory ganglion; stereociliary bundles
S37	130 ± 20	otic vesicle; pars inferior; endolymphatic duct; sensory ganglion; stereociliary bundles [hatchlings attach to surfaces by mucus from cement gland and hang along vertical axis]
S42	180 ± 30	Initial invaginations of otic vesicle (horizontal canal formation); pars inferior; pars superior; endolymphatic duct; cartilage; sensory ganglion; stereociliary bundles
S45	230 ± 30	increased invagination of otic vesicle (formation of semicircular canals); pars inferior (saccular macula); pars superior; endolymphatic duct; cartilage; sensory ganglion; stereociliary bundles [larvae feed]
S47	540 ± 40	sacculus; utricle; horizontal canal; anterior canal; posterior canal; lagena; endolymphatic duct; cartilage; otoconia; sensory ganglion; stereociliary bundles
S50	1080 ± 90	sacculus; utricle; horizontal canal; anterior canal; posterior canal; lagena; amphibian papilla; basilar papilla; endolymphatic duct; cartilage, otoconia; sensory ganglion; stereociliary bundles

¹ Size estimated in the anteroposterior dimension by multiplying the total number of sections by the thickness of sections in paraffin embedded tissue (mean ± S.D.; n = 3 for all stages). Actual size may be larger due to shrinkage artifacts incurred during tissue processing.