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Transplantation of *Xenopus laevis* ears reveals the ability to form afferent and efferent connections with the spinal cord

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ABSTRACT Previous comparative and developmental studies have suggested that the cholinergic inner ear efferent system derives from developmentally redirected facial branchial motor neurons that innervate the vertebrate ear hair cells instead of striated muscle fibers. Transplantation of *Xenopus laevis* ears into the path of spinal motor neuron axons could show whether spinal motor neurons could reroute to innervate the hair cells as efferent fibers. Such transplantations could also reveal whether ear development could occur in a novel location including afferent and efferent connections with the spinal cord. Ears from stage 24-26 embryos were transplanted from the head to the trunk and allowed to mature to stage 46. Of 109 transplanted ears, 73 developed with otoconia. The presence of hair cells was confirmed by specific markers and by general histology of the ear, including TEM. Injections of dyes ventral to the spinal cord revealed motor innervation of hair cells. This was confirmed by immunohistochemistry and by electron microscopy structural analysis, suggesting that some motor neurons rerouted to innervate the ear. Also, injection of dyes into the spinal cord labeled vestibular ganglion cells in transplanted ears indicating that these ganglion cells connected to the spinal cord. These nerves ran together with spinal nerves innervating the muscles, suggesting that fasciculation with existing fibers is necessary. Furthermore, ear removal had little effect on development of cranial and lateral line nerves. These results indicate that the ear can develop normally, in terms of histology, in a new location, complete with efferent and afferent innervations to and from the spinal cord.

KEY WORDS: *Xenopus laevis, ear, efferent innervation, transplantation*

Introduction

The vertebrate inner ear receives two kinds of fibers: afferents that come from placodally-derived sensory neurons (Rubel and Fritzsch, 2002) and efferents which come from brainstem neurons (Simmons, 2002). Whereas afferents carry information from the ear to the brain, efferents provide a feedback loop to modify mechanic stimuli information processing at the periphery. Efferents have been described in all vertebrates (Fritzsch, 1999) and the extensive branching of single fibers to reach both lateral line and inner ear hair cells has been extensively studied in amphibians (Hellmann and Fritzsch, 1996). The nature of these efferent cells has long been enigmatic and they have been referred to as reticular formation cells or branchial motornneurons given their close proximity in many vertebrates to the facial branchial motor neurons (Roberts and Meredith, 1992). More recent developmental studies have shown that efferent innervation of the ear is derived from facial branchial motor neurons (Fritzsch and Nichols, 1993) and that efferents may be guided to reach the ear by the transcription factor Gata3 that is uniquely expressed in these motor neurons and nearby reticular formation neurons (Karis *et al.*, 2001). In vertebrates, the ear develops at the rostral boundary of the anteriormost, first forming somite (Cooke, 1978; Chung *et al.*, 1989; Huang *et al.*, 1997) and paraxial mesoderm anterior to the ear does not form somites (Noden and Francis-West, 2006) but may form somitomeres. Furthermore, in vertebrates, the most rostral somite is reduced in size to accommodate the ear. Combined these data could be interpreted to imply that in vertebrates, the ear develops in place of a somite or somitomere. If true, the facial branchial motor neurons destined to innervate the somite- or somitomere-derived muscle fibers may have been rerouted to innervate the ear when the ear evolved in ancestral vertebrates

Abbreviations used in this paper: GFP, green fluorescent protein; TEM, transmission electron microscopy.

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(Fritzsch *et al.*, 2007).

This raises the question as to whether there is a unique property of cranial motor neurons to innervate hair cells of the ear or whether any motor neuron can innervate the ear if placed in its trajectory. In other words, is formation of efferents to the ear a chance event that happened to capture facial branchial motor neurons simply because the ear evolved in the trajectory of these motor neurons? Supporting evidence comes from the lateral line system in which branchial motor neurons associated with the glossopharyngeal nerve become efferent to the hair cells of the posterior lateral line (Hellmann and Fritzsch, 1996).

In the present study, we transplanted *Xenopus laevis* ears into the path of spinal motor neuron axons, revealing that spinal motor neurons have the ability to innervate the ear as efferent fibers and end on hair cells instead of muscle cells and probably modify hair cell function via the now well characterized nicotinergic acetylcholine receptors expressed in hair cells (Sugai *et al.*, 1992; Jagger *et al.*, 2000; Katz *et al.*, 2004; Derbenev *et al.*, 2005). Such transplantations could also reveal the potential for normal ear development in a novel location, including afferent connection with the spinal cord, a part of the CNS that never receives inner ear afferent innervations which is normally restricted to the alar plate of the brainstem (Maklad and Fritzsch, 2003).

Results

Assessment of transplantations

Success of transplantations is shown in Table 1 and in Fig. 1. Residual ear formation is shown in Table 2 for embryos in which the success of transplantation and ear regeneration were monitored. In some cases in which only a transplanted vesicle without otoconia formed, a new otic vesicle was observed in the native location (Fig. 1E). This was more frequent in transplantations of earlier embryos (stage 24) than in later stages (stage 26). It remains unclear whether this indicates a residual capacity for parts of the otocyst to regenerate. However, in many cases, the residual native 'ear' consisted of nothing more but an endolymphatic duct filled with calcium carbonate crystals (Fig. 1 C,I). In transplanted ears which formed otoconia, 75% did not form a residual ear nor endolymphatic duct in the original location (Fig. 1 B,H). However, formation of a residual native ear in transplants that had developed otoconia occasionally occurred (17%).

To determine whether manipulation of the otic placode affects further development, ears were removed, rotated 180°, and replaced. Even though for transplantations we sought to maintain orientation, it was not always the case and often the ears underwent some degree of rotation. Thus rotation of the ear and replacing it back into the head replicated many of the manipulations performed during transplantation and could serve as an additional control for the ability of a manipulated ear to develop normally. Success of ear rotations is shown in Table 1. Eight of the 13 ears were normal or near normal in appearance (Fig. 1 D,G) when compared with control embryos (Fig. 1A). Otoconia in nearly half of the ears were even found to be re-oriented so that they resumed native orientation. In the chick, anterior-posterior specification occurs after the formation of the otic placode (Bok *et al.*, 2005), thus this might explain the re-orientation of rotated *X. laevis* ears. Only one ear rotation failed to form otoconia. Thus we conclude that our manipulations themselves have little effect on normal ear development.

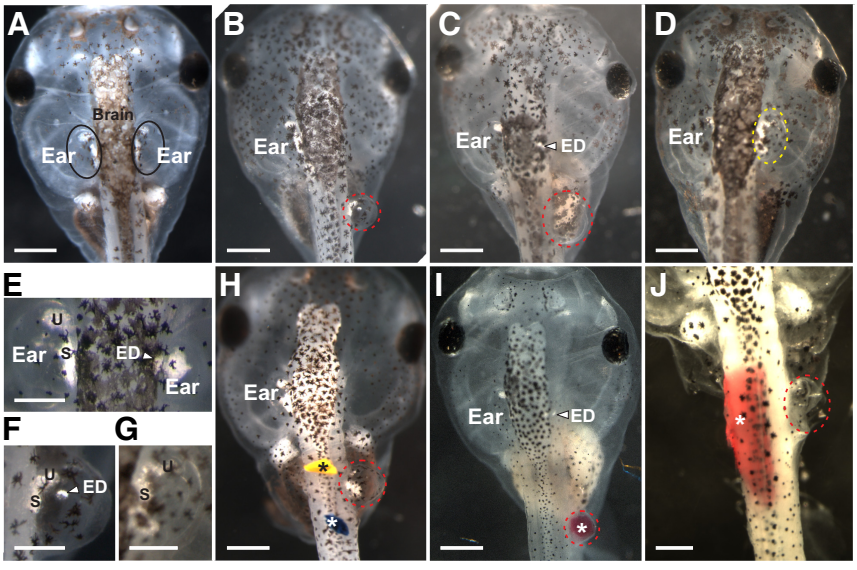

Fig. 1. Stage 46 *X. laevis* showing transplanted ears. (A) *Control, untransplanted embryo, ears are circled in black.* **(B)** *Embryo with a transplanted ear containing otoconia, outlined in a dotted red line.* **(C)** *Embryo with a transplanted empty vesicle, outlined in a dotted red line.* **(D)** *Embryo that had its ear rotated, outlined in a dotted yellow line.* **(E)** *Untransplanted ear, left, and residual ear formation, right.* **(F)** *Higher magnification of transplanted ear in (B).* **(G)** *Higher magnification of rotated ear in (D).* **(H)** *Lipophilic dye injections into the spinal cord rostral and caudal to the ear.* **(I)** *Dextran amine dye injection into the transplanted ear.* **(J)** *Lipophilic injection ventral to the spinal cord and contralateral to the ear. Native, unmanipulated ears are labeled. U, utricle; S, saccule; ED, endolymphatic duct. Asterisks indicate sites of dye injections. Scale bar is 0.5 mm for (A-D,H,I) and 0.25 mm for (E,F,G,J).*

Transplanted ears develop hair cells and ganglia

To assess normal development of the sensory epithelia in ears containing otoconia, *in situ* hybridization for miR-183, a conserved hair cell marker of ears (Pierce *et al.*, 2008), was performed on ears containing otoconia. In the untransplanted ears, miR-183 was localized in the sensory end organs: the utricle, saccule, and anterior, horizontal and posterior canals as well as in the placodally-derived ganglion cells: Trigeminal, Vestibular and Vagus (Fig. 2A). Transplanted ears were also miR-183 positive in the utricle, saccule, the 3 semicircular canals, and in the placodally-derived vestibular ganglion cells (Fig. 2B). To confirm the presence of hair cells, transplanted ears were immunostained with antibody against myoVI and acetylated tubulin. MyoVI-positive hair cells in patches of sensory epithelia were observed in transplanted ears (Fig. 2C), although some sensory epithelia contained only a few hair cells. These hair cells had kinocilia positive for tubulin (Fig. 2D). Serial microtome sections of plastic-imbedded transplanted ears also revealed hair cells with apical specializations as seen with a light microscope (Fig. 2F) and with transmission electron microscopy (Fig.

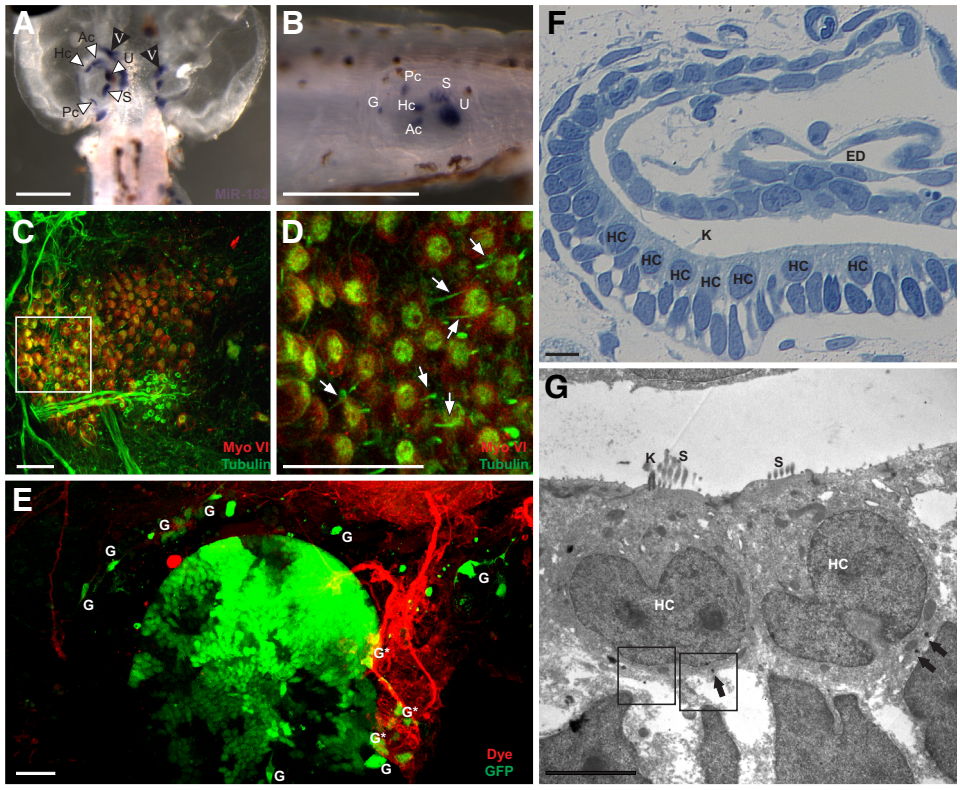

Fig. 2. Development of the transplanted ear. **(A)** *MiR-183 in sensory epithelia in the native ear and in surrounding cranial ganglia as well as ganglia next to the ablated ear (i.e. Trigeminal ganglia, V).* **(B)** *Lateral view of the transplanted ear with miR-183 positive sensory epithelia and ganglion cells; anterior is to the right.* **(C)** *Acetylated tubulin (green) and MyoVI (red) immunostaining of a transplanted ear showing hair cells.* **(D)** *Magnification of region in C show kinocilia (white arrows) on hair cells.* **(E)** *Green fluorescent protein (GFP)-labeled transplanted ear reveals GFP-positive delaminating sensory ganglion cells (G), a few of which (G*) are colocalized with lipophilic dye (red). Lipophilic dye injections were ventral to the spinal cord.* **(F)** *2 μm thick transverse section through the dorsal and lateral view of transplanted ear showing a sensory epithelium with hair cells and an endolymphatic duct.* **(G)** *Transmission electron micrograph of two hair cells in the transplanted ear with synaptic ribbons (black arrows). The boxed areas are shown at higher magnification in* Fig. *3 K,L. U, utricle; S, saccule; Ac, anterior canal crista; Hc, horizontal canal crista; Pc, posterior canal crista; G, ganglion cell(s); HC, hair cells K, kinocilium; S, stereocilia; ED, endolymphatic duct. Scale bar is 0.5 mm in (A,B); 25 μm in (C,D); 50 μm in (E), 0.1 mm in (E inset); 10 μm in (F) and 5 μm in (G).*

2G). These data suggest that the transplanted ears developed relatively normally with all sensory epithelia present that occur at that stage.

To confirm the presence of sensory ganglia in the transplanted ears, *in situ* hybridization for miR-124 was performed on transplanted ears. MiR-124 is expressed in the central nervous system (Mishima *et al.*, 2007) and in inner ear vestibular ganglion cells (Weston *et al.*, 2006). Cells positive for miR-124 were present in transplanted ears (data not shown). To further confirm the presence of ganglion cells, otic placodes from embryos injected with GFP were transplanted into uninjected individuals. Daily observation using a dissection microscope equipped with epifluorescence revealed GFP-positive delaminating ganglion cells migrating away from the ear. After a week, these delaminated cells were imaged with confocal microscopy (Fig. 2E), indicating that the ganglion cells originate from the transplanted otic placode and migrate outward during embryo development.

Afferent innervation of transplanted ears

Dextran amine and lipophilic dyes were injected into the spinal cord of embryos (Fig. 1H) containing otoconia in their transplanted ears to label innervations of these ears. Both types of dye labeled ganglion cells in the transplanted ear (Figs. 2E, 3A and 3B). The lack of dye in some ganglion cells in Figs. 2E and 3A indicates that not all ganglion cells sent projections to the spinal cord. Ganglion cells making connection with the spinal cord appear to do so by fasciculation with existing nerves innervating the surrounding muscle tissue (Fig. 3 A,B). In addition to making projections to the spinal cord, immunohistochemistry for acetylated tubulin revealed fibers (presumably from ganglion cells) closely apposed to hair cells (Fig. 3C). Transmission electron microscopy confirmed the presence of afferent terminals on hair cells (Fig. 3K).

Efferent innervation of transplanted ears

To determine whether spinal motor neurons project to hair cells in the transplanted ear, several methods were used. Trans-

TABLE 1

SUCCESS OF TRANSPLANTATION OF OTIC PLACODES

	Development of ear with otoconia	Development of ear without otoconia	No development of ear
Transplanted ears (109)	73*	29*	7
Rotated ears (13)	12	1	0

Numbers of transplants or rotated ears performed are indicated in parentheses.

*Examples of transplants with and without otoconia are shown in Figs 1B/1F and 1C respectively.

TABLE 2

FORMATION OF RESIDUAL EARS AND SUCCESS OF TRANSPLANTATION

Residual ear formation	Development of transplanted ear with otoconia	Development of transplanted ear without otoconia	No development of transplanted ear
No residual ear	30	0	0
ED only	3	10	0
Residual ear, no ED	7	0	0
Residual ear + ED*	0	2	3

ED, endolymphatic duct. * Residual formation of ear plus endolymphatic duct is shown in Fig. 1E.

planted ears were injected with Rhodamine dextran amine dyes to backfill to the spinal cord (Fig. 1I). In addition, for some embryos Flurosceine dextran amine dye or lipophilic dye (NeuroVue™ maroon) was applied rostral and caudal to the ear to label motor neurons that innervate muscle tissue to determine if any uncut nerves outside of the ear took up Rhodamine dextran amine that might then give a false positive; these cells would be double labeled. Cell bodies in the ventral spinal cord positive for only Rhodamine dextran amines were present in 10 of 16 ventral spinal cords examined, suggesting that motor neurons can innervate the ear if placed in their trajectory (Fig. 3D).

To further demonstrate that motor neurons do innervate the ear, lipophilic dye was injected immediately ventral to the spinal cord from the contralateral side as to not damage the ipsilateral nerves innervating the ear (Fig. 1J). The dye would only come into contact with spinal motor neurons exiting the spinal cord via ventral roots and thus any nerves labeled in the transplanted ear should be motor neurons that had rerouted to innervate the ear. Lipophilic dye label was observed in a few nerve fibers innervating several of the transplanted ears (Figs. 2E and 3G). In one example, lipophilic-dye filled nerve fibers that traveled around the ear on each side and also filled nerve fibers which sent projections to the hair cells (Fig. 3G). To further visualize the extent to which this ear was innervated, beyond the contribution from the spinal motor neurons, this ear was immunostained with antibody against tubulin. This revealed more extensive, presumably afferent innervations (Fig. 3H). The deeper ventral roots observed in Fig. 3G were faintly stained with tubulin, but not easily detected in the collapsed image (Fig. 3H). The reduction of staining of deeper neurons is likely due to the inability of the antibody to fully penetrate the tissue. In another example, a few of the ganglion cells sent projections along the presumed motor nerves as they colocalized with the dye (Fig. 2E, see G*) therefore, it was necessary to confirm motor innervation even when co-localization with ganglion cells was not apparent as in Fig. 3G.

To confirm that motor neurons innervate some transplanted ears, antibody against VAChT was used to show motor terminals on hair cells. Since antibody against VAChT had not yet been characterized in amphibians, we tested it on lateral line neuromasts and hair cells in untransplanted ears, two sources with known motor innervation (Will, 1982). The neuromasts and control ears were found to have puncta positive for VAChT on the hair cells (Fig. 3 E,F). In the transplanted ears, we found terminals positive for VAChT on hair cells (Fig. 3 I,J). Specifically, the transplanted ear that had shown extensive spinal motor neuron innervation when dye was injected ventral to the spinal cord (Fig. 3G)

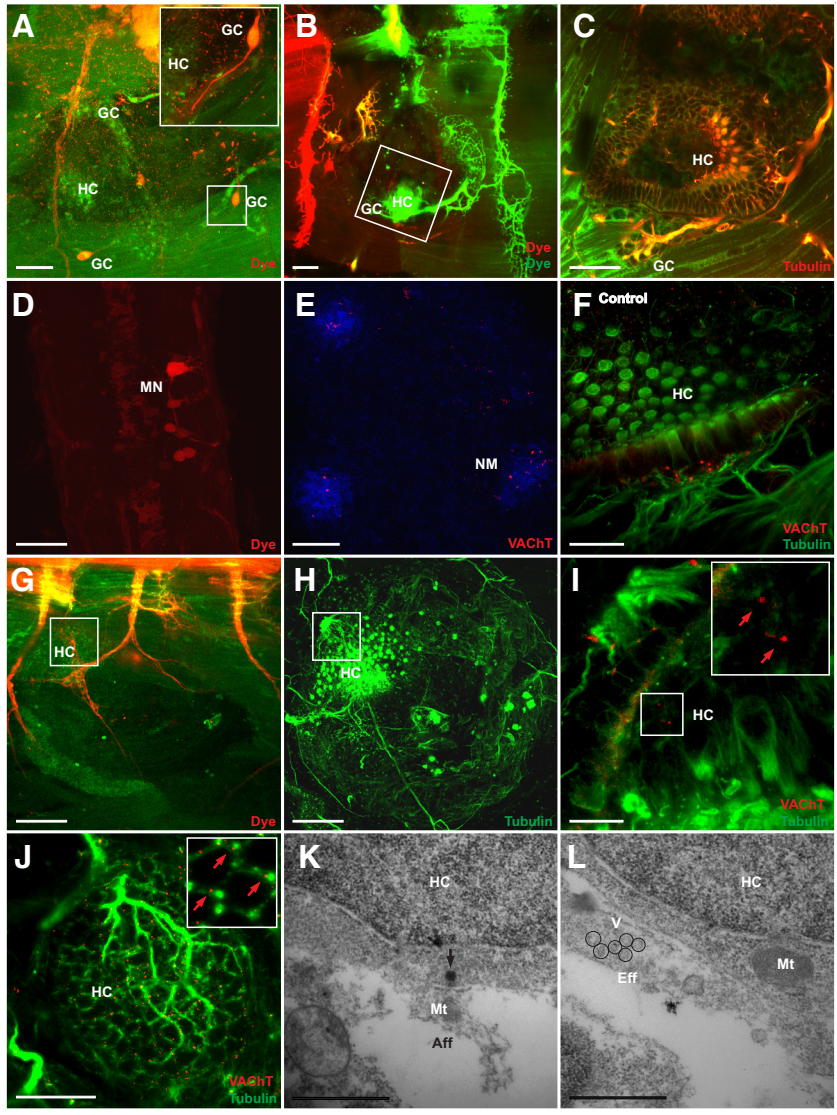

Fig. 3. Afferent and efferent innervation of transplanted ears. (A) *Transplanted ear that had dextran amine (red) injected into the spinal cord. Autofluorescence of the tissue is green. Inset: higher magnification of boxed area.* **(B)** *Transplanted ear that had lipophilic dyes injected into the spinal cord rostral (red) and caudal (green) to the ear.* **(C)** *Acetylated tubulin immunostaining (red) of boxed area in B showing ganglion cells apposed to hair cells. Autofluorescence of the tissue is green.* **(D)** *Cells in the ventral spinal cord positive for dextran amine dyes that had been injected into the transplanted ear.* **(E)** *Neuromasts in the skin showing vesicular acetylcholine transporter (VAChT)-positive terminals on the hair cells. Autofluorescence is blue.* **(F)** *Hair cells in a control ear immunostained for acetylated tubulin and VAChT.* **(G)** *Transplanted ear that had lipophilic dye injected ventral to the spinal cord show nerve fibers extending to the ear.* **(H)** *Transplanted ear in G immunostained for acetylated tubulin.* **(I)** *Transplanted ear in G immunostained for acetylated tubulin and VAChT. Inset: higher magnification of boxed area. Red arrows indicate VAChT immunostaining.* **(J)** *Transplanted ear immunostained for acetylated tubulin and VAChT. Inset: single optical section of VAChT staining (red arrows) at the base of hair cells.* **(K)** *Electron micrograph of an afferent terminal on a hair cell. A synaptic ribbon (arrow) is observed on the hair cell above the afferent terminal* **(L)** *Electron micrograph of an efferent terminal with vesicles (circled) making a synapse on a hair cell. These TEM images were obtained from ears previously processed for immunohistochemistry to confirm the presence of VAChT, hence poor preservation of membranes. GC, ganglion cell; HC, hair cell; MN, motor neuron cell body; NM, neuromasts; Aff, afferent terminal; Eff, efferent terminal; V, vesicles; Mt, mitochondria. Scale bar is 100 μm in (A,B,C,D,G,H); 25 μm in (E,F,I,J) and 1 μm in (K,L).*

also had VAChT-positive terminals on hair cells that co-localized with areas labeled by the lipophilic dye (Fig. 3I). To further validate the presence of efferent terminals on hair cells, transplanted ears that were positive for VAChT terminals on hair cells were imbedded in resin and sectioned for transmission electron microscopy. Efferent terminals were confirmed on hair cells by the presence of axon terminals containing vesicles (Fig. 3L), thus demonstrating that spinal motor neurons can innervate an ear if placed in its trajectory.

Cranial and lateral line nerves develop nearly normally

To assess the effect otocyst removal had on the development of the cranial nerves and lateral line nerves, heads from both control embryos and those having an ear transplanted were immunostained with antibody against acetylated tubulin. Fig. 4A shows the normal distribution of the cranial nerves. In embryos in which an ear was removed, near normal distribution of the cranial nerves was observed although the cranial nerves that would normally circle the ear now traverse the empty space (Fig. 4 B,C). Similar effects were observed with the lateral line nerves. Compared to control embryos (Fig. 4 D,F), in transplanted embryos the lateral line neuromasts spread out to cover the space not occupied by the ear (Fig. 4 E,G,H). In some instances, and more often when the ear was transplanted during the later stages, the parietal lateral line neuromasts failed to develop or were reduced (Fig. 4E). Otherwise the development of the lateral line nerves in transplanted embryos was normal and showed almost no quantitative differences in the number of neuromasts, in particular the supraorbital line (10.1 ± 0.5 neuromasts posterior to the parietal line for the control side versus 10.2 ± 0.5 for the transplanted side (mean ± standard error of the mean, n = 11)).

Discussion

Our results confirm and extend previous studies on ear transplantation (Fritzsch, 1996; Fritzsch *et al.*, 1998; Groves, 2005) in amphibians but add the hitherto unstudied aspect of afferent and efferent innervations. Below we will first discuss the degree of development we achieved in transplanted *Xenopus* ears, followed by a discussion of our tracing data.

Evidence that these transplanted ears could develop relatively normal in a novel location is the presence of hair cells in the transplanted ears, identified by morphology as well as *in situ* hybridization and immunohistochemistry for hair cell markers. This suggests that the epithelial end organs all develop and that either their development is likely not dependent upon the tissue surrounding the placode or that it was specified prior to transplantation, as recent work in chicks has demonstrated that the otic ectoderm becomes specified prior to placodal formation (Abelló *et al.*, 2010). On the other hand, while care was taken to remove only the otic placode, it cannot be ruled out that some surrounding mesoderm and ectoderm was also transplanted, thus influencing further development of the ear. Furthermore, in the GFP-labeled ears, while migration of ganglion cells was observed, it is also possible that some of the green cells observed surrounding the ear may be transplanted mesoderm in addition to the GFP-labeled delaminating ganglion cells. Taken together, these sets of data confirm that well-developed sensory epithelia form with hair cells. Thus, many transplanted ears develop fully normal with respect to otoconia formation, hair cell differentiation and segregation of several sensory epithelia (Fritzsch and Wake, 1988).

That the transplanted ear can develop normally would suggest that the fates of the cells in the otic placode were determined by stage 24-26. In most of the embryos where only a vesicle formed on the trunk, a partial or near normal ear redeveloped in the original location. Waldman *et al.* (2007), using *X. laevis*, ablated otic placodes at various stages to determine the ability of the surrounding tissue to regenerate an ear. Placodes ablated at

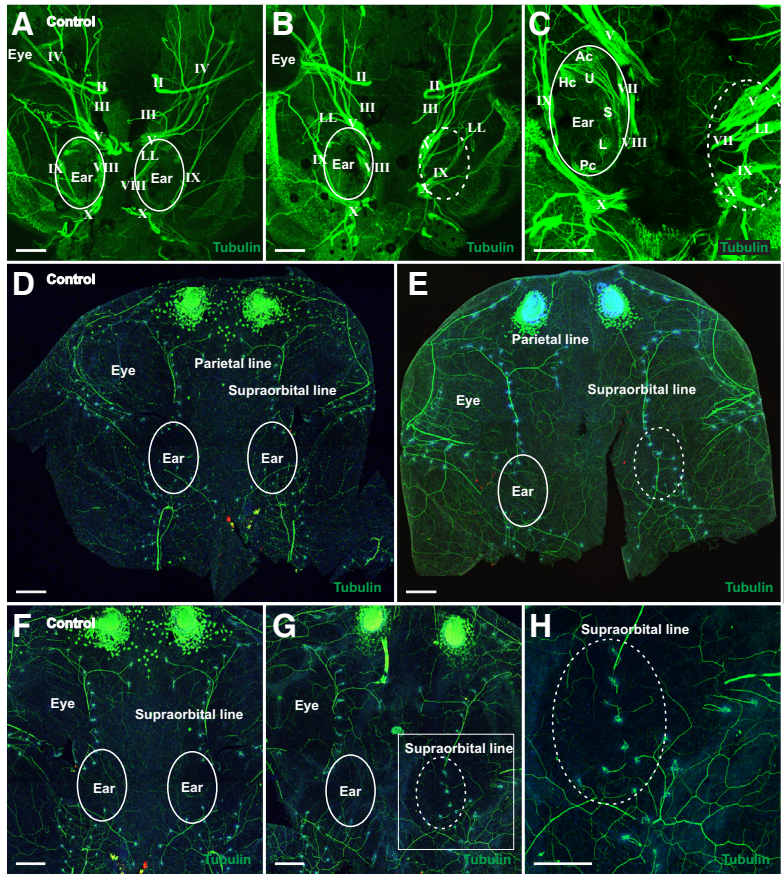

Fig. 4. Development of cranial and lateral line nerves. *Acetylated tubulin immunostaining of cranial nerves in a control embryo* **(A)** *and in embryos in which the ear was transplanted* **(B,C)**. **(D)** *Acetylated tubulin (green) immunostaining of lateral line nerves in the cranial skin of a control embryo* **(E)** *Acetylated tubulin (green) and Hoechst (blue) immunostaining of a later-stage transplanted embryo. Acetylated tubulin (green) immunostaining of lateral line nerves in the cranial skin of a control embryo* **(F)** *and an embryo in which the ear was transplanted* **(G)**. **(H)** *Higher magnification of boxed area in E. Areas where native ears are present and where ablated ears had occupied are circled with solid and dotted lines, respectively. Native ears are labeled 'Ear'. Eyes (A,B) and skin over the eyes (D,E,F,G) are labeled 'Eye'. Cranial nerves are labeled with Roman Numerals. LL, lateral line; U, utricle; S, saccule; L, lagena; Ac, anterior canal; Hc, horizontal canal; Pc, posterior canal. Scale bar is 250 μm.*

early stages (21-23) were able to regenerate completely normal ears whereas beyond stage 28, the ear rarely regenerates. Ablation at stages 24-27 sometimes resulted in regenerated, although abnormal ears (Waldman *et al.*, 2007). In a study with salamanders, ablation of the otic placode could result in regeneration of the inner ear, but only when ablation was done at early stages (Kaan, 1926). Therefore, in the present study, it could be that in the embryos in which an ear redeveloped, the embryo was at a stage in which the remaining tissue was competent to regenerate an ear. On the other hand, it is also possible that since the transplanted placode only formed a vesicle on the trunk and not a complete ear, that not the entire placode was completely transplanted. Waldman *et al.* (2007) also demonstrated that partial ablations could result in regeneration of the ears in some instances. Therefore, it could also be that the partial placode remaining in the head regenerated an ear. The fact that the partial placode in the trunk did not form a complete ear might be due to the novel environment that it developed in and that perhaps additional cues from the head might be necessary for restoring the otocyst. Support for the latter possibility, that residual ears formed when the placode was not completely transplanted, was that most of the transplanted ears that contained otoconia did not have a residual ear in the native location (Fig. 1B). Whereas, for transplanted ears containing only a vesicle and no otoconia or when no transplant was observed, some component of the residual ear, whether it be just the endolymphatic duct or an entire ear, formed in the native location (Fig. 1 C,E).

In addition to the ability of the ears to develop in a novel location, many ears were also innervated. Dye injections into the spinal cord or transplanted ears revealed afferent and efferent innervations respectively. Using these dye injections, we observed that some afferent ganglion cells sent projections to the hair cells and to the spinal cord along trajectories that likely do not bear any informational molecules used by inner ear afferents to navigate during development to reach the hindbrain (Rubel and Fritzsch, 2002). Furthermore, the random dispersal of delaminating ganglion cells away from the ear is also explained by an absence of these normal guidance molecules as there did not appear to be any formal organization of their migration (Fig. 2E). The observation that some, but not all, ganglion cells were filled with dye when injections were made into the spinal cord (Figs. 2E and 3A), suggests that connections to the spinal cord happen by chance and are only made if the growing ganglion cell axons happen to fasciculate with existing nerves. However, since many ganglion cells axons were apposed to hair cells, synaptic connections are likely made thus indicating that the mechanism for hair cell innervation develops normal in the novel location.

Immunostaining of transplanted ears with antibody against VAChT revealed puncta on the basal surface of the hair cells, demonstrating the presence of motor terminals on these hair cells. For transplanted ears that were also labeled from dye injections ventral to the spinal cord, VAChT puncta were found only on hair cells that were in close proximity to the labeled spinal motor neuron projections (Fig. 3 G,I). Furthermore, transmission electron microscopy confirmed efferent terminals on hair cells in transplanted ears as the presence of many synaptic vesicles in the axon terminal is a property of efferent axons, but is not found in afferent axons (Jones and Eslami, 1983; Simmons, 2002). The reduced integrity of the membranes was a result of utilizing ears previously processed for immunohistochemistry to confirm the presence of VAChT. Thus, the evidence presented here of efferent innervation of the transplanted ear demonstrates that some spinal motor neurons have the ability to reroute to innervate a new target if the new target replaces the previous target and is in the direct trajectory of the growing axon. This finding is consistent with the hypothesis that efferent innervation of the ear arose in evolution from rerouted facial branchial motor neurons.

Finally, immunohistochemistry for acetylated tubulin was performed to determine the effect, if any, transplantation had on the development of the cranial and lateral line nerves of the head. The results presented here demonstrated that ear transplantation had little to no effect on further cranial and lateral line development other than, for both the cranial nerves and lateral line neuromasts, they spread out to either traverse or fill in, respectively, the space left void by the ablated ear (Fig. 4). It is possible that for the lateral line neuromasts, either the ear suppresses expansion of the anterior and posterior lateral lines and that, in the absence of the ear, the neuromasts spread out to cover that space, or simply as the skin closes following the removal of the ear, the lateral line placode is stretched as the wound heals. One aberrant effect that was occasionally observed was the absence of the parietal lateral line on the transplanted side (Fig. 4E). In *X. laevis*, the lateral line placodes is formed by about stage 24 (Winklbauer and Hausen, 1985). At this stage, the inducibility of surrounding tissue to form the lateral line begins to decline (Schlosser, 2002). Therefore any disruption of the placode at stage 24 likely results in reinduction of surrounding tissue to form a new placode, whereas at stage 26, the surrounding tissue is less inducible, resulting in a possible loss of neuromasts in some places, such as the parietal line.

In conclusion, the results presented here demonstrate the potential for a motor neuron to reroute to innervate a novel target, supporting the idea that efferents may have arisen from rerouted facial branchial motor neurons during the evolutionary formation of the ear. Such rerouting to novel targets can be induced by ablation of innervations of a given muscle fiber inducing the attraction of nearby motoneuron axons to expand to denervated targets in *X. laevis* (Fritzsch and Sonntag, 1990, 1991), mice (Fritzsch *et al.*, 1995; Porter and Baker, 1997) and man (Engle *et al.*, 1997; Miyake *et al.*, 2008). We cannot exclude such near range attractive signaling from denervated hair cells. However, since hair cell innervation by spinal motor neurons occurred only when the hair cells were in the direct trajectory of the growing axons, this suggests that the rerouting of the facial branchial motor neurons occurred as a chance event when the ear developed in their trajectory. Future tests will determine whether reinnervation of the ear can occur by other cranial motor neurons and thus reflects a general motor neuron ability to engage in synaptic innervations in placode derived hair cells.

Material and Methods

Animals

Xenopus laevis embryos were obtained through induced breeding using human gonadotropin injection. Fertilized embryos were maintained in stock cultures in Petri dishes. Other than during manipulations, em-

bryos were kept in 90mm Petri dishes containing 0.1X Marc's Modified Ringer's Solution (MMR) (diluted from 1x MMR, see below) until stage 46. *X. laevis* stages were as described by Nieuwkoop and Faber (1994).

GFP mRNA injections

The jelly coat was removed using 2% cysteine in 0.1X MMR pH 7.8 (diluted from 1X MMR, see below) shortly after fertilization for embryos used in injections. Embryos were placed in a Ficoll solution (2% Ficoll 400, GE/Pharmacia, in 0.5X MMR) for 5 min. Green fluorescent protein (GFP) mRNA was diluted with distilled water so that the final amount injected was 1 ng. Embryos were injected at the 2 to 4 cell stage using a calibrated glass needle controlled by a Pico-Injector (Harvard Apparatus, Holliston, MA). Injections were made into each cell, keeping the total amount of mRNA per embryo constant.

For synthesis of GFP mRNA, plasmid template (pβGFP/RN3P) was linearized using *Sfi*I and purified using T3 RNA polymerase from the mMessage mMachine kit (Ambion). Protocol was followed according to manufacture's directions.

Transplantations

All transplantations were performed in 1x MMR pH 7.6-7.8, diluted from 10x stock (1M NaCl, 18mM KCl, 20mM $CaCl_2$, 10mM $MgCl_2$, 150mM Hepes). For ear transplantations, otic placodes from the right side of stage 24-26 embryos were removed and transferred ipsilaterally more caudal, replacing a somite. Embryos were kept in 1xMMR for about 10-15 min to promote healing before transfer to 0.1xMMR. Healing was confirmed visually as a fusion of the ectoderm superficial to the otocyst with the ectoderm of the insertion side.

Animals were reared and the transplantation was checked daily for continued growth. Appearance of otoconia was monitored and compared with the remaining untouched control ear. Success of transplantation was scored as three levels of success as follows: normal ear development with formation of otoconia; formation of an ear without recognizable otoconia; no ear development (Table 1).

To check the success rate of our transplantations, we also removed the ear and reimplanted it but rotated by approximately 180°. Those rotated ears were monitored and scored as above (Table 1). In contrast to the caudal transplantation our success rate of normal differentiation of rotated ears was greater (approximately 92% vs. 67%).

Dextran amine label

Texas Red, rhodamine, or fluorescein dextran amines were used to backfill from the spinal cord to the transplanted ear and from the transplanted ear to the spinal cord. Dextran amines were dissolved in distilled water and recrystallized on a tungsten needle (Fritzsch, 1993). Stage 46 embryos were anesthetized in 0.02% benzocaine (Crook and Whiteman, 2009). For dye filling of afferents and efferents to the ear, a small cut was made into the spinal cord adjacent to the transplanted ear and the dextran amine dye was applied. For transganglionic filling of afferents and retrograde filling of efferents, a small opening was made into the transplanted ear and the dextran amines were applied to the sensory epithelial surface inside the ear, which rapidly filled the entire ear vesicle with dye (Fig. 1I). The embryos were transferred to the above outlined 0.1X MMR buffer in a Petri dish to rinse off excess dye and then were transferred to a fresh Petri dish for 45 min to 1h. The wash dish was changed frequently. Finally, the embryos were re-anesthetized and fixed in 4 or 10% paraformaldehyde (PFA) by immersion.

Lipophilic dye label

Lipophilic dye-soaked filter paper (Fritzsch *et al*., 2005) was used to backfill from the spinal cord to the transplanted ear. Embryos were first fixed in 10% PFA following anesthesia. Skin was removed in preparation of injection. Small pieces of dye-soaked filter paper were injected into the spinal cord immediately rostral (NeuroVue™ Green) and caudal (NeuroVue™ Maroon) to the transplanted ear (Fig. 1H). For other embryos, small pieces of flattened dye-soaked filter paper (NeuroVue™ Red) were injected immediately ventral to the spinal cord, entering from the contralateral side so not to destroy the nerves innervating the ear (Fig. 1J). For this injection, the dye would only contact the spinal motor neurons exiting the ventral spinal cord. The embryos were kept in 0.4% PFA at 36°C for about 6h or at room temperature for 10-12h to allow for dye diffusion. For some embryos in which dextran amines were injected into the ear, lipophilic dye (NeuroVue™ Maroon) was injected rostral and caudal to the ear into the nerves innervating the muscle to backfill to the spinal cord. Embryos were mounted on a slide in glycerol and images were taken with a Leica TCS SPE confocal microscope or TCS SP5 multi-photon confocal microscope.

In situ *hybridization*

Whole-mount *In situ* hybridization for micro RNA (miR)-183 and miR-124 was performed on control and transplanted ears from stage 46 embryos as previously described (Pauley *et al*., 2003; Pierce *et al*., 2008). Briefly, locked nucleic acid probe for miR-183 and miR-124 (Integrated DNA Technologies, Coralville, IA) was labeled with digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). Fixed embryos were bleached overnight in 2ml Eppendorf tubes containing 3% hydrogen peroxide. Embryos were re-fixed with 4% PFA, washed in RNAse free PBS 3 times, digested with proteinase K treatment for 40 min for miR-183 or 5 min for miR-124 and then fixed again in 4%PFA. The embryos were washed again with PBS. After 1h incubation in hybridization mix at 60°C, 200μl salmon sperm and 100ng riboprobe were added and the embryos were kept overnight at 60°C. Embryos were then washed with 2X SSC and then PBS, after which 2μl RNAse A Enzyme (5mg/ml, 83U/mg) was added and embryos were kept at 37°C for 90 min. Embryos were then washed several times with 1X Wash solution (Roche), then incubated in 1X blocking buffer (Roche) for 1h and finally overnight in anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche; diluted 1:2000 in 1X blocking buffer). The antibody solution was discarded and replaced by 1X wash solution once per hour and then kept overnight in wash solution. Embryos were rinsed with 1X detection buffer (Roche) and then detected with BM Purple.

Immunohistochemistry

Control and transplanted ears were immunostained with antibody against acetylated tubulin (Farinas *et al*., 2001), myosin (Myo) VI (Kaiser *et al*., 2008), and vesicular acetylcholine transporter (VAChT) (de Castro *et al*., 2009). Cranial and lateral line nerves were immunostained with antibody against acetylated tubulin. Embryos were defatted in 70% ethanol from 30 min to overnight and then rehydrated in PBS for 1h. Embryos were blocked with 1ml Carnation block (2% Carnation powder, 0.1% Triton X-100 in PBS) for tubulin or with 500μl 1% Triton X-100 and 500μl 5% normal goat serum (NGS) for one hour for Myo VI or VAChT. When antibody against tubulin was used in conjunction with VAChT or Myo VI, the latter blocking buffer was utilized. Embryos were washed 3 times briefly in PBS and then incubated in either tubulin primary antibody (1:800; Cell Signaling Technology) in Carnation block solution, Myo VI (1:400; Proteus Biosciences; a specific hair cell marker) in diluted block (0.01% Triton X-100, 0.25% NGS in PBS), or VAChT (1:500; Sigma; identifies motor neuron terminals) in diluted block for 72h. Embryos were washed in PBS 3 times for an hour each and blocked in respective solutions for an hour. Embryos were then washed 3 times briefly in PBS and incubated in secondary antibody (goat-anti-mouse 1:500; Alexa, or goat-anti-rabbit 1:500; Alexa) for 24h in the same block solutions used for the primary antibody incubation. Embryos were washed 3 times for an hour each and then mounted on slides in glycerol. Confocal images were taken with a Leica TCS SPE confocal microscope or TCS SP5 multi-photon confocal microscope.

Plastic imbedding for light and transmission electron microscopy

Transplanted ears along with surrounding muscle tissue were fixed in

2.5% glutaraldehyde overnight and then washed in 0.1M phosphate buffer three times. Ears were then fixed in 1% osmium tetroxide for one hour and washed again in 0.1M phosphate buffer. Ears were then dehydrated in 70% ethanol overnight. Next, ears were further dehydrated in absolute ethanol with 5 washes, 10 min each, then put in a 1:1 solution of absolute ethanol to propylene oxide for 5 min, and finally propylene oxide, 5 washes, 10 min each. Ears were then infiltrated with a 1:1 solution of propylene oxide to resin overnight. Next, the propylene oxide was allowed to evaporate for 4 hrs and the resin was poured into a mold. This was incubated at 60°C for 24 hrs. Sections were cut with an ultratome using a diamond blade, 2μm thick for light microscopy and 100nm thick for electron microscopy. Thick sections were counterstained for light microscopic imaging with Stevenel's blue (2% potassium permanganate and 1.3% methylene blue) at 60°C (del Cerro *et al.*, 1980). Ultrathin sections were counterstained with uranyl acetate and lead citrate and imaged in a Jeol 1230 TEM microscope.

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