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Ears on rears : transplantation of ears reveals afferent pathfinding properties

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EARS ON REARS: TRANSPLANTATION OF EARS REVEALS AFFERENT PATHFINDING PROPERTIES

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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has been approved by the Examining Committee for
the thesis requirement for the Master of Science degree
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ABSTRACT

Afferent neurons transmit information from both external and internal origin into the central nervous system (CNS). Sensory organs are connected at the periphery to these neurons, which in turn project into specific regions in the CNS. In sensory organs, such as the vertebrate ear, which receive auditory and vestibular stimuli, establishing precise connections with central targets is necessary for discrete, simultaneous, and efficient processing. However, it is not clear how afferents of the inner ear establish central projections with their target nuclei in the hindbrain. Transplantation of ears in *Xenopus laevis* offer a method through which the navigational properties of inner ear afferents can be experimentally tested. Specifically, grafting of ears to novel locations allow us to assess the pathfinding capabilities of afferents following ectopic placement.

In transplanting ears adjacent to the spinal cord, we found that despite variable entry points along the dorsal-ventral axis, afferents projected dorsally, similar to projections of native ears in the hindbrain. Furthermore, these afferents were able to reach the hindbrain and project into vestibular nuclei. Late stage transplantations to the spinal cord revealed ear afferent fasciculation with afferents of the lateral line, indicating an alternative navigational route. Similarly, ventral transplantations to the heart region demonstrated ear afferent projection with the vagus nerve. These results collectively suggest that inner ear afferents are molecularly guided to reach their targets in the CNS once they are in proximity to it. However, they also display a capability to project along existing nerves both within the central and peripheral nervous systems. These results provide new information into how inner ear afferents navigate to connect with the CNS.

PUBLIC ABSTRACT

The ear allows you to hear by perceiving sound. Additionally, the ear is important in sensing your orientation in space, as well as for sensing gravity. After perceiving all of this information, the ear converts it into signals that are relayed into the hindbrain by sensory neurons. These neurons make very defined and precise connections with the ear as well as with specific targets in the hindbrain. How sensory neurons of the ear establish these specific connections is not well understood. To better understand how these connections are created, I transplanted developing ears in the frog *Xenopus laevis*, a well suited organism for transplantation work, into foreign areas to study how neurons of the ear navigate to reach, ultimately, their specific target areas.

I show that in transplantation of an ear to the spinal cord, ear sensory neurons are able to enter the spinal cord, a position they normally do not enter, and navigate dorsally to their hindbrain targets. Additionally, these neurons were observed to associate with sensory neurons of the lateral line, another sensory system present in *Xenopus.* Finally, in ears transplanted to the heart region, ear sensory neurons were found to follow with the vagus nerve. These results suggest that ear sensory neurons are guided molecularly in the central nervous system to reach their targets, but also demonstrate capability to follow with existing neurons both in the central and peripheral nervous system. These results add to our current knowledge of how the ear connects with the brain.

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INTRODUCTION

Central Nervous System Projection of Sensory Neurons

Afferent neurons transmit incoming information derived from both internal and external origin into central nervous system (CNS) (Bermingham et al., 2001). Peripheral input is provided by sensory organs, which transduce specific stimuli into signals capable of being transmitted by their associated afferents to the CNS. Within the CNS, these neurons terminate in distinct anatomic and modality specific regions (Fritzsch et al., 2005a; Liu et al., 2016; Maklad and Fritzsch, 2003). The connections that arise between sensory organs, afferents, and their CNS nuclei allow for efficient, discrete, and simultaneous processing of sensory information. In multimodal sensory organs, such as the vertebrate ear that serves the detection of angular and linear vestibular stimulation and auditory sensation (Fritzsch and Straka, 2014), afferents require distinct central targets to ensure segregated processing. This way, the vertebrate ear can selectively transduce auditory and vestibular mechanical stimuli. Transduction of such stimuli allow for the sensation of sound, gravity, and both linear- and angular- accelerations. Simultaneous but distinct processing of auditory and vestibular inputs is a demand placed on all vertebrates challenged with sensing and responding to the variable presence of those stimuli in their environment.

Ensuring properly targeted connections requires that developing afferents are able to navigate within the CNS to reach selectively their target nuclei and neurons within these nuclei. From the inner ear, afferents project into the hindbrain and connect with dorsally located vestibular or auditory nuclei (Torres and Giráldez, 1998), with no overlap between ventrally located trigeminal projections, or even more dorsal lateral

line/electroreceptive projections found in some amphibians and fishes (Fritzsch et al., 2005a). Most information about sensory afferent navigation has been provided from studies of the visual system, where components of the Eph/ephrin system establish the pathfinding properties of retinal ganglion cell (RGC) axons in the midbrain (Brown et al., 2000; Kullander and Klein, 2002). In contrast to the visual system, less understanding of the development of central projections of inner ear afferents is known (Maklad and Fritzsch, 2003), aside from work attributing guidance by incompletely understood molecular cues (Coate et al., 2015; Elliott et al., 2017; Jahan et al., 2010).

Extensive experimental evaluation of sensory neuron navigational characteristics has been conducted using the model system of *Xenopus laevis*. In a study where a developing eye was transplanted onto the spinal cord, retinal ganglion cell (RGC) axons were claimed to enter the CNS at the spinal cord and project within it, with eventual termination observed within the optic tectum (Giorgi and Van Der Loos, 1978). These data demonstrate the capability of sensory neurons to navigate from an ectopic location in the CNS to reach their intended targets, but were not conducted using modern tract tracing techniques (Fritzsch et al., 2005b) leaving the presented data up to interpretation. More recent work in transplanting an eye to the trunk demonstrated the ability of td-Tomato labeled afferents to provide successful sensory input into the CNS, but did not reveal exactly how afferent information reached the CNS (Blackiston and Levin, 2013; Blackiston et al., 2017). Collectively this work suggests the possibility of long range cues acting in RGC pathfinding to reach directly or indirectly the CNS.

Transplantation of developing ears in Xenopus laevis has provided insight into the connections established between the CNS and the ear (Elliott and Fritzsch, 2010; Elliott

et al., 2013; Elliott et al., 2015; Fritzsch et al., 1998). Such transplantation work demonstrated afferent projection into the spinal cord, a location where inner ear afferents do not normally project to, following transplantation adjacent to the spinal cord (Elliott and Fritzsch, 2010). Consistent with results from the transplanted eyes, these data suggest ear afferents are capable of navigating into the CNS from an ectopic entry point. However, the afferent projection patterns once in the spinal cord, and the location of axon termination, were not determined. As a result, the navigational properties of ear afferents upon foreign entry into the CNS remain unclear. In addition to local entry to the CNS following an altered entry point (Elliott and Fritzsch, 2010), similar ectopic ear placements revealed an apparent innervation of the transplanted ear adjacent to the spinal cord by afferents of the posterior lateral line (pLL) system (Fritzsch et al., 1998). Fasciculation of inner ear afferents with afferents of the pLL could potentially offer an alternative route for inner ear fibers to reach the hindbrain.

In this thesis, I address several unanswered questions regarding ear afferent navigation:

1) Do inner ear afferents project into the spinal cord in a topology comparable to native ear fibers in the hindbrain?

2) Are ear afferents projecting in the spinal cord capable of navigating within the CNS to reach the hindbrain and, once in the hindbrain, the vestibular nucleus?

3) Will inner ear afferents fasciculate along existing nerves to route themselves toward the hindbrain when the ear is positioned distant from the hindbrain?

I will present findings to these questions that will offer additional insight into the navigational properties of sensory neurons of the ear as they project centrally to their targets.

Development of the Inner Ear and Its Central Targets

The vertebrate inner ear develops from the otic placode, a thickening of ectoderm along the dorsolateral domain flanking the neural tube (Schlosser and Ahrens, 2004). The otic placode derives from a pan-placodal domain that gives rise to other cranial sensory organs and ganglia, such as those of the lateral line, trigeminal, and olfractory systems (Patthey et al., 2014; Schlosser, 2010). The pan-placodal domain is initially identified by the expression of sine oculus homeobox homolog 1 (*Six1*) (Pieper et al., 2011). In the otic placode, signaling from surrounding mesoderm and from the hindbrain influence its induction. Downregulation of BMP signaling is one such mechanism through which the ectoderm of the pan-placodal area becomes induced to give rise to the otic placode. Additionally, *fgf3* and *fgf8* both act in this process (Phillips et al., 2001). Following *Six1* expression, individual placodes express subsets of the paired box (*Pax*) genes (Pieper et al., 2011). *Pax2* and *Pax8*, for example, are expressed in the otic placode (Schlosser, 2005). *Pax2/8* expression induces increased cellular proliferation, which in turn promotes invagination of the placode to form the otic cup (Bouchard et al., 2010). Much like other placodal invaginations, the cup folds inward and closes off. Subsequently, separation from the overlying ectoderm establishes an ectodermal independent hollow ball of cells called the otic vesicle (Fritzsch et al., 2010). The otic vesicle will give rise to all cell types of the inner ear, including sensory neurons, hair cells and supporting cells.

Continued proliferation occurs after the formation of the vesicle, which acts to facilitate morphological changes that will give rise to the discreet anatomical regions of the inner ear (Kopecky et al., 2011).

As the ear proliferates and forms its anatomical 3D structure, the distinct neurosensory components begin to develop from subsets of proliferating otic vesicle cells. Formation of these neurosensory components begins first with vestibular and auditory sensory epithelia. Each epithelial region within the ear is composed of hair cells and surrounding support cells. Otic sensory neurons, of which vestibular arise first, followed by auditory (Matei et al., 2005) will form from neural precursor populations and leave the otocyst in a delamination process (Fritzsch et al., 2015). Thereafter, these newly formed neurons will extend dendritic processes toward the concomitantly or delayed forming hair cells, while also extending axonal processes centrally toward vestibular and auditory nuclei, respectively (Yang et al., 2011). Tightly regulated expression and activity of several transcription factors is a required step in coordinating expression of many genes that impact the formation of differentiated characteristics of cells within the inner ear (Fritzsch et al., 2015). Three basic-helix-loop-helix (bHLH) genes have been identified as being necessary in the formation of these cell types: Neurogenin 1 (*Neurog1*) for induction of afferents, Neuronal differentiation 1 (*Neurod1*) for differentiation of afferents, and Atonal homolog 1 (*Atoh1*) for hair cell differentiation (Fritzsch et al., 2010).

As inner ear afferents project toward their targets, specific genes promote the formation of terminal characteristics. Disruption of these genes has provided insight into their effects on afferent development. More specifically, conditional deletion of *Neurod1*

in mice resulted in aberrant entry points of cochlear and vestibular afferents in the hindbrain, suggesting Neurod1 functions in promoting navigational competency (Jahan et al., 2010). Therefore, proper development of neurosensory components of the inner ear, specifically sensory neurons, is a necessary first step in establishing functional connections within the hindbrain.

The neural tube is the precursor of the CNS, creating both the brain and spinal cord. At the most dorsal point of the neural tube, a subset of cells known as Neural Crest (NC) delaminate and migrate to regions of the developing embryo. NC cells give rise to the majority of the neurons and glia of the peripheral nervous system (PNS) (Theveneau and Mayor, 2012) except for those derived from placodes (O'Neill et al., 2012). A patterning process specifies differential precursor domains within the neural tube that will produce the diverse types of cells found in the CNS. Secreted signals from neural and non-neural sources act as morphogens, which diffuse into concentration gradients that influence the fate of cells within the neural tube (Hernandez-Miranda et al., 2016). For example, the protein Sonic Hedgehog (Shh) is secreted from the notochord and floor plate, which is a structure ventral to the neural tube. As a result, the Shh concentration gradient is high at ventral neural tube regions and progressively decreases toward more dorsal regions. Shh signaling in the portion of the neural tube that will become the spinal cord promotes the expression of ventral-specific transcription factors (e.g., Nkx2.2, Olig2) (Le Dréau and Martí, 2012). The expression of these and other ventral-specific transcription factors depends on the concentration of Shh and the duration of Shh production (Briscoe and Ericson, 2001).

Gradient-specified subtype specification is also observed in the dorsal regions of the neural tube. In a manner similar to Shh, the proteins BMP and Wnt operate to specify cell fate of dorsal-located cells. BMP and Wnt are secreted from the roof plate of the neural tube in a gradient characterized by dorsal-ventral concentration levels that are opposite of Shh expression (Lai et al., 2016). BMP/Wnt signaling promotes the expression of dorsal-specific transcription factors (e.g., Olig3, Atoh1, Ngn1). The expression of these transcription factors also depends on the concentration of BMP and Wnt received by neural tube cells ventral to the roof plate. Cell subtype production is likewise specified by these transcription factors. For example, Olig3, Atoh1 and Ngn1 progenitor domains give rise to dorsal-located interneurons (e.g. dI1 interneurons) (Hernandez-Miranda et al., 2016). The process by which diffusible concentration gradients establish regions of specific transcription factor expression, and thereby influence cell fate, is consistent through the hindbrain and the spinal cord (Hernandez-Miranda et al., 2016).

Pathfinding Properties of Neurons

Establishing functional connections with central and peripheral targets is a necessary step for neurons during development of the nervous system. As neurons become post-mitotic and adopt terminal differentiated characteristics, they extend their axons from their cell bodies in order to connect with their targets in the organism. The growth cone, a cellular extension located at the end of the axon guides outward growth of an axon. Axons use the growth cone to sense and integrate environmental guidance cues in order to navigate to their intended targets (de Ramon Francas et al., 2016). Guidance

cues can be present locally, mediating contact repulsion or attraction between axons and intermediate targets, or axons and other growing fibers. Additionally, long range guidance cues induce both chemoattractive and chemorepulsive responses in the growth cone, serving to instruct axon movement from distances (Kolodkin and Tessier-Lavigne, 2011). Concentration gradients of such long range guidance cues enable axons to read differential levels of an instructing signal, which allow for coordinated growth in a specific direction. A variety of molecular guidance cues have been implicated in the navigational properties of neurons. Molecules such as Netrins, Slits, Semaphorins, and Ephrins are well-established guidance cues for a variety of neuronal subtypes (Seiradake et al., 2016) and are interpreted by growth cone cell surface receptors that influence cellular machinery to enable proper stirring movement. Other cues implicated in axonal guidance are molecules that act as diffusible morphogens during embryonic development. Molecules such as Wnt, Shh, and BMP have all be demonstrated as diffusible guidance cues for axons (Salinas and Zou, 2008; Seiradake et al., 2016). In addition, select neurotransmitters have been shown to enhance neuron guidance, such as the monoamine serotonin demonstrated in frogs (Blackiston et al., 2017).

Guidance of axons within the CNS has been well characterized, particularly among dorsally located spinal commissural neurons, which make use of chemoattractive and chemorepulsive gradients of guidance cues both for navigating the dorsal-ventral and anterior-posterior axes (Martinez and Tran, 2015). At the periphery, inward guidance of CNS incoming afferents has been best studied in the visual system. The growth cones of retinal ganglion cells use concentration gradients of the surface receptor EphA and its

ligand ephrin-A to establish precise topographic connections between RGCs in the periphery and hindbrain nuclei centrally (Brown et al., 2000).

In contrast to the eye, relatively little is known about the development of central projections in the ear (Maklad and Fritzsch, 2003). Previous work has proposed a mechanism by which afferents establish central projections in a spatiotemporal manner. In such a system, early forming structures project centrally first, thereby leaving later forming structures to project into un-innervated CNS regions [e.g. vestibular projections forming first and projecting ventrally compared to more dorsal-projecting later forming auditory projections (Fritzsch et al., 2005a)]. However, an increasing amount of work is beginning to suggest molecular cues as the primary determinants of afferent navigation. Projection into the hindbrain occurs prior to the neurons of central nuclei (e.g. cochlear nuclei) becoming post mitotic, suggesting target differentiation is not required for afferents to navigate within the CNS (Jahan et al., 2010). Indeed, even if central neurons are molecularly ablated afferents can project and even develop some cochleotopic projections (Elliott et al., 2017). Additionally, transplantations of developing ears to the spinal cord show an ability to form local afferent and efferent connections, which suggests cues present in the spinal cord are sufficient to promote afferent growth into the spinal cord (Elliott and Fritzsch, 2010).

Transplantation Model to Evaluate Pathfinding Properties

In this thesis, I assessed ear sensory neuron navigation by using experimental embryology in *Xenopus laevis* embryos. In particular, I transplanted the developing ear to a novel location adjacent to the spinal cord. Doing so enabled me to evaluate projection

patterns of afferents as they enter the CNS ectopically. By transplanting an ear adjacent to the spinal cord, I was able to determine if inner ear afferents can grow into the spinal cord in the same topologic manner as native ear afferents do in the hindbrain. My results indicate that, while the dorsal-ventral entry point of inner ear afferents varied, they projected to the dorsal regions of the spinal cord after entering, suggesting afferents are being guided by dorsoventral molecular cues present longitudinally throughout the hindbrain and spinal cord. Additionally, these fibers are able to grow along dorsal located spinal fiber bundles to reach the hindbrain and approach and/or enter the vestibular nucleus once they reach the hindbrain.

In addition, by transplanting ears to more ventral locations, I was able to determine if inner ear afferents can navigate along other nerves (lateral line nerves, vagus nerve) to reach the hindbrain. My results indicate that, indeed inner ear afferents appear to navigate along other nerves, for example the lateral line and the vagus. I will present data on these findings and address their contribution toward the navigational properties of developing ear afferent projections. My results provide new insight into the important questions surrounding how neural connections are established between the ear and the CNS, implying nearly opportunistic fasciculation with existing nerves and fiber bundles, but also implying a short range navigation to dorsal parts of the spinal cord and, within the hindbrain, the vestibular nuclei. My data provide information that, if molecularly characterized, could benefit attempts to restore inner ear sensory neurons in those who suffer from sensorineural hearing loss or providing testable insights into pathfinding properties of inner ear afferents navigating in an unusual environment to ultimately reach their target in the hindbrain.

METHODS

Animals

Xenopus laevis embryos were obtained through induced ovulation by injection of human chorionic gonadotropin, followed with fertilization by sperm suspension in 0.3X Marc's Modified Ringer's Solution (MMR, diluted from 10X stock; 1M NaCl, 18mM KCl, 20 mM CaCl2, 10 mM MgCl2, 150 mM Hepes, ph 7.6-7.8). The jelly coat was removed with 2% cysteine in 0.1X MMR. Embryos were contained in 0.1X MMR until having reached the desired stage for manipulation (see below) and until reaching stage 46 as described by Nieuwkoop and Faber (1994).

Ear Transplantations

All surgical manipulations were performed in 1.0X MMR (diluted from 10X MMR) and performed at room temperature. Animals were anesthetized with 0.02% Benzocaine (Crook and Whiteman, 2006) prior to and during all manipulations. To determine the projection patterns of transplanted inner ear afferents into the spinal cord, and to evaluate navigational properties of transplanted afferents once in the CNS, otic placodes from donor embryos were removed and transplanted to recipient hosts at stage 25-27. Removed placodes were grafted adjacent to the spinal cord in place of a removed somite (Figure 1B) on one side of the embryo. Additionally, to determine if inner ear afferents are capable of using other existing nerve projections as substrate with which to fasciculate with, and reach the hindbrain, placodes were transplanted to the presumptive heart region, in the vicinity of the vagus nerve trajectory (Figure 1C). In assessing if spinal cord transplanted inner ear afferents are able to fasciculate with afferents of the

lateral line, and to determine if the capability to do so is dependent on the time of transplant with respect to lateral line placode migration, transplantations of an ear vesicle at stage 25-27 and at stage 32-36 were performed. As before, otic placodes (stage 25-27) or otic vesicles (stage 32-36) were grafted from a donor animal to a recipient, and placed adjacent to the spinal cord in place of a removed somite. Embryos were kept in 1.0X MMR after surgery for 15-30 minutes to allow healing. Following time to heal, animals were transferred into 0.1X MMR until stage 46 upon which they were anesthetized in 0.02% Benzocaine and fixed by immersion in either 4% paraformaldehyde (PFA) when used for immunohistochemistry or dextran (see below) or in 10% PFA when used for lipophilic dye tracing (see below). Successful development of the ear was confirmed at stage 46 based on the presence or absence of an ear in the region of transplantation and by the presence of otoconia (Figure 1, Table 1). Ear development was further assessed using anti-Myo6 antibody to label specifically hair cells and anti-tubulin to label nerve fibers (see immunochemical analysis below). Only animals with fully formed transplanted ears, as indicated by otoconia in position above sensory epithelia, were used for further analysis.

Lipophilic Dye Labeling

Axonal projections from transplanted ears were labeled using NeuroVue lipophilic dyes (Fritzsch et al., 2005b). NeuroVue™ Maroon, NeuroVue™ Red, and NeuroVue™ Jade dye-soaked filter paper pieces were cut to fit and were placed inside transplanted ears. Care was taken to place the dye on presumptive regions of sensory epithelia as determined by location of otoconia within the ears. Dye placed in

transplanted ears labels inner ear afferent axons through backfilling of dendritic processes, terminating on hair hells, into ganglion cell bodies. Dye was also placed into the spinal cord following transection, either rostral or caudal, to the adjacently transplanted ear to fill inner ear afferent axonal processes within the spinal cord as they project within it and into the hindbrain. To determine lateral line innervation patterns of an ear transplanted adjacent to the spinal cord, dye was placed into the posterior lateral line ganglia caudal and adjacent to the native ear, filling lateral line afferents to neuromast (lateral line) organs along the trunk of the animal. In the same animals, dye was placed into the spinal cord to label afferents entering the CNS. Native ear afferent projections into the hindbrain were labeled with dye inserted into each ear.

Dye diffusion: Following dye insertions, animals were kept in 0.4% paraformaldehyde and incubated at 60°C or 36°C to permit diffusion. Dye placed in the spinal cord or posterior lateral line ganglia were incubated at 60°C for 60 hours. Dye placed into transplanted ears near the spinal cord were incubated for 18 hours at 36° to determine the spinal cord entry point or for 60 hours at 60° to assess hindbrain innervation. Ears transplanted to the heart region were incubated for 3 days at 60°. Native ear dye placements were incubated for 18 hours at 36°. Following diffusion, the brain and spinal cord was dissected out and the specimens were mounted in glycerol for imaging on a TCS SP5 Multi-photon confocal microscope using excitation emission settings specific for the different lipophilic dyes used (Tonniges et al., 2010).

Dextran amine Labeling

Dextran amine dye injections into ears transplanted adjacent to the spinal cord were used to evaluate inner ear afferent projection in the CNS. Entry points of inner ear afferents into the spinal cord as well as their projections into the hindbrain were evaluated using Texas red, tetramethylrhodamine, Alexa Fluor 647, and Alexa Fluor 488 dye. A small incision was made into the transplanted ear of anesthetized animals (0.02% Benzocaine) and a recrystallized drop of the labeling dye on a tungsten needle was inserted (Fritzsch, 1993). Care was taken to fill the ear entirely with the dye. Animals were washed in 0.1X MMR three times in succession and kept in a dish containing 0.1X MMR for 2-3 hours. Afterwards, the embryos were reanesthetized in 0.02% Benzocaine and fixed in 4.0% PFA. After fixation, the brain and spinal cord was dissected out and the specimens were mounted in glycerol for imaging on a TCS SP5 Multi-photon confocal microscope using appropriate excitation/emission filter settings.

Immunohistochemistry

To determine presence of sensory epithelia in transplanted ears, as well as local innervation of the ear and its surroundings, fixed stage 46 animals were dissected to remove the lower jaw and skin and were dehydrated in 70% ethanol overnight. Animals were washed in 1X PBS three times for 10 minutes each before being blocked in 5.0% normal goat serum (NGS) with 0.1% Triton-X 100 for 1 hour. Following a brief wash in 1X PBS, primary antibodies of neuronal marker acetylated tubulin (1:800, Cell Signaling Technology) and hair cell marker Myosin VI (1:400, Proteus Biosciences) were incubated with the embryos overnight at 36°C. Animals were washed three times for 10 minutes

and blocked in 5.0% NGS + 0.1% Triton X 100 for 1 hour prior to incubation with species-specific secondary antibodies (1:500, Alexa) along with nuclei marker Hoechst 33342 overnight. Animals were washed in 1X PBS six times for 15 minutes each and mounted in glycerol for imaging on a TCS SP5 Multi-photon confocal microscope.

RESULTS

Success of Transplantations

Success of transplantations was assessed based on the presence and degree of development of an ear with otoconia at the place of transplantation (to the trunk adjacent to spinal cord and more ventrally to the heart region) (Table 1, Figure 1). While most transplants were successful in that they developed ears with otoconia, in some instances ears developed without otoconia or did not develop at all (Table 1), consistent with data from similar placements adjacent to the cord to assess the ability of spinal motor neurons to become efferents to inner ear hair cells (Elliott and Fritzsch, 2010). Similar rates of success were found for ears transplanted adjacent to the spinal cord and ventrally near the heart; 81 and 73 percent, respectively, of animals had transplanted ears with otoconia (Table 1, Figure 1). Only ears that contained otoconia were used for further analysis as presence of otoconia coincides with hair cell formation (Elliott and Fritzsch, 2010).

Table 1. Success of transplantations

Ear Transplantations	Development with otoconia	Development without otoconia	No ear development
Ears adjacent to the spinal cord early* (114)	95	11	8
Ears adjacent to the spinal cord late** (30)	22	1	7
Ears to the heart region (11)	8	0	3

Total number of transplants indicated in parentheses

*Ear transplantations performed at stages 25-27

**Ear transplantations performed at stages 32-36

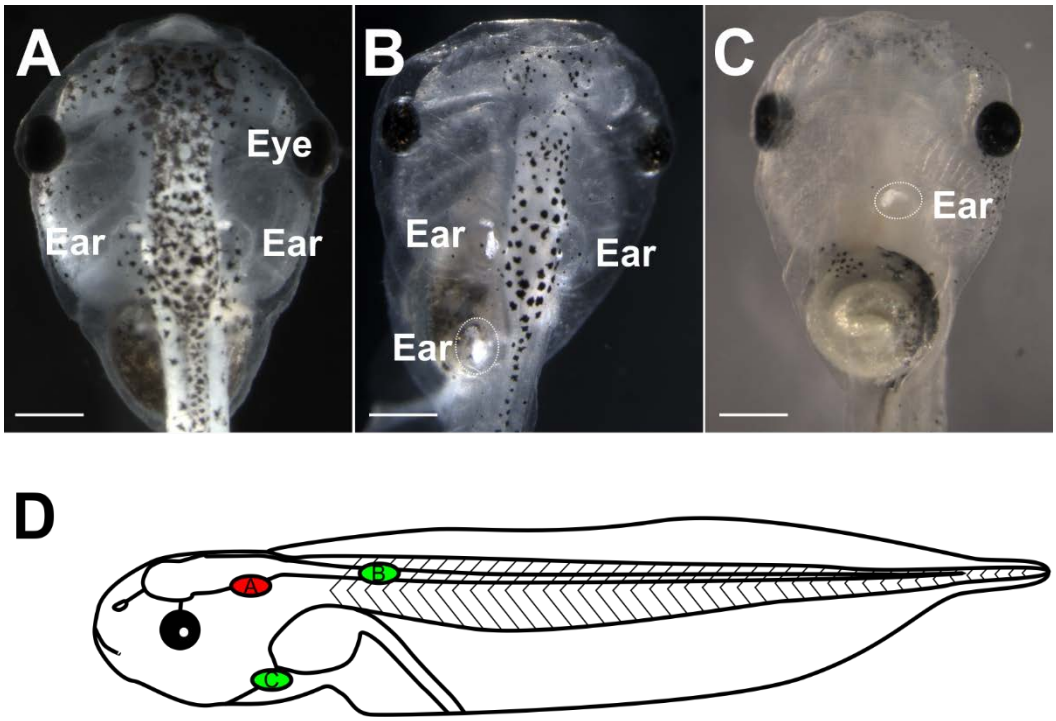

Figure 1. Evaluation of ear transplantations (A-D) Stage 46 X. laevis embryos showing positions of native ears (labeled) and transplanted ears (circled). **(A)** Control animal. **(B)** Embryo with a third ear transplanted adjacent to the spinal cord. **(C)** Ventral view of embryo with a third ear transplanted next to the heart. **(D)** Schematic diagram representing a lateral view of stage 46 X. laevis demonstrating the positions of the native ear (red, A) and the two different transplantations (green, B,C). Labels correspond to panels A-C, respectively. Scale bars are 0.5 mm.

Ears Transplanted Adjacent to the Spinal Cord Develop Hair Cells and Neurons

To assess development of sensory epithelia in ears transplanted adjacent to the spinal cord, ears were immunostained with antibodies against myoVI and acetylated tubulin, which are markers for hair cells and neurons, respectively. Positive myoVI staining revealed the presence of hair cells in transplanted ears (Figure 2A). Hair cells were found to be in discreet clusters within the ear, indicating distinct sensory epithelia. The presence of ganglion cells with the transplanted ear was confirmed by acetylated tubulin staining, as shown by a collection of cell bodies with neuronal projections

innervating regions of sensory epithelia (Figure 2B, C). Innervation of hair cells by afferent processes occurred on the basal side of hair cells, as confirmed by kinocilium present (labeled by acetylated tubulin) on the opposite apical side (Figure 2C). These results indicate that ears transplanted adjacent to the spinal cord are capable of developing hair cells and neurons, and that peripheral connections are established between the two cell types. This is consistent with previous work demonstrating that ears transplanted ectopically in *X. laevis* develop sensory epithelia and afferents relatively normal (Elliott and Fritzsch, 2010; Elliott et al., 2013).

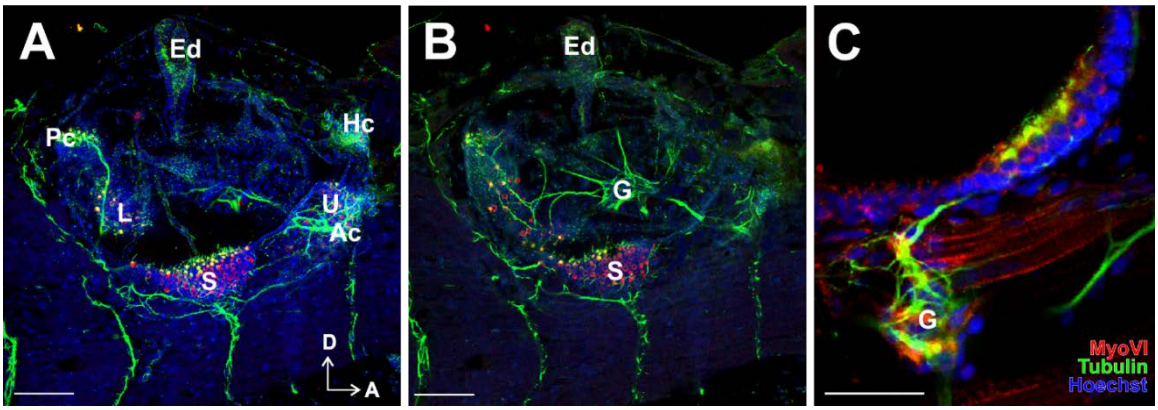

Figure 2. Development of transplanted ears (A) Lateral view of an ear transplanted adjacent to the spinal cord at stage 25-27 labeled with antibodies against myoVI (red) and tubulin (green) demonstrating the presence of hair cells in six distinct epithelia and neurons, respectively. **(B)** Single optical section of the ear in A showing ganglion cells associated with the ear (G). **(C)** Single optical section demonstrating afferent innervation of hair cells in an ear transplanted adjacent to the spinal cord. S Saccule, L Lagena, Ac Anterior canal, Pc Posterior canal, Hz Horizontal canal, U Utricle, Ed endolymphatic duct. Scale bars are 100 µm.

Ears Transplanted Adjacent to the Spinal Cord Project Dorsally Regardless of Entry Point

Since ear afferent connections with the spinal cord in identical transplants have been observed previously by retrograde labeling of ganglion cells from dye injection into

the spinal cord (Elliott and Fritzsch, 2010), and having verified that transplanted ears develop hair cells and neurons, afferent axon projections were traced from the ear using lipophilic or dextran amine dyes (Fritzsch, 1993; Fritzsch et al., 2005b) to label afferent projections to the spinal cord. We aimed to determine if inner ear afferents from an ear transplanted adjacent to the spinal cord enter and project as native afferent fibers do in the hindbrain, indicating a potential conservation of molecular cues between the hindbrain and spinal cord that guide dorsoventral guidance of sensory afferents.

Following labeling of afferent projections from the ear, the brain and spinal cord were dissected from the embryo and the entry point along the dorsal-ventral (D-V) axis of the spinal cord was determined (Figure 3A). In assigning the plane of entry, the midline was defined as the center of the z-series stack encompassing the entirety of brain and spinal cord. Above and below this point was defined as dorsal and ventral, respectively. 14 of 20 animals had projections with a dorsal entry point. 1 animal had afferents enter at the midline, and 5 animals had a ventral entry point (Figure 3B).

Plane of projection within the spinal cord was assessed in a similar manner and defined by the D-V plane where fibers were observed to project. All 20 animals examined had afferents projecting dorsally within the spinal cord (Figure 3B). Additionally, these projections extended both rostral and caudal from the entry site. These results suggest that regardless of entry point along the dorsal-ventral axis, inner ear afferents entering the spinal cord will project dorsally, as they do in the hindbrain.

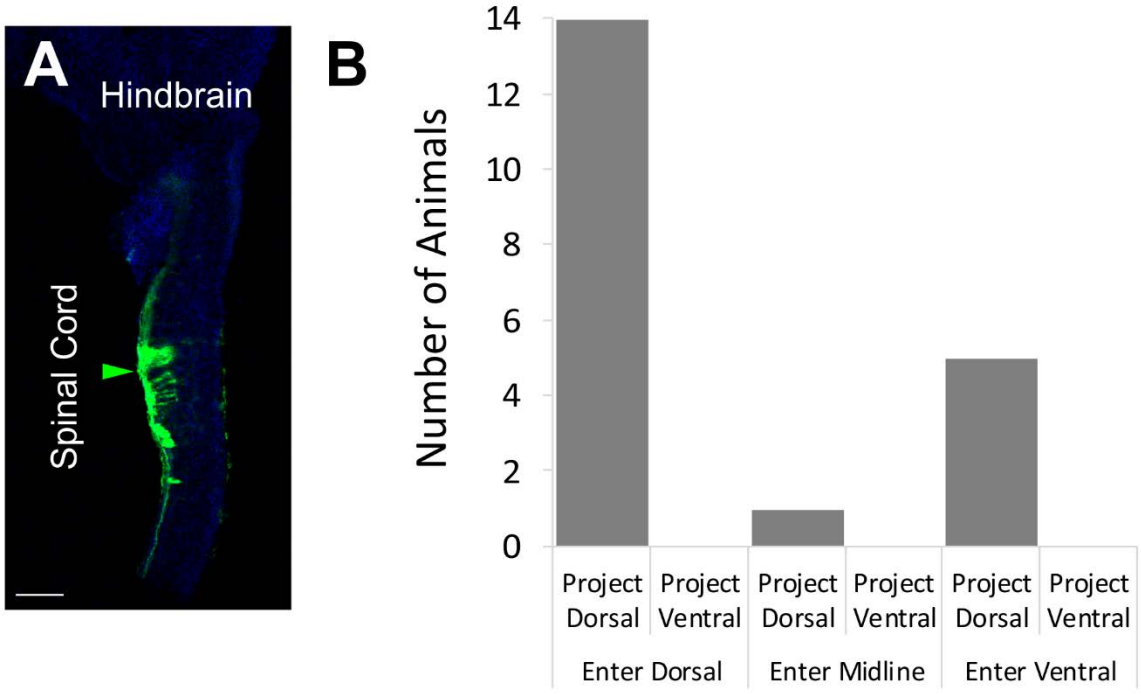

Figure 3. Ear afferent innervation of the spinal cord (A) Single optical section of an X. laevis brain and spinal cord in the dorsal plane following injection of dye into an adjacently transplanted ear (green) shows afferents entering the spinal cord dorsally. **(B)** Analysis of entry point and plane of projection for animals with ears transplanted adjacent to the spinal cord. Serial optical sections were analyzed for entry point of labeled fibers (dorsal, midline, ventral) and for plane of projection (dorsal, ventral). n=20. Green arrowhead indicates inner ear afferent projections. Scale bar is 100 µm.

Ears Transplanted Adjacent to the Spinal Cord Project to the Hindbrain

Since ear afferent projections into the spinal cord appear to project dorsally regardless of the D-V entry point (Figure 3), we next sought to identify if the afferent fibers projected into, and established connections with, the dorsally located vestibular nucleus in the hindbrain. Dyes were placed either into the transplanted ear itself or injected rostral to the ear into the spinal cord. In both instances ascending spinal tracts were labeled along with inner ear afferents, demonstrating fasciculation between the two (Figure 4C-D'). Labeling of the ascending spinal tracts also labels the trigeminal (V)

nucleus afferents (Figure 4B, B'), as there exists a continuity between the ascending spinal tracts and the descending tract of V (Maklad and Fritzsch, 2003). Labeling of native ear projections into the hindbrain identify the vestibular nucleus (Figure 4B, B').

In animals with inner ear afferents projecting to the spinal cord, fibers appear to fasciculate with the ascending spinal tracts and once reach the trigeminal nucleus, reroute into the vestibular nucleus (Figure 4D, D'). Of 8 animals analyzed in this manner, all were found to have fibers approaching and/or projecting directly into the vestibular nucleus. In contrast, in control animals there is no projection from the spinal cord into the vestibular nucleus (Figure 4A-4B'). Collectively these data show that inner ear afferents that enter the hindbrain from the spinal cord are capable of projecting to the vestibular nucleus, suggesting vestibular afferents are being navigationally instructed through yet unknown molecular cues once entering the hindbrain.

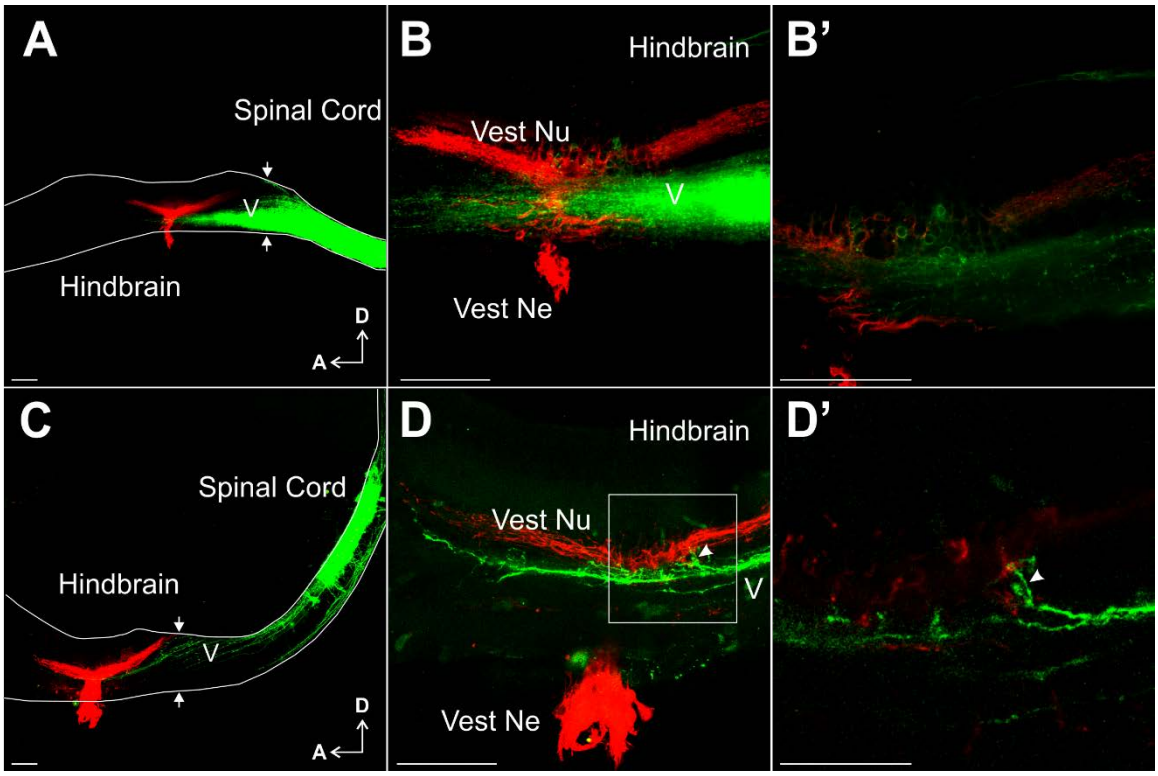

Figure 4. Afferent innervation of the hindbrain by ears transplanted adjacent to the spinal cord (A) Control hemisection of the brain and spinal cord showing ascending spinal fibers (green) enter the hindbrain and fill the descending tract of trigeminal nucleus (V, unlabeled). Note the lack of overlap between the trigeminal nucleus and vestibular nucleus at higher magnification of A **(B)** and of B **(B')**. Native ear projections (red) into the hindbrain are labeled. Arrows denote the hindbrain/spinal cord boundary. **(C)** Hemisection showing ascending spinal tracts and spinal cord transplanted ear afferent fibers projecting into the hindbrain (green) along the descending tract of V (unlabeled). **(D)** Higher magnification of C showing inner ear afferents projecting into the vestibular nucleus from the trigeminal nucleus (arrowhead). **(D')** Higher magnification of box in D showing projections into the vestibular nucleus (arrowhead). 8 animals were analyzed. Scale bars are 100 µm in A,B,C,D and 50 µm in B', D'. Vest Ne vestibular nerve, Vest Nu vestibular nucleus.

Lateral Line Interaction with Ears Transplanted Adjacent to the Spinal Cord

Previous work in transplanting ears to the spinal cord revealed an apparent innervation of the transplanted ear by afferents of the posterior lateral line (pLL) system (Fritzsch, 1998). This could potentially offer an additional route for ear afferents to project to the hindbrain. Since transplants in this study were done when embryos were at

stage 36, a time when the posterior lateral line placode extends from the otic region caudally, we used this approximate stage for our transplantations to assess if there was a preference for the lateral line or spinal cord for inner ear afferents to project along. Immunostaining of animals with ears transplanted at the late (stage 32-26) stages revealed that pLL afferents associate with the ear when transplanted in its projection pathway, as well as having neuromasts dorsal to the transplanted ear (Figure 5C).

To identify whether inner ear afferents have a preference for the lateral line and/or spinal cord, and also if transplanted ears are innervated by lateral line fibers, dye was implanted into the pLL and spinal cord. Labeling in this manner revealed inner ear afferent projections into the spinal cord as shown by backfilling of ganglion cells (Figure 5D). In addition, the pLL appears to project into the ear itself (Figure 5D-F), as shown by fibers projecting deep within the ear (Figure 5E). Additionally, an apparent overlap between pLL and spinal cord-projecting inner ear fibers was observed in one out of five animals examined, suggesting that these inner ear afferents could also be fasciculating with the pLL (Figure 5F) but with no apparent directional preference. Together these data indicate that inner ear afferents project centrally into the spinal cord or peripherally along the pLL, however it is unclear if there exist a preference for either routes or where terminations occurs when following pLL afferents.

Given that the lateral line also passes dorsal to the transplanted ear when the ear was transplanted at stage 25-27 (Figure 5B) as it does at the later stages (Figure 5C), we need to determine if there is an effect of timing of the transplant on a preference for direct projection into the hindbrain or navigation along the lateral line.

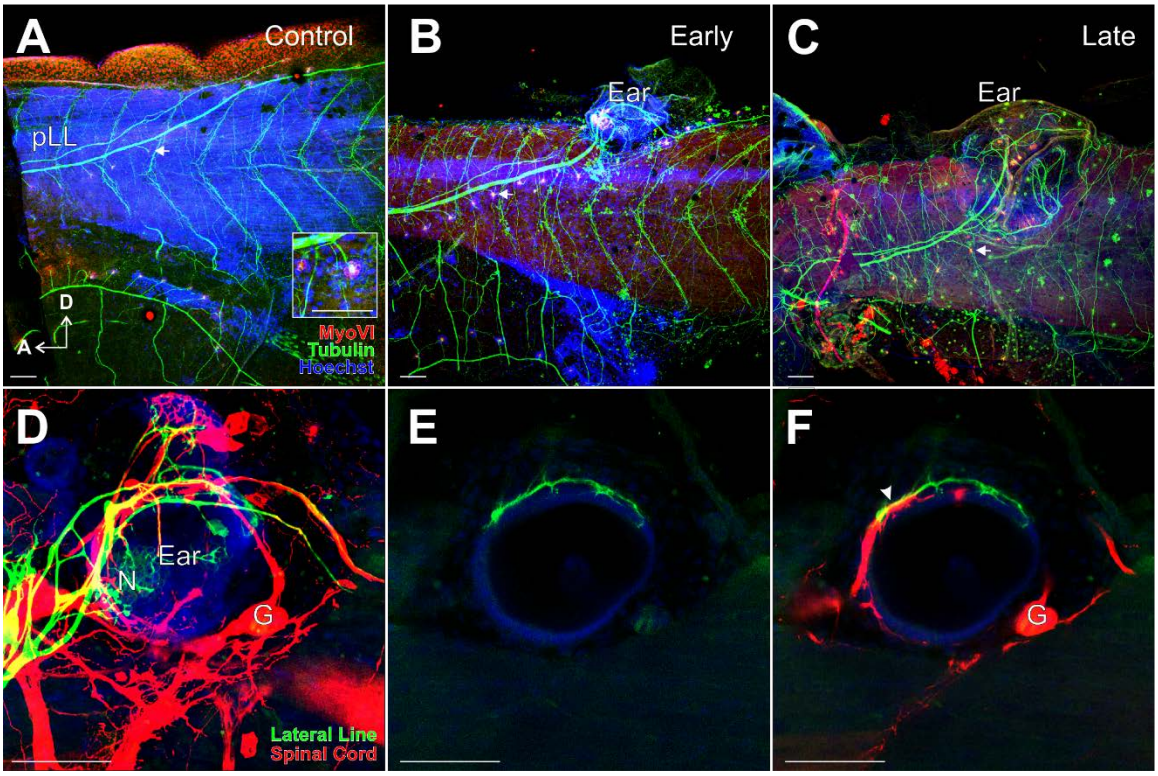

Figure 5. Lateral line interaction with ears transplanted adjacent to the spinal cord **(A)** Lateral view of the trunk of a control animal labeled with antibodies against myoVI (red) and tubulin (green) demonstrating the presence of hair cells and neurons of the posterior lateral line (pLL) neuromasts (arrows, also inset) and afferents, respectively. **(B)** Animal with an ear transplanted adjacent to the spinal cord at stage 25-27. **(C)** Animal with an ear transplanted adjacent to the spinal cord at stage 32-36. **(D)** Lateral view of ear transplanted adjacent to the spinal cord at stage 32-36 demonstrating ear afferent innervation of the spinal cord (red, spinal cord dye placement) and afferents of the pLL projecting into the ear (green, pLL ganglia dye placement). **(E)** Single optical section of D showing an apparent innervation of the ear by pLL afferents (green). **(F)** Same optical section in E merged with spinal cord dye signal demonstrating an apparent fasciculation of spinal cord-projecting inner ear afferents with pLL afferents (arrowhead). G, inner ear ganglia. Scale bars are 100 µm.

Ears Project along the Vagus Nerve when Transplanted to the Heart Region

Ears transplanted even further ventrally into the region of the developing heart were used to determine if inner ear afferents are capable of using other nerves, specifically the vagus, to fasciculate on and reach the hindbrain. Dye labeling of these transplants identified afferents of the inner ear projecting with the vagus nerve (Figure

6A), identified in 5 animals. Labeled fibers were found to project dorsally from the heart region and into the vagus ganglia (Figure 6B). Given the close proximity of posterior lateral line and vagus ganglia, transcellular diffusion of the dye labeled both (Figure 6B). Central projections, in one animal of the five, of lateral line fibers were observed from this labeling (Figure 6C). It is unclear from this data if afferents of the inner ear are reaching the hindbrain after projecting along the vagus. Further work would need to be done to determine this.

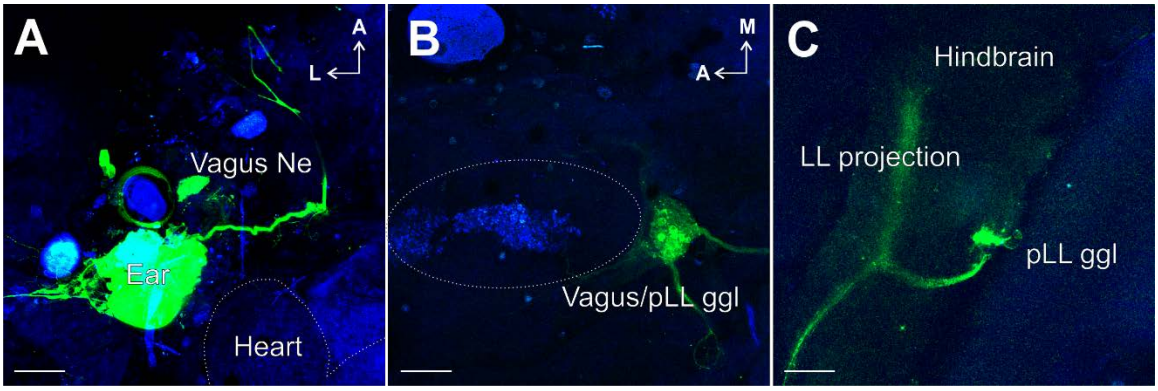

Figure 6. Inner ear afferent fasciculation along the vagus nerve (A) Ventral view of an animal with an ear transplanted into the heart region showing ear afferents projecting with the vagus nerve (Vagus Ne). Heart is outlined with a dotted line. **(B)** Dorsal view of the same animal in A showing projections entering the Vagus ganglia (Vagus ggl), while also transcellularly filling posterior lateral line ganglia (pLL ggl). Native ear is circled with a dotted line. **(C)** Brain from animal in A and B showing lateral line projections entering the hindbrain. Scale bars are 100 µm.

DISCUSSION

Through basic embryological manipulations, I have experimentally examined the navigational properties of afferents of the inner ear if transplanted to novel foreign areas caudal to its normal position. The results presented here provide evidence of the navigationally capabilities of primary vestibular neurons, and in doing so add to our current understanding of the processes that connect the vertebrate ear to the central nervous system, specifically the connections to vestibular nuclei and the cerebellum of the hindbrain.

Similar rates of success of overall ear development, judged by presence of otoconia and immunostaining for hair cells, were found for all different transplantations performed. That successful development of ectopically placed ears was observed is consistent with previous otic placode and vesicle (Fritzsch et al., 1998) transplantations. Instances where ears formed without otoconia, or no ear developed at all, potentially represent perturbations as a consequence of the grafting process (Elliott and Fritzsch, 2010).

In the present study I transplanted developing ears in Xenopus laevis adjacent to the spinal cord. Dye tracing data confirmed afferent connections with the spinal cord and revealed similar dorsal projection patterns as observed in native fibers projecting within the hindbrain. While entry into the spinal cord was variable along the dorsal-ventral axis, it was found that despite the entry location, fibers projected dorsally after entering. It is possible but was not elucidated here, that afferent entry points reflect fasciculation along dorsal and ventral root fibers. Since spinal projecting inner ear afferents revealed a capability to enter ventrally and at the midline, yet project dorsally once within the spinal

cord, these data indicate that navigational information, most likely a diffusible attraction factor, is dorsally derived. Alternatively, entering fibers could potentially be repelled by ventral factor(s). Dorsal projection of native inner ear afferents is readily observed in the hindbrain (Rubel and Fritzsch, 2002; Straka et al., 2014; Torres and Giráldez, 1998). That spinal entering ear fibers project in this manner suggest inner ear afferents are being guided molecularly, and that these guidance cues are conserved between the spinal cord and the hindbrain.

Longitudinal columns of identical dorsal gene expression establish a continuity between the hindbrain and the spinal cord (Gowan et al., 2001; Ma et al., 1997; Maklad and Fritzsch, 2003). Discreet domains of Atoh1 and the ventrally adjacent Ngn1/2 are the sites of termination for native auditory and vestibular afferents, respectively (Fritzsch et al., 2006; Maricich et al., 2009) and extend through the hindbrain and spinal cord (Bermingham et al., 2001; Gowan et al., 2001). These domains are considered to be established by dorsally produced diffusible gradients of BMP and Wnt molecules (Hernandez-Miranda et al., 2016; Lai et al., 2016; Le Dréau and Martí, 2012; Ulloa and Marti, 2010) as well as ventrally produced Shh molecules (Litingtung et al., 2002). Combined, these morphogens are potentially acting in an instructive manner to guide inner ear afferents to dorsal regions of the hindbrain. Given the currently understood roles of Shh, BMP and Wnt in axon pathfinding (Fenstermaker et al., 2010; Seiradake et al., 2016; Yam and Charron, 2013), it is conceivable that they may also function in such a manner in the hindbrain and spinal cord. Indirect evidence to support this claim is given by selective deletion of *Neurod1* in the ear, demonstrating that despite an aberrant common entry point instead of a distinct vestibular and auditory entry to vestibular and

cochlear nuclei, respectively, auditory and vestibular fibers mostly segregate once within the hindbrain (Jahan et al., 2010). A feature that is similar to the projection patterns observed after variable entry points of spinal projecting ear afferents described above.

Inner ear afferents projecting into the spinal cord could be traced to the hindbrain, specifically in the vestibular nucleus. Labeling of ascending spinal tracts with afferents of the inner ear was expected given dorsal projection of both. After reaching the hindbrain, inner ear afferents continued along the descending tract of trigeminal nerve fibers to enter the hindbrain, which itself is continuous with the ascending spinal tracts of spinal fibers. Following entry into the hindbrain, collaterals of inner ear afferents reroute from the descending tract of trigeminal and connect with the vestibular nucleus (Figure 4). These results indicate that upon novel entry into the hindbrain, afferents of the inner ear will navigate to the vestibular nucleus. Rerouting from ventral trigeminal tracts to the dorsal vestibular nucleus is consistent with our contention of dorsally produced attractants or ventral repellants guiding afferents of the inner ear. Similar to the observed projection patterns within the spinal cord, dorsal attraction or ventral repulsion is likely mediated by gradients of diffusible morphogens. However, we cannot rule out the role of other guidance contributors such as components of the Eph/ephrin system, which are expressed in vestibular sensory neurons (Bianchi and Liu, 1999). Another explanation could be an integration of both diffusible morphogens and the Eph/ephrin system in driving guidance in these neurons.

In addition to spinal projecting inner ear afferents, generation of three eared frogs in *X. laevis* demonstrated overlap between afferents of a native and additional rostral placed ear in the vestibular nucleus (Elliott et al., 2015). Furthermore, in ears transplanted

to the orbit in place of an eye, entry with afferents of the trigeminal nerve revealed similar rerouting to reach vestibular nuclei (Elliott et al., 2013). While the former indicates independent entry into the hindbrain, the latter suggests inner ear afferents are able to fasciculate along existing nerves to reach the hindbrain. Regardless of the mechanism used to enter the CNS, navigation to vestibular nuclei strongly support our molecular basis of central projection development. In the present study, fibers entering the spinal cord from a transplanted ear likely project along existing afferents as they enter the CNS. Dorsally entering ear afferents presumably do so by fasciculation with incoming somatosensory fibers (Todd, 2010) of Rohon-Beard cells, the predecessors to later forming dorsal root ganglion cells (Clarke et al., 1984). Ventral entry of fibers from transplanted ears are possibly entering by following along motor neurons, which themselves project ventrally from the spinal cord. Indeed, following of fibers that innervate the surrounding musculature is likely the ventral (or dorsal) pathway taken, an observation noted here and previously (Elliott and Fritzsch, 2010), especially given the ability of any motor neuron to innervate the ear (Elliott et al., 2013; Fritzsch and Elliott, 2017). In one instance entry at the midline was observed. The lack of any normal outgoing or incoming native spinal cord fibers at this position potentially conclude inner ear afferents in this animal are not entering with other fibers. Collectively however, this data is interpreted to suggest that inner ear afferents from spinal cord transplanted ears are potentially associating with existing fibers, either sensory or motor, to enter the CNS. Given this, I suggest that reaching the CNS is primarily an opportunistic association with other PNS fibers. Whether the absence of random inner ear afferent projections at this late stage reflect a neurotropic directional growth to the CNS or a neurotrophic pruning of

non-CNS projecting afferents requires studying of labeled fibers in earlier stages (Blackiston et al., 2017).

In mice lacking Schwann cells, spiral ganglion neurons displayed errant peripheral targeting, demonstrating a tendency to navigate along Schwann cell covered fibers or Schwann cells (Mao et al., 2014). Additionally, formation of peripheral fascicles in auditory regions of the ear are dependent on Eph/ephrin interactions (Coate et al., 2012), indicating auditory afferents appear to have partiality in navigating along existing fibers. Disruption of components of the Eph/ephin present in vestibular afferents (Allen-Sharpley et al., 2013) can cause altered efferent projections (Cowan et al., 2000). Such work lends support to the notion that spinal entering ear afferents would navigate along existing fibers to enter the CNS.

Late stage transplantations suggested the capability of afferents of the inner ear to project centrally into the spinal cord, while also fasciculate peripherally with posterior lateral line afferents. These data attest to further fasciculation capability of afferents of the inner ear, supporting the assumption that they are able to associate with existing nerves to project along. Presumably, fasciculation with pLL afferents could offer an additional route with which fibers of the transplanted ear could use to reach the hindbrain. We are currently unable to report on this prediction, though our expectations would be that they are capable, especially given novel entry with afferents of the trigeminal (Elliott et al., 2013). Fasciculation with pLL afferents could be the result of shared placodal origin (Schlosser, 2002, 2005; Schlosser and Northcutt, 2000). Indeed, that the lateral line and otic placodes are induced similarity (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005) and express similar subsets of genes, such as *Pax 2/8*

(Pieper et al., 2011), could indicate why fiber association is observed when given the chance. Alternatively, both lateral line and vestibular organs possess evolutionarily conserved cell types, imparted by shared gene regulatory networks (Duncan and Fritzsch, 2012), indicating that such derived traits could be why they fasciculate. Finally, given the sample size, opportunistic association with accessible PNS bundles is another possibility. That lateral line afferents project into the transplanted ears may equally reflect opportunistic fasciculation, enhanced once the ear is approached by the neurotrophins released form sensory epithelia (Fritzsch et al., 2016). Our sample size did not allow us to identify if there exists a preference between the alternative explanations. Future work would need to be done to assess this.

Our early transplantations also demonstrated pLL association with this ear. However, we have not yet identified if early transplanted ears have pLL afferents projecting into the ear or if inner ear fibers fasciculate with them. Delamination of neurons from the otic vesicle occurs around stage 31 in *Xenopus laevis* (Quick and Serrano, 2005), and the caudal migration of trunk lateral line placodes reaches the base of the dorsal fin at stage 40 (Nieuwkoop and Faber, 1994; Winklbauer, 1989). As such, early stage transplantations might have a preference to project into the spinal cord, given the considerable distance between the migrating placodes and its ganglia, which we have shown freely project into the spinal cord. Ganglia of late transplantations are exposed to both afferents of the lateral line and native spinal projecting fibers, offering either route to navigate on, potentially restricting the amount of fibers entering the spinal cord. Further work would need to be done to determine if these predictions are observed.

In transplantations to the heart region, inner ear afferents fasciculate along fibers of the vagus nerve. That inner ear afferents project with the vagus nerve demonstrates an additional situation where afferents of the inner ear opportunistically navigate along existing nerves. If inner ear fibers projecting in this manner are able to enter the hindbrain is unclear. However, labeled projection of vagus fibers to its ganglia suggests this is a possibility. Posterior lateral line ganglia are also labeled in this manner, likely due to proximity between the two collections of ganglion cells. Central projection into dorsal regions of the hindbrain was observed from this labeling, representing projections of the lateral line based on relative position (Fritzsch, 1981; Fritzsch et al., 2005a). At this time is it unclear if afferents from an ear transplanted in the heart region are able to reach the hindbrain.

CONCLUSION

Altogether, my evaluation of the pathfinding properties of inner ear afferents has provided novel data on how sensory neurons from the ear can connect with the central nervous system. My data suggest that inner ear afferents reach the CNS primarily by fasciculation along existing nerves and may do so in an opportunistic manner, perhaps mediated in part by some not yet fully evaluated bias toward placodally derived neurons. Once transplanted ear afferents reach the spinal cord, no matter the entry point, my results implicate a principal of molecular guidance in instructing centrally projecting afferents of the ear to navigate to dorsal areas in the spinal cord, and within the hindbrain to reach vestibular nuclei. Eyes grafted to the spinal cord indicate a similar mechanism of long range guidance (Giorgi and Van Der Loos, 1978). Interestingly however, my results also suggest a robustness in the pathfinding capabilities of vestibular afferents. Entering the spinal cord by following existing nerves, such as processes of somatosensory afferents or motor neurons, represents a measure of plasticity in afferents of the ear. Indeed, apparent fasciculation with afferents of the lateral line and the vagus nerve to presumably navigate to the hindbrain would also indicate these neurons are capable of navigating to their targets even when disadvantaged to do so.

Such capabilities may not be a representative feature of all sensory neurons. Olfactory placode transplantations demonstrated terminating local innervation rather than long range navigation (Magrassi and Graziadei, 1985). Similarly, eyes grafted to the trunk establish functional connections even without clearly defined projections (Blackiston et al., 2017). Regardless of conservation, the pathfinding properties of vestibular afferents discussed here provide additional insight into how connections are

established between the ear and the brain. This is of benefit to those who suffer from sensorineural hearing loss or vestibular disorders by adding more information to our growing knowledge of establishing functional connections that provide meaningful sensory input to compensate for such losses.

REFERENCES

Allen-Sharpley, M.R., Tjia, M., Cramer, K.S., 2013. Differential Roles for EphA and EphB Signaling in Segregation and Patterning of Central Vestibulocochlear Nerve Projections. PLoS One 8, e78658.

Baker, C.V.H., Bronner-Fraser, M., 2001. Vertebrate Cranial Placodes I. Embryonic Induction. Developmental Biology 232, 1-61.

Bermingham, N.A., Hassan, B.A., Wang, V.Y., Fernandez, M., Banfi, S., Bellen, H.J., Fritzsch, B., Zoghbi, H.Y., 2001. Proprioceptor Pathway Development Is Dependent on MATH1. Neuron 30, 411-422.

Bianchi, L.M., Liu, H., 1999. Comparison of Ephrin-A ligand and EphA receptor distribution in the developing inner ear. The Anatomical Record 254, 127-134.

Blackiston, D.J., Levin, M., 2013. Ectopic eyes outside the head in Xenopus tadpoles provide sensory data for light-mediated learning. J Exp Biol 216, 1031-1040.

Blackiston, D.J., Vien, K., Levin, M., 2017. Serotonergic stimulation induces nerve growth and promotes visual learning via posterior eye grafts in a vertebrate model of induced sensory plasticity. npj Regenerative Medicine 2, 8.

Bouchard, M., de Caprona, D., Busslinger, M., Xu, P., Fritzsch, B., 2010. Pax2 and Pax8 cooperate in mouse inner ear morphogenesis and innervation. BMC Dev Biol 10, 89.

Briscoe, J., Ericson, J., 2001. Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol 11, 43-49.

Brown, A., Yates, P.A., Burrola, D.O., Vaidya, A., Jessell, T.M., Pfaff, S.L., O'Leary, D.D.M., Lemke, G., 2000. Topographic Mapping from the Retina to the Midbrain Is Controlled by Relative but Not Absolute Levels of EphA Receptor Signaling. Cell 102, 77-88.

Brugmann, S.A., Moody, S.A., 2005. Induction and specification of the vertebrate ectodermal placodes: precursors of the cranial sensory organs. Biology of the Cell 97, 303-319.

Clarke, J.D., Hayes, B.P., Hunt, S.P., Roberts, A., 1984. Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of Xenopus laevis. The Journal of physiology 348, 511-525.

Coate, Thomas M., Raft, S., Zhao, X., Ryan, Aimee K., Crenshaw Iii, E.B., Kelley, Matthew W., 2012. Otic Mesenchyme Cells Regulate Spiral Ganglion Axon Fasciculation through a Pou3f4/EphA4 Signaling Pathway. Neuron 73, 49-63.

Coate, T.M., Spita, N.A., Zhang, K.D., Isgrig, K.T., Kelley, M.W., 2015. Neuropilin-2/Semaphorin-3F-mediated repulsion promotes inner hair cell innervation by spiral ganglion neurons. eLife 4.

Cowan, C.A., Yokoyama, N., Bianchi, L.M., Henkemeyer, M., Fritzsch, B., 2000. EphB2 guides axons at the midline and is necessary for normal vestibular function. Neuron 26, 417-430.

Crook, A.C., Whiteman, H.H., 2006. An Evaluation of MS-222 and Benzocaine as Anesthetics for Metamorphic and Paedomorphic Tiger Salamanders (Ambystoma tigrinum nebulosum). American Midland Naturalist 155, 417-421.

de Ramon Francas, G., Zuniga, N.R., Stoeckli, E.T., 2016. The spinal cord shows the way - How axons navigate intermediate targets. Dev Biol In Press

Duncan, J.S., Fritzsch, B., 2012. Evolution of Sound and Balance Perception: Innovations that Aggregate Single Hair Cells into the Ear and Transform a Gravistatic Sensor into the Organ of Corti. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology 295, 1760-1774.

Elliott, K.L., Fritzsch, B., 2010. Transplantation of Xenopus laevis ears reveals the ability to form afferent and efferent connections with the spinal cord. Int J Dev Biol 54, 1443-1451.

Elliott, K.L., Houston, D.W., Fritzsch, B., 2013. Transplantation of *Xenopus laevis* Tissues to Determine the Ability of Motor Neurons to Acquire a Novel Target. PLoS One 8, e55541.

Elliott, K.L., Houston, D.W., Fritzsch, B., 2015. Sensory afferent segregation in three-eared frogs resemble the dominance columns observed in three-eyed frogs. Sci. Rep. 5.

Elliott, K.L., Kersigo, J., Pan, N., Jahan, I., Fritzsch, B., 2017. Spiral Ganglion Neuron Projection Development to the Hindbrain in Mice Lacking Peripheral and/or Central Target Differentiation. Front Neural Circuits 11, 25.

Fenstermaker, A.G., Prasad, A.A., Bechara, A., Adolfs, Y., Tissir, F., Goffinet, A., Zou, Y., Pasterkamp, R.J., 2010. Wnt/planar cell polarity signaling controls the anterior-posterior organization of monoaminergic axons in the brainstem. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 16053-16064.

Fritzsch, B., 1981. The pattern of lateral-line afferents in urodeles. A horseradish-peroxidase study. Cell Tissue Res 218, 581-594.

Fritzsch, B., 1993. Fast axonal diffusion of 3000 molecular weight dextran amines. Journal of Neuroscience Methods 50, 95-103.

Fritzsch, B., Barbacid, M., Silos-Santiago, I., 1998. Nerve dependency of developing and mature sensory receptor cells. Annals of the New York Academy of Sciences 855, 14-27.

Fritzsch, B., Eberl, D.F., Beisel, K.W., 2010. The role of bHLH genes in ear development and evolution: revisiting a 10-year-old hypothesis. Cell Mol Life Sci 67, 3089-3099.

Fritzsch, B., Elliott, K.L., 2017. Evolution and Development of the Inner Ear Efferent System: Transforming a Motor Neuron Population to Connect to the Most Unusual Motor Protein via Ancient Nicotinic Receptors. Frontiers in Cellular Neuroscience 11.

Fritzsch, B., Gregory, D., Rosa-Molinar, E., 2005a. The development of the hindbrain afferent projections in the axolotl: evidence for timing as a specific mechanism of afferent fiber sorting. Zoology (Jena) 108, 297-306.

Fritzsch, B., Kersigo, J., Yang, T., Jahan, I., Pan, N., 2016. Neurotrophic Factor Function During Ear Development: Expression Changes Define Critical Phases for Neuronal Viability, in: Dabdoub, A., Fritzsch, B., Popper, A.N., Fay, R.R. (Eds.), The Primary Auditory Neurons of the Mammalian Cochlea. Springer New York, New York, NY, pp. 49-84.

Fritzsch, B., Muirhead, K.A., Feng, F., Gray, B.D., Ohlsson-Wilhelm, B.M., 2005b. Diffusion and imaging properties of three new lipophilic tracers, NeuroVue Maroon, NeuroVue Red and NeuroVue Green and their use for double and triple labeling of neuronal profile. Brain Res Bull 66, 249-258.

Fritzsch, B., Pan, N., Jahan, I., Elliott, K., 2014. Inner ear development: building a spiral ganglion and an organ of Corti out of unspecified ectoderm. Cell and Tissue Research, 1-18.

Fritzsch, B., Pauley, S., Beisel, K.W., 2006. Cells, molecules and morphogenesis: the making of the vertebrate ear. Brain Res. 1091, 151-171.

Fritzsch, B., Straka, H., 2014. Evolution of vertebrate mechanosensory hair cells and inner ears: toward identifying stimuli that select mutation driven altered morphologies. Journal of Comparative Physiology A 200, 5-18.

Giorgi, P.P., Van Der Loos, H., 1978. Axons from eyes grafted in Xenopus can grow into the spinal cord and reach the optic tectum. Nature 275, 746-748.

Gowan, K., Helms, A.W., Hunsaker, T.L., Collisson, T., Ebert, P.J., Odom, R., Johnson, J.E., 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31, 219-232.

Hernandez-Miranda, L.R., Müller, T., Birchmeier, C., 2016. The dorsal spinal cord and hindbrain: From developmental mechanisms to functional circuits. Developmental Biology In Press.

Jahan, I., Kersigo, J., Pan, N., Fritzsch, B., 2010. Neurod1 regulates survival and formation of connections in mouse ear and brain. Cell and tissue research 341, 95-110.

Kolodkin, A.L., Tessier-Lavigne, M., 2011. Mechanisms and molecules of neuronal wiring: a primer. Cold Spring Harbor perspectives in biology 3.

Kopecky, B., Santi, P., Johnson, S., Schmitz, H., Fritzsch, B., 2011. Conditional deletion of N-Myc disrupts neurosensory and non-sensory development of the ear. Developmental Dynamics 240, 1373-1390.

Kullander, K., Klein, R., 2002. Mechanisms and functions of eph and ephrin signalling. Nat Rev Mol Cell Biol 3, 475-486.

Lai, H.C., Seal, R.P., Johnson, J.E., 2016. Making sense out of spinal cord somatosensory development. Development 143, 3434-3448.

Le Dréau, G., Martí, E., 2012. Dorsal–ventral patterning of the neural tube: A tale of three signals. Developmental Neurobiology 72, 1471-1481.

Litingtung, Y., Dahn, R.D., Li, Y., Fallon, J.F., Chiang, C., 2002. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature 418, 979-983.

Liu, Z., Hamodi, A.S., Pratt, K.G., 2016. Early development and function of the Xenopus tadpole retinotectal circuit. Current Opinion in Neurobiology 41, 17-23.

Ma, Q., Sommer, L., Cserjesi, P., Anderson, D.J., 1997. Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. The Journal of neuroscience : the official journal of the Society for Neuroscience 17, 3644-3652.

Magrassi, L., Graziadei, P.P.C., 1985. Interaction of the transplanted olfactory placode with the optic stalk and the diencephalon in Xenopus laevis embryos. Neuroscience 15, 903-921.

Maklad, A., Fritzsch, B., 2003. Development of vestibular afferent projections into the hindbrain and their central targets. Brain Res Bull 60, 497-510.

Mao, Y., Reiprich, S., Wegner, M., Fritzsch, B., 2014. Targeted deletion of Sox10 by Wnt1-cre defects neuronal migration and projection in the mouse inner ear. PLoS One 9, e94580.

Maricich, S.M., Xia, A., Mathes, E.L., Wang, V.Y., Oghalai, J.S., Fritzsch, B., Zoghbi, H.Y., 2009. Atoh1-lineal neurons are required for hearing and for the survival of neurons in the spiral ganglion and brainstem accessory auditory nuclei. The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 11123-11133.

Martinez, E., Tran, T.S., 2015. Vertebrate spinal commissural neurons: a model system for studying axon guidance beyond the midline. Wiley Interdisciplinary Reviews: Developmental Biology 4, 283-297.

Matei, V., Pauley, S., Kaing, S., Rowitch, D., Beisel, K.W., Morris, K., Feng, F., Jones, K., Lee, J., Fritzsch, B., 2005. Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. Developmental dynamics : an official publication of the American Association of Anatomists 234, 633-650.

Nieuwkoop, P., Faber, J., 1994. Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Publishing, Inc, New York.

O'Neill, P., Mak, S.S., Fritzsch, B., Ladher, R.K., Baker, C.V., 2012. The amniote paratympanic organ develops from a previously undiscovered sensory placode. Nat Commun 3, 1041.

Patthey, C., Schlosser, G., Shimeld, S.M., 2014. The evolutionary history of vertebrate cranial placodes – I: Cell type evolution. Developmental Biology 389, 82-97.

Phillips, B.T., Bolding, K., Riley, B.B., 2001. Zebrafish fgf3 and fgf8 Encode Redundant Functions Required for Otic Placode Induction. Developmental Biology 235, 351-365.

Pieper, M., Eagleson, G.W., Wosniok, W., Schlosser, G., 2011. Origin and segregation of cranial placodes in Xenopus laevis. Developmental Biology 360, 257-275.

Quick, Q.A., Serrano, E.E., 2005. Inner ear formation during the early larval development of Xenopus laevis. Developmental Dynamics 234, 791-801.

Rubel, E.W., Fritzsch, B., 2002. Auditory system development: primary auditory neurons and their targets. Annual review of neuroscience 25, 51-101.

Salinas, P.C., Zou, Y., 2008. Wnt signaling in neural circuit assembly. Annual review of neuroscience 31, 339-358.

Schlosser, G., 2002. Development and evolution of lateral line placodes in amphibians I. Development. Zoology 105, 119-146.

Schlosser, G., 2005. Evolutionary origins of vertebrate placodes: Insights from developmental studies and from comparisons with other deuterostomes. J Exp Zool 301B, 347-399.

Schlosser, G., 2010. Chapter Four - Making Senses: Development of Vertebrate Cranial Placodes, in: Kwang, J. (Ed.), International Review of Cell and Molecular Biology. Academic Press, pp. 129-234.

Schlosser, G., Ahrens, K., 2004. Molecular anatomy of placode development in Xenopus laevis. Developmental Biology 271, 439-466.

Schlosser, G., Northcutt, R.G., 2000. Development of neurogenic placodes in Xenopus laevis. J Comp Neurol 418, 121-146.

Seiradake, E., Jones, E.Y., Klein, R., 2016. Structural Perspectives on Axon Guidance. Annual review of cell and developmental biology 32, 577-608.

Straka, H., Fritzsch, B., Glover, J.C., 2014. Connecting Ears to Eye Muscles: Evolution of a ‘Simple' Reflex Arc. Brain, Behavior and Evolution 83, 162-175.

Theveneau, E., Mayor, R., 2012. Neural crest delamination and migration: From epithelium-to-mesenchyme transition to collective cell migration. Developmental Biology 366, 34-54.

Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11, 823-836.

Tonniges, J., Hansen, M., Duncan, J., Bassett, M.J., Fritzsch, B., Gray, B.D., Easwaran, A., Nichols, M.G., 2010. Photo- and bio-physical characterization of novel violet and near-infrared lipophilic fluorophores for neuronal tracing. J Microsc 239, 117-134.

Torres, M., Giráldez, F., 1998. The development of the vertebrate inner ear. Mechanisms of Development 71, 5-21.

Ulloa, F., Marti, E., 2010. Wnt won the war: antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube. Developmental dynamics : an official publication of the American Association of Anatomists 239, 69-76.

Winklbauer, R., 1989. Development of the lateral line system in Xenopus. Prog Neurobiol 32, 181-206.

Yam, P.T., Charron, F., 2013. Signaling mechanisms of non-conventional axon guidance cues: the Shh, BMP and Wnt morphogens. Curr Opin Neurobiol 23, 965-973.

Yang, T., Kersigo, J., Jahan, I., Pan, N., Fritzsch, B., 2011. The molecular basis of making spiral ganglion neurons and connecting them to hair cells of the organ of Corti. Hearing Research 278, 21-33.