Affecting Wnt3a in the Inner Ear of Xenopus laevis

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Affecting Wnt3a in the Inner Ear of Xenopus laevis

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Honors Research Thesis
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**Abstract**

Problems associated with the development of the ear can result in vertigo and partial or complete hearing loss. Efforts to better understand the development of the inner ear have been made in mouse, frog, chick, and other species. However, there is still relatively little known about the mechanisms that control the processes during development. This research has used the African Clawed Frog, *Xenopus laevis*, to study embryonic and inner ear development. The gene Wnt3a was previously determined to be expressed in the dorsal half of the developing otocyst. This suggested a possible role for Wnt3a in inner ear development, but little has been done to determine the specific purpose of Wnt3a. To examine the role of Wnt3a in the development of the inner ear we wanted to inject Wnt3a and antagonists to affect inner ear development without disturbing the formation of the dorsoventral axis. Therefore we developed an animal cap assay to alleviate this problem. We extracted animal caps at late blastula and soaked them in noggin protein to induce the formation of the head. Our results showed that noggin protein is capable of rescuing animal cap cells. An *in situ* hybridization with a probe for Wnt3a was used to determine if the ears produced via this method expressed the gene of interest. No results were obtained from the *in situ*. In the future it will be necessary to repeat the hybridization. Our hypothesis is that injection of antagonists and activators of Wnt will affect the formation of the inner ear, supporting Wnt3a’s role in its development. From this further work could determine what genes lie upstream and downstream of Wnt3a, which would provide a more accurate picture of inner ear development.
development, therefore opening more avenues of treatment for hearing impaired individuals.

Introduction

Over 28 million Americans suffer from hearing impairment and of those children born deaf, at least one in two thousand cases is hereditary (Isaacson and Vora, 2003; Steel and Brown, 1994). Problems associated with the ear do not only affect those with the disability but they also take a toll on the lives of their family, friends, and co-workers. Taking this into consideration, nearly 100 million Americans are affected by hearing impairment. Because hearing disabilities affect a significant fraction of the population it is imperative to more accurately understand the function and development of the ear in order to provide possible solutions, such as gene therapy.

In order to understand the development of the inner ear in humans, studies have looked at the cellular and physiological mechanisms responsible for hearing and balance in the inner ear of the amphibian embryo (Duellman and Trueb, 1986) as well as in other species such as mouse, opossum, chick, zebrafish, and the northern cat (for review see Quick and Serrano, 2005). Characteristics of the development of the inner ear are relatively conserved throughout these species making it possible to study one and perhaps relate the results to the other. Although there has been extensive research devoted to genes, pathways, and tissues involved in inner ear development there is still much to be learned in order to understand hearing disabilities that arise due to problems associated with early development. This research focused on the African Clawed Frog, Xenopus laevis, in order to more properly understand the mechanisms involved in embryonic and inner ear development.
Embryonic Development in *Xenopus laevis*

Embryonic development in *Xenopus laevis* consists of multiple events that lead to the formation of the ear. Early development of *Xenopus* occurs through steps such as fertilization, cleavage, gastrulation, organogenesis and maturation. Before fertilization the egg consists of two hemispheres: the animal hemisphere which contains less yolk and lies in the upper half; and the vegetal hemisphere which contains more yolk and lies on the bottom half. At this time the egg is symmetrical about the animal vegetal axis. After the egg has completed its first meiotic division it can be released for fertilization. Fertilization accomplishes completion of the second meiotic division as well as initiating a 30° rotation of the egg cortex with respect to the inner cytoplasm. Vincent *et al.* (1986) determined that a displacement of the animal and vegetal subcortical cytoplasm relative to the surface is the primary event leading to the 30° rotation. It was further suggested by Elinson and Rowling (1988) that the cortical rotation is generated by a microtubule-based motor. The result of the cortical rotation is the relocation of cytoplasm from the vegetal pole towards the equatorial region (between the vegetal and animal hemispheres) which activates molecules known as dorsal determinants that specify the dorso-ventral axis (Holowacz and Elinson, 1993).

In their research Scharf and Gerhart (1980) found that UV-irradiation prevents cortical rotation and the formation of the dorso-ventral axis. However, they also determined that, if the embryos are tipped during the first cell cycle, cortical rotation can be induced by gravity and the embryos will develop normally. Therefore cortical rotation was determined to be the initial event in dorsal-ventral patterning leading to proper axis formation (Gerhart *et al.*, 1989). The signals leading to correct dorsal-ventral patterning
were further examined by Holowacz and Elinson (1993). When injected into UV-ventralized embryos, significantly more dorsalizing activity was found in vegetal dorsal cytoplasm compared to animal dorsal cytoplasm. Because the vegetal dorsal cytoplasm was capable of rescuing axis formation they determined that the dorsal-determining information resides in the vegetal pole prior to cortical rotation. Therefore it is the initial event of fertilization that essentially sets up the egg to produce the necessary signals to establish the dorso-ventral axis and the tissues required to form the correct embryonic body plan.

As previously stated, the vegetal hemisphere of the egg contains more yolk than the animal hemisphere. Therefore cleavage begins at the animal hemisphere, working its way down through the vegetal pole. As shown in Figure 1, more rapid cell division occurs in the upper half of the embryo as opposed to the slower division of the bottom half (Gilbert, 2003). Due to the location of the third cleavage the cells in the vegetal hemisphere are larger than those in the animal hemisphere. During cleavage a cavity forms inside the animal hemisphere known as the blastocoel which can be seen in Figure 1, H. The embryo is now named blastula.

Figure 1: Cleavage of a frog egg

Figure 1: Cleavage number represented by Roman numerals. Order of appearance designated in A-H. (A,B) The 2nd division (II) begins in the animal region before first (I) has divided vegetal cytoplasm. (C) Third division also occurs in animal pole. (D-H) The vegetal hemisphere has larger and fewer divisions because it is more yolky. (H) Cross section mid-blastula stage embryo. (from Gilbert 2003, after Carlson 1981)
The blastocoel permits cell migration during the next step of development, as well as preventing the vegetal cells from prematurely interacting with the cells above it. After the formation of the blastocoel different genes begin to be transcribed in different cells giving the cells specific “fates” as to what tissues they will form. Therefore by late blastula, cells have formed the three germ layers: endoderm (middle), ectoderm (outer), and mesoderm (inner); however, these layers still need to be moved to their proper locations through gastrulation.

Gastrulation is initiated 180° opposite the sperm entry point on the future dorsal side of the embryo. Just below the equator, at the marginal zone (where the animal and vegetal hemispheres meet) the cells invaginate and form a slit-like blastopore known as the blastopore lip. Cell movement begins in the marginal zone as cells migrate through the dorsal blastopore lip to the animal pole and become dorsal mesoderm and then the notochord, somites, and prechordal plate (presumptive head region). Those cells that remain on the outside form the second germ layer, ectoderm, while the larger yolky cells at the vegetal hemisphere become endoderm. Therefore gastrulation has resulted in the correct placement of the three germ layers: ectoderm (precursor to epidermis and nervous system) on the outside, endoderm (precursor to gut lining) on the inside, and mesoderm (precursor to muscle, kidney, blood, and skeleton).

Organogenesis is the next stage of development which begins with the formation of the notochord from mesoderm cells in the most dorsal portion of the embryo. The ectodermal cells above the notocord form a tube and stretch to form the neural tube/nervous system. Those cells between the epidermis and central nervous system become the neural crest cells which form the cartilage of the face, pigment cells, and
peripheral neurons. Meanwhile, the mesoderm tissue adjacent to the notochord to become somites, precursors to the muscles, dermis, and spinal vertebrae, after which the embryo forms a mouth and anus completing a tadpole like figure. It has been suggested that signals responsible for establishing the neural plate such as the BMP antagonists noggin and chordin (for review see Brugmann and Moody, 2005) are also responsible for inducing the non-neural ectoderm located in the anterior region of the embryo lateral to the neural plate and neural crest known as pre-placodal ectoderm (PPE). The PPE forms the hypophyseal placode, the olfactory placode, the lens placode, and the otic placode which then forms the inner ear. Once the muscles and gills have formed the embryo hatches from its jelly coat and undergoes metamorphosis to lead to the final body plan of the frog (Gilbert, 2003). It is evident that the final body plan of *Xenopus* occurs through a series of complex interconnected events, therefore it is important to examine some of the specific details involved with each of the above steps in order to truly understand the mechanisms involved in development and more importantly to appreciate how embryonic development correlates with the formation of the inner ear.

**Important Mechanisms in Embryonic Development**

At cleavage mesoderm precursors exist in the deep layer of the cells whereas ectoderm and endoderm precursors reside in the superficial layer of the embryo. One group of cells responsible for the induction of these precursors is known as the Nieuwkoop center. The Nieuwkoop center is composed of the dorsal-most vegetal cells and induces marginal zone cells (including the dorsal lip cells) to form another group of cells known as the Spemann organizer. The dorsal pathway triggered by cortical rotation produces the Nieuwkoop center (Darras *et al*., 1997) which then induces the Spemann
organizer which is proven to be the source of mesoderm patterning and neural information (Woo and Fraser, 1997). This region is referred to as the organizer because it produces a signal which converts adjacent mesoderm from ventral to more dorsal mesoderm; known as dorsalization (Jones et al., 1995) and organizes the tissues into an embryo with the proper dorso-ventral and anterior-posterior axes (Gilbert, 2003). Research has tried to determine which genes are produced by these regions. The transcription factor VegT and the TGF-β family paracrine factor Vg1 are located in the vegetal cells during cleavage and are known to be responsible for proper organization of the tissues leading to correct axis formation (Gilbert, 2003). It has been suggested that there are actually two main signaling activities involved in the induction of the early genes of the Spemann organizer: one being activin/Vg1 pathway that mediates the mesoderm inducing activity produced by the Nieuwkoop center; and the other is a Wnt signaling domain that is active on the dorsal side of the embryo that acts through maternal levels of the molecule β-catenin (for review see Cease et al. 1998). The Wnt/β-catenin pathway was further examined along with the BMP/Smad1 pathway and the VegT/Xnr-derriere/Smad2 pathway in 2002 by Xanthos et al. Their research determined that all three pathways work in conjunction to achieve proper organizer formation.

After fertilization β-catenin accumulates on the dorsal side of the cytoplasm and through this contributes to dorso-ventral axis formation (for review see Liu et al., 1999). In their research Larabell et al. (1997) found that β-catenin levels are up-regulated by ectopic Xwnt-8 and inhibited by glycogen synthase kinase-3, suggesting its expression is controlled by the wingless/Wnt signaling pathway (Larabell et al., 1997). The canonical or “standard” Wnt pathway is a signaling pathway by which Wnt binds to the gene
receptor Frizzled, which releases Dishevelled, inhibiting GSK3β, therefore allowing β-catenin to induce a change in gene activity (Gilbert, 2003). The Wnt pathway is shown in Figure 2.

Figure 2: The canonical Wnt pathway

Stabilization of the β-catenin protein as shown on the right in Figure 2 is an essential role in the Wnt signaling pathway because it is a co-activator for the T cell factor (TCF)/lymphocyte enhancer factor family. In the absence of Wnt, Frizzled, or Dishevelled, β-catenin forms a complex with APC, Axin, and Serine/threonine kinase GSK3β at which point GSK-3β phosphorylates β-catenin degrading its future activity (for review see Liu et al., 1999). It was therefore suggested that inhibition of GSK3β on the dorsal side of the embryo is responsible for the accumulation of β-catenin leading to the dorso-ventral polarity (Larabell et al., 1997). Support of this hypothesis was provided by Miller and colleagues (1999) who investigated the subcellular localization of another Wnt signaling molecule, Dishevelled (Dsh) in fertilized *Xenopus* eggs, revealing that Dsh
associated with organelles found within the prospective dorsal side of the embryo after cortical rotation, suggesting that the translocation of Dsh plays a role in activating the maternal Wnt pathway in early development. Because Dsh was present in the dorsal side of the embryo but not on the ventral side, β-catenin could instruct vegetal mesoderm to create and Nieuwkoop center. Some of the molecules that are known to carry out the described functions of the organizer include chordin, siamois, follistatin, Xnr1, noggin (for review see Lustig et al., 1996), and Xnr3 (Smith et al., 1995). While some of these molecules are activated by the activin/Vg1 signal others, such as Xnr3 and siamois are induced by Wnt signaling (Crease et al., 1998).

Noggin was the first molecule shown to have the ability to mimic the events initiated by the Spemann organizer. Previous research had shown that vegetal cells were capable of rescuing axis formation in UV-ventralized embryos. Based on this, Smith and Harland (1991) set out to isolate molecules that participate in axis formation. They found that injection of poly(A)⁺ RNA from gastrula stage embryos into UV-treated embryos could partially rescue dorsal development. From this they performed an assay to isolate molecules with axis inducing activity. In their experiment Smith and Harland (1992) used a cloning strategy for isolating cDNAs with dorsalizing activity in Xenopus embryos. They used their novel technique of sib selection where they started with 10,000 clones and from this determined the dorsalizing factor to be in a group of 1,000 clones, which was then consolidated to 100 clones, etc. In the end they successfully isolated Xwnt-8, suggesting it was the endogenous dorsalizing factor responsible for the axis rescue. Experiments that injected ectopic Wnt-1 into the embryo lead to the development of a secondary axis suggesting that Wnt is involved in the specification of dorsal
mesoderm and therefore axis formation (McMahon and Moon, 1989) so the role of Xwnt-8 as the endogenous inducing signal seemed appropriate. However, because Xwnt-8 is expressed on the ventral side of the embryo after dorsal axial tissue has formed they concluded that it was not likely to be the endogenous axis-inducing activity. Therefore Smith and Harland repeated the screen looking for another dorsalizing activity. In this screen they isolated a second dorsalizing RNA named noggin. Not only could injected noggin mRNA promote the formation of a vegetal dorsalizing center like that of the Nieuwkoop center, but it is endogenously expressed in the dorsal mesoderm (the Spemann organizer) during the blastula phase. Figure 3 shows an in situ hybridization performed by Smith and Harland (1992) to show noggin expression in *Xenopus* embryos. Noggin staining appears dorsally in late blastula A and at gastrula is found near the blastopore lip in the Spemann organizer (C). This is consistent with their hypothesis of noggin as an important signaling molecule during gastrulation.

Figure 3: Endogenous Noggin Expression

![Figure 3: noggin in situ hybridization. Whole embryos hybridized with antisense noggin RNA probes. (A) stage 9, vegetal pole view. Staining restricted to dorsal side of embryo. (B) Stage 9, side view. Staining in dorsal marginal zone. (C) Stage 10.5 side view. Arrowhead indicates staining in dorsal lip of blastopore. (Smith and Harland, 1992)](image)

Noggin has been hypothesized to act by regulating other molecules, allowing for the formation of neural identities. Noggin is one of the genes responsible for neural induction in *Xenopus* by interfering with BMP (Chang *et al.*, 1999). It was suggested by Baker *et al.* (1999) that a Wnt signal contributes to an early repression of bone morphogenic protein (Bmp4) on the dorsal side of the embryo which sensitizes the
ectoderm to respond to neural inducing signals from the organizer, thereby forming neural tissue. In a review by Stern (2005) it is reported that there are likely a complex series of events that leads to the formation of the embryonic neural plate. Again BMP’s act through Wnt molecules (specifically Xwnt-3a as found by Wolda et al., 1992) to establish the appropriate neuronal fates leading to the formation of the neural tube (Chesnutt et al., 2004). It is those tissues that are not destined to form neural tissue that go to form the pre-placodal ectoderm.

The pre-placodal ectoderm forms various placodes of the frog embryo. Studies have shown that there may be three mechanisms involved in the induction of PPE: neural induction, border zone interactions, and regulatory genes (for review see Brugmann and Moody, 2005). As previously described neural induction occurs by antagonizing signaling molecules that instruct for an epidermal fate such as noggin, chordin and FGF’s. Fibroblast growth factors have also been found to be capable of direct neural induction (for review see Brugmann and Moody, 2005). Another mechanism found to be involved in formation of PPE occurs through interactions between the neural plate and epidermis that create a border zone. In Xenopus studies have suggested that dlx genes help to promote a border zone which is then capable of expressing neural crest and PPE fates. Finally, the regulatory genes six and eya have been identified as playing possible roles in the establishment of PPE (for review see Brugmann and Moody, 2005). Together these activities establish the PPE which then subdivides into individual placodes which give rise to specific structures.
**Formation of the Inner Ear**

The otic placode is near the hindbrain and is responsible for the formation of the inner ear. There have been multiple molecules and actions acting at gastrula and neurula suggested to be involved in otic placode induction in *Xenopus* (for review see Koebernick *et al.*, 2003). During the later half of gastrulation naïve ectoderm is induced by surrounding tissues to form the otic placode. The hindbrain lies next to the region destined to form the otic placode making it a likely candidate as a source for induction. Members of the Fibroblast Growth Factor family (FGF) are expressed in the hindbrain during mid-late gastrulation making them likely otic inducing factors (for review see Phillips *et al.*, 2004). In their research Phillips *et al.* (2004) found that FGF’s work with Wnt-8 to induce development of the otic placode from PPE. Another source of tissue induction was found in the research done by Kil *et al.* (2005) that suggested that cranial paraxial mesoderm (beneath the presumptive otic placode) is also necessary for otic placode induction. The genes located in the cranial paraxial mesoderm and responsible for induction were examined by Groves and colleagues (2000) who showed that cranial ectoderm becomes specified to form the otic placode through the expression of four genes: Pax-2, Sox-3, BMP-7, and Notch. In addition to this Sox9 expression is detected in the cells immediately adjacent to the lateral neural crest which corresponds to the prospective otic placode making it another likely candidate in otic placode specification (Saint-Germain *et al.*, 2004). Once the inductive signals have committed the placodal ectoderm to an inner ear fate the otic placode gives rise to an invagination of a thickened epithelium known as the otic cup (for review see Brugmann and Moody, 2005). As the
cup forms the epithelium thickens three fold (for review see Brigande et al., 2000) to form the otic pit (Saint-Germain et al., 2004) and then the rim of the otic pit fuses and pinches off to form the otic vesicle as shown in Figure 4 (Quick and Serrano, 2005).

Figure 4: Formation of the Otic Vesicle

Like that of the otic placode, the formation of the otic vesicle is obtained through the interaction of many genes. Previously it was suggested that members of the fibroblast growth factor gene family, FGF3 and FGF10, have redundant roles acting with neural signals for correct otic vesicle formation in mouse (Alvarez et al., 2003). Sox9 expression is present from the formation of the otic placode throughout that of the otic vesicle. It is under positive control of Wnt and FGF, further suggesting that these pathways are involved in development of the vesicle (Saint-Germain et al., 2004). In addition to these molecules Hedgehog (Hh) signaling activity (Hammond et al., 2002) and Six1 expression (Ozaki et al., 2004) are critical for patterning of the otic vesicle.

The otic vesicle is necessary for cytodifferentiation and morphogenesis of the otic capsule which is the bony labyrinth/capsule surrounding the inner ear (for review see Frenz, 2001). Members of FGF, transforming growth factor-beta (TGFβ) and BMP guide the induction and cytodifferentiation of the capsule. FGF’s and TGFβ’s are present during the time corresponding to the completion of the otic capsule and regulate cells
through different stages of its differentiation (for review see Frenz, 2001). It was determined by Ficker et al., (2004) that molecules secreted by the otic epithelium induce and control otic capsule chondrogenesis (hardening) from the surrounding periotic mesenchyme. After the formation of the otic vesicle and that of the bony labyrinth specific structures of the inner ear begin to develop through intense cell growth and differentiation (Saint-Germain et al., 2004, Kil and Collazo, 2001).

The inner ear consists of epithelial cells where the sense organs for hearing and balance are found surrounded by mesenchymal cells which are encased by the otic capsule (Brigande et al., 2000). Through development unspecified cells obtained distinct identities to form sensory and non-sensory structures (for review see Cole et al., 2000). In Xenopus, inner ear compartments form in a two week period, between stages 45 and 50 (Quick and Serrano, 2005). As the otic vesicle grows it forms the three semicircular canals, the amphibian papilla, basilar papilla, the endolymphatic duct, the vestibule and ganglion (Stevens et al., 2003). The Xenopus inner ear has eight sensory organs (for review see Quick and Serrano, 2005). The vestibular sensory organs include four maculae (the utricle, lagena, macula neglecta, and saccule) which sense gravity and linear acceleration (Raft et al., 2003) and three cristae for each of the semicircular canals (Stevens et al., 2003) that respond to angular acceleration (Raft et al., 2003). The lagena, which senses vibrations, forms in the posterior section of the inner ear (Quick and Serrano, 2005). The basal papilla is the auditory organ responsible for hearing (for review see Kil and Collazo, 2001). These structures are shown in Figure 5.

Figure 5: Structures of the Inner Ear of Xenopus
The formation of the inner ear in *Xenopus* was closely examined by Quick and Serrano (2005) through histological data collected from stages 28-47. They found that the process of inner ear compartmentalization occurs about 15 days after fertilization. As shown in Figure 6 (A), the otic vesicle is present at stage 28, lateral to the central nervous system and pinched off from the epidermis. Over time it is obvious that the otic vesicle increases in size. In stage 31 (B) it is evident that the sensory ganglia are derived from the otic vesicle due to their presence within the vesicle by stage 37 (C). In stage 42 (D1) the pars superior and pars inferior are present and serve to form the vestibular and auditory structures. Capsule formation also occurs at this stage. Invagination of the otic vesicle occurs by stage 45 and marks the initial formation of the compartments of the semicircular canals (E2). The developing endolymphatic duct can also be seen at this time (E2).
Figure 6: Development of the *Xenopus laevis* ear

Quick and Serrano (2005) also looked at the compartmentalization of the organs of the inner ear. In stage 47 the utricle, anterior canal, horizontal canal, and saccus can be seen (Figure 7 A1-4). A1 and A2 show that utricle and crista of the anterior canal and horizontal canal are within the same compartment whereas the saccus is forming in another compartment (A2). From the general sphere found at stage 28 additional offshoots form the endolymphatic duct, semicircular canal, and auditory sense structures by stage 50.
This research by Quick and Serrano (2005) provided evidence that the formation of the inner ear of *Xenopus* is indeed a very complex process that is likely regulated by multiple genes and signals. In their research Brigande *et al.* (2000) found that the entire dorsal rim of the otic cup becomes the endolymphatic duct and the posterioventral rim becomes the lateral otocyst wall possibly due to signaling across compartment boundaries (invisible cell walls). This suggested that there are restrictions on cells movements, creating boundaries that force cells in specific regions to form specific organs. This, however, was contradicted in lineage analysis performed by Kil and Collazo (2001) which showed that all regions of the otic placode and otocyst give rise to the sensory organs of the inner ear, but a specific region would give rise to cells found in multiple sensory organs. Therefore it is likely that cells move and communicate with one another in order to determine who forms what. One way this is thought to be accomplished is through the Pax-Six-Eya-Dash gene network which is known to direct proper sensory
organ formation. More specifically Six1 is expressed in the otic vesicle as well as other organs and functions as a key regulator of otic vesicle patterning and the expression of genes responsible for inner ear structures (Ozaki et al., 2004). In addition it has been suggested that BMP4 and other BMPs are required for the various phases and processes of inner ear development (Chang et al., 1999). In conjunction with BMP4 expression, presumptive sensory organs arose due to expression of the sensory organ markers Lunatic Fringe (L-fng) and chicken Serrate1 (Ser1) which are components of the Notch signaling patterning (Cole et al., 2000). In addition to these genes, the role of Wnt in inner ear development has also been explored in studies of mouse, frog, and other species.

**The Significance of Wnt**

The Wnt family consists of 19 genes which have been reported to have multiple effects on cellular differentiation and growth (Kanazawa et al., 2005). Wnt-1 is the original gene found to be part of a group of genes that are expressed during embryonic development, some of which are found in the inner ear (McMahon, 1992). Since then, the significance of Wnt signaling for the specification of the inner ear has been explored in quail (Kil et al., 2005), mouse (Riccomagno et al., 2005), chick (Stevens et al., 2003), and frog (Wolda et al., 1992; Gregorius, 1999).

In their studies Wolda and colleagues (1993) presented the full-length coding sequence for Xwnt3a and its expression pattern relative to Xwnt1 using whole-mount in situ hybridization. Xwnt3a expression was observed during embryonic and inner ear development. Xwnt3a was first detected at the neurula stage at the neural fold, which gives rise to the head structures. It is later, at stage 22, detected along the full length of the anterior/posterior axis. At stage 27 Xwnt3a is expressed along the mesencephalon
and the anterior portion of the neural tube. Also at this time Xwnt3a is expressed on the dorsal surface of the otic vesicle which persists through the tailbud stage. Because of the expression seen in the otic vesicle, Wolda et al. (1993) investigated the early expression of Xwnt3a in the otic placode. They found that expression is detected in the neural ectoderm, in the region of the neural tube. At stage 21-24 Xwnt3a expression is also observed in the dorsal part of the invaginating otic placode. This suggested that Xwnt3a could have a role in the induction and patterning of the otic vesicle. Wnt3a expression in the inner ear was therefore examined by Gregorius (1999) and colleagues at the University of Redlands. *In situ* hybridization was used to determine Wnt3a expression in the developing otic vesicle of *Xenopus* from stage 26 to stage 45. As indicated by the arrow, Wnt3a expression occurs in the dorsal half of the otic vesicle (Figure 8).

**Figure 8:** Wnt3a expression in the inner ear of *Xenopus*

Because Wnt3a expression was present in the otocyst throughout formation of the inner ear, this research helped to support that done by Wolda *et al.* (1993), further suggesting a role for Wnt3a in formation of the otic vesicle.
Wnt3a expression has also been examined in other species. In 2003 Stevens et al. looked at the role of Wnt/β-catenin signaling in chicken ear development. They used retrovirus-mediated gene transfer to overexpress a truncated form of *Xenopus* β-catenin which activates the canonical Wnt pathway, or a full-length copy of chicken Wnt3a. The misexpression of Wnt3a altered the development of the inner ear and led to the formation of ectopic vestibular patches. Their data suggests that Wnt/β-catenin signaling is involved in inner ear formation through establishing the sensory and nonsensory regions and specifying vestibular and auditory sense organ identity (Stevens et al., 2003).

A similar result was found by Kil et al., (2005) in quail. Their research examined the role of the hindbrain and cranial paraxial mesoderm in the formation of the inner ear. They used the vitamin-A-deficient (VAD) quail model system which lack endogenous retinoids. Due to this VAD embryos have a hindbrain defect. As previously discussed the hindbrain has been proposed to play a role in induction of the inner ear. Their research showed that VAD quail embryos, lacking the posterior half of the hindbrain that is next to the inner ear, had incorrect Wnt3a expression and the sensory patches and vestibular organs were reduced or absent. This is consistent with their hypothesis of the role of Wnt3a in inner ear development. Research was performed by Riccomagno et al. (2005) which focused on determining the signaling pathways required to regulate the expression of the genes Dlx5/6, Hmx2/3, and Gbx2 that are present in the developing otocyst and are responsible for correct vestibular organ development in mouse. Their results showed that Dlx5/6 and Gbx2 are targets of Wnt signaling in the dorsal otic vesicle. Furthermore it was determined that the Wnt signal came from the dorsal hindbrain, unlike the expression observed in frog, and Wnt1 and Wnt3a were the specific
ligands required for this signaling and are restricted by sonic hedgehog. Wnt1 and Wnt3a expression was observed in the dorsal neural tube which is also in contrast to the expression of Wnt3a in frog.

It is now evident that Wnt/Wnt3a is likely involved in the formation of the inner ear in *Xenopus*; however, there has not been much done to explore the results of affecting Wnt3a in the inner ear. Traditionally genes of interest or their inhibitors are injected directly into the embryo and then their expression is visualized. For example, one method used to determine the role of Wnt3a was performed by Stevens *et al.* (2003) in chick where Wnt3a could be injected through a virus directly into the embryo and then the results were visualized through *in situ*. Previous methods have injected molecules into the embryo to study its effects on the ear because a method has not been devised to inject the molecule directly into the ear. In the case of *Xenopus* neither of these two methods can be used to affect and understand the role of Wnt3a in the inner ear. If Wnt3a were to be injected directly into the egg it would interfere with the canonical Wnt pathway involved in the β-catenin accumulation on the dorsal half of the embryo and therefore interfere with the formation of the dorso-ventral axis due to the fact that the cell would be receiving a double dorsalizing signal. This research aimed to circumvent this problem by injecting Wnt ligands, such as Wnt3a, into the egg, then removing the animal cap cells before they received any dorsalizing signals from the Spemann organizer located in the vegetal tissue. As previously discussed, noggin protein is capable of mimicking the organizer and can therefore be used to induce animal cap cells to form heads and subsequently ears even in the presence of an exogenous Wnt3a signal.
The primary goal of this research was to affect the role of Wnt3a in the development of the otic vesicle in order to understand its full function. First it was necessary to determine what concentration of noggin would be adequate to mimic the Spemann organizer and induce the establishment of the dorso-ventral axis as well as to visualize the structures of the inner ear to determine if it was normal. 0.7 µg/ml noggin was a sufficient amount of protein to induce axis formation in late blastula animal caps. Then in situ hybridization was used to visualize Wnt3a expression in the otic vesicle of the rescued animal cap embryos. However, no results were obtained by the in situ. In the future it would be essential to use perform another in situ to determine Wnt3a expression in the otic vesicle. It would also then be possible to inject activators and inhibitors of the Wnt pathway and visualize the effects. Our hypothesis is that interference in the Wnt pathway will result in missing or reduced structures of the inner ear, meaning Wnt3a plays a significant role in inner ear development and it would then be possible to determine its specific function. This will also present an opportunity to determine what genes may be upstream and downstream from Wnt3a which could provide possible solutions to hearing impairments and open avenues for gene therapy to assist with the prevention of inherited hearing disabilities.

**Materials and Methods**

*Induced ovulation*

Using a 1-mL sterile syringe, 600 units of Human Chorionic Gonadotropin (HCG) was injected directly under the skin above the dorsal lymph sac of female *Xenopus*. The dorsal lymph sacs are located approximately 1 inch above the cloaca and directly to the left and right of the dorsal lateral line. After the injection the syringe was slowly
extracted parallel to the body and the wound was lightly massaged. The female’s eggs were collected approximately 15 hours post injection.

Obtaining testes

The male *Xenopus* was placed in 1.2% MS222 for 20 minutes or until anesthetized. *Xenopus* was then placed on ice, ventral side up, with his head and limbs covered with ice. Scissors were then used to cut through the skin and muscle layer directly parallel to the ventral lateral line. The fat bodies were carefully pulled out of the body cavity and the testes were removed and placed in ice cold 1X MMR solution. The testes were stored at 4°C until use.

Fertilization

Eggs were collected in a Petri dish by holding the female and applying pressure on the posterior half of her abdomen. A sliver of one testis was removed and shredded with forceps into a small amount of 0.1X MMR. The 0.1X MMR came from a stock solution of 1 Liter of 10X Marc’s Modified Ringer (MMR) solution, pH 7.4, which was made with 1 M NaCl, 20 mM KCl, 20 mM CaCl₂, 10 mM MgCl₂, and 50 mM Hepes. This solution was then placed directly onto the eggs for 5 minutes. After fertilization, the eggs were immersed completely in 0.1X MMR.

Removal of the Jelly Coat

2.5% cysteine hydrochloride pH 7.8-7.9 was made fresh. The embryos were placed in the solution. Once the jelly coat had been removed the embryos were rinsed three times in 0.1X MMR and separated into groups of 20-40 embryos, then placed in a 16°C incubator.
**Staging**

Embryos were visualized using the medium power of a dissecting microscope and staged according to Nieuwkoop and Faber (1975). Also at this time unhealthy/dead embryos were removed so as to not infect the rest of the group. Embryos were collected at mid to late blastula (stage 7-9) for animal cap removal. To decrease growth rate they were placed in a 16°C incubator until they reached the appropriate stage.

**Removal of animal caps**

Embryos were placed on a medium size petri dish with an agar bottom and 0.1 x MMR. The procedure was performed under a dissecting microscope. Forceps were used to rotate the embryo so it was lying vegetal hemisphere up. One forcep was used to penetrate the vegetal hemisphere. At the point of penetration the two forceps were used to pull open the embryo so the cells were lying flat on the dish. The vegetal and marginal cells were removed from the animal cap cells by size differentiation. Once only animal cap cells remained a mouth pipette was used to transfer the animal cap into a 25 cell dish coated with agar. This process was repeated for the rest of the embryos giving a total of 10 to 15 animal caps.

**Preparation of noggin protein**

The noggin stock was 1 mg/ml. It was diluted to 1μg/ml concentration noggin with 1X MMR and 0.65% pen and strep. This was then further diluted to 0.25μg/ml, 0.3 μg/ml, 0.4μg/ml, 0.5μg/ml, 0.6μg/ml, and 0. μg/ml with 1X MMR and stored in sterile eppendorf tubes at -80°C.
Noggin treatment

Five-hundred µl of dilute noggin protein was placed in 1 well of a 25 cell microliter plate coated with agar. To this 1-2 animal caps were added per well and soaked overnight in the noggin, after which they were removed from the noggin and placed in 0.1 X MMR. The controls were placed in 0.1 X MMR.

Fixing

The animal caps were placed individually in short form vials in MEMFA, 1% formaldehyde, 8% pure water, 10X MEMFA (1 M MOPS pH 7.4, 20 mM EGTA, and 10 mM MgSO₄, pH 7.4) and placed on a shaker for 1 hour. After they were fixed the majority of the fixing solution was removed and the animal caps were washed in 50% methanol 2-3 times then in 100% methanol 2-3 times and stored in a small volume of 100% methanol in -20°C.

Single in situ

Embryos into Probe: To rehydrate through EtOH into Ptween we removed the fixing solution from each vial with a sterile pipette and added enough 75% EtOH/25% H₂O to cover the embryos. The next wash was 50% EtOH/50% H₂O. Each wash was 5 minutes. Ptween was prepared with 1XPBS and 0.1% Tween 20 to make 1 Liter. The next wash was done in 25% EtOH/75% PTw, and 3 washes were done with 100% PTw all for 5 minutes each. PTw was removed from each vial and 66µl proteinaseK in 100 ml PTw was added to each for 25 minutes after which it was rinsed twice for 5 minutes each in Triethanolamine (0.1 M Triethanolamine pH 7-8 made up in sterile bottle and autoclaved). The solutions were removed and 0.25% acetic anhydride in triethanolamine was added to cover the embryos for 10 minutes. After 5 minutes 0.25% acetic anhydride
was added to the vials and rocked for 5 minutes. The acetic anhydride treatment was removed and 2 rinses of PTw were done for 5 minutes each. To refix in paraformaldehyde, 20% paraformaldehyde (made 20% in water, neutralized with NaOH and heated at 65°C with shaking until clear and stored cold) in PTw was added to the vials for 20 minutes. Then there were 3 rinses for 5 minutes each in PTw. A pipette removed the solutions from the vials and each was filled with hybridization solution and placed at 60°C for 10 minutes. The hybridization solution was made with 50% formamide, 5X SSC (DEP treated), 1 mg/ml yeast RNA in DEP water, 100 μg/ml Heparin in DEP water, 1X Denhardt’s solution, 0.1% w/v Tween 20 in DEP water, 0.1% w/v CHAPS, and 5 mM EDTA (DEP treated) to make 100 ml volume with DEP water stored at -70°C. The DEPC water was prepared to 0.05% by diluting the DEPC stock 1:10 in EtOH and adding 0.5% in 1 Liter of H2O and autoclaved. Each vial’s solution was replaced with fresh hybridization buffer and incubated at 60°C for approximately 6 hours. The solution was replaced with hybridization solution (probe diluted to 1μg/ml in hybridization).

Competent cells for BMP, Wnt, and Serrate were thawed on wet ice. After gently mixing the cells, 100 ul was transferred into a chilled polypropylene tube. Added 10ng of DNA to the competent cell mixture. The cells were then put in ice for 30 minutes then placed in a 42°C water bath for 45 seconds to heat-shock. They were then placed on ice for 2 more minutes. 0.9ml of room temperature S.O.C. medium was added to the cells and then the cells were shaken at 225 rpm at 37°C for 1 hour. 100ul of the cells were then spread on each LB+AMP plate and incubated over night. The embryos soaked in the hybridization solution overnight at 60°C with the plate sealed in parafilm.
Into First Antibody: The probe containing hybridization solution was removed from each vial and stored at -80°C. Hybridization buffer was added to the vials for 10 minutes at 60°C. The solutions were removed with sterile pipettes and 2X SSC (from 20X SSC, pH 7.0 for 500 mls made with 87.65 g NaCl and 44.1 g Na Citrate, autoclaved) was added to each for 20 minutes at 60°C. This rinse was done 3 times. The solutions were removed and to them was added 0.2X SSC for two rinses 30 minutes each at 60°C. These solutions were removed and the embryos were soaked in 1X MAB (for 1 Liters of 5X made with 500 mM maleic acid and 750 mM NaCl) twice for 15 minutes each. This reaction was blocked with MAB + 2% Boehringer Mannheim Blocking Reagent for 1 hour. This was then blocked with MAB + 2% BMB blocking reagent + 20% heat treated sheep or lamb serum. The antibody incubation was performed by replacing the solutions with the same solution containing a 1:1000 dilution of the antidigoxygenin-alkaline phosphatase antibody for 4 hours at room temperature. An antisense mRNA probe for Wnt3a was created via a two-hour incubation at 37°C of 0.05 μg/ml Wnt3a DNA (RNase free), 1:10 digoxigenin-UTP RNA labeling mix (contained 1 mM NTPs and labeled UTP, Roche), 1X transcription buffer (from 10X stock, Roche), and 1:10 T7 RNA polymerase (from E. coli, Roche), diluted in DEPC water and stored -70°C. The vials were washed 5 times for 60 minutes each in 1X MAB. They were then stored overnight.

Washes and Chromogenic Reaction: The solutions were removed and replaced with 2 rinses 5 minutes each of alkaline phosphatase buffer made with 100 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20, 1 mM levamisole for 200 mls. The solutions were replaced with wash buffer containing chromogenic substrates of 3.5 μl/ml
BCIP and 4.21 μl/ml NBT. The color reaction was allowed to develop for the entire day but there was no color reaction so the *in situ* was not visualized.

**Results**

*Noggin protein treatment*

The first aim of our study was to determine what concentration of noggin protein would result in axis formation by mimicking the Spemann organizer. Previous research had used 0.25 μg/ml noggin to induce axis formation. Therefore we diluted our stock concentration of 1 μg/ml to 0.25 μg/ml and treated 5 animal cap cells of *Xenopus laevis* overnight. Five animal caps were placed in 0.1 X MMR as a control. After 24 hours of treatment the animal caps were transferred with a transfer pipette to 0.1 X MMR and visualized under a dissecting microscope. The animal caps were checked for two days after this but only two survived and none of them appeared to develop a dorso-ventral axis (results not shown).

Because the 0.25 μg/ml concentration of noggin was not adequate to induce axis formation we repeated the experiment using two embryos each treated at 0.3 μg/ml, 0.4 μg/ml, 0.5 μg/ml, and 0.7 μg/ml concentrations of noggin as well as two control animal caps which were placed untreated in 0.1 X MMR (Figure 9). The concentrations 0.3 μg/ml, 0.4 μg/ml, and 0.5 μg/ml showed little or no extension movements giving very little indication of the noggin treatments being sufficient enough to induce axis formation. However, in the case of 0.7 μg/ml, one embryo clearly developed an axis which was indicative of sufficient noggin treatment to mimic the organizer in animal cap cells. This was significantly greater than the concentration of noggin (0.25 μg/ml) expected to induced axis formation.
Figure 9: The effects of various noggin concentrations on animal cap cells

0.3 µg/ml  0.4 µg/ml  0.5 µg/ml  0.7 µg/ml

Figure 9: 0.3, 0.4, 0.5, 0.7 µg/ml noggin protein concentrations were used. All embryos treated at stage 7-9.
Extension movements can be seen in 0.3 and 0.4. Most obvious extension in 0.7. Control was animal cap placed in
1XMMR.

A similar procedure was used to repeat the experiment but this time 10 animal
caps removed at 7 stage blastula were treated with 0.7 µg/ml concentrated noggin. Two
animal caps were placed in 0.1 X MMR as controls and 2 embryos were also placed in
0.1 X MMR as staging references. By stage 27 all noggin treated animal caps showed
obvious extension along the dorso-ventral axis. The degree of extension is comparable to
the 0.7 µg/ml treatment shown in Figure 9 which indicated the noggin treatment was
successful (results not shown). Both control animal caps, untreated, died after 48 hours.
The embryonic controls survived through tailbud approximately stage 50. Eleven of the
treated animal caps were fixed and used in the in situ hybridization the following day. To
determine the extent of embryonic development that could occur in the noggin treated
animal caps the remaining treated animal caps were allowed to develop for an additional
5 days in a 14°C incubator. Over the 5 days the embryos appeared to reach their
maximum extension at about stage 40. After this cell death appeared to rapidly increase until the 5\textsuperscript{th} day when all animal caps had died.

**In Situ Hybridization**

Noggin treated animal cap cells were fixed at stage 26-28 and in situ hybridization with a Wnt3a probe was used to determine Wnt3a expression in noggin treated animal caps. The in situ did not result in any staining of the animal caps and therefore Wnt3a expression could not be observed (results not shown).

**Discussion**

To understand the role Wnt3a plays in the development of the inner ear in *Xenopus laevis* we need to alter its expression or action. Because the Wnt pathway is known to play a role in dorso-ventral axis formation we cannot simply inject Wnt3a into the 1 or 2 cell embryo. This would disrupt normal development and it is unlikely that the inner ear would even form. Therefore we have used Noggin protein treatment of stage 7 blastula animal cap cells to induce axis formation by essentially mimicking the Spemann organizer. Because animal cap cells receive no signal and are destined to form ectoderm we could remove the animal cap and supply a dorsalizing signal, like that of the Spemann organizer, by soaking it in noggin protein. This allows us to inject Wnt3a into the embryo without disrupting embryonic development. We expect that animal caps derived from an egg injected with Wnt3a will have altered inner ear formation.

The first aim of our research was to determine the ideal concentration of Noggin protein that would be capable of mimicking the organizer in animal cap cells removed at blastula stage 7. The concentration that appeared to be most successful in its treatment
was 0.7 µg/ml (Figure 2). Treatment at this concentration resulted in embryo elongation which was assumed to be due to the establishment of the dorso-ventral axis. Ideally we would have liked to seen embryos resembling a truncated tadpole. To improve on the noggin treatment it would be possible to repeat the experiments with a fresh batch of noggin to insure the concentrations were correct. It would also be useful to try a greater dose of noggin. In order to lengthen the survival time embryonic care should be increased. By forming more “normal” embryos it is likely that the structures of the inner ear would be similar to untreated embryos and therefore interference with the Wnt3a pathway would give more accurate results.

An in situ hybridization was used to determine the expression of Wnt3a in the rescued animal cap embryos. We hoped to find that noggin treated animal caps were capable of forming ears and Wnt3a expression would be seen in the developing otic vesicle. However, the in situ hybridization did not result in any staining so we were unable to determine the extent of Wnt3a expression. Because the formation of the probe is a technical process that had not been previously preformed by our research group it is likely that there may have been a problem with the probe or getting it into the cell. Another possibility is that the Alkaline Phosphatase Buffer used in the Chromogenic reaction of day 3 was prepared incorrectly and would have given poor results.

In the future it is necessary to repeat the in situ hybridization. Sectioning would also be another helpful procedure to determine the extent of structure formation in the inner ear of rescued animal cap embryos. In order to claim success of this method it would be crucial for the structures of the noggin treated animal cap ears to be similar to those of untreated embryos. In addition Wnt3a expression also needs to be observed in
the dorsal otocyst of the developing noggin treated animal cap. If development is only slightly altered using this assay then it would be possible to use other genes that are known to be involved in the Wnt pathway in order to attempt to affect the pathway and further understand the role of Wnt3a in inner ear development. Genes that could be used to do this include a dominant/negative Dishevelled as well as β-catenin. Our hypothesis is that affecting Wnt3a through the use of activators and inhibitors of the Wnt pathway will result in improper development of the otic vesicle, altering the vestibular and sensory structures of the inner ear. This would prove Wnt3a’s role in the development of the otic vesicle as well as provide insight to the genes upstream and downstream of Wnt3a during development. By further understanding the role of various genes in inner ear development we can open the doors to medical exploration that may one day provide possible solutions to and preventions of hearing disabilities.

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