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Abstract

Vertebrate nervous system development requires a complex series of events to transform a flat neuroepithelium into complex structures containing specialized cell types. The anterior neuroectoderm gives rise to the brain and is the origin for some of the first neurons that differentiate. It is also the origin of the cranial neural crest cells that form craniofacial features. This thesis focuses on the embryonic development of two tissues that arise from the zebrafish anterior neuroectoderm, the epithalamus, a region of the dorsal forebrain, and the mandible, the lower jaw.

Early in development, the flat neural plate folds into a neural tube. The pineal gland, an organ involved with circadian rhythms, begins as two precursor domains at the lateral edges of the neural plate that converge into a single tissue when the neural tube closes. The pineal gland, along with the parapineal gland and habenula nuclei, form the epithalamus in the dorsal forebrain. In embryos with open neural tubes, the left and the right sides of the pineal and surrounding epithalamus are widely spaced. I found that despite this displacement, pineal cell types differentiate normally and initiate their rhythmic function. Conversely left-right asymmetry in the epithalamus was lost; both sides exhibited left-sided characteristics. Further, this loss of asymmetry in the epithalamus was correlated to severity in neural tube defects. Embryos with left isomerism had significantly wider pineal anlage domains than those with normal or reversed asymmetry.

Cranial neural crest cells from the dorsal neural tube migrate to form craniofacial structures, including the cartilaginous precursor to the mandible, Meckel’s cartilage. The bigtime (bti) mutant exhibits reductions in mandibular
development. I found that although cranial neural crest cells localize normally to the lower jaw region in these mutants, they fail to differentiate into functional chondrocytes that secrete a sufficient amount of collagenous extracellular matrix.
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I. Introduction

Understanding the mechanisms that instruct cellular differentiation is one of the central themes of developmental biology. Throughout this thesis, I describe both spatial and signaling requirements for differentiation and patterning of cells that originate in the zebrafish neuroectoderm. While described in the context of zebrafish development, many of these mechanisms are applicable to a range of biological patterning. For instance, concentration gradients of diffusible signaling proteins pattern the developing neural tube as they instruct neuronal subtype differentiation (Nguyen et al., 2000a; Nishi et al., 2009; Alaynick et al., 2011). On an ecosystem level, nutrient concentration gradients pattern species richness within and across landscapes (Müller, 1998; Pausas, 2001). Another mechanism, feedback loops, may be responsible patterning zebrafish left-right brain asymmetry through spatially regulating protein signals to the left side only (Lu et al., 2013). Similarly, nutrient cycling by soil microbial communities works in feedback loops to promote plant abundance (Bardgett et al., 2005). Thus, the mechanisms highlighted through my research are not only applicable at the developing tissue level, but they also play a role in biological patterning ranging from individual cells to ecosystems.

The role of neural tube closure in zebrafish epithalamic development

Development of the neuroectoderm

Early in vertebrate development, the process of gastrulation transforms a single layered mass of undifferentiated cells, the blastula, into three distinct cell layers from which all other tissues will be derived (Kaneda and Motoki, 2012;
Solnica-Krezel and Sepich, 2012). The outer cell layer of the embryo, the ectoderm, gives rise to the nervous system and epidermis, or the outer covering of the organism. Muscle and bone are derived from the middle germ layer called the mesoderm. The innermost cell layer, called the endoderm, gives rise to the gastrointestinal tract (Arnold and Robertson, 2009).

Bone Morphogenetic Protein (BMP), one of the many Transforming Growth Factor-β (TGFβ) proteins, plays an inductive role in establishing the embryo’s dorsal-ventral axis. TGFβ proteins are a large family of proteins that regulate cell differentiation and proliferation (Alaynick et al., 2011). Throughout the embryo, BMP signaling induces a ventral fate and lack of BMP signaling induces a dorsal fate (Weinstein and Hemmati-Brivanlou, 1999). BMP inhibitors, Chordin, Noggin, and Follstatin, are found in the presumptive dorsal side of the embryo (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999).

The vertebrate nervous system is derived from the dorsal ectoderm. In the ectoderm, BMP signaling induces an epidermal fate while lack of BMP signaling induces a neural fate (Weinstein and Hemmati-Brivanlou, 1997; Weinstein and Hemmati-Brivanlou, 1999). These dorsal cells form the neural plate, the precursor to the nervous system, as they elongate to into columnar cells and become distinct from the flatter epidermal ectoderm cells (Weinstein and Hemmati-Brivanlou, 1999). In Xenopus embryos, overexpression of BMP in the ectoderm results in reduction of dorsal structures while overexpression of the BMP inhibitor Noggin results in expansion of dorsal tissues, including the brain (Dale et al., 1992; Neave et al., 1997).
Primary neurulation forms the anterior neural tube

The vertebrate nervous system forms from two neural tubes that develop separately and then fuse together (Lowery and Sive, 2004; Saitsu and Shiota, 2008). The anterior, or primary, neural tube develops first and will become the brain and rostral spinal cord (Orahilly and Muller, 1994). The posterior, or secondary, neural tube forms second and will become the caudal region of the spinal cord (Griffith et al., 1992; Lowery and Sive, 2004).

The primary neural tube forms through the process of primary neurulation, during which a flat neuroepithelium folds and converges at the dorsal midline of the embryo to form a hollow neural tube (Figure 1). This neural tube extends from the cranial region through the thoracic region of the embryo (Lowery and Sive, 2004). Secondary neurulation occurs in the tail bud of the developing embryo after the anterior neural tube has formed (Lowery and Sive, 2004). During secondary neurulation, mesodermal mesenchymal cells underlying the ectoderm coalesce into a neural rod called the medullary cord. This neural rod then cavitates to form a neural tube (Griffith et al., 1992; Lowery and Sive, 2004).

The formation of the epithelium prior to neural tube formation distinguishes primary neurulation from secondary neurulation (Lowery and Sive, 2004). An epithelium is a sheet of cells tightly joined by a number of cell junctions. These junctions allow for the coordinated movements and direct cell-cell communication that are required to fold the neural plate into the neural tube (Lowery and Sive, 2004; Suzuki et al., 2012). Conversely, a mesenchyme is unorganized loose associations of
cells (Griffith et al., 1992). During secondary neurulation these cells must undergo mesenchymal to epithelial transformations to adhere to one another (Figure 1) (Lowery and Sive, 2004) (Griffith et al., 1992).

In this thesis, I am concerned with the process of primary neurulation as I examined the role that anterior neural tube closure plays in development of the dorsal forebrain. The open neural tube phenotype discussed throughout this thesis results from incomplete primary neurulation.

Shortly after gastrulation, primary neurulation proceeds in a four step process (Colas and Schoenwolf, 2001). First, in response to inhibition of BMP, the cells of the dorsal ectoderm elongate and thicken to form the neural plate, also called the neuroectoderm (Weinstein and Hemmati-Brivanlou, 1999; Colas and Schoenwolf, 2001). The neural plate then elongates along the anterior-posterior axis of the embryo via convergent extension movements (Moury and Schoenwolf, 1995; Colas and Schoenwolf, 2001).

In mammals and birds, the neural plate bends at discrete hinge points as it folds (Schoenwolf and Franks, 1984; Moury and Schoenwolf, 1995; Colas and Schoenwolf, 2001). Folding first occurs at a medial hinge point at the midline of the neural plate, and then at two dorsolateral hinge points. Next, the cells of the folding neural plate converge at the dorsal midline and fuse to form the neural tube (Figure 1) (Schoenwolf and Franks, 1984; Colas and Schoenwolf, 2001). During primary neurulation in zebrafish, the neural plate keels to form a neural rod. The neural rod cavitates from the anterior to posterior end forming the lumen of neural tube (Figure 1) (Lowery and Sive, 2004; Nyholm et al., 2009)
In order to form a distinct structure, cells of the neural tube must detach from the overlying epidermal ectoderm. This task is accomplished through regulation of adhesion proteins involved in epithelial cell junctions. Cadherins are cell adhesion proteins that dimerize with cadherins of adjacent cells and anchor to the cytoskeleton within the cell (Detrick et al., 1990; Levine et al., 1994). E-cadherin is the main adhesion protein of the epidermal ectoderm while N-cadherin is found in the neural
tube (Detrick et al., 1990; Levine et al., 1994). Cells of the neural tube separate from the epidermal ectoderm by downregulating E-cadherin and upregulating N-cadherin (Detrick et al., 1990). Because N-cadherin of neural plate cells does not dimerize with E-cadherin of epidermal cells, the neural tube detaches from overlying epidermal epithelium cells.

Once the neural tube forms a distinct structure, the anterior neural tube bulges to form the three primary regions of the brain. The forebrain, called the prosencephalon, includes two secondary regions called the telencephalon rostrally and diencephalon caudally (Eagleson and Harris, 1990; Stern, 2001). The cerebral hemispheres, olfactory lobes and hippocampus develop in the telencephalon. The epithalamus, thalamus, and hypothalamus develop in the diencephalon (Kiecker and Lumsden, 2005). The midbrain, called the mesencephalon, gives rise to the cerebral peduncles and the corpora quadrigemina. The hindbrain, called the rhombencephalon, consists of the pons, medulla oblongata, and cerebellum (Moens and Prince, 2002).

Dorsal-ventral patterning of the neural tube

The dorsal and ventral midlines of the neural tube are important signaling centers for establishing signal gradients that initiate cellular differentiation along the dorsal-ventral axis of neural tube (Tanabe and Jessell, 1996; Nishi et al., 2009). The epidermal ectoderm overlying the closed neural tube secretes BMP4, which in turn initiates BMP4 expression in the dorsal region of the neural tube, called the roof plate. BMP4 in the roof plate induces a cascade of BMP4 signaling that diffuses ventrally establishing gradient in the neural tube (Figure 2) (Nguyen et al., 2000a). At the same
time Sonic hedgehog (Shh) is secreted from the notochord, the mesodermal rod that underlies the neural tube. Shh induces a second signaling center in the ventral neural tube, called the ventral brain in the head and the floor plate in the spinal cord. The ventral brain secretes Shh that diffuses dorsally creating a second signaling gradient (Figure 2) (Nishi et al., 2009; Alaynick et al., 2011). Cells throughout the neural tube are exposed to varying concentrations of these protein signals, which in turn instruct transcription factor synthesis and cellular differentiation. For example, motor neurons differentiate in the ventral third of neural tube in response to high Shh and low BMP4 signaling (Alaynick et al., 2011).

**Figure 2. BMP4 and Shh gradients pattern the dorsal-ventral axis of the neural tube.** A BMP4 gradient extends ventrally from the dorsal neural tube while a Shh gradient extends dorsally from the ventral brain. Exposure to varying concentrations of these signals instructs cellular differentiation throughout the neural tube.
Neural tube defects are common birth defects in humans

Neural tube defects, which occur in 1 out of every 1000 human births, result when the primary neural tube fails to close properly (Greene et al., 2009). There are number of neural tube defects in mammals that range from mild to severe. Spina bifida, a protrusion of the spinal cord through the spinal column resulting from failure of posterior neural tube closure, can often be surgically corrected. In contrast, anterior neural tube defects such as exencephaly, in which the forebrain is located outside of the skull and anencephaly, in which the forebrain is missing, are lethal (Figure 3) (Greene and Copp, 2005; Greene et al., 2009).

A number of nutritional and genetic factors are linked to neural tube defects. For example, genes involved in regulation of one-carbon synthesis, or folate, may contribute to neural tube defect susceptibility, and folic acid supplementation prior to and during pregnancy decreases the likelihood of having neural tube defects (Czeizel and Dudas, 1992; Kumar et al., 2012)(Dunlevy et al., 2006). 5,10-methylene tetrahydrofolate reductase (MTHFR), a gene associated with increased risk of neural tube defects, generates 5-methylTHF, an enzyme that remethylates homocysteines (Frosst et al., 1995). Individuals with the A222V polymorphism demonstrate reduced MTHFR function, which results in elevated homocysteine levels, reduced DNA methylation and higher incidence of neural tube defects (Greene et al., 2009). DNA methylation and nucleotide biosynthesis are important for rapid cellular proliferation, required during the formation of the neural tube. Thus, inhibition of DNA methylation has been suggested as a possible cause of neural tube defects. (Greene et al., 2009).
**Figure 3. Neural tube defects in humans.** Anencephaly is a lethal neural tube defect that results when the anterior neural tube fails to close. Spina bifida results when the posterior neural tube fails to close and can be surgically corrected. Figure from Neurosurg Focus, American Association of Neurological Surgeons, 2004.

*Conserved role for mesoderm in neural tube closure*

A number of mammalian studies have demonstrated that the tissue underlying the anterior neural tube is required for neural tube closure. In the developing vertebrate embryo, the mesoderm lies directly below the neuroectoderm (Arnold and Robertson, 2009). In mice, head mesenchyme is a tissue layer that directly underlies then neural plate (Chen and Behringer, 1995). Head mesenchyme contains head mesoderm and neural crest cells (Chen and Behringer, 1995). The neural crest is a multipotent region of epithelial cells that become mesenchymal and migrate from the lateral edges of the neural plate and gives rise to cranial cartilage as well as neurons of the peripheral nervous system and other cells types (Huang and Saint-Jeannet, 2004).
Knockout of mesenchyme associate genes *Twist, Cart1* or *Cited2* results in neural tube defects (Copp et al., 2003) (Greene and Copp, 2005). *Twist* is expressed in head mesenchyme and in the neural plate before it begins to fold. *Twist* promotes differentiation and development of mesenchyme (Chen and Behringer, 1995). Knock out of *Twist* in the mesenchyme causes exancephaly while knock out of *Twist* in the neural plate does not result in an open neural tube (Copp et al., 2003). Further, knock down of either *Cart1* or *Cited2* genes causes excessive apoptosis in the anterior mesenchyme as well as open neural tube phenotype (Greene and Copp, 2005).

The role of mesodermal tissues in signaling anterior neural tube folding is conserved in non-mammalian vertebrates as well. Zebrafish head mesendoderm contains both mesodermal and endodermal derivatives and directly underlies the anterior neural plate (Feldman et al., 2000). Zebrafish mesendoderm is also required for closure of the anterior neural tube (Aquilina-Beck et al., 2007).

Embryos lacking a TGFβ receptor complex protein, One-eyed pinhead (Oep), lack head mesendoderm and exhibit neural tube defects (Aquilina-Beck et al., 2007). Oep is translated from maternal mRNA prior to the Midblastula Transition, the point in development at which embryos begin transcribing their own zygotic mRNA (Schier et al., 1997). In zebrafish, the Midblastula Transition occurs around 2.75 hours post fertilization (hpf). Embryos lacking only maternal *oep* mRNA (*M*oep) or embryos lacking only zygotic *oep* mRNA (*Z*oep) can be normal as they retain some production of the Oep protein. However, embryos lacking both maternal and zygotic *oep* mRNA (*M*Zoep) lack all anterior mesendoderm and always exhibit open neural tube phenotypes (Aquilina-Beck et al., 2007).
Anterior mesendoderm can be rescued in MZoep embryos with injections of TaramA*, a constitutively active TGFβ receptor (Mathieu et al., 2002). The progeny of cells injected with TaramA* will adopt a mesodermal fate. Approximately half of the MZoep embryos injected with TaramA* into one cell at the 16 cell stage have closed neural tubes (Aquilina-Beck et al., 2007).

**Nodal signaling is required for neural tube closure**

Zebrfish embryos with Nodal signaling deficiencies lack anterior mesendoderm and exhibit open neural tube phenotypes (Aquilina-Beck et al., 2007). In zebrfish there are three Nodal signaling proteins, Squint (Sqt), Cyclops (Cyc) and Southpaw (Aquilina-Beck et al., 2007). All three Nodal ligands form homodimers that interact with a TGFβ receptor complex that includes TGFβ Type I and Type II subunits as well as the Oep protein (Liang and Rubinstein, 2003b). This receptor complex also interacts with Lefty1 (Lft1), a Nodal inhibitor. Binding of a Nodal ligand to the TGFβ receptor complex results in phosphorylation of the receptor and subsequent phosphorylation of cytoplasmic Smad proteins. Phosphorylated Smad proteins translocate to the cell nucleus and initiate expression of Nodal target genes (Figure 4) (Liang and Rubinstein, 2003b).
Figure 4. The Nodal Signaling Pathway. Nodal signaling initiates expression of nodal genes in a positive feedback loop. Nodal signaling further initiates expression of the Nodal inhibitor gene \( lft1 \) in a negative feedback loop. Adapted from J. O. Liang and A. L. Rubinstein, 2003.

Embryos with Nodal signaling deficiencies exhibit neural tube defects with varying severity (Aquilina-Beck et al., 2007). Some embryos with a mutation in the gene that encodes the Sqt protein appear normal with closed neural tubes while others exhibit cyclopia and loss of somites (the mesodermal regions located along the sides of the neural tube that give rise to dermal, muscular and vertebral tissues) (Aquilina-Beck et al., 2007)(Lu et al., 2013). Embryos with a mutation in the \( cyc \) gene have closed neural tubes but lack ventral brains and exhibit cyclopia (Hatta et al., 1991). Further, embryos with mutations in \( cyc \) and \( sqt \) genes exhibit severe open neural tube defects, cyclopia, and complete loss of somites (Dougan et al., 2003; Aquilina-Beck et al., 2007; Lu et al., 2013).
**Pineal gland development is an indicator of neural tube closure**

In zebrafish, the pineal gland is a photoreceptive organ located in the dorsal diencephalon (Masai et al., 1997; Concha and Wilson, 2001). The pineal is part of the pineal complex, which also includes the left-sided parapineal organ. The pineal complex, along with the left and right habenulae nuclei, makes up a region of the brain called the epithalamus (Concha and Wilson, 2001). The pineal gland begins as two precursor regions at the lateral edges of the neural plate. When the neural tube closes, these precursors converge into a single, oval tissue located dorsally in the brain (Figure 5) (Masai et al., 1997; Concha and Wilson, 2001; Aquilina-Beck et al., 2007)

![Figure 5. Pineal gland formation in the Zebrafish.](image)

(A) Pineal precursor domains (red) begin at the lateral edges of the neural plate and (B) move towards one another as the neural plate keels. (C) Precursors converge into a single domain (D) at the anterior dorsal midline of the brain. p = pineal gland. (A-C) Adapted from Kimmel et al., 1995 & Papan and Campos-Ortega, 1994.

In normal embryos, pineal cell differentiation begins prior to neural tube closure. The upstream transcription factor of pinealocyte differentiation, *floating head*
(flh), is expressed in the pineal precursor domains (Masai et al., 1997). Embryos that lack flh have reduced production of projection neurons and photoreceptors, the two cell types found in the pineal gland (Masai et al., 1997). Projection neurons extend axons to different regions of the brain, as opposed to having localized axons. The projection neurons of the pineal gland send axons to ventral regions of the brain (Wilson and Easter, 1991b; Wilson and Easter, 1991a). The photoreceptors in the pineal gland contain the photopigment Exorhodopsin (Exorh) that can respond to environmental light cues (Mano et al., 1999; Pierce et al., 2008).

When the neural tube fails to close, the pineal precursor regions do not converge into a single tissue (Aquilina-Beck et al., 2007). In embryos with mild neural tube closure defects, the pineal gland precursors partially fuse into a laterally elongated tissue. In embryos with severe open neural tube phenotypes, the pineal precursors remain divided (Aquilina-Beck et al., 2007). For example, zebrafish embryos with a mutation in the sqt gene not only have variable neural tube defects but have variable pineal gland phenotypes as well. Mild sqt mutants can have oval pineal glands while more severe mutants have elongated or divided pineal glands (Aquilina-Beck et al., 2007).

Wingless integrated 1 (wnt1) is expressed in converging cells of the neural plate (Krauss et al., 1992; Kelly and Moon, 1995). In WT and sqt embryos with oval pineal glands, wnt1 is expressed in one domain along the dorsal midline. However, in sqt embryos with elongated or divided pineal glands wnt1 is expressed in two domains indicating that the neural tube is not closed (Figure 6) (Aquilina-Beck et al.,
Interestingly, rescue of the Nodal signaling pathway in mutant embryos also rescues pineal gland phenotype (Aquilina-Beck et al., 2007).

**Figure 6. Embryos with Nodal deficiencies exhibit a range of open neural tube defects.** (A-B) Dorsal view of WT and sqt embryos with one, oval pineal gland and single wnt expression domains at the dorsal midline of the brain indicated neural tube closure. (C-E) sqt embryos with elongated, and divided pineal phenotypes with two wnt expression domains along the dorsal midline of the brain indicating that the neural tube is open. Adapted from Aquilina-Beck, 2007.

*Asymmetric development in the vertebrate body and brain*

The vertebrate brain and viscera develop with prominent structural and functional left-right asymmetries (Corballis, 2009). For example, the external vertebrate body is bilaterally symmetric while the internal arrangement of organs is asymmetric (Corballis, 2009). This arrangement likely evolved as an efficient means of packaging organs into a confined space. Symmetric development is default and therefore asymmetric development requires a symmetry-breaking event (Corballis, 2009).

Cilia in the node, called Kupffer's vesicle in zebrafish, are responsible for breaking this symmetry in mammals and fish (Nonaka 1998; Essner et al., 2005). The organizer is formed during gastrulation at the future dorsal side of the embryo and is responsible for establishing the embryonic axes (Downs, 2009). Each cell in
Kupffer’s vesicle has a cilium that rotates clockwise, creating a leftward flow of extracellular fluids (Nonaka 1998; Takeda et al., 1999). In response to signal molecules carried by this flow, Nodal pathway genes are expressed in the left lateral plate mesoderm of the embryos. In this way, Nodal signaling acts to establish the left identity in viscera (Hamada et al., 2002).

The epithalamus, the region of the brain containing the pineal gland, exhibits the first detectable asymmetry in the zebrafish brain (Concha and Wilson, 2001; Halpern et al., 2003). First, Nodal pathway genes cyc, lftl, and paired-like homeodomain 2 (pitx2) are expressed on the left side of the pineal gland (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). The parapineal organ then develops to the left of the pineal (Borg, 1983; Concha and Wilson, 2001; Gamse et al., 2003). The left and right habenulae nuclei then develop with characteristic asymmetries (Gamse et al., 2003; Halpern et al., 2003). The left habenula has higher neuropil density and higher expression of the potassium channel tetramerisation domain containing 12.1 (kctd12.1) gene than the right habenula, which expresses kctd12.1 at a low level (Figure 7) (Gamse et al., 2003).

Habenular identity is determined by the position of the parapineal (Gamse et al., 2003). Embryos that exhibit reversed brain situs have a parapineal that develops to the right of the pineal anlage. In these embryos, kctd12.1 is expressed at high levels in the right habenula and low levels in the left habenula (Gamse et al., 2003). Further, when the pineal is ablated, both the left and right habenulae express low levels of kctd12.1, consistent with right-sided identity (Gamse et al., 2003).
Figure 7. Asymmetric development in epithalamus. In WT embryos, Nodal related genes are expressed on the left side of the pineal gland and a parapineal develops to the left of the pineal gland. The parapineal directs asymmetric development of the left and right habenula nuclei. The left habenula exhibits higher expression of the left-associated gene *kctd12.1* Adapted from Lu et al. 2013.

**The contribution of cranial neural crest to development of the mandible**

*Neural Crest arises from the neuroectoderm*

In vertebrates, a specialized subset of neuroepithelial cells called neural crest delaminates from the neural tube and migrates to highly predictable regions of the body. The neural crest is a multipotent stem cell population that gives rise to a number of cell types and tissues including craniofacial structures such as the upper and lower jaws, neurons and glia of the peripheral nervous system, and melanocytes (Huang and Saint-Jeannet, 2004; Dupin and Sommer, 2012; Milet and Monsoro-Burq, 2012).

The neural crest arises from a region of the neural plate called the neural plate
border. Prior to neurulation, the ectodermal germ layer is comprised of the neuroectoderm centrally and the epidermal ectoderm laterally (Figure 8) (Klymkowsky et al., 2010). The boundary of these two cell types forms the neural plate border. Neural crest induction requires BMP signaling to initiate an epithelial-mesenchymal transition. BMP signaling from the dorsal neural tube is limited at the neural plate border by Chordin and Noggin, BMP inhibitors produced by the somites early in development. When the somites stop producing Chordin and Noggin, BMP signaling activates $wnt$ genes in the presumptive neural crest (Selleck et al., 1998). Wnt signaling initiates expression of the neural crest specific genes $snail$ and $rho$ that are required for neural crest migration (Liu and Jessell, 1998).

The neural crest is divided into trunk neural crest and cranial neural crest. Trunk neural crest cells migrate both dorsolaterally and ventromedially to give rise to melanocytes and a variety of ganglion, neurons, and glia. Cranial neural crest cells migrate anteriorly to give rise to craniofacial tissues such as the mandible (lower jaw), the hyoid, and bones of the middle ear. The premigratory position of cranial neural crest cells determines their segmental fate (Klymkowsky et al., 2010). For example cranial neural crest cells that migrate from the posterior midbrain form the precursor to the mandible and cranial neural crest cells that migrate from the anterior hindbrain form the precursor to the hyoid bone.
**Figure 8. The neural crest originates in the neuroectoderm.** During neurulation, cells at the neural plate border undergo an epithelial to mesenchymal transition and delaminate from the neural tube. Called the neural crest, these cells migrate to specific locations throughout the body to form craniofacial cartilages, peripheral nervous system structures and melanocytes. Adapted from Gammill & Bronner-Fraser, 2003.

**The zebrafish bigtime mutant exhibits habenular asymmetry defects**

The zebrafish *bigtime (bti)* mutant was identified in a screen for fish with habenular asymmetry defects (Doll et al., 2011). The cells that give rise to neurons of the dorsal habenulae nuclei originate in the ventricle of the dorsal diencephalon (Aizawa, 2007). Within the dorsal habenulae, early differentiating ventricular cells form the neurons of the lateral subnuclei while the neurons that form the medial subnuclei differentiate later (Aizawa, 2007). In *bti* mutants, the lateral subnuclei are expanded while the medial subnuclei are reduced (Doll et al., 2011). Further, left-right habenular asymmetry is lost (Doll et al., 2011). This suggests that ventricular
cells differentiate prematurely in these mutants (Doll et al., 2011). The bti mutation is in the sec61al1 gene, which encodes an endoplasmic reticulum pore-forming component of the endoplasmic reticulum (ER) translocation channel (Rapoport, 2007; Doll et al., 2011). The Sec61al1 protein is involved with transporting polypeptides across the ER membrane and into the ER lumen (Rapoport, 2007). This nonsense mutation truncates the Sec61al1 protein and likely reduces ER pore function. Ventricular cells in bti mutants lose their epithelial nature earlier than those in WT embryos suggesting that sec61al1 may be required to maintain neural progenitors in their non-neural state in the neuroectoderm (Doll et al., 2011). In contrast to habenular loss of asymmetry, pineal Nodal signaling and parapineal development are normal in bti mutants (P.-N. Lu, and S. Brannan, unpublished data).

Research Design

*Embryos with open anterior neural tubes have normal differentiation and function of pineal gland cells.*

Nodal signaling is required for closure of the anterior neural tube and embryos that lack Nodal signaling exhibit open neural tube phenotypes. In these embryos, the pineal gland is elongated or fully divided as the pineal precursor domains fail to fully converge. Chapter 2 of this thesis examines pinealocyte differentiation and initiation of function in embryos with open neural tubes. Signals from the dorsal midline of the neural tube are important for dorsal-ventral patterning throughout the neural tube. Thus, it was possible that pinealocyte differentiation would be disrupted in embryos with open neural tubes. I demonstrated the two pineal
neuronal types, projection neurons and photoreceptors, differentiated in embryos with open neural tubes. This suggested that differentiation of pineal cells does not require localization to the dorsal midline of the brain. Further, pineal cells initiated the rhythmic function characteristic of pineal cells. This work demonstrated that neural tube closure is not required for pinealocyte differentiation or initiation of rhythmic function. These results suggest that initiation of pineal development precedes neural tube closure in WT embryos. This work was published in Lu, P.-N., C. Lund, S. Khuansuwan, A. Schumann, M. Harney-Tolo, J. T. Gamse, J. O. Liang. Failure in closure of the anterior neural tube causes left isomerization of the zebrafish epithalamus. *Dev Biol.* 2013 Feb 15;374(2):333-44.

*Embryos with open neural tubes exhibit left isomerism of the epithalamus*

In contrast to normal pineal cell differentiation, embryos with open neural tubes exhibited loss of asymmetry in the epithalamus. When the pineal gland was elongated or divided, both sides often expressed characteristic left sided genes such as *lft1* and *pitx2*. Bilateral parapineals developed and the left and right habenula nuclei both exhibited high expression of *kctd12.1*. Both Nodal deficient embryos (*sqt* mutants and Lft1 overexpressing embryos) and N-cadherin (*cdh2*) mutants with open neural tubes exhibited incomplete penetrance of the left isomerized phenotype. Further, the penetrance was higher in the *sqt* mutants than in the generally milder *cdh2* mutants.

Chapter 3 of this thesis examines the correlation between the left isomerized phenotype and the severity of open neural tube defects. I demonstrated that embryos
with widely elongated and divided pineal domains were significantly more likely to exhibit left isomerism than those with narrower pineal domains. Further, I demonstrated that parapineal positioning was disrupted cdh2 mutants with divided pineal anlage and bilateral parapineals. This research suggests that asymmetric development in the zebrafish epithalamus is correlated to the close proximity of the left and right sides. It is possible that in WT embryos, a diffusible signal from the left side of the pineal prevents the right side from developing with left-sided characteristics. In embryos with widely spaced pineal anlage, left isomerism results when this signal fails to diffuse far enough to the right side of the pineal. This work was also published in Lu, P.-N., C. Lund, S. Khuansuwan, A. Schumann, M. Harney-Tolo, J. T. Gamse, J. O. Liang. Failure in closure of the anterior neural tube causes left isomerization of the zebrafish epithalamus. Dev Biol. 2013 Feb 15;374(2):333-44.

**Characterization of the bigtime (bti) mutant jaw phenotype**

In addition to loss of habenular asymmetry, bti mutants also have defects in craniofacial development. When compared to WT siblings, bti mutants exhibit reductions in Meckel’s cartilage, the precursor to the mandible. The cranial neural crest cells that form Meckel’s cartilage originate in the dorsal neural tube and migrate to the lower jaw region where they differentiate into chondrocytes that secrete a collagenous extracellular matrix. Previous members of our lab demonstrated that Meckel’s cartilage is reduced or lost in bti mutants (P.-N. Lu, and S. Brannan, unpublished data).
Chapter 4 of this thesis describes ongoing work to characterize the jaw phenotype of the *bti* mutant. We crossed the *sox10*:GFP transgenic line, which expresses GFP in migrating cranial neural crest cells, with the *bti* mutant line to examine cranial neural crest movement over time. I demonstrate that early cranial neural crest migration is normal in these mutants. Further, cartilage specific genes are expressed in the presumptive Meckel’s cartilage. This suggests that the reduced jaw phenotype results from failure of the cranial neural crest cells to differentiate into functional chondrocytes.
II. Embryos with open anterior neural tubes have normal differentiation and function of pineal gland cells

The vertebrate central nervous system develops from a flat neuroepithelium that folds into a closed neural tube, the anterior portion of which becomes the brain. The pineal gland, located in the dorsal forebrain, begins as two precursor domains at the lateral edges of the neural plate that converge into a single tissue when neurulation is complete. In zebrafish embryos with open anterior neural tubes the left and right sides of the pineal gland often remain divided. Despite their displaced location, pineal cells differentiate into projection neurons and photoreceptors. Further, differentiated pineal cells initiate rhythmic expression of the melatonin biosynthetic gene *aanat2*. These results support a model in which initiation of pinealocyte differentiation precedes neural tube closure, and therefore neural tube closure is not required for pineal cell differentiation. Further, rhythmic gene expression results suggest that differentiated pineal photoreceptors are functionally capable of responding to environmental light stimuli.

Introduction

Formation of the vertebrate brain involves a series of coordinated cell movements that fold a flat neural plate into a neural tube (Lowery and Sive, 2004). At the same time, signaling pathways initiate differentiation of the specific cell types that will differentiate in the developing brain. It is likely that coordination of the movements and the signaling are likely required to instruct differentiation of many cell types in the developing brain. The pineal gland develops in the dorsal
diencephalon of the zebrafish brain and is involved with the entrainment and regulation of circadian rhythms (Masai et al., 1997; Mano et al., 1999; Concha and Wilson, 2001). Early in development, the pineal gland begins as two precursor domains at the lateral edges of the neural plate. These domains converge into a single gland at the dorsal midline of the brain when neurulation is complete (Masai et al., 1997; Concha and Wilson, 2001; Aquilina-Beck et al., 2007). During this process, cells of the precursor domains initiate the differentiation pathways that result in the mature pineal cell types, such as photoreceptors and projection neurons.

The main function of the photoreceptors and pineal neurons is to regulate the gland’s function in circadian rhythms (Masai et al., 1997; Mano et al., 1999). Circadian rhythms are changes in physiology and behavior that cycle within a roughly 24 hour period (Pegoraro and Tauber, 2011). Melatonin, the hormone that initiates the feeling of drowsiness in many animals, is produced by the pineal gland at high levels during the night and low levels during the day. In response, many animals sleep during the night and are awake during the day (Gothilf et al., 1999). The circadian clock that drives these rhythms consists of three components: environmental stimuli, an endogenous oscillator and regulated circadian outputs (Merrow, 2005). These circadian rhythms are largely entrained by environmental cues but persist when these stimuli are absent as the endogenous oscillator maintains rhythmic outputs even in constant conditions. This entrainment allows organisms to anticipate changes in the environment as opposed to passively responding to stimuli (Merrow, 2005).

The molecular basis of the vertebrate circadian clock, including the clock in the zebrafish pineal, consists of a feedback loop involving the transcription factor
complex CLOCK/BMAL (Ishida et al., 1999). CLOCK/BMAL activates expression
of the transcription factor period (per) and (cry) genes. PER and CRY proteins inhibit
CLOCK/BMAL activity through binding the complex and per2 specifically is
required for the onset of rhythmic gene expression in the zebrafish pineal (Ziv et al.,
2005). However, the PER and CRY proteins bound to CLOCK/BMAL degrade over
time, releasing to the complex to be active again (Ishida et al., 1999; Okamura et al.,
2002). In most vertebrates, this occurs over a roughly 24 hour period.

The pineal gland is a light sensitive structure in many non-mammalian
vertebrates (Mano et al., 1999). The photoreceptive molecule Pinopsin, which is
similar to retinal opsins, regulates light entrainment of circadian rhythms in the pineal
in chickens, pigeons and chameleons (Deguchi, 1979; Max et al., 1995; Kawamura
and Yokoyama, 1996). Exhorodopsin (Exorh) is a similar molecule produced in the
zebrafish pineal gland by photoreceptor cells (Mano et al., 1999). Similarly to
Pinopsin, Exorh mediates entrainment of the pineal circadian rhythm in zebrafish
(Mano et al., 1999; Pierce et al., 2008)

In mammals, pineal circadian rhythms are centrally regulated by the
Suprachiasmatic nucleus (SCN) in the ventral brain, where clock genes are
continually expressed (Whitmore et al., 1998). Photoreceptive cells in the retina of
the eye mediate entrainment of the body’s circadian clock. These photoreceptors
produce opsins, which are g-protein coupled receptors that induce a signaling cascade
to transduce light signals neuronally from the retina to the SCN. Neurons of the SCN
synapse with neurons of the pineal to regulate its circadian output (Korf, 1994;
Halstenberg et al., 2005).
Pineal photopigments share similarities with retinal photopigments (Mano et al., 1999). Pineal Exorh shares over 70% sequence identity with retinal Rhodopsin and the *exorh* gene in pineal photoreceptors and *opsin* genes in retinal photoreceptors are both expressed in rhythmic patterns (Mano et al., 1999; Halstenberg et al., 2005; Pierce et al., 2008). Further, Orthodenticle homeobox 5 (Otx5), a transcription factor that regulates *exorh* expression in the pineal gland, is only expressed in the retina and pineal gland in larval zebrafish, suggesting it regulates retinal photopigment expression as well (Gamse et al., 2002; Pierce et al., 2008). Despite these similarities, the retinal photopigments function to transduce neuronal signals while pineal exorh functions to transduce environmental light stimuli into a neuroendocrine signal through the release of the hormone melatonin (Arendt et al., 1995; Yanez and Meissl, 1996; Ekstrom and Meissl, 1997; Gothilf et al., 1999; Ekstrom and Meissl, 2003).

Melatonin is produced rhythmically in the pineal gland at high levels during the night and low levels during the day (Arendt et al., 1995; Gothilf et al., 1999). *serotonin N-acetyltransferase 2 (aanat2)* is a gene that encodes the penultimate enzyme in the melatonin biosynthetic pathway. The Aanat2 enzyme transfers an acetyl group from acetyl coenzyme A to serotonin forming N-acetylserotonin, the precursor to melatonin (Klein et al., 1971). Consistent with the timing of melatonin production, *aanat2* expression in pineal cells is high at night and low during the day (Gothilf et al., 1999) (Gothilf et al., 2002).

In addition to its hormonal circadian output, the pineal gland regulates circadian rhythms through neural connections. Pineal projection neurons surround the pineal photoreceptors and send axons ventrally to premotor and precerebellar targets.
including the thalamus, hypothalamus and optic tectum, where they likely modulate circadian rhythms (Wilson and Easter, 1991a; Wilson and Easter, 1991b; Yanez et al., 2009).

Differentiation of both pineal photoreceptors and projections neurons requires the homeobox transcription factor Floating head (Flh) (Masai et al., 1997). flh mutants have reduced neuron development. While the majority of the brain develops normally, the epiphyseal region in these mutants remains a single-layered epithelium with only a few differentiated pineal cells. Interestingly, the aanat2 gene is still rhythmically expressed in the remaining pineal cells of flh mutants (Gamse et al., 2002).

The timing of flh expression suggests that pineal cell differentiation may be initiated prior to neural tube closure. In WT embryos, the neural plate forms in the ectoderm at 90% epiboly. During epiboly, the epithelial ectoderm spreads to surround the yolk and deeper germ layers of the embryo (Beate Schmitz, 1993). flh is expressed in the two pineal precursor domains at the lateral edges of the neural plate also at 90% epiboly, suggesting that pineal cell differentiation may be initiated while the cells are in two distinct domains in the neural plate (Wilson and Easter, 1991a; Masai et al., 1997).

Consistent with this model of early initiation of differentiation, the first islet-1 expressing cells in the folding neural plate colocalize with flh expression. The islet-1 gene is expressed in some neurons immediately prior to or at their final mitotic event and is expressed in both differentiating projection neurons and photoreceptors of the pineal gland (Masai et al., 1997). Thus islet-1 expression colocalized with flh
expression demonstrates that pineal neurons are the likely first neurons to 
differentiate in the zebrafish brain (Masai et al., 1997).

*flh* expression is spatially regulated to the presumptive pineal regions in the 
neural tube by Wnt/β-catenin signaling (Masai et al., 1997). When Wnt/β-catenin 
signaling in disrupted in the forebrain of *masterblind* (*mbl*) zebrafish mutants, the 
presumptive pineal region extends rostrally into the telencephalon (Masai et al., 1997; 
Heisenberg et al., 2001; Sanders and Whitlock, 2003). These mutants develop two to 
three times as many pineal projection neurons and photoreceptors as WT embryos 
(Masai et al., 1997; Heisenberg et al., 2001).

The *mbl* mutation affects the *axin1* gene, a component of the Wnt/β-catenin 
signaling pathway (Shimizu et al., 2000; Heisenberg et al., 2001; Sanders and 
Whitlock, 2003). Wnt signaling plays a crucial role in induction of the vertebrate 
head and axis development. Overexpression of Wnt causes axis duplication while 
subdivision of the forebrain requires suppression of Wnt signaling (Zeng et al., 1997; 
Heisenberg et al., 2001). The protein kinase Gsk3 functions in multicomponent 
complex to phosphorylate β-catenin, a downstream transcription factor of Wnt (Ikeda 
et al., 1998). Phosphorylation of β-catenin prevents activation of Wnt target genes.

In *mbl* mutants Axin1, a scaffolding protein that allows Gsk3 to colocalize 
with and inhibit β-catenin, fails to properly bind Gsk3 and Wnt signaling is not 
suppressed (Ikeda et al., 1998). Thus, the telencephalon develops with diencephalon 
identity (Heisenberg et al., 2001). Consistent with this, *flh* expression is expanded 
rostrally in *mbl* mutants as well. This suggests that in WT embryos, Wnt/β-catenin 
promotes *flh* expression in the developing brain. Thus, the *mbl* gene likely functions
to limit \textit{flh} expression to the presumptive pineal region through inhibition of Wnt/β-catenin signaling in the telencephalon (Masai et al., 1997).

In embryos with open neural tubes, the pineal precursor domains fail to converge at the dorsal midline of the brain but rather remain in elongated or fully divided domains. My results demonstrated that both pineal photoreceptors and projection neurons differentiated in embryos with open neural tubes despite the displaced location of the pineal anlage. I further demonstrated that pineal cells in embryos with divided pineal anlage initiated rhythmic expression of \textit{aanat2}; expression is high at night and low during the day. This suggests that localization to the dorsal midline of the brain is not required for pinealocyte differentiation or initiation of function. These results support a model where pineal photoreceptor and projection neuron differentiation is initiated prior to neural tube closure by \textit{flh} expression in the pineal precursor domains. Consistent with this, previous studies have demonstrated that \textit{flh} is still expressed in both elongated and divided pineal anlage of embryos with open neural tubes, suggesting that pineal cell types may still differentiate (Aquilina-Beck et al., 2007). Once differentiated, pineal photoreceptors are functional and respond to environmental light stimuli through production of Exorh and rhythmic expression of \textit{aanat2}. 
Methods

Zebrafish

Zebrafish stocks were maintained at 28.5°C in a 14:10 light:dark cycle according to standard procedures (Westerfield, 2000). Stocks included the WT strain Zebrafish Danio Rerio (ZDR) (Aquatica Tropicals, Plant City, FL), Tg(foxd3:GFP)zf104 (Gilmour et al., 2002), sqt") (Feldman et al., 1998) and sqt") (Feldman et al., 1998) (Schier et al., 1996). WT, transgenic, and mutant embryos were obtained through natural breeding (Westerfield, 2000).

Whole mount in situ hybridization (WISH)

Single and double WISH was carried out as previously described (Liang et al., 2000) (Thisse and Thisse, 2004) (Thisse et al., 1993). Antisense RNA probes included: serotonin N-acetyl transferase (aanat2) (Gothilf et al., 1999), and exorhodopsin (exorh) (Mano et al., 1999).

Whole mount antibody staining

Whole mount antibody staining was performed as described (Pierce et al., 2008). The 4D2 monoclonal antibody against the N-terminus of bovine Rhodopsin was used at a dilution of 1:60 to detect Exorh protein (Noche et al., 2011). Goat anti-mouse antibody coupled to Alexa Fluor 546 (Invitrogen) was used at a dilution of 1:2000.
Photography

Bright field and fluorescent images were obtained on a SPOT camera or a CoolSnap ES camera connected to a Nikon Eclipse 80i microscope and on a Leica DM6000B microscope.

mRNA Injections

Embryos from the ZDR and foxd3:GFP lines were injected with 0.63 pg to 5 pg of lft1 mRNA or GFP mRNA at the one cell stage. Embryos with the same neural tube phenotype were pooled for further experiments disregarding the amount of mRNA injected. All injections were done using a Harvard Apparatus PLI-90 nitrogen picoinjector.

Results

*Injection of lft1 mRNA can phenocopy Nodal signaling mutant phenotypes.*

In embryos with open neural tubes, the pineal precursor domains fails to converge properly and embryos have laterally elongated or divided pineal anlage. For instance, Nodal signaling deficient embryos with a mutation in the gene that encodes the Sqt protein have open neural tubes pineal phenotypes as well as cyclopia, and somite loss (Thisse and Thisse, 1999; Aquilina-Beck et al., 2007). However, these phenotypes are variable and some sqt mutants develop with normal pineal phenotypes. In these studies, we used injections of lft1 mRNA, encoding the Nodal inhibitor Lft1, to produce a large number of Nodal deficient embryos in order to examine pineal cell differentiation in embryos with elongated and divided pineal phenotypes. Injections
of lft1 mRNA phenocopy the Nodal mutant phenotypes in WT embryos and allowed us to generate large numbers of embryos with elongated or divided pineal glands (Figure 9) (Thisse and Thisse, 1999; Feldman et al., 2002a).

WT embryos were injected with lft1 mRNA at the one to four cell stage. lft1 mRNA injected embryos exhibit elongated and divided pineal glands, cyclopia, and variability in somite loss (Figure 9 A-D). Roughly half of embryos injected with lft1 mRNA exhibit a Nodal deficient phenotype (Figure 9 E). We used lft1 mRNA injected embryos as well as sqt mutants in these studies.

Figure 9. Injection of lft1 mRNA induces Nodal signaling deficient phenotypes in WT embryos. (A) Normal phenotype (indistinguishable from WT). (B) Class I (cyclopia, loss of some somites, similar to sqt mutants with mild phenotypes). (C) Class II (cyclopia, loss of over half of somites, similar to sqt mutants with severe phenotypes). (D) Class III (cyclopia, complete loss of somites, similar to cyc; sqt double mutants). (E) Percentage of embryos injected with 5 pg lft1 mRNA with each phenotypic class. These phenotypes are based on the original characterization of Lft1 overexpression phenotypes in zebrafish (Thisse and Thisse, 1999). Scale bar = 200 μM.
**Pineal photoreceptors and projection neurons differentiate in embryos with open neural tubes.**

In embryos with open neural tubes, *flh* is still expressed in elongated and divided pineal anlage (Aquilina-Beck et al., 2007). We hypothesized that pineal cell types would differentiate in embryos with elongated and divided pineal anlage. To assay for pineal cell differentiation, we examined photoreceptor marker Exorh and projection neuron marker FoxD3 in *sqt* and *lft1* mRNA injected embryos with open neural tube phenotypes. In WT embryos fixed at 2 dpf, *exorh* mRNA was expressed in oval pineal domains (Figure 10 A). Similarly, *exorh* mRNA was present in elongated and divided pineal domains in *sqt* mutants of the same age (n=7) (Figure 10 B, C).

FoxD3 is a transcription factor found in migratory neural crest cells as well as neurons of the pineal gland. The *foxd3*:GFP transgene is expressed in pineal projection neurons and some pineal photoreceptors (Gilmour et al., 2002; Lein et al., 2007; Morris, 2009). In WT and control mRNA injected embryos fixed at 2 dpf, *foxd3*:GFP was expressed in an oval domain (Figure 11 A and Figure 12 A). *foxd3*:GFP was also expressed in elongated and divided domains in *lft1* mRNA injected embryos of the same age (n=10 elongated, n=108 divided, 100%) (Figure 11 B, C and Figure 12 B, C).

Given the sequence similarity of the zebrafish pineal Exorh protein with retinal rhodopsins, the Bovine Rhodopsin antibody 4D2 can be used to label Exorh in the zebrafish pineal (Mano et al., 1999; Noche et al., 2011). We used the 4D2 antibody to examine Exorh protein in the pineal anlage of embryos from the
foxd3:GFP line. Antibody staining with 4D2 in control and lft1 mRNA injected embryos from the foxd3:GFP line revealed that the photopigment is present in oval, elongated and divided pineal anlage in embryos expressing the foxd3:GFP in these same domains (Figure 12 D-F). However, an overlay of these two markers demonstrated Exorh and foxd3:GFP were expressed in distinct cells in control mRNA and lft1 mRNA injected embryos at 2 dpf. This suggests that the foxd3:GFP transgene is expressed in projection neurons only at this stage of development (n=4 elongated, n=11 divided, 100%) (Figure 12 H-J). Roughly half of all embryos injected with lft1 mRNA exhibited elongated or divided pineal domains (Figure 12 I).

<table>
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<tr>
<th>Pineal Phenotype</th>
<th>oval</th>
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<td>exorh mRNA</td>
<td>A</td>
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**Figure 10. Photoreceptors differentiate in Nodal deficient embryos with open neural tubes.** Dorsal views of embryos fixed at 48hpf. Anterior to the top with position of eyes (e) indicated on (A) WT embryo. (A) exorh is expressed in WT embryos with oval pineal glands. (B-C) exorh is expressed in elongated and divided domains in sqt and lft1 mRNA injected embryos with open neural tubes phenotypes. All embryos were fixed at 48 hpf. All images are dorsal views, anterior to the top (n = 21 oval, n = 2 elongated, n = 6 divided) Scale = 25µm
Figure 11. Projection neurons differentiate in Lft1 overexpressing embryos with open neural tubes. (A) The foxd3:GFP transgene in expressed the oval pineal glands of control mRNA injected embryos. (B-C) foxd3:GFP is expressed in elongated and divided domains in lft1 mRNA injected embryos with open neural tube phenotypes (n=108) All embryos were fixed at 48 hpf. All images are dorsal views, anterior to the top. Scale = 50µm.
Figure 12. Both projection neurons and photoreceptors differentiate in the same Lft1 overexpressing embryos with open neural tubes. Embryos were analyzed for both foxd3::GFP transgene expression and Exorh protein expression, and a representative embryo with an oval pineal (A, D, G), with an elongated pineal (B, E, H), and a divided pineal (C, F, I) are shown. (A-C) are images of the foxd3::GFP expression, (D-F) are images of the Exorh immonostaining, and (G-I) are overlays of the two expression patterns. Note that in (G-I), foxd3::GFP and Exorh are expressed in distinct regions of the pineal, suggesting the foxd3::GFP transgene is primarily found in pineal projection neurons at this stage of development (n = 36 oval, n = 4 elongated, n = 11 divided). (J) Percentage of lft1 mRNA injected embryos expressing the foxd3::GFP transgene in an oval pattern (closed anterior neural tube), or elongated or divided pattern (open neural tube) n > 120 embryos. All embryos were fixed at 48 hpf. All images are dorsal views, anterior to the top. Scale bar = 25 μm.
**Pineal cells initiate rhythmic function in embryos with open neural tubes.**

In zebrafish and other vertebrates, the pineal gland is a main site of melatonin production. Melatonin, the main circadian hormone, is expressed at high levels during the night and low levels during the day (Cahill, 1996; Gothilf et al., 1999; Gothilf et al., 2002). In conjunction with this, the gene for the melatonin biosynthetic enzyme *aanat2* is expressed at high levels during the night and low levels during the day (Gothilf et al., 1999; Gothilf et al., 2002). In control mRNA injected embryos, *aanat2* is expressed at high levels in oval pineal domains of embryos fixed at night and at low levels in oval pineal domains of embryos fixed during the day (n = 10 for each time point) (Figure 13 A). This rhythmic expression pattern persists in *lfi1* mRNA embryos with divided pineal gland anlage (Figure 13 B).
Figure 13. Rhythmic expression of *aanat2* is initiated in pinealocytes of embryos with open neural tubes. Dorsal views of embryos raised in 14:10 hour light:dark cycle fixed at indicated time points. (A) GFP mRNA injected control embryos with oval pineal glands. (B) *lft1* mRNA injected embryos with divided pineal glands. Closed arrowheads indicate pineals with high *aanat2* expression; open arrowheads indicate pineals with low *aanat2* expression. ZT indicates time within the circadian cycle, lights turning on at ZT = 0 and lights turning off at ZT = 14. Dark bars indicate lights off and light bars indicate lights on. For each time point, \( n \geq 10 \) for embryos with oval shaped pineal and embryos with divided pineal. Scale bar = 25 \( \mu \) m.

Discussion

*Pinealocyte differentiation pathways do not require localization to the dorsal midline of the brain.*

Results of this study demonstrated that pineal projection neurons and photoreceptors differentiate in embryos with open neural tubes despite lateral displacement of the pineal precursor domains. The presence of *exorh* mRNA as well as *Exorh* protein demonstrates that photoreceptors were present. *foxd3*:GFP expression in distinct cells from *Exorh* expression demonstrates that projection neurons were also present. These results suggest that neural tube closure and
localization to the dorsal midline of the brain are not required for pinealocyte differentiation.

It likely that flh expression is sufficient to initiate pineal projection neuron and photoreceptor differentiation in the neuroepithelium. flh expression is required for normal differentiation of pineal neurons (Masai et al., 1997). In embryos that lack flh, the presumptive pineal region remains a single-layered neuroepithelium and only a few pineal neurons differentiate by later embryonic stages. When flh expression is expanded in mbl mutants, the extent of differentiated pineal cells is also expanded (Masai et al., 1997). Both pineal projection neurons and photoreceptors differentiate rostrally in the telencephalon (Masai et al., 1997).

flh is expressed in the presumptive pineal domains at the neural plate stage in WT embryos (Masai et al., 1997). Further, flh is expressed in divided and elongated pineal domains in sqt mutants with open neural tubes (Aquilina-Beck et al., 2007). Consistent this, we found that pineal cell types differentiate in embryos with these elongated and divided pineal phenotypes. Together, these data support a model in which flh, spatially regulated by mbl, initiates pineal cell production in the neuroepithelium prior to neural tube closure.

**Pineal photoreceptors are functional in embryos with open neural tubes**

Aanat2 is the penultimate enzyme in the melatonin biosynthetic pathway and expression of the aanat2 gene reflects melatonin’s rhythmic production; expression is high at night and low during the day. aanat2 is expressed rhythmically in the divided pineal anlage of embryos with open neural tubes despite their laterally displaced
location, suggesting pineal cells not only differentiate but also initiate rhythmic function in embryos with open neural tubes.

In the zebrafish pineal, initiation of rhythmic aanat2 expression requires light stimuli as aanat2 expression does not initiate normally in embryos raised in constant dark (Ziv et al., 2005). As a light-sensing molecule, Exorh likely mediates transduction of light signals though a signaling cascade that initiates aanat2 expression. Depletion of Exorh in the pineal results in a significant decrease in aanat2 expression (Pierce et al., 2008). Expression of circadian clock gene per2 is light-induced and initiates rhythmic expression of aanat2 in the pineal gland (Ziv et al., 2005; Ziv and Gothilf, 2006). When per2 is depleted, the rhythmic nature of aanat2 expression is reduced. It is possible that per2 acts an intermediate between Exorh and rhythmic aanat2 expression in the pineal gland. (Ziv et al., 2005; Ziv and Gothilf, 2006).

Exorh expression in the pineal anlage of embryos with open neural tubes demonstrated that photoreceptors differentiated, and suggested that aanat2 may also be expressed. Our results demonstrated that aanat2 was expressed in embryos with a divided pineal anlage. Further, the rhythmic nature of aanat2 expression leads to the prediction that per2 was also likely expressed. These studies demonstrated that differentiated pineal photoreceptors in embryos with open neural tubes are capable of responding to environmental light stimuli.
**Examination of pineal neural connections in embryos with open neural tubes.**

Pineal projection neurons send their axons to targets in the ventral brain including the hypothalamus, thalamus, and optic tectum, where they likely regulate circadian outputs (Wilson and Easter, 1991b; Wilson and Easter, 1991a; Yanez et al., 2009). Expression of foxd3:GFP in embryos with open neural tubes demonstrated that projection neurons differentiated, suggesting that these axonal projections may also form. Further examination of these axonal projections is needed to determine if they reach their ventral targets. Proper connections of these pineal projections to ventral brain targets would suggest that the pineal neural outputs are likely functional in embryos with open neural tubes.

**Circadian studies in embryos with open neural tubes**

Though these data demonstrated that differentiated pineal photoreceptors were functional in embryos with open neural tubes, these embryos were raised in a 14/10hr light/dark cycle. Rhythmic aanat2 expression may reflect a direct response to environmental light changes as opposed to true entrainment of the circadian clock. In zebrafish embryos, the circadian clock is entrained after roughly 72 hpf when raised in a light/dark cycle (Noche et al., 2011). Once transferred to constant conditions, embryos demonstrate rhythmic expression of aanat2 and other circadian-regulated genes even in the absence of environmental light changes (Noche et al., 2011). Further analysis of rhythmic gene expression in embryos with open neural tubes that are raised in constant conditions after 72 hpf would determine the extent of circadian entrainment in cells of divided and elongated pineal anlage. If circadian rhythms
have initiated, it would suggest that differentiated pineal tissues are capable of
circadian entrainment despite failure to form a single pineal gland.

III: Left isomerism correlates with severity of the open neural tube phenotype in embryos with open neural tubes

The zebrafish epithalamus exhibits left-right asymmetries in structure and gene expression that have implications in behavioral characteristics such as laterality in eye use. In embryos with severe open anterior neural tubes, the left and right sides of the pineal anlage in the epithalamus remain separated and all of the epithalamic left-right asymmetries become left isomerized. Both sides of the brain exhibit left-sided characteristics. However, the left isomerized phenotype is incompletely penetrant as some embryos with open neural tubes develop asymmetry in the epithalamus. We demonstrated that the pineal widths of embryos with open neural tubes and left isomerism are significantly wider than those of embryos with open neural tubes and normal left-right asymmetry. These results suggest that normal establishment of asymmetry in the zebrafish epithalamus requires close proximity of the left and right sides of the brain. Further, this suggests that there is a signal that diffuses from the left to the right side of the pineal gland that prevents the right from developing with left-sided characteristics. This signal likely has a limited diffusion range as the mechanism fails when the neural tube is widely open.

Introduction

Structural and functional asymmetries are prominent in the vertebrate viscera and brain (Corballis, 2009). While the vertebrate body is externally symmetric, the internal arrangement of organs is asymmetric (Corballis, 2009). Similarly, the vertebrate brain contains numerous morphological and functional left-right
asymmetries (Corballis, 2009). For example, in humans a region of the cerebral cortex called the planum temporale is generally larger in the left hemisphere than in the right. Consistent with this structural asymmetry, language processing is generally localized to the left hemisphere (Foundas et al., 1994; Raquel Dorsaint-Pierre, 2006). It is likely that left-right brain asymmetries provide adaptive advantages (Corballis, 2009). Not only is brain lateralization an efficient use of space, but unilateral hemispheric control likely allows for faster information processing whereas duplication may lead to conflict between the hemispheres (Foundas et al., 1994; Ringo et al., 1994; Corballis, 2009).

The zebrafish epithalamus in the dorsal diencephalon of the brain exhibits left-right asymmetries in structure and gene expression that have behavioral implications (Barth et al., 2005; Schier, 2009). The pineal gland anlage in the dorsal epithalamus is the first tissue in the zebrafish brain to demonstrate left-right asymmetry (Concha and Wilson, 2001; Halpern et al., 2003). Nodal signaling pathway genes such as cyc, lft1, and pitx2 are expressed only on the left side of the pineal gland (Rebagliati et al., 1998a; Rebagliati et al., 1998b; Sampath et al., 1998b; Concha et al., 2000; Liang et al., 2000). Nodal signaling in the left side of the pineal gland directs formation of the parapineal gland to the left of the pineal. Cells that form the parapineal gland originate with pineal cells but migrate leftward to form a morphologically distant structure (Concha et al., 2000; Snelson et al., 2008). By 4 dpf the parapineal is located ventrally and posteriorly to the left of the pineal gland (Gamse et al., 2003).
Flanking the pineal complex in the epithalamus are the left and right habenula nuclei (Concha et al., 2000). The parapineal innervates the left habenula, which has higher neuropil density and expresses high levels of \textit{kctd12.1}. Conversely, the right habenula expresses low levels of \textit{kctd12.1} and high levels of \textit{kctd12.2} (Gamse et al., 2003). The parapineal gland directs asymmetric development of the left and right habenula nuclei (Gamse et al., 2003; Gamse et al., 2005). The habenula that forms on the same side as the parapineal develops with left identity. In embryos with reversed epithalamic situs, a parapineal develops to the right of the pineal and the right habenula expresses high \textit{kctd12.1}. When the parapineal is ablated, both the left and right habenulae develop right identity (Gamse et al., 2003; Gamse et al., 2005).

Asymmetric projections from the epithalamus to the midbrain play an important role in the laterality of eye usage in zebrafish (Barth et al., 2005). In fish with normal brain asymmetry, projections from both of the habenulae extend ventrally to the Interpeduncular nucleus (IPN) in the midbrain (Gamse et al., 2005). Projections from the left habenula extend to the dorsal and ventral IPN while projections from the right habenula extend to the ventral IPN only (Gamse et al., 2005). When placed in a tank with a mirror, zebrafish larvae with normal epithalamic asymmetry examine themselves with their right eye first and their left eye second (Barth et al., 2005). In fish with reversed epithalamic asymmetry, habenular projections to the IPN are reversed as well. When placed in a tank with a mirror, larvae with reversed habenular asymmetry first use their left eye to examine themselves before switching to the right (Barth et al., 2005).
Nodal signaling deficient (*sqt* mutants and *lft1* mRNA injected embryos) and N-cadherin deficient (*cdh2*) embryos with open anterior neural tubes exhibit elongated or divided pineal gland phenotypes (Aquilina-Beck et al., 2007). These embryos also often exhibit left isomerization of all epithalamic left-right asymmetries. Nodal related genes *lft1* and *pitx2* are expressed in both the left and right sides of pineal anlage in Nodal deficient embryos with elongated or divided pineal glands. Further, bilateral parapineals form on either side of the brain midline in both Nodal deficient and *cdh2* mutants with divided pineal anlage. Consistent with the presence of bilateral parapineals, the left-sided gene *kctd12.1* expression is high in both the left and right habenulae while the right-sided gene *kctd12.2* is expressed at low or undetectable levels in Nodal deficient embryos.

Interestingly, the left isomerized phenotype is not fully penetrant and decreases as the epithalamus develops. While 93% Nodal deficient embryos with divided or elongate pineal anlage have bilateral expression of *lft1* in the pineal, only 70% of these embryos develop bilateral parapineals and 56% have bilateral *kctd12.1* expression. Further, only 41% of *cdh2* mutants with open neural tubes develop bilateral parapineals. *cdh2* mutants generally exhibit mild open neural tube defects in comparison to Nodal deficient embryos, leading to the hypothesis that these penetrance differences were correlated to neural tube defect severity. I demonstrated that in both *sqt* and *cdh2* mutants, pineal anlage were significantly wider in embryos with left isomerism than embryos with normal or reversed symmetry. These results suggest that establishment of asymmetry in the epithalamus requires close proximity of the left and right sides. Further, this suggests a model in which a diffusible signal
from the left side of the pineal prevents the right side from developing left-sided characteristics. This signal likely has a limited diffusion range as this mechanism fails when the left and right sides of the pineal anlage are widely spaced.

**Methods**

**Zebrafish**

Zebrafish stocks were maintained at 28.5°C in a 14:10 light:dark cycle according to standard procedures (Westerfield, 2000). Stocks included the WT strain Zebrafish Danio Rerio (ZDR) (Aquatica Tropicals, Plant City, FL), *n-cadherin/cdh2<sup>vum125</sup>* (Birely et al., 2005) (von der Hardt et al., 2007), *sqt<sup>ex35</sup>* (Feldman et al., 1998), and *sqt<sup>ex35;cyc<sup>m294</sup>* (Feldman et al., 1998). WT, transgenic, and mutant embryos were obtained through natural breeding (Westerfield, 2000).

**Whole mount in situ hybridization (WISH)**

Single and double WISH was carried out as previously described (Thisse et al., 1993; Liang et al., 2000; Thisse et al., 2004) Antisense RNA probes included: *orthodenticle homeobox 5 (otx5)* (Gamse et al., 2002) and *growth factor independent 1.2 (gfi1.2)* (Dufourcq et al., 2004).

**Photography**

Bright field and fluorescent images were obtained on a SPOT camera or a CoolSnap ES camera connected to a Nikon Eclipse 80i microscope and on a Leica DM6000B microscope.
**Data Analysis**

Digital images with dorsal views of the epithalamus were measured using ImageJ software. Pineal width was measured from the most lateral points of the *otx5* pineal expression, regardless of whether the pineal was oval, elongated, or divided. Pineal organs were considered elongated if their width was longer than their length and their width was greater than the upper 95% confidence interval of WT pineal organ width (n = 46 WT pineal organs measured). Statistical analysis was performed using Tukey-Kramer comparison and Student’s T-test using JMP 10.0 software.

**Results**

*The left isomerized phenotype is correlated with the severity of the open neural tube defect.*

Both *sqt* mutants and *cdh2* mutants with open neural tubes exhibit variability in pineal phenotype (Aquilina-Beck et al., 2007). Some embryos have widely spaced divided pineal anlage while other have mildly elongated pineal domains. Both mutants also exhibit left isomerization of the epithalamus with incomplete penetrance. To determine if the left isomerized phenotype was correlated to pineal anlage width, I examined pineal and parapineal phenotypes using WISH with antisense probe for *otx5*, a pineal marker, and *gfi1.2* a parapineal maker in *sqt* and *cdh2* embryos fixed at 3 dpf. Parapineal phenotype was used as an indicator of asymmetry as one-sided parapineal is indicative of asymmetric development while bilateral parapineals are indicative of left isomerization.
sqt and cdh2 mutants were grouped separately into three classes based on pineal and parapineal phenotypes. The first class consisted of mutant embryos with oval pineals and one-sided parapineal, the second included mutants with elongated or divided pineals and one, sided parapineal, and the third consisted of mutants with elongated or divided pineals and bilateral parapineals. In comparing each class with one another, paired T-tests demonstrated that the pineal widths of sqt mutants with elongated or divided pineal anlage and bilateral parapineals (n=9) were significantly greater than those of embryos with elongated or divided pineals and one, sided parapineal (n=29) (p < 0.0001) (Figure 14).

Paired T-tests also demonstrated that pineal widths of cdh2 mutants with elongated or divided pineal anlage and bilateral parapineals (n=2) were significantly greater than those of embryos with elongated or divided pineals and one, sided parapineal (n=17) (p < 0.0001). Further, the majority of sqt and cdh2 embryos with left isomerism had pineal widths greater than 100 µm (sqt n=8/9; cdh2 n=2/2) (Figure 14).
Figure 14: The differences in left isomerization frequency between Nodal deficient and cdh2 mutant embryos are caused by differences in the severity of the neural tube phenotype. sqt and cdh2 mutant embryos at 3 dpf were assayed for parapineal phenotype and pineal width. Each data point on the graphs indicates the result from a single embryo. The short horizontal lines indicate the average of each group, with error bars indicating standard deviation. For Nodal deficient embryos, n = 12 for oval, unilateral; n = 29 for elongated/divided, 19 unilateral, and n = 9 for elongated/divided, bilateral. For cdh2 mutants, n = 2 for oval, unilateral, n = 17 elongated/divided, bilateral, and n = 2 for elongated/divided, bilateral.

Abnormal positioning of bilateral parapineals

Among embryos with divided pineal domains and bilateral parapineals, we noticed that there was variation in the position of the parapineals. Parapineal positioning was analyzed by grouping parapineals based on their relative position to the pineal and whether or not the parapineal overlapped the pineal at any of these positions. Bilateral parapineals in Nodal deficient embryos developed in the same anterior-posterior position as the pineal domains (n=15/16) but with variability with
respect to the lateral-medial axis. However, the bilateral parapineals of \textit{cdh2} mutants tended to develop anterior to the pineal domains (n=4/6) (Figure 15). These results suggest that the anterior-posterior polarity of the neural tube is more disrupted in \textit{cdh2} mutants than in Nodal deficient embryos.

\textbf{Figure 15: The parapineal position is disrupted in embryos with the left isomerized phenotype.} Relative position of the parapineals to the pineal anlage in (A) WT embryos with oval pineal anlage and (B) \textit{lft1} mRNA injected embryos and (C) \textit{cdh2} mutant embryos with divided pineal anlage. Shaded areas represent the location of the pineal anlage. Numbers within the center of the shaded area represent the position of parapineals that fully overlapped the pineal anlage. Numbers within the outer perimeter of the shaded area represent the positions of parapineals that partially overlapped the pineal anlage and numbers outside the shaded area represent parapineals that did not overlap the pineal anlage. Note the high rate of situs inversus in the WT embryos (n=9/31, 30\%). This high rate of reversed brain asymmetry has been observed in another study (Liang et al., 2000).
Discussion

This work demonstrated that the pineal widths of *sqt* and *cdh2* mutants with left isomerism were significantly wider than those embryos with normal or reversed epithalamic asymmetry. These results suggest that establishment of asymmetry in the zebrafish epithalamus requires close proximity of the left and right sides of the brain. In WT embryos, Nodal gene expression on the left side of the pineal gland directs placement of the parapineal gland to the left of the pineal (Concha et al., 2000; Snelson et al., 2008; Schier, 2009). We propose that a diffusible signal with limited diffusion range originates in the left, Nodal-expressing side of the pineal gland and prevents the right side from developing with left-sided characteristic. When the pineal domain is widely spaced this signal cannot diffuse far enough to the right and both sides develop with left-sided characteristics.

*Lft1 is a candidate for the signal that prevents the right side of the pineal from developing left characteristics*

Nodal signaling is involved in the establishment of left-right asymmetry in the zebrafish viscera (Concha et al., 2000; Chen and Schier, 2001; Muller et al., 2012). Nodal genes are expressed only in the left lateral plate mesoderm shortly after gastrulation (Sampath et al., 1998b). Similarly, Nodal genes are expressed only on the left side of the zebrafish pineal gland in during epithalamic development (Liang et al., 2000). It is possible that Nodal signaling establishes left-right asymmetry in the zebrafish epithalamus with Lft1 being the signal that prevents the right side of the brain from developing with left identity. *cyc* and *lft1* are expressed on the left side of
the pineal and then secreted (Liang et al., 2000). Cyc has a diffusion range of a few cell diameters while Lft1 can diffuse many cell diameters. Any Cyc signaling on the right side would quickly be inhibited by diffusing Lft1 (Chen and Schier, 2002).

**Lft1 and Cyc may work in a reaction-diffusion mechanism to pattern left-right asymmetry in the epithalamus**

Nodal signaling acts in a reaction-diffusion mechanism during left-right axis development in vertebrate viscera (Muller et al., 2012). In reaction-diffusion mechanisms, patterns are generated when cells produce a morphogen that diffuses slowly and an inhibitor that diffuses quickly. The morphogen in this model must positively regulate both its own expression and the expression of its inhibitor (Gierer and Meinhardt, 1972; Turing, 1990; Meinhardt, 2008). In early zebrafish development, *cyc* is expressed in the left lateral plate mesoderm, where it positively regulates both its own expression and *lft1* expression (Sampath et al., 1998a). Cyc protein, acting as the activator, has a shorter diffusion range than its inhibitor Lft1 (Chen and Schier, 2001; Chen and Schier, 2002; Muller et al., 2012). While both Cyc and Lft1 originate in the left mesoderm, Lft1 diffuses farther to adjacent regions. This inhibits Nodal signaling in these surrounding areas generating spatial restrictions to cell fate (Muller et al., 2012).

It is possible that a similar Nodal signaling based reaction-diffusion mechanism establishes left-right asymmetry in the zebrafish epithalamus. In WT embryos, *cyc* and *lft1* are expressed on the left side of the pineal anlage (Liang et al., 2000). Though both Cyc and Lft1 can diffuse, the farther diffusing Lft1 will quickly
inhibit any Cyc that diffuses the right side of the pineal, maintaining asymmetry between the two sides (Figure 16 A).

In Nodal signaling deficient embryos with closed neural tubes, \textit{cyc} is initially expressed in both sides of the pineal. However, these embryos develop with randomized asymmetry suggesting that initial bilateral expression of \textit{cyc} is corrected. Similarly, in embryos with mild open neural tubes, \textit{nodal} genes are initially expressed bilaterally in the pineal anlage and these embryos develop with randomized asymmetry. In both cases, the side that endogenously produces more Cyc also produces more Lft1 as Cyc positively regulates both itself and Lft1 (Figure 16). Lft1 produced on both sides diffuses across the pineal anlage and combines to inhibit Cyc signaling in the side with endogenously lower Cyc production. These embryos develop with randomized asymmetry as either the left or the right side can have endogenously higher Nodal activity (Figure 16 B).

When the pineal anlage is divided and the two sides are widely spaced, both the left and right sides again initially express Nodal genes. However, Lft1 cannot diffuse far enough from either side to inhibit Cyc signaling on the opposite side (Figure 16 C). Thus, Cyc signaling in both sides directs formation of bilateral parapineals. This in turn directs development left habenular identity on both sides.
Figure 16: A possible reaction-diffusion model involving Nodal signaling patterns left-right asymmetry in the zebrafish epithalamus. (A-C) Graphic representations of Cyc and Lft protein concentration in the pineal gland. (A) In embryos with closed neural tube, Cyc and Lft1 are produced on the left (L) side of the pineal. More Lft1 diffuses to the right, inhibiting Cyc signaling on the right (R). (B) In embryos with mild open neural tubes Cyc and Lft1 are produced on both sides of the pineal anlage. Diffusing Lft1 from both sides with inhibit Cyc signaling on the side with endogenously less Cyc production. (C) In embryos with severely open neural tubes Cyc and Lft are produced on both sides of the pineal anlage. Lft1 does not diffuse far enough from either side to inhibit Cyc on the opposite side.

While a reaction-diffusion mechanism involving Cyc and Lft1 is a good model to explain the establishment of zebrafish epithalamic asymmetry, it is possible that deficiencies in the Nodal signaling pathway itself directly result in left
isomerization of the epithalamus. However, this is unlikely as cdh2 mutants demonstrate the left isomerized phenotype despite intact Nodal signaling pathways.

**Insights into the role of contact between the left and right sides of the epithalamus**

While our data supports the a reaction-diffusion model involving Cyc and Lft in the pineal gland, we cannot rule out the role of a contact-dependent mechanism in establishing left-right asymmetry in the epithalamus. Both Nodal deficient embryos and cdh2 mutants have reduced N-cadherin mediated cell adhesion throughout the neural tube (Lele et al., 2002; Aquilina-Beck et al., 2007). Convergence and intercalation of cells in the developing neuroepithelium as well as oriented cell divisions are disrupted in embryos that lack N-cadherin (Hong and Brewster, 2006).

Mispositioning of parapineals with respect to the anterior-posterior axis in cdh2 mutants with left isomerism supports the requirement of N-cadherin for proper orientation of cells in the neural tube. A reduction in N-cadherin facilitated polarity in the neuroepithelium may also disrupt the development of left-right asymmetry. However, Nodal deficient embryos with left isomerism have normal anterior-posterior parapineal position despite reduced N-cadherin. This suggests that polarity of the neural tubes may require another mechanism, such as a reaction diffusion mechanism involving Cyc and Lft1.

IV: Characterization of the bigtime (bti) mutant jaw phenotype

The cranial neural crest is a specialized subset of stem cells that migrates from the dorsal anterior neural tube to form craniofacial structures, including Meckel’s cartilage, the cartilaginous precursor to the mandible. Embryos from the bigtime (bti) mutant line have reduced Meckel’s cartilage formation. These mutants have a truncation of the endoplasmic reticulum (ER) pore component protein Sec61α11, which is involved with protein transport into the ER’s lumen. With reduced protein translocation into the ER, we hypothesize that the cranial neural crest derived chondrocytes in bti mutants likely fail to secrete the collagen protein needed to form the extracellular matrix of cartilage tissues. This ultimately results in a reduced Meckel’s cartilage. My characterization of fluorescently labeled, migrating neural crest cells demonstrated that early cranial neural crest migration was normal in bti mutants. Further, collagen genes were expressed in differentiating cranial neural crest cells in the presumptive Meckel’s cartilage region. This collagen expression suggests that bti mutant chondrocytes likely synthesize collagen protein, however the expression pattern is inconsistent with WT collagen expression. Collagen genes are expressed in bilateral rows that do not converge to form the shape of Meckel’s cartilage in bti mutants. This is consistent with the hypothesis that the reduction in Meckel’s cartilage in bti mutants results from failure of chondrocytes to secrete collagen protein into the extracellular matrix.
Introduction

The neural crest is a multipotent stem cell population in vertebrates that arises at the border of the neural plate and the epidermal ectoderm (Gammill and Bronner-Fraser, 2003) (Figure 17). Neural crest cells undergo an epithelial to mesenchymal transition, detach from surrounding epithelial cells, and migrate to highly predictable locations throughout the body (Gammill and Bronner-Fraser, 2003; Duband, 2010). Neural crest cells include the precursors to melanocytes (pigment cells), chondrocytes (cartilage making cells), and many neurons and glia of the peripheral nervous system (Dupin and Sommer, 2012). Cranial neural crest cells originate in the dorsal anterior neuroectoderm and migrate throughout the head to give rise to various craniofacial structures including the mandible (the lower jaw in vertebrates), hyoid (a small bone located superior to the larynx) and bones of the middle ear (Santagati and Rijli, 2003; Dupin and Sommer, 2012).
Figure 17. The neural crest originates in the neuroectoderm. During neurulation, cells at the neural plate border undergo an epithelial to mesenchymal transition and delaminate from the neural tube. Called the neural crest, these cells migrate to predictable locations throughout the head. Adapted from Gammill and Bronner-Fraser, 2003.

Cranial neural crest cell migratory paths are first determined by their premigratory positions in the segmented neuroectoderm (Santagati and Rijli, 2003; Klymkowsky et al., 2010). The developing brain is divided into the forebrain, midbrain, and hindbrain. The hindbrain is further divided into segmented regions called rhombomeres (Eagleson and Harris, 1990)(Lumsden et al., 1991). Cranial neural crest cells migrate in separate streams from the midbrain and hindbrain rhombomeres to predictable destinations throughout the head (Lumsden et al., 1991; Santagati and Rijli, 2003; Knight and Schilling, 2006a). For example, the stream of cranial neural crest cells originating in the posterior midbrain and rhombomere one of
the hindbrain migrate to the region of the head just dorsal and posterior to the eye. These cells are determined to give rise to chondrocytes of the maxilla (the upper jaw) and the mandible (Figure 18) (Lumsden et al., 1991; Knight and Schilling, 2006a).

After migrating out from the neural tube, the cranial neural crest cells that will give rise to craniofacial structures localize to the pharyngeal arches (Knight and Schilling, 2006a). Pharyngeal arches are mesodermal structures that give rise to the pharynx in humans and the branchial arch support for the gills in fish (Knight and Schilling, 2006a). The anterior-posterior identity of the migrating cranial neural crest cells is regulated by hox gene expression in the pharyngeal arches. hox expression induces the gene transcription responsible for positional specification of the cranial neural crest cells (Santagati and Rijli, 2003). The anterior identity of pharyngeal arch one results from a lack of hox expression, meaning the premandibular cranial neural crest cells also do not express hox genes (Figure 18) (Gendronmaguire et al., 1994; Santagati and Rijli, 2003).

In zebrafish and other vertebrates, Hox paralogue group 2 genes, hoxa2 and hoxb2, are expressed in pharyngeal arch two, which gives rise to the hyoid bone (Figure 18)(Santagati and Rijli, 2003). Ectopic expression of hox2 genes in the first pharyngeal arch results in the loss of the mandible and a duplication of the hyoid bone (Hunter and Prince, 2002) (Pasqualetti et al., 2000). Conversely, loss of hox2 genes in the second pharyngeal arch results in lost of the hyoid and duplication of the mandible (Gendron-Maguire et al., 1993; Rijli et al., 1993; Hunter and Prince, 2002; Miller et al., 2004).
Figure 18. Cranial neural crest cell migration in zebrafish. Cameria lucida drawing of a lateral view of a zebrafish embryo at 24 hpf. Cranial neural crest cells from the midbrain and hindbrain rhombomeres (r2 – r7) migrate to the pharyngeal arches (p1 – p7). The anterior- posterior identity of the pharyngeal arches is regulated by hox expression. P1 has no hox expression and gives rise to the mandible. P2 is characterized by hox2 expression gives rise to the hyoid bone. P3-7 are characterized by hoxb expression and give rise to the branchial arches. Adapted from Knight and Schilling, 2006.

In zebrafish, cranial neural crest cells with a mandibular fate originate at the level of the posterior midbrain and anterior hindbrain and migrate to the first pharyngeal arch (Figure 18) (Schilling and Kimmel, 1997; Knight and Schilling, 2006a). Once localized, cranial neural crest cells move ventrally and rostrally to differentiate and form Meckel’s cartilage, the mesenchymal precursor to the mandible (Figure 19). These Meckel’s cartilage precursors must undergo chondrocyte differentiating, during which they form condensations and secrete collagen protein into the extracellular matrix (Schilling and Kimmel, 1997; Knight and Schilling, 2006b).
In zebrafish, craniofacial cartilage formation begins at approximately 2 dpf (Schilling and Kimmel, 1997). Initial chondrocyte differentiation in the pharyngeal arches begins at 45 hpf and Meckel’s cartilage chondrification occurs between 53 and 57 hpf (Schilling and Kimmel, 1997). Chondrification occurs almost simultaneously on the right and left sides of the developing jaw with the first condensations of cartilage arising near the site of articulation with the palatoquadrate cartilage (precursor to the maxilla or upper jaw). As these condensations enlarge they expand rostrally from the joint, elongating the lower jaw (Schilling and Kimmel, 1997) (Figure 19).

**Figure 19: Chondrification in the Pharyngeal arches.** Alcian blue staining demonstrating the progress of chonrification of pharyngeal cartilages at (A) 52hpf, (B) 68 hpf and (C) 96 hpf. White arrows indicate progression of Meckel’s cartilage (mc) development. Ventral views with anterior to the left. Scale = 100 µm. Adapted from Schilling and Kimmel, 1997.
Mandibular development in zebrafish *bti* mutants is reduced (Doll et al., 2011) (P.-N. Lu and S. Brannan – Unpublished data). *bti* fish have mutation in a gene that encodes the endoplasmic reticulum (ER) pore-component protein Sec61α1 (Doll et al., 2011). The Sec61α1 protein is part of the ER pore which moves newly synthesized proteins across the ER membrane into the lumen of the ER (Rapoport, 2007). The truncated Sec61α1 protein in *bti* mutants likely renders this ER pore nonfunctional (Doll et al., 2011). Consistent with the ER pore being nonfunctional, the ER volume is reduced in the chondrocytes of *bti* mutants (Doll et al., 2011). The reduction in ER likely results from decreased amount of protein transported into the ER.

To form cartilage, chondrocytes must secrete large amounts of collagen into the extracellular matrix (Lang et al., 2006). We hypothesized that the reduction in Meckel’s cartilage development seen in *bti* mutants resulted from failure of the cranial neural crest derived chondrocytes to secrete a sufficient amount of collagen protein. It possible that cranial neural crest cells migrate to the pharyngeal arches and initiate chondrification in *bti* mutants. However, these chondrocytes may be unable to secrete a sufficient amount of collagen protein.

Consistent with this hypothesis, previous members of our laboratory demonstrated that early cranial neural crest migration appears to be normal in *bti* mutants (P.-N. Lu and S. Brannan, unpublished data). The neural crest marker *dlx2* was expressed in normal patterns in *bti* mutants up to 28 hpf (Figure 20). However, Alcian blue, which stains proteoglycans attached to the collagen in cartilage, demonstrated that the mutants have a reduction in Meckel’s cartilage by 4 dpf (P.-N.
Lu and S. Brannan, unpublished data). Lack of staining by 6 dpf further demonstrated that cartilage is not produced at later stages in bti mutants, suggesting that the reduction in Meckel’s cartilage is not due to a developmental delay (Figure 21).

Figure 20. Early neural crest migration marker is normal in bti mutants. dlx2 expression in (A-B) WT and (C-D) bti mutants at 28 hpf. (A,C) dorsal views and (B,D) lateral views, both with anterior to the left. Scale = 100µm. (P.-N. Lu and S. Brannan – Unpublished data)
To further test our hypothesis, I examined fluorescently labeled cranial neural crest cell migration over time in live \textit{bti} mutant embryos. Consistent with our previous studies, early cranial neural crest migration was normal (P.-N. Lu and S. Brannan unpublished data). However, beginning at approximately 44 hpf, bilateral streams of cranial neural crest cells in \textit{bti} mutants do not migrate anteriorly and to form the Meckel’s cartilage shape seen in WT embryos at the same stages. To determine if cranial neural crest cells initiated chondrification in \textit{bti} mutants, I examined expression of two cartilage-specific collagen genes, \textit{col27a1} and \textit{col2a1}. I demonstrated that these genes were robustly expressed in \textit{bti} mutants in patterns consistent with fluorescently labeled cell patterns. This collagen expression suggests that cranial neural crest derived chondrocytes likely synthesize collagen. These data, in conjunction with previous Alcian blue cartilage staining, suggest that the reduced
mandibular development in bti mutants results from an inability of chondrocytes to secrete collagen into the extracellular matrix.

Methods

Zebrafish

Zebrafish stocks were maintained at 28.5°C in a 14:10 light:dark cycle according to standard procedures (Westerfield, 2000). Stocks included the WT strain Zebrafish Danio Rerio (ZDR) (Aquatica Tropicals, Plant City, FL), sec61a1c163 (Doll et al., 2011), Tg(sox10:GFP) (Dutton et al., 2008). WT, transgenic, and mutant embryos were obtained through natural breeding (Westerfield, 2000).

Whole mount in situ hybridization (WISH)

Single and double WISH was carried out as previously described (Liang et al., 2000; Thisse et al., 2004; Thisse et al., 1993). Antisense RNA probes included: collagen27alpha-1a (col27a1) (Thisse, 2001) and collagen2alpha-1a (col2a1) (Thisse, 2001).

Photography

Embryos were raised individually in twenty well plates to allow us to follow cranial neural crest migration in each embryo over a period of three days. Prior to imaging, embryos were anesthetized in Tricaine and placed in methylcellulose for proper orientation. After imaging, embryos were rinsed and replaced into their respective wells.
Bright field and fluorescent images were obtained on a SPOT camera or a CoolSnap ES camera connected to a Nikon Eclipse 80i microscope and on a Leica DM6000B microscope.

Results

*Early cranial neural crest migration is normal in bti mutants*

The cartilaginous precursor to the zebrafish mandible, Meckel’s cartilage, forms from cranial neural crest cells that migrate from the dorsal neural tube to the first pharyngeal arch. Previous work demonstrated that *bti* mutants exhibit reductions in development of the cranial neural crest derived Meckel’s cartilage (Doll et al., 2011)(P.-N. Lu and S. Brannan – Unpublished data). To determined whether or not the reduced Meckel’s cartilage in *bti* mutants resulted from a failure of cranial neural crest cells to migrate properly, we compared migration of these cells from the dorsal neural tube to the pharyngeal arches in WT embryos and *bti* mutants.

We first examined cranial neural crest migration in WT embryos from 22 to 96 hpf to determine a normal migration pattern (Figure 22). This was accomplished using the *sox10*:GFP transgenic line, in which cranial neural crest cells are fluorescently labeled (Dutton et al., 2008). From 22 to 40 hpf, the *sox10*:GFP expressing cells moved bilaterally from the neural tube towards the pharyngeal arches (Figure 22). Beginning at roughly 40 hpf, cranial neural crest cells migrated anteriorly along the ventral side of the head in two lateral streams. By 66 hpf, these bilateral rows of *sox10*:GFP expressing cells converged anteriorly in pattern consistent with the shape of Meckel’s cartilage (Figure 22). These movements are consistent with
previous studies demonstrating cranial neural crest migration form the neural tube to the lower jaw (Schilling and Kimmel, 1997; Knight and Schilling, 2006a).

Next, we examined *sox10*:GFP expression in *bti* mutants and their WT siblings in parallel from 22 hpf to 66 hpf (Figure 22). The pattern of *sox10*:GFP expression in *bti* mutants was indistinguishable from WT patterns up to 44 hpf. As previously stated, after 44 hpf two lateral streams of fluorescently labeled cells moved anteriorly on the ventral surface of the head in WT embryos. Two similar streams of *sox10*:GFP cranial neural crest cells moved rostrally in *bti* mutants. However, the cells failed to converge into the shape of Meckel’s cartilage even by 66 hpf. Instead, the cells remained in bilateral rows medial to the eyes (Figure 22).
Figure 22. Migration of sox10:GFP expressing neural crest cells in WT embryos and bti mutants. Lateral views with anterior to the left (22 hpf – 33 hpf) and ventral views with anterior to the top (44 hpf – 66 hpf) of sox10:GFP expression in live WT (n = 13) and bti (n = 4) mutants. Images are representative of all embryos examined. WT and bti images are from the same embryo followed over time. mc = presumptive Meckel’s cartilage, e = eyes, o = otic vesicles. Scale = 100 µm
Collagen genes are expressed in the presumptive Meckel’s cartilage in bti mutants

Cartilage is a tissue that consists of few chondrocytes and large amounts of collagenous extracellular matrix (Lang et al., 2006). These chondrocytes produce and secrete a high level of collagen protein into the extracellular matrix (Lang et al., 2006). We hypothesized that chondrification was initiated in the presumptive mandible of bti mutants. However, we examined collagen gene expression as it was possible that cranial neural crest cells did not differentiate into chondrocytes in bti mutants. WISH with antisense probe to collagen genes col27a1 and col2a1 revealed that cells of the developing Meckel’s cartilage expressed the chondrocyte-specific genes in bti mutants (Figure 23, col2a1 not shown). Both col27a1 and col2a1 were expressed in bti mutants at 2 and 3 dpf (Figure 23, col2a1 not shown). Further, both collagen gene expression patterns throughout the pharyngeal cartilages were consistent with sox10:GFP expression patterns in both WT and bti mutants of the same age. In WT embryos, collagen genes were expressed in a pattern consistent with the shape of the developing Meckel’s cartilage. However, collagen genes are expressed in bilateral rows that do not meet to form the presumptive Meckel’s cartilage shape in bti mutants (Figure 22 and 23).
Figure 23: Collagen genes are expressed in \textit{bti} mutants. Ventral views of \textit{col27a1} expression in fixed WT embryos and \textit{bti} mutants at 2 dpf (n = 3 WT, n = 3 \textit{bti}), 3 dpf (n = 18 WT, n = 24 \textit{bti}), and 4 dpf (n = 10 WT, n = 9 \textit{bti}). Anterior to the top. mc = Meckel’s cartilage. Scale = 100 µm.

\textbf{Discussion}

In WT embryos, the \textit{sec61a1} gene is expressed in highly secretory tissues. One such tissue is cartilage, which is largely comprised of collagen protein secreted by chondrocytes (Lang et al., 2006; Doll et al., 2011). The truncated Sec61a1 ER pore component protein present in \textit{bti} mutants likely prevents the ER pore from functioning. It is likely that newly synthesized collagen proteins are not transported into the ER, from where they are secreted into the extracellular matrix. Consistent with this hypothesis the ER volume is reduced in \textit{bti} mutant chondrocytes (Doll et al., 2011).
In order to successfully form Meckel’s cartilage, cranial neural crest cells must first migrate properly to the first pharyngeal arch and then differentiate into the chondrocytes that secrete a substantial amount of collagen protein into the extracellular matrix (Schilling and Kimmel, 1997; Knight and Schilling, 2006a; Doll et al., 2011). In our studies, sox10:GFP expression demonstrated that early cranial neural crest migration to the first pharyngeal arch was normal in bti mutants. However, beginning at 44 hpf, sox10:GFP expression in bti mutants suggests that cranial neural crest cells do not fully form Meckel’s cartilage. While bilateral streams of cranial neural crest cells move anteriorly and articulate to form the presumptive jaw cartilage in WT embryos, these cells remain in bilateral rows that do not articulate in bti mutants.

The timing of the bti mutant mandiblar phenotype suggests that the defect arises from an inability of bti mutants to form cartilage, as the onset of abnormal development coincides with the initiation of chondrification of the pharyngeal cartilages. sox10:GFP expression in bti mutants deviated from the WT pattern beginning at roughly 44 hpf while chondrification of the pharyngeal arches begins at roughly 45 hpf (Schilling and Kimmel, 1997).

Examination of cartilage specific collagen genes col27a1 and col2a1 demonstrated that cranial neural crest cells in the mandibular region initiated chondrification. Both genes were robustly expressed in the mutants even when expression patterns no longer coincided with those of WT embryos. However, previous work in our laboratory, using staining of the cartilage protein associated proteoglycans, demonstrated that the collagenous extracellular matrix itself is greatly
reduced in Meckel’s cartilage of bti mutants (P.-N. Lu and S. Brannan unpublished data). Collagen expression data suggests that collagen protein is likely synthesized in the chondrocytes of the presumptive Meckel’s cartilage of bti mutants. However, the collagen protein produced cannot enter the ER through and thus is not secreted into the extracellular matrix.

Further examination of the Meckel’s cartilage in bti mutants would determine the extent to which collagen protein is produced. Alcian blue stains the proteoglycans on collagen proteins and thus is indicative of collagen presence (Klymkowsky and Hanken, 1991). However, quantitative examination of collagen protein with Western Blots would determine the extent of collagen production in bti mutants.

It is also possible that the jaw phenotype in bti mutants results from disruptions in late cranial neural crest migrations that are related to extracellular morphogen signaling. For example, Sonic hedgehog (Shh) signaling at the midline of the prechordal plate mesoderm is known to play a role in mediating morphogenesis and chondrification of the craniofacial neural crest cells where they converge at the midline of the head (Wada et al., 2005). Thus, it is possible that cranial neural crest cells fail to articulate in bti due to disruptions in Shh signaling as well as other morphogen signaling. However, it is still likely that a decrease in collagen protein secretion contributes to the bti jaw phenotype as the sec61al1 mutation likely disrupts the ability of chondrocytes to transport proteins into the ER.
V. Discussion

Through the studies described in this thesis, I examined spatial requirements for development of the zebrafish pineal complex and surrounding epithalamus. In Chapter 2, my work demonstrated that localization to the dorsal midline of the brain is not required for differentiation of pineal cell types (Lu et al., 2013). This finding supports a model for autonomous specification of pineal cell types, initiated by expression of the transcription factor flh in the neuroectoderm prior to neural tube closure. Further, differentiated photoreceptors are functional in embryos with open neural tubes.

The research described in Chapter 3 demonstrated that establishment of left-right asymmetry within the zebrafish epithalamus does require neural tube closure. Embryos with widely open neural tubes exhibited left isomerization of all the characteristic left-right asymmetries within the epithalamus (Lu et al., 2013). This finding suggests the mechanism that establishes left-right asymmetry in the zebrafish brain involves a short-range diffusible signal that prevents the right side from developing with left-sided characteristics. I propose that Lft1 may be this diffusible signal and that Nodal signaling may work in a reaction-diffusion mechanism to pattern left-right asymmetry in the brain.

Chapter 4 of my thesis describes preliminary characterization of the abnormal jaw phenotype in zebrafish bti mutants. I demonstrated that while early neural crest cell migration to the pharyngeal arches is normal, late migration to form the jaw is disrupted. However, chondrification is initiated in the jaw as collagen genes were expressed. We hypothesize that the reduction in Meckel’s cartilage formation in bti
mutants results from the failure of cranial neural crest derived chondrocytes to secrete the collagenous extracellular matrix required to form Meckel’s cartilage.

**Autonomous specification of pineal cell types in the neuroectoderm.**

The vertebrate brain is a valuable model tissue in which to study spatial requirements for cell differentiation as it begins as a flat neural plate epithelium that folds into a neural tube (Lowery and Sive, 2004). While an undifferentiated cell in the neural plate begins in a given location with respect to the left-right and dorsal-ventral body axes, it often differentiates in a new position after the neural plate folds into the neural tube. For instance, pineal precursor cells that begin at the lateral edges of the neural plate eventually converge into one tissue at the dorsal midline of the brain (Masai et al., 1997; Concha and Wilson, 2001; Aquilina-Beck et al., 2007). Because these spatial changes subject the cells to different concentrations of signaling molecules, such as those that originate in the overlying epidermal ectoderm or underlying notocord, localization to the dorsal midline may play an important role in correctly initiating their differentiation into mature projection neurons and photoreceptors.

Differentiation of many cells within the dorsal ventral axis of the neural tube relies on the interplay of BMP and Shh morphogen gradients (Tanabe and Jessell, 1996; Nguyen et al., 2000b; Nishi et al., 2009; Alaynick et al., 2011). Given the dorsal position of the pineal gland in the neural tube, one hypothesis is that these gradients are involved with differentiation of pineal cell types. BMP signaling in the overlying ectoderm initiates BMP expression in the roof plate of the neural tube. As
BMP diffuses ventrally throughout the neural tube, it initiates a BMP signaling cascade that is high in the dorsal neural tube and low in the ventral neural tube (Nguyen et al., 2000b). Similarly, Shh signaling in the ventral brain diffuses dorsally creating a second, opposing gradient (Nishi et al., 2009; Alaynick et al., 2011). Cells of the dorsal neural tube are exposed to high levels of BMP signaling at an earlier time than those of the ventral neural tube. These cells are specified as neural crest or sensory neurons (Nguyen et al., 2000b). Conversely, neural tube cells adjacent to the floor plate are exposed to high Shh and virtually no BMP signaling, which establishes ventral identity. The neural tube cells that lie just dorsal to these are exposed to less Shh and slightly more TGFβ signaling, which induces motor neuron identity (Nishi et al., 2009; Alaynick et al., 2011).

Loss of either BMP or Shh results in loss of associated neuron types (Nguyen et al., 2000b). Embryos that are deficient in BMP signaling have reduced neural crest and dorsal sensory neurons of the spinal cord but exhibit no change to the number of the ventral motor neurons (Nguyen et al., 2000b). Embryos that are deficient in Shh exhibit loss of ventral and motor neurons with an increase in neural crest and dorsal sensory neuron production (Nguyen et al., 2000b).

These BMP and Shh signaling gradients could act to conditionally specify pineal cells. In this model, the pineal precursors cells would require interaction with adjacent cells for correct specification. If this hypothesis is correct, lateral displacement of pineal precursors in embryos with open neural tubes may disrupt BMP signaling and therefore pineal cell differentiation.
However, pineal cells are some of the first neurons in the brain to differentiate. Thus, a second hypothesis is that pineal cell differentiation is initiated prior to neural tube closure by flh expression in the pineal precursor domains (Masai et al., 1997). flh expression in the neural plate is required for differentiation of most pineal cells (Masai et al., 1997). In mbl mutants with expanded flh expression, pineal cell production is also expanded. Conversely, pineal cell production is reduced in embryos that lack flh (Masai et al., 1997). In WT embryos, flh is expressed in the pineal precursor domains at the time of neural plate formation in the ectoderm, suggesting that initiation of pineal cell differentiation may also occur at this time (Masai et al., 1997).

My work suggests that localization to the dorsal midline of the brain is not required for pineal cell differentiation. Both projection neurons and photoreceptors differentiated in laterally displaced pineal anlage in embryos with open neural tubes (Lu et al., 2013). Further, differentiated photoreceptors were functional as they responded to light cues, as evidenced by rhythmic expression of aanat2 in divided pineal anlage (Lu et al., 2013). These results support the model in which flh initiates pineal cell differentiation prior to neural tube closure. Consistent with this, flh is expressed in the elongated and divided pineal domains of embryos with open neural tubes (Aquilina-Beck et al., 2007). This suggests that pineal cell types are specified autonomously through flh expression and not conditionally through interaction with adjacent cells.

However, it is possible that signaling from the dorsal midline of the brain may play a role in differentiation of a subset of pineal neurons. While flh initiates
differentiation of most pineal cells, some still differentiate in embryos that lack \textit{flh}. The pineal remains as a single-layered neuroepithelium with only a few differentiated pineal cells (Masai et al., 1997). These \textit{flh} independent cells are at least partially functional as they express \textit{aanat2}. Further, this \textit{flh} independent subset of cells is capable of circadian entrainment and rhythmic \textit{aanat2} expression persists in constant dark conditions (Gamse et al., 2002).

Examination of pineal cell differentiation in embryos with open neural tubes that also lack \textit{flh} would determine if neural tube closure is required for differentiation of \textit{flh} independent pineal cells. \textit{flh/sqt} double mutants should exhibit both open neural tube phenotypes as well as reduced production of pineal neurons (Feldman et al., 1998; Gamse et al., 2003). Examining differentiation of these \textit{flh} independent cells in embryos that exhibit open neural tube phenotypes would determine if this subset of cells requires localization to the dorsal midline of the brain for differentiation (Masai et al., 1997).

Differentiation of this \textit{flh} independent subset of neurons in \textit{flh/sqt} mutants with open neural tubes would suggest that localization to the dorsal midline is not required for differentiation of any pineal cells. This would further suggest that a BMP signaling gradient may not play a role pineal cell differentiation. However, if no pineal neurons in \textit{flh/sqt} mutants differentiate, this would suggest that the \textit{flh} independent pineal cells do require localization to the dorsal midline of the brain for differentiation. This requirement of neural tube closure in the differentiation of \textit{flh} independent pineal cells suggests that BMP singling may initiate their differentiation.
Further examination of BMP signaling in embryos with open neural tubes would determine if this gradient is actually disturbed in embryos with open neural tubes.

**Zebrafish pineal tissue may function autonomously**

One of the pineal gland’s major functions is the rhythmic production of the circadian hormone melatonin (Gothilf et al., 1999). The *aanat2* gene, which encodes the penultimate enzyme in the melatonin biosynthetic pathway, is also expressed in rhythmic patterns (Klein et al., 1971). Expression of *aanat2* is high at night and low during the day, a reflection of high melatonin production at night and low production during the day (Gothilf et al., 1999; Gothilf et al., 2002). In the zebrafish pineal, initiation of *aanat2* expression and entrainment of these rhythms requires dark to light transition stimuli. These light signals are transduced into neurendocrine signals by the pineal photopigment Exorh (Mano et al., 1999; Pierce et al., 2008).

It is possible that circadian rhythms are established in pineal tissues of embryos with open neural tubes. My work demonstrated that *aanat2* was rhythmically expressed in the divided pineal anlage of embryos with open neural tubes (Lu et al., 2013). This expression, in conjunction with the presence of Exorh, indicated that pineal photoreceptors were functional and responded to light cues in embryos with open neural tubes. However, as explained in Chapter 2, we cannot determine if this rhythm is entrained and circadian in nature as these embryos were raised in a light/dark cycle. Given the photosensitive capabilities of the zebrafish pineal gland, it is possible that this rhythmic expression of *aanat2* is a passive response to light/dark transitions.
To determine the extent of circadian entrainment in the pineal anlage of embryos with open neural tubes, it is necessary to conduct constant condition experiments and analyze rhythmic gene expression. Persistence of rhythmic *aanat2* expression in embryos with open neural tubes raised in all light or all dark conditions would demonstrate that differentiated pineal tissues are capable of circadian entrainment despite failure to form a single tissue. Conversely, loss of *aanat2* rhythmic expression would suggest that convergence of the pineal precursors and formation of one tissue is necessary for establishment of circadian rhythms.

It is also possible that the pineal circadian rhythms function autonomously. Some embryos with open neural tubes exhibit a rare fragmented pineal phenotype in which pineal cells are found in three or more domains throughout the dorsal forebrain. Examination of circadian entrainment in these embryos would provide further information as to the autonomy of pineal tissues. Entrained rhythmic gene expression in three or more pineal domains would further suggest that pineal cells do not require convergence into a single tissue to initiate circadian function.

*A potential role for Lft1 in the establishment of epithalamic asymmetry*

While localization to the dorsal midline is not required for pinealocyte differentiation, it does play an important role in patterning left-right asymmetry in the pineal gland and surrounding epithalamus. In embryos with open neural tubes and widely spaced pineal domains, both sides develop with left-sided characteristics (Lu et al., 2013). However, not all embryos with open neural tubes exhibit left isomerism. Some embryos develop with normal or reversed epithalamic asymmetry (Lu et al.,
We noticed that the penetrance of the left isomerized phenotype was higher in the more severe Nodal deficient embryos compared to the milder cdh2 mutants. This led to the hypothesis that the severity of the open neural tube defect correlated to loss of asymmetry. My work demonstrated that, in both sqt and cdh2 mutant, embryos with left isomerism had pineal domains that were significantly wider than those embryos with asymmetry (Lu et al., 2013).

The correlation between pineal width and left isomerism suggests that the distance of the pineal anlage plays a role in establishment of zebrafish epithalamic asymmetry. In Chapter 3 of this thesis, I suggested a model in which a signal from the left side of the pineal gland diffuses to inhibit the right side from developing with left-sided characteristics. This signal must have a limited diffusion range as the mechanism fails when the pineal domains are widely spaced. Further, I proposed Lft1 is a good candidate to be this signaling and that Cyc and Lft1 may be acting in a reaction diffusion mechanism in the pineal gland. Testing the hypothesis that Lft1 is the signal that prevents left isomerism in the epithalamus is an important future direction for this research.

In order to examine the hypothesis that Lft1 prevents left isomerism in the epithalamus, it is first necessary to examine Cyc and Lft1 protein in the pineal gland. While our current results demonstrate where cyc and lft1 genes are expressed, the diffusible nature of these proteins means they are not confined to the region in which their mRNAs are located. Immunostaining for Cyc and Lft1 protein would provide a clearer picture of where these proteins are acting in WT embryos and embryos with open neural tubes. Both cyc and lft1 are initially expressed only on the left side of the
pineal gland in WT embryos. If Cyc and Lft1 are acting in a reaction diffusion mechanisms, we predict that Cyc protein will remain largely in the left side of the pineal gland as it has a short diffusion range (Chen and Schier, 2002). We further expect that Lft1 protein will be diffuse throughout both the left and right sides as it has a diffusion range of many cell diameters (Chen and Schier, 2002). Lft1 protein diffusing across the pineal domain would quickly inhibit any Cyc protein that diffused into the right, maintaining Nodal signaling asymmetry between the left and right sides.

In embryos with mild open neural tube defects, Nodal related genes are initially expressed in both sides of the pineal gland. However, later in development these embryos develop randomized sidedness, with either the left or right side developing a parapineal. We predict that in these embryos with mild open neural tube defects, Cyc will be higher on one side than the other but present in both sides. Similarly, we predict that Lft1 will be present on both sides but in relatively equal levels because of its higher rate and distance of diffusion. The side with higher Cyc protein would eventually develop with left sided identity as Lft1 diffusing from both sides will inhibit Cyc signaling on the side with endogenously less Cyc. In embryos with severe open neural tube defects we would expect to see Cyc and Lft1 again in both the left and right sides of the pineal. However, Lft1 protein should be diffuse across the distance between pineal anlage.

To further determine if Lft1 is the signal that prevents left isomerism in the epithalamus, we need to examine development of epithalamic asymmetry in embryos that lack Lft1. We hypothesize that these embryos should exhibit left isomerism at a
high rate in embryos with closed neural tubes. Specifically, in a \textit{lft1} mutant, \textit{cyc} should be expressed in both sides of the closed pineal with no Lft1 to spatially restrict Cyc signaling. Bilateral Nodal signaling would instruct formation of bilateral parapineals, which in turn would instruct development of two left-identity habenulae. Two \textit{lft1} mutants have been generated but they are not yet available to the public (Zebrafish Model Organism Database; http://zfin.org/action/mutant/mutant-list?zdbID=ZDB-GENE-990630-10).

While a \textit{lft1} mutant would be a useful tool for examination of epithalamic asymmetry, it may present challenges. Lft1 protein is important for controlling development of the embryonic organizer, the structure that directs development of the body axis, during gastrulation stages (Meno et al., 1999; Feldman et al., 2002b). Transplantation experiments in mice, xenopus, and zebrafish have demonstrated that a second organizer induces formation of an entire second body axis in the developing embryo (Beddington, 1994)(Saude et al., 2000). High Nodal signaling on the dorsal side of the embryos induces organizer formation in dorsal mesoderm (Gritsman et al., 2000). Nodal inhibitors Lft1 and Lft2 are necessary to spatially restrict Nodal signaling to this region during formation of the organizer (Bisgrove et al., 1999; Meno et al., 1999; Feldman et al., 2002b). Nodal expression in the dorsal mesoderm is likely to be expanded in a \textit{lft1} mutant, resulting in an expanded organizer (Feldman et al., 1998; Meno et al., 1999; Gritsman et al., 2000; Feldman et al., 2002b) When the organizer is expanded in embryos with overexpression of \textit{nodal} genes, dorsal and anterior mesodermal structures are also expanded while ventral and posterior structures are reduced (Schier and Talbot, 2001).
Expansion of the organizer and dorsalization in \textit{lft1} mutants is not likely to be a problem for studying development of asymmetry in the epithalamus. Embryos depleted of both Nodal inhibitors, Lft1 and Lft2, with Morpholino injections also exhibit an expanded organizer due to loss of Nodal regulation (Feldman et al., 2002b). These embryos exhibit a loss of the ectoderm and expansion of mesoderm and most die within the hours following gastrulation (Feldman et al., 2002b). However, embryos depleted of only Lft1 exhibit a milder phenotype, which includes a reduced head size (Feldman et al., 2002b).

It is likely that the epithalamus will still develop in \textit{lft1} mutant embryos. \textit{cyc} and Nodal target gene \textit{pitx2} are expressed bilaterally in the brain in Lft1 depleted embryos (Feldman et al., 2002b). This suggests that the tissues in which we are interested still develop when Lft1 is absent during gastrulation. Further, it suggests that \textit{cyc} is likely expressed in both the left and right sides of the brain in \textit{lft1} mutants. Development of bilateral parapineals in a \textit{lft1} mutant would suggest that Lft1 is necessary for development of left-right asymmetry development of asymmetry in the epithalamus.

\textit{Reduced collagen production likely causes reduced jaw development in \textit{bti} mutants.}

In Chapter 4 of this thesis I described preliminary work examining the reduced jaw phenotype of the zebrafish \textit{bti} mutant line. Alcian blue cartilage staining by previous members of our lab demonstrated cartilage is reduced in the lower jaws of \textit{bti} mutants (P.-N. Lu and S. Brannan, unpublished data). Little to undetectable amounts of cartilage formed between 3 to 6 dpf in \textit{bti} mutants. However, early
migration of the cranial neural crest cells, from which Meckel’s cartilage is derived, was normal (P.-N. Lu and S. Brannan, unpublished data).

The timing of the reduced bti jaw phenotype suggests that cranial neural crest cells fail to undergo complete chondrification. While early cranial neural crest cell migration to the pharyngeal arches was normal in bti mutants, the pattern of migration deviated from the WT pattern beginning at 44 hpf. Bilateral streams of cells moved anteriorly from the pharyngeal arches to articulate in the shape of Meckel’s cartilage by 66 hpf in WT embryos. These bilateral streams of cells remained separate in bti mutants and the pattern did not form the presumptive Meckel’s cartilage shape even by 66 hpf. The timing of this phenotype coincides with the initiation of chondrification in the pharyngeal arches, which initiates at roughly 45 hpf in WT embryos (Schilling and Kimmel, 1997).

To determine if a disruption in chondrification results in reduction of Meckel’s cartilage, I examined cranial neural crest-derived chondrocyte differentiation. Robust expression of both collagen col27a1 and col2a1 genes in the presumptive pharyngeal cartilages, including Meckel’s cartilage, suggests that cranial neural crest cells initiate differentiation into chondrocytes. However, the sec61al1 mutation likely reduces ER pore function and newly synthesized collagen proteins are not transported into the ER. Consistent with this, ER volume is reduced in bti mutants (Doll et al., 2011). This preliminary work provides strong evidence for the hypothesis that the bti mutant jaw phenotypes results from an inability of cranial neural crest derived chondrocytes to secrete collagen into extracellular matrix, which is necessary
for cartilage formation. Thus, further examination of collagen protein is necessary to characterize the \textit{bti jaw} phenotype.

While we predict that there should be an overall decrease in collagen protein production in the \textit{bti} mutants, robust \textit{col27a1} and \textit{col2a1} expression and Alcian blue cartilage staining suggest that some collagen protein is likely still produced. Planned Western blotting experiments will allow us to quantify differences in collagen protein between WT embryos and \textit{bti} mutants. Both \textit{col27a1} and \textit{col2a1} genes are expressed only in the head, of zebrafish embryos at 2 to 4 dpf. Thus, we can remove the heads and examine collagen in the craniofacial cartilages only.

Further, immunostaining for collagen would give us a clearer idea of where collagen protein is present in \textit{bti} mutants. If inability to secrete collagen into the extracellular matrix causes the \textit{bti} jaw phenotype, it is possible that collagen protein is produced but is not secreted. Staining embryos with an ER marker in addition to collagen antibody would determine if the collagen that is produced enters the ER from where it can be secreted. If collagen protein remains outside the ER, this would suggest that it cannot be moved across the ER membrane. This would support the hypothesis that the \textit{sec61al1} mutation renders the ER pore nonfunctional and demonstrate further that the \textit{bti} jaw phenotype results likely from decreased collagen protein secretion.

\textit{Patterning as an integrating principle in biology}

In this thesis, I have described the way in which Nodal signaling proteins are involved in both positive and negative feedback mechanisms in the developing
embryo (Liang and Rubinstein, 2003a). Further, I have proposed a reaction-diffusion model by which these Nodal feedback mechanisms may pattern the zebrafish epithalamus. Similarly, feedback mechanisms can pattern ecosystems. Nutrient use by below ground soil microbes has been suggested as a feedback mechanism that patterns plant abundance (Bardgett et al., 2005). Fungal soil food webs have a higher rate of nutrient conservation and cycling. The abundance of soil nutrients positively feedback to promote plant abundance (Bardgett et al., 2005). This abundance in turn positively affects decomposition and fungal nutrient cycling. Conversely, bacterial soil food webs have a higher rate of nutrient loss than fungal webs. This loss of nutrients negatively influences plant abundance and therefore decomposition (Bardgett et al., 2005).

Throughout the developing embryo, concentration gradients of signaling proteins instruct cellular differentiation and tissue patterning. For example, BMP proteins produced in the dorsal neural tube diffuse ventrally creating a gradient in the neural tube while Shh proteins from the ventral neural tube diffuse dorsally, patterning cells within the neural tube (Nguyen et al., 2000b; Nishi et al., 2009; Alaynick et al., 2011). Similarly, ecological gradients pattern microbial, plant, and animal populations (Müller, 1998; Pausas, 2001). Altitude, temperature, water, and nutrient gradients all pattern species richness in predictable manners. For example, low nutrient availability typically corresponds to lower species richness while moderate availability corresponds to higher species richness (Pausas, 2001).
Understanding patterning mechanisms is an integrating principle throughout biology. In this thesis, I examined mechanisms of differentiation and patterning of cells that originate in the zebrafish neuroepithelium. My work demonstrates the relevance of patterning at the cell and tissue level in the zebrafish embryo. However, some of the mechanisms of patterning I identified are applicable to a spectrum of biological areas. Thus, my work identifying of the underlying mechanisms that pattern tissues in the developing embryo will be relevant for studies of the mechanisms that pattern cells, tissues, animal and plant populations, and ecosystems.
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