ZEBAFISH EPITHALAMUS AS A MODEL SYSTEM FOR STUDYING CIRCADIAN RHYTHMS AND LEFT-RIGHT ASYMMETRY

by

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To my family,

for their support and love
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Zebrafish Epithalamus as a Model System for Studying Circadian Rhythms and Left-Right Asymmetry

Abstract

by

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This dissertation is composed of three major contributions focusing on the circadian and asymmetric properties of zebrafish. First we demonstrated that the gene expression rhythms could be established in the pineal organ of cyc mutant larvae lacking the Suprachiasmatic nucleus (SCN) structure. We also found that such rhythmic gene expression pattern could be maintained under constant environments. However, the amplitudes of the expression rhythms were decreasing by the end of the second day under constant environments. These findings indicate that the zebrafish SCN is not required for establishing the circadian rhythms of the pineal organ, but may be needed for maintaining the rhythms. These findings also further support the theory that unlike the highly centralized mammalian circadian system, circadian rhythms in zebrafish are decentralized [Chapter 2 and (Noche et al., 2011)].

We then demonstrated that when the neural tube failed to close and resulted in divided epithalamic structures, the left-right (L/R) asymmetric characteristics of the
epithalamus become left-isomerized. Normally asymmetrically expressed Nodal pathway genes became bilaterally expressed on both sides of the divided pineal organ. Also, normally left-located parapineal organ were observed on both sides, and both habenula nuclei exhibited a “left” pattern of gene expression. In contrast, other aspects of pineal development, such as expression of photoreceptor, projection neuron, and circadian related genes, remained normal. These findings indicate that a closed neural tube is required for the establishing of epithalamus left-right asymmetry. Our results also imply a previously unknown short-ranged mechanism that prevents the target tissues from acquiring left characteristics during the epithalamus L/R asymmetry establishment (Chapter 3).

We also contributed in characterizing a novel zebrafish mutation sec61al1c163, which was originally identified for affecting the habenular complex L/R asymmetry. In this ongoing study we demonstrated that the sec61al1c163 mutation only has minor effects on the pineal organ structure. We also found that the neural crest derived craniofacial structures in the mutant embryos were severely defective, likely caused by failure in chondrocyte differentiation (Chapter 4).
Chapter 1

Introduction
The role of suprachiasmatic nucleus (SCN) in the decentralized zebrafish circadian system

Introduction to circadian rhythms

Circadian rhythms are changes in gene expression, biochemistry, physiology and behavior that cycle with a roughly 24 hour period (Merrow et al., 2005; Pegoraro and Tauber, 2011). Circadian rhythms can be found in almost every species from prokaryotic organisms (Huang and Grobbelaar, 1995) to plants (Gardner et al., 2006) to animals (Pando and Sassone-Corsi, 2002). One of the most obvious examples of circadian rhythms is that diurnal (day-active) animals are active during the day and sleep during the night. Many other parameters in different species are also oscillating with circadian character. For example, in diurnal mammals, body temperature is higher during the day (Refinetti and Menaker, 1992). In plants, leaf movement and photosynthetic activity are rhythmic (McClung, 2006). In life forms as simple as the cyanobacterium Synechococcus, a prokaryotic organism, the processes of photosynthesis and nitrogen fixation are separated to opposite times of the day. Therefore, individual compartments are not required to separate these incompatible processes (Huang and Grobbelaar, 1995).

Circadian rhythms are the external expression of an internal timing mechanism that are entrained by daily environmental cues

The essential elements to compose a functional circadian rhythm include the ability to entrain the phase of rhythmic characters with environmental cues, the presence of an endogenous oscillator with a period of approximately 24 hours, and clock-controlled outputs that direct all the downstream physiological rhythms. It is important to
note that while the circadian rhythms can be entrained by environmental cues, circadian rhythms are not just passive responses to daily environmental changes. Instead the endogenous oscillator is capable of driving the rhythmic characteristics in constant conditions without environmental cues.

The endogenous oscillator provides adaptive advantages since the rhythms allow an individual to predict and prepare for the daily changes in the environment, instead of only passively respond after the changes have happened (Merrow et al., 2005). The importance of circadian rhythms is supported by the fact that certain forms of circadian characters can be found in almost every species, even though the clock component proteins between plants, fungi and bacteria are largely unrelated by sequence, suggesting convergent evolution (Merrow et al., 2005).

While circadian rhythms can persist in constant conditions, the rhythms may gradually become desynchronized with the normal daily environmental changes because the endogenous clock often does not run on a perfect 24 hours cycle. For example when zebrafish larvae were placed in constant environment, the average length of time between the onset of the peaks of locomoter (moving from place to place) activity is 25.6 hours, with standard deviation up to one hour (Cahill et al., 1998). In an extreme case reported recently, a Somalian cavefish *Phreatichthys andruzzii*, which has evolved in complete darkness for millions of years, has lost the ability of being entrained by light but still retains certain behavioral and biochemical rhythms. For example feeding behavior and circadian expression pattern of several clock component genes can be observed, although the rhythms have a 47 hours period, which is significantly longer than the usual 24-hour cycle and is not responsive to light/dark environmental cues (Cavallari et al., 2011).
In normal conditions, to prevent the endogenous clock from becoming out of synchronization with the environment, certain input pathways have to entrain the endogenous clock. While photoreception is likely the most intensively studied mechanism, other environmental cues are also known to play roles. For example, feeding, temperature and sound are capable of entraining circadian rhythms in different animal species (Lopez-Olmeda et al., 2010; Menaker and Eskin, 1966; Sehadova et al., 2009).

**Molecular bases of the endogenous clock in vertebrates**

The current model for the vertebrate circadian rhythms mechanism is composed of feedback loops, in which the transcription factor complex CLOCK/BMAL (BMAL1 in mammals) activates the transcription of multiple *period* (*per*) and *cryptochrome* (*cry*) genes. The PER and CRY proteins then form complexes and bind to the CLOCK/BMAL complex and inhibit their transcription factor activity (Figure 1) (Ishida et al., 1999). Due to differences in protein stability, the PER/CRY complexes eventually degrade after binding to the CLOCK?BMAL complex and the CLOCK/BMAL complex can be active again (Okamura et al., 2002).

Zebrafish homologs of all the major mammalian clock component genes including *clock*, *bmals* (*bmal1*, *bmal2*), *periods* (*per1*, *per2*, *per3*) and *cryptochromes* (*cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3*, *cry4*) have been discovered (Cahill, 2002). Based on current results, it is argued the molecular mechanism of the endogenous clock in zebrafish has much in common with the mammalian system, but is different in details (Cahill, 2002).
One example of the differences between the homologous zebrafish and mammalian clock component genes is that mammalian *clock* is constantly expressed in the suprachiasmatic nucleus (SCN), the central pacemaker of mammalian circadian system. In contrast, zebrafish *clock* mRNA is known to be rhythmically expressed in all tissues tested, with an expression peak in the early night and a low point near dawn (Whitmore et al., 1998).

Another example of the differences between the homologous zebrafish and mammalian clock component genes is the rhythmic expression patterns of *bmal*. When comparing the expression of mammalian and zebrafish *bmal* genes, while both the mammalian and zebrafish *bmals* are rhythmically expressed, the peak and low points of the mammalian and zebrafish *bmals* are significantly different (Honma et al., 1998; Shearman et al., 2000). Furthermore, within zebrafish, the expression patterns of *bmal1* and *bmal2* are also roughly synchronized with the expression of *clock* (Cahill, 2002) but with minor difference between each other (Cermakian et al., 2000). From the aspect of protein activities, although both of zebrafish BMAL proteins have transcriptional activity and can bind to zebrafish CLOCK protein, their CLOCK-binding affinities and transcriptional activities are not the same, suggesting that the two *bmal* genes are not fully redundant (Cermakian et al., 2000).

Three *per* genes and six *cry* genes have been identified in zebrafish. While all three *per* genes are expressed rhythmically, the rhythmic expression of zebrafish *per2* is light-dependent and cannot be maintained in constant conditions, which is different from the case of mammals (Shearman et al., 1997). The six zebrafish *cry* genes are also expressed rhythmically with three different circadian expression patterns (Cahill, 2002).
Although zebrafish are known to have two paralogs of many mammalian single-copy genes due to a whole-genome duplication event during evolution (Postlethwait et al., 1998), this large number of cry genes with diverse expression patterns and functions suggests that zebrafish cry genes are not simply redundant. However, the detailed roles of each of the zebrafish cry genes are not fully understood.

**Mammalian circadian rhythms are highly centralized**

The SCN is part of the hypothalamus of the brain. In mammals, SCN is often described as the major endogenous oscillator, or the pacemaker, that coordinates the circadian rhythms of the whole organism (Merrow et al., 2005). For example, introduced lesions in the rat hypothalamic area (where the SCN locates) leads to the loss of circadian rhythms in drinking behavior and locomotor activity (Stephan and Zucker, 1972). Moreover, when the SCN tissue from a donor hamster with a circadian period different from the recipient was transplanted to another SCN ablated hamster, the circadian rhythms were restored and exhibited the period of the donor (Lehman et al., 1987).

One of the mechanisms for mammalian SCN to synchronize circadian rhythms with environmental cues is the photoneuroendocrine pathway. In the photoneuroendocrine pathway, the photoreceptor cell in retina receives light signals from the environment and sends the information to the major endogenous oscillator, the SCN. SCN then controls the melatonin biosynthesis in the pineal organ via a complex chain of neuronal transduction (Korf, 1994; Korf et al., 1998; Moore, 1996). The increased level of melatonin consequently leads to night time behaviors including drowsiness and lowering of the body temperature in diurnal mammals.
While the mammalian SCN is crucial for the generation of biological rhythms throughout the organism, the self-sustainable rhythmic expression of clock component genes is not unique to the mammalian SCN. It is known that rhythmic expression of some of the clock component genes can be found in mammalian peripheral tissues including liver, endocrine tissues, the heart and the skeletal muscles (Bell-Pedersen et al., 2005; Shearman et al., 1997; Yamazaki et al., 2000; Zylka et al., 1998). Such results suggest that on the level of molecular mechanism, these peripheral oscillators are similar to the SCN oscillator.

However, one crucial difference between the SCN and other peripheral oscillators is that SCN contains the only known mammalian oscillators that can be entrained by light, suggest a hierarchical relationship between the SCN and other peripheral oscillators (Bell-Pedersen et al., 2005). Also, in SCN-lesioned mice, circadian rhythms of gene expression within different tissues are remained, but the synchrony of phase among the tissues of individual animals is lost (Yoo et al., 2004). Another evidence supporting the hierarchical model is that mammalian peripheral oscillations are phase-delayed by 4–12 hours relative to the CSN (Zylka et al., 1998), suggesting that the role of SCN is to coordinate the internal circadian mechanisms with the outside world (Bell-Pedersen et al., 2005).

**Zebrafish circadian rhythms are decentralized**

Contrary to the highly centralized mammalian circadian mechanism, evidence suggests that the circadian mechanism is more decentralized in zebrafish. Cultured tissues and cell lines from heart, kidney, spleen, eye, pineal and other parts of brain not only
exhibit rhythmic gene expression, but can also be directly entrained by light (Cahill, 2002; Pando et al., 2001; Whitmore et al., 2000; Whitmore et al., 1998). For example, manipulating the phase of light-dark cycle led to altered expression pattern of clock in cultured zebrafish heart and kidney tissues (Whitmore et al., 2000). Also, results from zebrafish embryonic cell line revealed that per1, per2, and per3 all response to an altered light-dark cycle (Pando et al., 2001). Furthermore, while opsin proteins, the light sensitive molecular responsible for photoreception, are only found in the retina of mammals (Shichida and Matsuyama, 2009), other opsins, for example the teleost multiple tissue (tmt) opsin can be found in a wide range of tissues including brain, heart, liver and kidney in zebrafish (Kojima and Fukada, 1999; Mano et al., 1999; Moutsaki et al., 2003).

**Research design**

In mammals, the SCN is required for the entraining and maintaining the circadian rhythms in the peripheral, while in vitro results suggest that zebrafish tissues with circadian rhythms can be entrained by light-dark changes directly. Interestingly, a SCN-like area has been described in zebrafish brain and both the retina and the pineal organ project to this SCN-like area (Yanez et al., 2009). However, before the studies described in this dissertation, the role of zebrafish SCN as a major endogenous oscillator was rarely investigated.

Chapter 2 of my dissertation describes our investigation of the role of SCN in pineal circadian rhythms in developing zebrafish. We took advantage of the mutant line cyclops (cyc), which is missing the ventral brain structures (Sampath et al., 1998). We found that the lost brain structures in the cyc mutants include the anterior hypothalamus
that are proposed to be the precursors of the SCN. We also found that despite the loss of SCN, the pineal rhythms in cyc larvae, similar to the WT larvae, are still retained under normal light-dark cycles. Furthermore, when kept in constant light or constant dark environments after being entrained by normal light-dark cycles, both WT and cyc larvae retained the pineal circadian rhythms, suggesting that SCN is not required to initiate the pineal circadian rhythms. Our results also indicate that when the embryos are kept in constant environment the peak of the rhythms decrease more quickly in cyc embryos. Such finding suggests that SCN may be required for maintaining the pineal circadian rhythms.

**Neural tube closure and the establishment of epithalamic asymmetry**

*Left-right (L/R) asymmetric characters of vertebrates*

For free moving animals, it is logical to hypothesize that bilaterally symmetric structure is adaptive since symmetrically placed limbs are efficient for linear movement. Also, structurally asymmetric animals with a “weak side” are not likely to be favored by the environment with conserved parity (Corballis, 2009). However, while the appearance of most vertebrate bodies are roughly left-right symmetric, their internal structures are significantly asymmetric. For example, during cardiac lateralization, an embryonic heart is transformed from a straight tube-like structure into a c-shaped loop (Taber, 2006). Theoretically, the convexity of this loop can point toward either side of the embryo. However in all vertebrate species studied, a majority of the individuals have the convexity of the heart loop points toward the right side (Manner, 2004). The structure of the lungs and the positions of the liver and gastrointestinal tract are also left-right
asymmetric (Varlet and Robertson, 1997). It is argued that such visceral asymmetry may allow more efficient packaging of the organs (Corballis, 2009; Hamada et al., 2002).

Structural and functional asymmetries in the brain are also observed in a wide variety of vertebrates including fish, birds and mammals (Corballis, 2009). It is argued that since brain left-right asymmetries are relatively common in different vertebrate species, such asymmetries likely lead to certain adaptive advantages (Corballis, 2009). One theory is that bilaterally symmetric control requires the relatively slow communication between hemispheres, while unilateral computations can be carried out with greater speed (Ringo et al., 1994). Another theory is that for highly complex tasks, which already require a significant amount of neural connection, duplication in the two hemispheres may lead to waste of the brain process power or inter-hemispheric conflict (Foundas et al., 2003).

**Cellular and molecular bases of vertebrate asymmetry**

The conceptual mechanism of establishing left-right asymmetry can be divided into four steps (Brown et al., 1990, 1991; Hamada et al., 2002) (Figure 2, based on Hamada et al., 2002). In the first step, the initial symmetry is broken. The second step is a relaying mechanism that transfers the initial break of symmetry events to the third step, asymmetric gene expression pattern. In the fourth step, the asymmetric morphology is formed.

Based on the current model of how asymmetry is established in mammals, the anterior tip of the primitive streak that forms during the gastrulation, also called the node, is responsible of the initial breaking of the left-right symmetry (Hamada et al., 2002;
Takeda et al., 1999). Each cell on the ventral side of the node has a monocilium which rotates clockwise (Sulik et al., 1994; Takeda et al., 1999). The clockwise rotation creates a leftward flow of extra-embryonic fluid. The flow then leads to increased Ca$_2^+$ concentration in one side of the node and initiates the downstream signaling pathways to establish left-right asymmetry (Hamada et al., 2002; Hirokawa et al., 2006).

While this clockwise rotation of the monocilium in the node or its equivalent is conserved in fish, birds and mammals (Essner et al., 2002; Hirokawa et al., 2006), evidences suggest that for amphibians and birds, the rotation of the monocilium is unlikely to produce a fluid flow due to the position of the cilia (Essner et al., 2002). Also, left-right asymmetry is already established before the node equivalent structure is presented in frog and chick (Hirokawa et al., 2006; Yost, 1991). It has been observed in frog that maternal mRNA of H$^+/K^+$-ATPase is already asymmetrically localized in the eggs during the initial two cleavages, and this asymmetric distribution of H$^+/K^+$-ATPase is essential for determining the left-right asymmetry (Levin et al., 2002). It is also hypothesized that the membrane potential asymmetry caused by the asymmetric distribution of the H$^+/K^+$ ATPase transporters is responsible for establishing the early left-right asymmetry in amphibians (Hirokawa et al., 2006; Levin et al., 2002).

In the case of fish, both the nodal flow at neurulation stages and the differential activity of H$^+/K^+$ ATPase at early cleavage stages mechanisms are required for correctly establishing L/R asymmetry (Essner et al., 2005; Kawakami et al., 2005; Kramer-Zucker et al., 2005). Also, the disrupting the H$^+/K^+$ ATPase activity led to L/R asymmetry defects without affecting the nodal flow, supporting the hypothesis that the two mechanisms are independent (Kawakami et al., 2005).
It is argued that the differences between fish, bird and mammal demonstrate that in the evolutilional common ancestor of modern vertebrates, the breaking of left-right symmetry started as two mechanisms. However during the course of evolution different species retained different subsets of these mechanisms (Hirokawa et al., 2006). For example fish kept both mechanisms while birds use only the asymmetrically distributed ATPase transporters and mammals use only the nodal flow mechanism.

The second step of establishing left-right asymmetry, the mechanism that relays the initial symmetry-breaking event to downstream asymmetric gene expression is still poorly understood. However, both of the known early symmetry-breaking events later lead to a highly conserved system which can be considered the third step of building the visceral L/R asymmetry. The system is composed of the asymmetric expression of Nodal, a TGF-β family protein, Lefty proteins, an group of antagonists of the Nodal signaling, and Pitx2, a downstream protein with transcriptional activity (Hamada et al., 2002; Schier, 2009). nodal, lefty and pitx2 genes are expressed in roughly the same region in the left side of the lateral plate mesoderm (LPM) during a short period of somitogenesis. lefty is also expressed in the left of the prospective floor plate and notochordal plate (Hamada et al., 2002; Schier, 2009).

Based on the general model of the Nodal signaling system, Nodal activity upregulates the expression of Nodal gene, which leads to even higher Nodal activity, thus creating a positive feedback loop. However, Nodal activity also upregulates the expression of the Lefty genes, which translates into the Nodal antagonist Lefty protein. Lefty then inhibits the activity of Nodal and creates a negative feedback loop. The interaction between the positive and negative feedback mechanisms acts as the regulator
to restrict Nodal activity to a specific time and site (Figure 2). Lefty is also expressed in
the notochord as a molecular barrier to further prevent Nodal activity from spreading to
the right side (Figure 2). (Hamada et al., 2002; Schier 2009).

This Nodal signaling system is known to be necessary for establishing the visceral
asymmetries. In mammals, disrupting the components of Nodal signaling system leads to
complex phenotypes. Nodal defective phenotypes include randomized visceral situs, in
which about 50% of the individuals exhibit reversed L/R asymmetry, and left or right
isomerization, in which the asymmetric characteristics are lost and both sides exhibit the
same sidedness (Hamada et al., 2002). While the details of Nodal signaling defects are
not described here, generally tissues receiving Nodal signal will assume a left identity
while cells without Nodal signaling will assume a right identity. Thus, Nodal is the factor
that biases tissues towards left identity and morphology.

Roles of L/R brain asymmetries in humans

A key study conducted by the French neurologist Paul Pierre Broca during the
second half of the nineteenth century provided one of the first evidence of the functional
aspect of human brain structural asymmetry (Broca, 1861; Tommasi, 2009). Broca
discovered that a lesion in the left frontal lobe was responsible for a patient's inability to
produce articulate speech (Broca, 1861; Tommasi, 2009). Modern studies further
revealed other forms of brain structural and functional asymmetry. For example in
humans, planum temporale, a region in the temporal lobe of the cerebral cortex, normally
has a larger volume, a greater extent of myelination and other cellular differences in the
left hemisphere (Hugdahl, 2005; Steinmetz, 1996). A study found that all human subjects
who had language lateralized to the left hemisphere, based on magnetic resonance imaging (MRI) scan, had a leftward asymmetry of the planum temporale (Foundas et al., 1994).

Abnormality or lack of brain asymmetry can be linked to functional defects in humans including language disorders, Alzheimer’s disease and auditory hallucinations (Leonard and Eckert, 2008; Levitan et al., 1999; Villain et al., 2008). For example, changes in the asymmetry in the planum temporale are associated in some cases to dyslexia, although this relationship is not linear. Specifically, increased differences between the left and right sides, lack of left-right asymmetry, and normal left-right asymmetry have all been found in affected individuals, suggesting that changes in morphology of the brain are only part of the etiology of dyslexia (Leonard and Eckert, 2008).

**Epithalamus as a model system for L/R brain asymmetries**

The vertebrate epithalamus is a brain structure with variable L/R asymmetry in different species. The types of asymmetries that can be found in vertebrate epithalamus include differences in gene expression, neurochemistry, neuron organization, neural connectivity, and size between the left and right sides (Concha and Wilson, 2001). However, the details of the left-right asymmetries are highly variable between different species (Concha and Wilson, 2001). For example, while the size of the habenular complex, one of the two major structures of the epithalamus, is left-right asymmetric in nearly all species of teleost fish, in some species including zebrafish and European eel the left habenula is larger and in other species including rainbow trout and European smelt
the right habenula is larger (Braitenberg and Kemali, 1970; Concha and Wilson, 2001; Holmgren, 1920).

The pineal complex, the other major structures of the epithalamus, is a structure located in a dorsal posterior segment of the diencephalon in the vertebrate brain. It is further composed of a pineal organ and a parapineal organ (Figure 3). For the pineal organ, the structural L/R asymmetry is subtle and most of the results are from zebrafish studies. For example, the pineal organ of an adult zebrafish is usually slightly biased to the left (Liang et al., 2000). However for the parapineal, which forms as an outgrowth of the diencephalic roof more anterior and separated from the pineal organ, the parapineal is located on the left side to the pineal organ in many teleost species including zebrafish (Concha and Wilson, 2001). In reptiles, the parapineal forms a partial eye at the top of the skull. While the partial eye is medially located, the neurons project only to a subdivision of the left medial habenular nucleus (Engbretson et al., 1981).

The term habenula was originally used to describe the stalk of the pineal organ (“pineal habenula”). In modern definition, the habenular complex indicates the structure located in the dorsal diencephalon on both sides of the third ventricle and is associated with the pineal complex. There are many different left-right asymmetric characters exhibited by the habenular complex. In fish and amphibians, one side of the habenular nuclei, usually the left (including in the case of zebrafish), has higher neuropil density than the contralateral habenular nucleus, and the gene expression patterns are different between the nuclei on the left and right sides. The habenular nuclei on left and right sides both project their axons to the interpeduncular nucleus (IPN) (Aizawa et al., 2007; Bianco and Wilson, 2009; Kuan et al., 2007). However, the axons from the left habenular
nucleus target the dorsal IPN while the axons from right habenula nucleus primarily target the ventral IPN (Halpern et al., 2003; Snelson and Gamse, 2009). In birds and mammals the asymmetric properties of the habenular complex are more subtle and variable. For example, in chick, the structural asymmetry of the medial habenular nuclei was only detected in males. However, when testosterone was injected to the females, the volume of the right habenular nuclei was increased. Such results suggest that the habenular asymmetry is sex dependent in chick (Gurusinghe et al., 1986). In mammals, the rat habenula is larger on the left, and the mouse habenula is larger on the right (Concha and Wilson, 2001).

Functional aspects of epithalamus asymmetry

Results from animal models and human brain imaging studies indicate that habenular complex regulates many behaviors including the regulation of sleep, reward and maternal behaviors (Klemm, 2004; Sutherland, 1982). Also, evidence suggests that abnormal habenular complex activity can be linked with schizophrenia in humans (Lecourtier and Kelly, 2007).

In fish, including zebrafish, both the epithalamus and IPN play roles in regulating many behaviors including feeding, mating, eye usage, and escape behavior (Bianco and Wilson, 2009). For example in zebrafish, the right eye is preferred when examining unfamiliar or complicate targets and the left eye is for familiar targets (Andrew et al., 2009; Bianco and Wilson, 2009; Miklosi and Andrew, 1999; Miklosi et al., 1997). This sided eye usage can be linked with structural asymmetries in the epithalamus and IPN. Specifically, zebrafish with the frequent situs inversus (fsi) mutation have a high rate of
situs inversus in the epithalamus and the viscera. When placed in front of a mirror, *fsi* mutant fish with reversed epithalamic asymmetry also exhibits reversed eye use behavior (Barth et al., 2005).

**Current model for establishing zebrafish epithalamus asymmetry**

Results from zebrafish studies have revealed some of the mechanisms that establish epithalamic L/R asymmetries, which is very similar to the pathway involved in visceral asymmetry in all vertebrates. During somitogenesis, *cyclops* (*cyc*), a gene encoding a Nodal signaling protein, is expressed in the left side of the presumptive pineal (Liang et al., 2000; Rebagliati et al., 1998a; Rebagliati et al., 1998b). *Lefty* (*lft1*), the gene encoding a Nodal antagonist, and *pitx2*, the gene encoding a downstream effector of the Nodal signaling, are also expressed in the similar area (Concha et al., 2000; Liang et al., 2000).

The parapineal originates as a cluster of cells from the pineal complex anlage to the left side of the brain, consists with the L/R asymmetric expression of Nodal related genes (Concha et al., 2000; Snelson et al., 2008). It has been demonstrated that parapineal ablation lead to the loss of habenular asymmetry, suggesting that it is the parapineal that guides the downstream asymmetries in the zebrafish habenular complex (Gamse et al., 2003).

The expression pattern of Nodal signal related genes are strikingly similar between visceral system and epithalamus. The asymmetrically expressed Nodal signaling related genes are among the earliest observable asymmetric characteristics in the zebrafish epithalamus and in the viscera of all vertebrates. Thus, it seems logical to
hypothesize that the establishing of visceral asymmetry and epithalamus asymmetry share a similar mechanism.

Based on current model of the establishing of L/R asymmetry in the zebrafish epithalamus (Figure 4A), initially the Nodal signaling related genes in the pineal anlage are asymmetrically expressed. The pineal asymmetry consequently guides the parapineal to form on the left side of the brain. The left sided parapineal then mediates the L/R asymmetries in the habenular nuclei (Figure 4A). However mutations that affect left-right asymmetry in the epithalamus suggest more complex mechanisms. For example in the mutants which the asymmetric gene expression patterns in the presumptive pineal are lost due to the lack of midline structures or endoderm (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000), a single parapineal is almost always formed on one random side of the pineal (Concha et al., 2000). Consequently, the habenula locates on the side with a parapineal will exhibit the characteristics of “leftness” (Concha et al., 2000; Gamse et al., 2003; Halpern et al., 2003) (Figure 4B). Such fact is particularly interesting since it suggests that epithalamus asymmetries can still rise when the initial pineal asymmetry is lost. In other words, while the pineal asymmetry may be responsible of guiding the sidedness of the downstream asymmetry, such pineal asymmetry is not likely the only “local symmetry-breaking” mechanism during the establishing of epithalamus asymmetry.

**Research design**

In the research described in the Chapter 3 of my thesis, we investigated the role of left-right interaction in the establishing of epithalamic asymmetry by observing the effects of neural tube closure defects. During the development of a WT zebrafish embryo,
pineal precursors originate in two domains at the edges of the neural plate. These precursors then converge towards the midline of the dorsal diencephalon and fuse to form a single pineal complex as the neural tube forms {Aquilina-Beck, 2007 #519}. We found that when the neural tube does not close properly and the epithalamus structure is divided into two parts, one on the left side and one on the right, the normally left biased parapineal also becomes divided, resulting in a parapineal forms on the left and one on the right sides. We also found that the two parapineals consequently direct the habenular nuclei on both sides to develop left-like properties. Such left isomerization phenomenon indicates the existence of a previously unknown mechanism between the left and right sides. This mechanism, likely a short-ranged signal that inhibits the effected tissue to assume left identity, is required for directing the sidedness of the epithalamus left-right asymmetry.

**Figure Legends**

**Figure 1: The negative feedback model of molecular biological clock.** LOCK/BMAL dimer activates the expression of *period (per)* and *cryptochrome (cry)* genes by binding to the E-box region. PER and CRY proteins then inhibit the activity of CLOCK/BMAL. Consequently, the production of PERs and CRYs is inhibited and the activity of CLOCK/BMAL complex can be restored.

**Figure 2: A general model of the molecular pathway for the determination of visceral left-right symmetry.** The symmetry breaking event initially takes place in or near the node at the neural fold stage. The breaking of symmetry leads to the left-sided
expression of Nodal. Nodal induces the expression of Lefty proteins which act as feedback inhibitors of Nodal to restrict Nodal activity within the left side. Note that the positive feedback mechanism is indicated by blue lines while the negative feedback loops are indicated by red lines. Nodal also induces the expression of Pitx2, a transcription factor that is responsible of generating left-side morphology. (Based on Hamada et al., 2002)

**Figure 3: The epithalamus of vertebrates.** Diagram of a sagittal section of a basal vertebrate. Several medial evaginations are present along the epithalamic roof of the diencephalon, the two most significant being the photoreceptive pineal and parapineal organs. Habenular commissure connects the left and right parts of the habenular nuclei (not shown in the figure). PAR, paraphysis; SD, saccus dorsalis; III third ventricle. (Modified from Concha and Wilson, 2001, modified from Kardong, 1995)

**Figure 4: Current model for laterality in the zebrafish epithalamus.** In WT embryos, left-right asymmetry of the brain is first apparent in the left-sided expression of the Nodal pathway genes cyc, lft1, and pitx2 in the pineal anlage (blue). Subsequently, the parapineal (green) is formed on the left side of the brain, adjacent to the pineal anlage. The left sided position of the parapineal influences the formation of differences between the left and right habenula nuclei, such as the more extensive expression of kctd12.1 in the left habenula (purple). In embryos with midline defects, the expression of cyc, lfty1, and pitx2 becomes bilateral. Despite this, the parapineal and habenula still develop left-
right asymmetry, although half of the embryos have situs inversus. Thus suggests that there is an inhibitory factor (T bar) that prevents both sides from becoming left.

References


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Figure 1

A diagram illustrating the relationships between CLOCK, BMAL, PERs, CRYs, E-box, and period or cryptochrome.
Figure 3

- PARAPINEAL ORGAN
- PINEAL ORGAN
- Choroid Plexus
- III
- EPITHALAMUS
- HABENULAR COMMISSURE
Expression of Nodal pathway genes in the pineal Parapineal organ

A Wildtype

B Midline defect

Wildtype expression in the habenula nuclei

Midline defect expression in the habenula nuclei

Expression of Nodal pathway genes in the pineal
Parapineal organ
Expression of kctd12.1 in the habenula nuclei

normal asymmetry
randomized asymmetry

Figure 4
Step 1:
The breaking of L/R symmetry

Step 2:
A relaying mechanism

Step 3:
The asymmetric expression of signalling moleculars

Step 4:
The side specific morphology

Figure 2
Chapter 2

Circadian rhythms in the pineal organ persist in zebrafish larvae that lack ventral brain

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Authors’ Contributions

RRN conceived of and designed the study, performed experiments on Exorh protein expression and antibody staining, and studies on aanat2 rhythms, shh, cry, and otx5 expression in cyc mutants, and wrote the first draft of the manuscript. PL helped optimize the antibody staining protocol and carried out studies on cry1b and cry3 expression during development, exorh mRNA rhythms in cyc mutants, and parts of the constant light and constant dark experiments. LGK assisted in designing and in performing the experiments on aanat2 rhythms and otx5 expression in cyc mutants raised in L/D. EG cloned and generated the probe for avpl and performed WISH on cyc mutants. JOL helped design and coordinated the study, carried out parts of the constant light and constant dark studies and the avpl in situ. All authors helped draft the manuscript and have approved the final version.
Abstract

The mammalian suprachiasmatic nucleus (SCN), located in the ventral hypothalamus, is a major regulator of circadian rhythms in mammals and birds. However, the role of the SCN in lower vertebrates remains poorly understood. Zebrafish cyclops (cyc) mutants lack ventral brain, including the region that gives rise to the SCN. We have used cyc embryos to define the function of the zebrafish SCN in regulating circadian rhythms in the developing pineal organ. The pineal organ is the major source of the circadian hormone melatonin, which regulates rhythms such as daily rest/activity cycles. Mammalian pineal rhythms are controlled almost exclusively by the SCN. In zebrafish and many other lower vertebrates, the pineal has an endogenous clock that is responsible in part for cyclic melatonin biosynthesis and gene expression.

We find that pineal rhythms are present in cyc mutants despite the absence of an SCN. The arginine vasopressin-like protein (Avpl, formerly called Vasotocin) is a peptide hormone expressed in and around the SCN. We find avpl mRNA is absent in cyc mutants, supporting previous work suggesting the SCN is missing. In contrast, expression of the putative circadian clock genes, cryptochrome 1b (cry1b) and cryptochrome 3 (cry3), in the brain of the developing fish is unaltered. Expression of two pineal rhythmic genes, exo-rhodopsin (exorh) and serotonin-N-acetyltransferase (aanat2), involved in photoreception and melatonin synthesis, respectively, is also similar between cyc embryos and their wildtype (WT) siblings. The timing of the peaks and troughs of expression are the same, although the amplitude of expression is slightly decreased in the mutants. Cyclic gene expression persists for two days in cyc embryos transferred to constant light or constant dark, suggesting a circadian clock is driving the rhythms. However, the amplitude of rhythms in cyc mutants kept in constant conditions decreases more quickly than in their WT siblings.
Our data suggests that circadian rhythms can be initiated and maintained in the absence of SCN and other tissues in the ventral brain. However, the SCN may have a role in regulating the amplitude of rhythms when environmental cues are absent. This provides some of the first evidence that the SCN of teleosts is not essential for establishing circadian rhythms during development. Several SCN-independent circadian rhythms have also been found in mammalian species. Thus, zebrafish may serve as a model system for understanding how vertebrate embryos coordinate rhythms that are controlled by different circadian clocks.

Introduction

Circadian rhythms are biological cycles in behavior, physiology, and biochemistry that occur approximately every 24 hours. These oscillations are present in almost every organism, from cyanobacteria to plants to humans (Pando and Sassone-Corsi, 2002). All circadian rhythms are regulated by a timing system composed of intracellular clocks with periods of approximately 24 hours, environmental cues, and clock-controlled outputs. An important characteristic is that circadian clocks are able to drive output rhythms even in the absence of environmental cues. However, environmental influences such as light and temperature are required to entrain or re-set the circadian oscillators so that they stay in synchronization with the organism’s surroundings.

Regulation of vertebrate circadian rhythms is best understood in mammals. The SCN, a group of neurons in the hypothalamus, is among the most intensely studied cellular site of circadian oscillators. Targeted bilateral lesion of the SCN abolished circadian oscillations in activity and drinking rhythms (Stephan and Zucker, 1972). Conversely, transplantation of a donor SCN into a host with a lesioned SCN restored circadian cycling (Ralph et al., 1990). Further, the new locomotor activity rhythms of the host matched the circadian cycle of the donor SCN, indicating that the SCN was the major influence on
the phase of the restored rhythms (Ralph et al., 1990). The SCN controls rhythms in other tissues through a variety of mechanisms, including the secretion of peptide hormones and synaptic signaling (Piggins and Loudon, 2005). For instance, rhythmic production of the circadian hormone melatonin in mammals is controlled almost entirely by a multi-synaptic pathway leading from the SCN to the cells of the pineal gland (Korf et al., 1998).

Although the mammalian SCN is important in controlling many rhythms in numerous organs, mammals also have circadian oscillators in other tissues as well as rhythms that are SCN-independent (Bell-Pedersen et al., 2005; Honma and Honma, 2009). For instance, the mammalian retina has an endogenous oscillator that controls local rhythms such as visual sensitivity and retinal melatonin synthesis (Ruan et al., 2006). This indicates that the mammalian circadian system may have similarities to the circadian systems of many lower vertebrates, which often have many oscillators and many sites of photoreception (Cahill, 2002; Tosini et al., 2001; Underwood et al., 2001).

A structure anatomically equivalent to the SCN has been described in zebrafish embryos and adults (Burrill and Easter, 1994; Mathieu et al., 2002; Rink and Wullimann, 2004; Wulliman et al., 1996; Yanez et al., 2009). However, the function of the zebrafish SCN in regulating circadian rhythms is unknown (Kaneko et al., 2006). This question is particularly interesting as many different isolated zebrafish cells and organs, such as the pineal, eyes, heart, spleen, and kidney, have endogenous oscillators and circadian photoreceptors that are able to generate cyclic gene expression (Cahill, 1996; Kaneko et al., 2006; Whitmore et al., 2000; Whitmore et al., 1998). For example, in adult zebrafish the pineal organ contains an endogenous circadian oscillator that is sufficient to drive rhythms in melatonin synthesis as well as photoreceptive neurons that entrain this oscillator (Cahill, 1996). However, whether pineal rhythms are also influenced by signals from other tissues, such as the SCN, is unknown in zebrafish and many other lower vertebrates. Interestingly, in some avian species, pineal rhythms are
controlled both by an endogenous pineal oscillator and by input from the SCN, raising the possibility that multiple tissues could regulate pineal circadian rhythms in zebrafish (Abraham et al., 2003; Brandstatter and Abraham, 2003; Underwood et al., 2001).

Here, we provide evidence that the SCN is not required for pineal rhythms in developing zebrafish. To do this, we took advantage of zebrafish cyc mutants, which have a mutation in one of three zebrafish nodal genes (Rebagliati et al., 1998; Sampath et al., 1998). Lack of Cyc/Nodal signaling results in a complete absence of the hypothalamus, including the regions that give rise to the SCN, the retro-chiasmatic nucleus, and the infundibulum (Hatta et al., 1991; Mathieu et al., 2002; Rebagliati et al., 1998; Sampath et al., 1998). Consistent with this earlier work, we find that expression of the avpl gene, which is typically expressed in and around the SCN (Balment et al., 2006; Caldwell et al., 2008), is absent in cyc mutants. Despite this, expression of the putative clock component genes cry1b and cry3, as well as the structure and size of the pineal organ was indistinguishable between cyc embryos and their WT siblings. The phase of gene expression of two pineal rhythmic genes, exorh and aanat2 persisted in the mutants. However, semi-quantitative analysis suggested that the amplitude of the rhythms was slightly reduced. aanat2 mRNA levels maintained their rhythmic changes in cyc embryos placed in constant conditions. This suggests that transcriptional rhythms were not being controlled by the environment, but rather by an internal cellular clock, perhaps the endogenous clock within the pineal cells. Since cyc mutants never have an SCN, this work indicates daily rhythms within the developing zebrafish pineal can be initiated in the absence of the developing hypothalamus, including the SCN anlage.
Methods

Zebrafish

Zebrafish embryos and larvae were obtained by natural matings and were raised at 28.5 °C at 14:10 hour (h) L/D cycle according to standard procedures (Kimmel et al., 1995). Developing fish were placed in a Sanyo MIR-153 incubator (Amsterdam, The Netherlands) with heating and cooling capabilities for maximum temperature stability. Light consisted of a single 60 watt Globe EnerSaver light bulb placed within the incubator. Stocks used were Oregon AB (WT), ZDR (WT) (Aquatica Tropicals, Plant City, FL), and cycm294 (Kimmel et al., 1995; Malicki et al., 1996; Sampath et al., 1998; Schier et al., 1996).

Whole mount RNA in situ hybridization (WISH)

WISH was carried out as described by Liang et al. (Liang et al., 2000). Antisense RNA probes included aanat2 (Gothilf et al., 1999), exorh (Mano et al., 1999), shh (Krauss et al., 1993; Liang et al., 2000), cry1b (Kobayashi et al., 2000), cry3 (Open Biosystems, Huntsville, AL), otx5 (Gamse et al., 2002), and avpl (Eaton et al., 2008).

To generate cry1b probe, cry1b cDNA in plasmid pME-18S-FL3 was subcloned into plasmid pBSK(+) using a 5’ EcoRI and a 3’ NotI restriction sites. Plasmid pBSK+ cry1b was linearized with EcoRI and was used for probe synthesis with T3 RNA polymerase. cry3 probe was PCR amplified from the cry3 cDNA in plasmid pME-18S-FL3 using primers and cycling conditions in ZFIN [http://zfin.org/cgi-bin/webdriver?MIval=aa-markerview.apg&OID=ZDB-CDNA-040425-188]. cry3 was made with T3 RNA polymerase.

Whole mount antibody staining
Whole mount antibody staining was done as described (Pierce et al., 2007). A mouse monoclonal antibody (4D2) against the N-terminus of bovine Rhodopsin (Hicks and Molday, 1986; Laird and Molday, 1988) was used to detect Exorh protein at a dilution of 1:60. A goat anti-mouse secondary antibody coupled to Oregon Green 488 (Invitrogen Molecular Probes, Carlsbad, CA) was used at a dilution of 1:2000.

**Morpholino and mRNA injections**

Control and translation blocking morpholinos (MO) against *exorh* (exorh atg MO) were obtained from Gene Tools (Philomath, OR). Sequences of the MO used were: control MO, 5’-CCT CTT ACC TCA GTT ACA ATT TATA-3’ and exorh atg MO, 5’-AGT TGG GTC CCT CCG TCC CGT TCAT-3’. One-cell stage embryos were injected with either 1.5 nanograms (ng) of control or exorh atg MO using a Harvard Instruments PLI-90 Pico-Injector (Holliston, MA).

To generate *exorh* mRNA for overexpression, the entire coding sequence of the zebrafish *exorh* gene (from plasmid pCR2.1-full-length *exorh*) [GenBank Accession Number: AB025312] was subcloned into pGEMHE plasmid (Liman et al., 1992; Mano et al., 1999). Donor and host plasmids were digested with EcoRI. The resulting EcoRI-digested full-length *exorh* coding sequence was ligated non-directionally into pGEMHE (plasmid pGEMHE-full-length *exorh* hereafter pGEMHE-flex) using the TAKARA DNA Ligation Kit version 1 (Madison, WI). The ligation reaction was transformed into Z-Competent *E. coli* cells (Zymo Research, Orange, CA). Clone orientation was verified via restriction digestion (double digestion with SacI and BanII) and DNA sequencing. DNA sequencing was done using forward and reverse sequencing primers (forward primer: 5’-TTT TTG CAG AAG CTC AGA ATA-3’; reverse primer: 5’-CAT TTT GTA AAG TGT AAG TTG GTAT-3’). DNA sequences were verified by doing a BLAST search (Altschul et al., 1990).
To synthesize full-length *exorh* mRNA, pGEMHE-flex plasmid was linearized with SphI. Linearized pGEMHE-flex was used for mRNA synthesis with an Ambion mMESSAGE mMACHINE kit (Austin, TX). Full-length *exorh* mRNA was verified through RNA formaldehyde agarose gel-electrophoresis. One blastomere of 8-16 cell stage embryos were injected with 400 picograms (pg) of beta-galactosidase (control) or full-length *exorh* mRNA and were fixed 7 h post-injection. Injected and fixed embryos were processed for whole mount antibody staining as described above.

**Photography and Image Analysis**

Embryonic and larval zebrafish samples were imaged using a Zeiss Axioplan 2 Imaging Microscope (Thornwood, NY) or Nikon Eclipse 801 Epifluorescent Microscope (Melville, NY) connected to a Spot RTke7.4 Slider digital camera together with Spot 4.5.9.1 software (Diagnostic Instruments, Sterling Heights, MI). Resulting images were processed and quantified for optical density using ImageJ 1.36b and 1.42q software [Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009]. Calibration was performed following developer’s instructions [http://rsb.info.nih.gov/ij/docs/examples/calibration/]. For in situ hybridization data, images were first converted to 8-bit grayscale and an oval encompassing the expression domain was drawn using the Specify tool. Optical Density (OD) was calculated by multiplying the oval area with the average intensity, both of which were obtained through the Analyze tool. Two-way ANOVA statistical analysis was carried out using Microsoft Excel.

For quantification of *aanat2* expression in the constant light and constant dark experiments, the background value was subtracted as follows. A small circle with diameter of 20 pixels was selected close to, but not touching the anterior edge of the WISH expression signal. The circular area and optical density was determined as above. Background-corrected OD values were calculated by subtracting the
(OD value of small circle x pineal oval area/small circle area) from the OD of oval around the pineal expression domain. All OD data are either means ± standard deviation or means + standard deviation only. Representative images with ODs closest to the mean were chosen.

Fluorescent images were analyzed as above with the exception that 8-bit grayscale images were first processed using the Inverted tool prior to choosing the oval area to be quantified for OD. For Exorh protein temporal expression, fluorescent image data was tested for significance using One-way Analysis of Variance (ANOVA) and Tukey’s test using Origin Software Version 7.5 SR4 (Northampton, MA).

To measure the dimensions of the otx5 expression domain, lines covering the length or the width of the pineal were drawn using ImageJ and the corresponding pixel length or width were converted to micrometers by calibrating the number of pixels in a 20 micrometer line.

**Results**

cyc mutants lack avpl gene expression in the ventral brain

We sought to define the function of the zebrafish SCN in regulating circadian rhythms during embryogenesis. Zebrafish cyc mutants are missing the ventral brain and the spinal cord, including expression of the genes sonic hedgehog (shh), emx2, and nk2.1 in the hypothalamus (Hatta et al., 1991; Mathieu et al., 2002; Rohr et al., 2001; Sampath et al., 1998). This strongly suggests that cyc mutants could also be lacking the hypothalamus-derived precursors to the SCN. Consistent with previous data, we found that cyc mutants are missing the shh-positive neurons in the anterior hypothalamus that are proposed to be the precursors to the SCN (Figure 1A, B) (Mathieu et al., 2002; Puelles, 1995).

To further characterize the ventral brain defect in cyc mutants, we examined the expression of the avpl gene. In mammals, vasopressin is expressed by the SCN neurons with a strong circadian rhythm, and has been linked to changes in hormone secretion from the pituitary, regulation of
reproduction in females, and behaviors such as daily rhythms in wheel running activity in nocturnal rodents (Caldwell et al., 2008). In non-mammalian vertebrates, vasopressin is replaced by Avpl, which is expressed in many ventral brain neurons including cells in and around the SCN (Balment et al., 2006). In zebrafish, avpl mRNA is found in two domains of the ventral brain: the dorsal preoptic area and the anterior aspect of the ventral hypothalamus (Eaton et al., 2008). Based on the location of the avpl positive cells in the ventral hypothalamus, these cells likely correspond to the zebrafish SCN. In cyc embryos, both avpl expression domains were undetectable (Figure 1C-J). This suggests that neurons expressing avpl, including those in the SCN, are missing.

**exorh mRNA is expressed rhythmically in the pineal organs of cyc mutants**

We next wanted to examine the expression of rhythmic genes in a tissue outside of the SCN (Figure 2). The developing pineal organ was ideal for this analysis. The pineal contains an endogenous oscillator and photoreceptors that are able to entrain, or reset, this oscillator in response to photic input (Cahill, 1996). Further, pineal rhythms can be easily followed by assaying the expression of circadian-regulated pineal genes (Appelbaum et al., 2005; Appelbaum et al., 2004; Gamse et al., 2002; Gothilf et al., 1999; Gothilf et al., 2002; Stenkamp et al., 1998; Triqueneaux et al., 2004; Ziv et al., 2005).

To determine what happens to pineal rhythms in cyc mutants, we first assayed the embryos for expression of exorh mRNA and protein. Exorh is a putative light-sensing, G-protein coupled receptor that is expressed in the pineal (Asaoka et al., 2002; Falcon et al., 2003; Mano et al., 1999; Vuilleumier et al., 2006). exorh mRNA is synthesized with a daily rhythm, with highest levels during the night and lower levels during the day (Pierce et al., 2008; Vuilleumier et al., 2006).

Embryos were raised in a standard 14:10 h light/dark (L/D) cycle, with all other environmental parameters held constant. Rhythms in exorh mRNA were present in the pineal organs of both WT and
cyc fish (Figure 2A, C). Further, the timing of the troughs and peaks of expression were similar between both sets of embryos (Figure 2A, C).

The amplitude of exorh expression was slightly lower at almost every time point tested (Figure 2C). To rule out the possibility that this was due to smaller pineal organs in cyc embryos, we measured the length and width of the pineal organs labeled for expression of orthodenticle homeobox 5 (otx5), a gene that is constitutively expressed in all pineal cells (Gamse et al., 2002). We found that the pineal size was not significantly different between WT and cyc fish (Figure 2E, F). This suggests that the decrease in the amplitude of exorh expression in cyc embryos was not due to smaller pineal organs.

**A gene involved in melatonin biosynthesis is expressed rhythmically in cyc mutants**

*aanat2* encodes the penultimate enzyme in the melatonin biosynthetic pathway (Gothilf et al., 1999). In zebrafish and many other vertebrates, *aanat* genes are expressed in the pineal with dramatic differences between day and night levels (Klein, 2006). Thus, *aanat2* expression serves as a very sensitive readout of pineal circadian cycling. In WT and cyc embryos maintained in a L/D cycle, *aanat2* transcripts cycled with indistinguishable periods and phases (Figure 2B, D). However, as was the case for exorh, the amplitude of *aanat2* expression was slightly diminished compared to WT siblings raised in parallel (Figure 2D). Thus, the nocturnal expression of *aanat2* in the pineal was largely unaffected by the loss of the putative SCN in cyc mutant embryos.

**Rhythmic expression of aanat2 persists in cyc embryos placed in constant environmental conditions**

The pineal organs of zebrafish, as well as other tissues, contain functional photoreceptors (Cahill, 1996; Carr and Whitmore, 2005; Kaneko et al., 2006; Whitmore et al., 2000; Whitmore et al., 1998). Further, we found that the Exorh protein was expressed normally in cyc mutants, suggesting that the
pineal photoreceptors could be functional in these mutants (Figure 3 and Supplemental Figure 1). Thus, one possibility was that the mRNA rhythms in the pinealocytes of cyc embryos were due to direct responses to changing light conditions, not to the presence of a circadian clock. To rule out this possibility, cyc embryos and their WT siblings were raised for two or three days in a standard L/D cycle, and then transferred to either constant light or constant dark for an additional two days (Figure 4 and 5).

When the embryos were exposed to a L/D cycle for only two days before transfer to constant conditions, the expression level of the aanat2 gene declined rapidly in the constant environment in both WT and cyc mutants, suggesting that circadian cycling was not fully established by such a short period of entrainment (Figure 4). In contrast, when embryos were maintained instead for three days in a L/D cycle, rhythmic expression of aanat2 persisted in WT and in cyc larva after transfer to either constant light or constant dark conditions (Figure 5). Under constant dark conditions, a slight shift in the peak of expression to the right was apparent, consistent with previous studies that showed the endogenous period of the clock that controls zebrafish pineal rhythms is slightly longer than 24 hours (Figure 5B) (Cahill, 2002; Gamse et al., 2002; Gothilf et al., 1999; Kazimi and Cahill, 1999). However, this shift was not readily apparent in fish in constant light (Figure 5B). The persistence in cyclic changes in expression after transfer to constant lighting conditions suggests that the rhythms are not due to responses to rhythmic environmental changes, but instead due to a functioning circadian clock in the cyc embryos.

Although the overall rhythm in gene expression persisted in constant conditions, there were some significant differences between the cyc mutants and their WT siblings. As in the L/D experiments (Figure 2), the levels of mRNA were slightly lower in the cyc embryos at most time points (Figure 5). Further, after approximately 48 hours in constant conditions, for both constant light and constant dark, the aanat2 expression was notably lower in the cyc embryos than in their WT siblings (Figure 5).
**cry gene expression is present in cyc mutants**

To gain insight into other potential changes in cyc embryos, we examined the expression of putative clock components during embryogenesis. The vertebrate circadian clock is composed of complex positive and negative feedback loops that take approximately 24 hours to go through one cycle. Cry proteins are essential components of this clock, in which they act as transcriptional repressors in the negative feedback loop. Zebrafish have six cry genes, all of which are expressed with a daily rhythm (Kobayashi et al., 2000; Lahiri et al., 2005).

We found that the expression of cry1b and cry3 was indistinguishable between cyc embryos and their WT siblings (Figures 6 and 7). In both types of embryos, cry1b was expressed widely in the developing brain (Figure 6). cry3 was also expressed in the brain, and more strongly in the ear, liver, and in retinal cells near the lens (Figures 6 and 7). These results indicate that transcription of cry1b and cry3 is present even when the ventral brain is absent.

**Discussion**

In mammals and birds, the SCN has an important role in regulating circadian rhythms throughout the organism. Retinal photoreceptors entrain circadian oscillators within the SCN, and the SCN then regulates pineal rhythms through a multisynaptic pathway (Liu et al., 2007). Although the retina-SCN-pineal pathway is present in fish, amphibians, and reptiles, its function is not well understood (Bertolucci et al., 2000; Burrill and Easter, 1994; Foa et al., 2006; Kramer et al., 2002). Here we provide evidence that pineal circadian rhythms are established and have normal phase in zebrafish cyc mutants, which lack ventral brain. This suggests that the SCN and the retinal-hypothalamic pathway are not essential for the onset and of pineal circadian rhythms during embryogenesis. However, the amplitude of
pineal rhythms is slightly, but significantly decreased, in cyc mutants. This suggests that the SCN or other tissues that are missing in these mutants, which include other regions of the ventral brain, may have a role in regulating rhythms during development.

**Pineal rhythms persist in cyc mutants**

The SCN has been defined anatomically in zebrafish embryos and adults (Burrill and Easter, 1994). We used cyc mutants to test the function of the zebrafish SCN. These mutants are missing the whole ventral brain including the entire hypothalamus, and thus, the precursors to the SCN (Hatta et al., 1991; Mathieu et al., 2002). We now demonstrate that expression of avpl, which is usually present in and around the SCN, is lacking in cyc mutants (Figure 1). This provides strong evidence that the SCN is completely absent in cyc mutants.

We found that rhythmic expression of aanat2 and exorh are present in cyc mutants during the first few days of development (Figure 2). The phases of expression of these genes are indistinguishable between cyc mutants and WT siblings raised in parallel in a L/D cycle. Further, the aanat2 expression rhythms persist after the embryos are transferred to constant dark or constant light, indicating they are being driven by a circadian clock (Figure 5).

**Diminished amplitude of gene expression in cyc mutants**

The expression amplitudes of aanat2 and exorh mRNA levels are slightly reduced in the cyc mutants compared to WT siblings raised in parallel. This was true for the fish kept in a normal L/D cycle and the fish transferred to constant dark or constant light conditions from a normal L/D condition (Figures 2 and 5). In addition, the decrease in amplitude of expression became more pronounced when the fish approached the third day in constant conditions (Figure 5).
cyc mutants have many developmental defects, making it difficult to precisely identify the cause of the decrease in gene expression. It is unlikely that the change is due to defects in pineal development, as the size of the pineal was not significantly decreased in cyc mutants (Figure 2). However, the lower amplitude of gene expression could be due in part to poor health or subtle changes in the pineal that we have not detected (Hatta et al., 1991; Rebagliati et al., 1998; Sampath et al., 1998).

A more interesting possibility is that the SCN or other missing tissues in the ventral brain contribute to the regulation of the amplitude of pineal rhythms. Because the phenotype of cyc mutants becomes more severe as the fish get older, we kept the fish in a L/D cycle for only a short time before being assayed. Thus, one possibility is that the SCN/ventral brain functions to help establish self-sustaining rhythms during development. This role for the SCN could explain why the amplitude of rhythms were lower in the cyc pineal overall, and why the rhythms in the cyc pineal started to dampen sooner after transfer to constant conditions. Testing this hypothesis will require a mutant that more specifically affects the SCN or a way to specifically inhibit the function of SCN cells.

Tissues that could be promoting circadian rhythms in cyc mutants

We do not completely understand what is driving pineal rhythms in cyc mutants. One possibility is that these rhythms are driven by clocks within the zebrafish pineal. Persistent circadian rhythms in melatonin synthesis were observed previously in isolated adult zebrafish pineals cultured for several days (Cahill, 1996). This suggests that the pineal oscillators in adult zebrafish are self-sustained. Clocks in the embryonic pineal may work in the same way. In support of this, a previous study showed that light input very early in development, long before the differentiation of an SCN, is necessary for onset of pineal rhythms (Ziv and Gothilf, 2006).
Oscillator proteins present in the pineal are also in many other zebrafish cells and tissues. Likewise, circadian rhythms have been detected in isolated cultured zebrafish embryonic and adult tissues (Kaneko et al., 2006; Whitmore et al., 2000; Whitmore et al., 1998). Thus, it is possible that other tissues present in cyc mutants are interacting to promote circadian cycling in the pineal.

We also cannot rule out a role for the lateral eyes. When entrained for only two days in a normal L/D condition, even WT fish cannot maintain a robust pineal rhythm when moved to constant conditions is particularly interesting (Figure 4). Based on current understanding of zebrafish pineal and retina development, the expression of exorh is observed in the presumptive pineal as early as 16 to 19 hpf (Vuilleumier et al., 2006) while eye photoreceptors can be detected only after 2.5 dpf (Branchek and Bremiller, 1984; Kljavin, 1987). It is logical to assume that a photoreceptive retina is required for establishing the self-sustainable pineal rhythms. Based on such model when the embryos are only entrained for the first 2 dpf, the photoreceptive retina is not yet established and consequently the pineal rhythm is not self-sustainable. Interestingly, since the pineal rhythm in cyc mutants also requires three days of entraining to be self-sustainable, if the photoreceptive retina does play a role in establishing pineal rhythms, the role must not be the commonly known retina-SCN-pineal pathway, but a novel SCN/ventral brain independent mechanism. Previous work suggested that the lateral eyes are not required for establishing rhythms in the pineal. Rhythms in pineal aanat2 expression are present in zebrafish chokh (chk) mutants, which lack lateral eyes (Kennedy et al., 2004). Further, chokh mutants can re-entrain to a shifted L/D cycle (Kennedy et al., 2004). However, the persistence of pineal rhythms in chokh fish after transfer to constant lighting conditions has not yet been tested, raising the possibility that they could have defects similar to those we found in cyc mutants.

A final possibility is that pineal rhythms are present in cyc mutants because there are some residual SCN neurons. However, this is unlikely, as the whole ventral brain is absent in cyc mutants.
(Figure 1A-B) (Hatta et al., 1991; Mathieu et al., 2002; Rebagliati et al., 1998; Rohr et al., 2001; Sampath et al., 1998). chk mutants are missing both eyes and hypothalamic avpl expression (Tessmar-Raible et al., 2007). Despite this, they have rhythms in gene expression of two clock components (clock and period4) and pineal aanat2 (Dickmeis et al., 2007; Kennedy et al., 2004). These results are consistent with our findings that loss of neurons expressing avpl, including those in the SCN, does not alter pineal rhythms.

*NORMAL CRY GENE EXPRESSION IN CYC MUTANTS*

Cry proteins are required for regulation of circadian rhythms in both plants and animals (Cashmore, 2003). These proteins were shown to play two major roles in regulation of circadian rhythms: as a photoreceptor to entrain the clock and as a repressor of Clock/BMAL-induced circadian transcription (Cashmore, 2003). Six Cry proteins are present in zebrafish (Ishikawa et al., 2002). By heterologous expression, it was shown that Cry1b, but not Cry3, is capable of blocking Clock/BMAL-dependent transcription (Ishikawa et al., 2002). This suggests that Cry1b and Cry3 have different functions. We find that cry1b and cry3 mRNAs are found in specific tissues of zebrafish embryos and larvae (Figures 5 and 6). Further, we demonstrate that cry1b and cry3 expression in developing brain, ear, and liver are present in cyc mutants (Figures 5 and 6). Although the function of the cry genes in zebrafish is not yet fully understood, the presence of cry1b and cry3 transcripts in the cyc brain suggests that the circadian clock may not be severely disrupted.

*EXORH PROTEIN EXPRESSION IS NORMAL IN CYC MUTANTS*

exorh was originally identified as a rhodopsin class gene expressed in the zebrafish and salmon pineal organs (Mano et al., 1999; Philp et al., 2000). Exorh has been predicted to be a G-protein coupled
receptor with ~70% amino acid sequence identity with vertebrate Rhodopsin (Mano et al., 1999). Our group and others have previously demonstrated the rhythmic expression of *exorh* mRNA (Pierce et al., 2008; Vuilleumier et al., 2006). In contrast to *exorh* mRNA, we find that Exorh protein expression does not follow a significant daily rhythm in WT embryos, nor is its expression affected in *cyc* mutants (Figure 3 and Supplemental Figure 1).

The functional significance of this difference in temporal expression pattern is unknown. There are other cases where mRNA displays cyclic expression, while the corresponding protein does not. In the chicken pineal gland, the mRNA encoding the photopigment Pinopsin shows a rhythm that is dependent upon activation by light (Takanaka et al., 1998). In zebrafish, *interphotoreceptor retinoid-binding protein (irbp)* mRNA expression is circadian-regulated, while and IRBP protein levels are constant (Rajendran et al., 1996; Stenkamp et al., 1998). In the case of IRBP, it is proposed that the higher expression of mRNA during the day is necessary to maintain the constant levels of protein, as protein turnover is much higher during the day than during the night (Rajendran et al., 1996; Stenkamp et al., 1998). This could be the explanation for the rhythmic pattern of *exorh* and *pinopsin* mRNA expression as well. Consistent with this, Pinopsin protein and mRNA levels decline rapidly when the embryos are placed in constant darkness (Takanaka et al., 1998).

**Conclusion**

More than two decades ago, a structure anatomically equivalent to the SCN was first described in a teleost fish (the goldfish) (Springer and Gaffney, 1981; Springer and Mednick, 1984). Several years later, a morphologically similar group of neurons was described in zebrafish (Burrill and Easter, 1994). However, no experimental evidence exists for a pacemaker role of the SCN in zebrafish or in other teleosts (Kaneko et al., 2006). Here, we establish that the zebrafish SCN is not required to establish
circadian rhythms in the embryonic pineal. However, the amplitude of circadian gene expression was slightly reduced, and so we cannot rule out a role for the SCN or other tissues missing in cyc mutants in helping to establish or maintain rhythms in developing zebrafish. This will form a strong foundation for future studies that explore the communication between different circadian tissues during development, and for comparative studies of pineal biology and circadian regulation between vertebrate species.

**Acknowledgements**

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**Figure Legends**

**Figure 1. vasotocin expression is absent in cyc mutants.** Embryos were fixed at (A, B) 24 hpf, (C-F) 46 hpf, and (G-I) 72 hpf and then processed for WISH with antisense probes for the indicated mRNAs. *shh*-expression in the ventral brain, including the hypothalamus (h, closed arrowhead) is (A) apparent in WT embryos but (B) absent in cyc mutants. (C, E, G, and I) *vasotocin* is expressed in the dorsal preoptic area (open arrowheads) and the ventral hypothalamus (closed arrowheads). (D, F, H, and J) Both *vasotocin* expression domains are eliminated in cyc mutants. (A-D, G, and H) are lateral views and (E, F, I, and J) are ventral views. The images in panels (C-J) are representative embryos of three independent experiments (n=3 fish per experiment). *vasotocin* expression was also completely absent in cyc mutants at 47 hpf, ZT23.5; 52 hpf, ZT 5.5; 62 hpf, ZT 15.5, and 71.5 hpf, ZT 23.5. No circadian rhythm in vasotocin expression was detected in the WT larva processed in parallel (n>4 larva per time point). Scale bars=50 µm.

**Figure 2. Rhythmic expression of pineal genes persists in cyc mutants.** Embryos were raised in a 14:10 h L/D cycle and then sets of embryos were fixed at the indicated stages and ZT and processed for WISH for expression of (A) *exorh* or (B) *aanat2*. Note the time of peak (closed arrowheads) and trough (open arrowheads) expression is the same between both sets of embryos. (C, D) Quantification of the WISH signal indicates that the amplitude of *aanat2* and *exorh* gene expression is slightly lower in cyc mutants than in their WT siblings. Two-way ANOVA reveals that the difference in the strength of the *aanat2* signal is significantly different between cyc and WT (p<0.01), which the strength of the *exorh* signal is not (p=0.14). (E, F) In contrast, the expression of *otx5*, a gene expressed in all pineal cells, is comparable between WT and cyc mutants. (F) At 54 hpf, ZT6 (left graph) and 78 hpf, ZT6 (right graph), length of *otx5* gene expression domain is similar between WT and cyc mutants, while the width of the
pineal at each stage is slightly bigger in the mutants, perhaps due to changes in the morphology of the cyc head (54 hpf, pineal length, p=0.11; 54 hpf, pineal width, p=0.0045; 78 hpf, pineal length, p=0.52; 78 hpf, pineal width, p=0.14, n≥3 embryos per time point). In A, B, and E, light conditions are indicated by the white (light period) and black (dark period) bars. All images are dorsal views, anterior to the top. Each experiment was repeated three times, and representative images are shown. Scale bars=20 µm.

**Figure 3. Pineal Exorh protein expression is indistinguishable between cyc mutant and WT embryos.** Embryos were fixed and processed for whole mount antibody staining with the anti-Rhodopsin antibody 4D2. Quantification of fluorescent antibody signal shows that optical density of the pineal immunostaining is not significantly different at either time point (46 hpf, ZT 22, n=10 embryos, p=0.41; 72 hpf, ZT 0., n=10 larvae, p=0.67). Images are dorsal views with anterior to the top. Experiment was repeated two times with similar results, and representative embryos are shown. Scale bars=20 µm.

**Figure 4: Two days in a L/D cycle is not sufficient to initiate robust circadian cycling of aanat2 expression.** Embryos were raised in a 14:10 h L/D cycle. At 47 hpf, ZT 23.5, a set of embryos was transferred to constant dark, constant temperature conditions. Embryos were fixed at the indicated stages and ZT and processed for WISH for expression of aanat2. Note the time of peak (closed arrowheads) expression is similar between the cyc embryos and their WT siblings. All images are dorsal views, anterior to the top. For the samples in a L/D cycle, position within the photoperiod is indicated by ZT time and light conditions by the white (light period) and black (dark period) bars. For the constant dark samples, the original L/D cycle is indicated by the ZT time and the black (original dark period) and dark
grey (original light period) bars. Experiment was repeated two times with similar results, and representative images from one of the experiments are shown. Scale bar=20 µm.

**Figure 5. Rhythmic expression of aanat2 persists in constant conditions.** Embryos were raised in a 14:10 h L/D cycle. At 71.5 hpf, ZT 23.5, a set of embryos was transferred to constant dark, constant temperature conditions. At 77.5 hpf, ZT 5.5 a set of embryos were transferred to constant light, constant temperature conditions. Embryos were fixed at the indicated stages and ZT and processed for WISH for expression of (A) aanat2. Note the time of peak expression is similar between the cyc embryos and their WT siblings. All images are dorsal views, anterior to the top. (B, C) Quantification of the WISH signal indicates that the amplitude of aanat2 gene expression is slightly lower in cyc mutants that in their WT siblings in constant conditions. Two-way ANOVA reveals that the differences between aanat2 signal is significantly different between cyc and WT (p<0.01 for both constant light and constant dark, n≥10 larva per time point, images of 4 larva per each experimental condition and time point used for statistical analysis). For the samples in a L/D cycle, position within the photoperiod is indicated by ZT time and light conditions by the white (light period) and black (dark period) bars. For the constant light samples, the original L/D cycle is indicated by the ZT time, and the white (original light period) and light grey (original dark period) bars. For the constant dark experiment, the original L/D cycle is indicated by the ZT time, and the black (original dark period) and grey (original light period) bars. Experiment was repeated two times with similar results, and representative images from one of the experiments are shown in A. Scale bar=20 µm.

**Figure 6. Expression of cry genes in zebrafish larvae.** WT larvae were fixed at the indicated stages and then processed for WISH with an antisense probe for cry1b or cry3. (A-C) cry1b is expressed in the
brain from 3 to 5 dpf (closed arrowheads). (D, G) At 3 dpf, cry3 is expressed in the brain (closed arrowheads), ear (open arrowheads) and in a region of the retina around the lens (black arrows). (E, H) At 4 dpf, cry3 is expressed in the brain (closed arrowheads) and ear (open arrowheads). (F, I) By 5 dpf, cry3 transcripts appear in the liver (gray arrowheads), the ganglion cell (black arrows) and inner nuclear layers (white arrows) of the retina, and persist in the brain (closed arrowheads). WISH at each stage was repeated two times and representative images are shown (n≥5 embryos for the experiment shown).

Panels (A-F) are lateral views, anterior to the left and panels (G-I) are dorsal views, anterior to the top. Scale bars=500 µm.

**Figure 7. cry gene expression is present in cyc mutants.** cyc embryos and their WT siblings were raised in a 14:10 h L/D cycle, fixed at 72 hpf, ZT 0, and then processed for WISH. In both (A-C, G-I) WT embryos and (D-F, J-L) cyc mutants, cry1b and cry3 are expressed in the (A, D, G, J) brain (closed arrowheads), (B, E, H, K) liver (gray arrowheads), and (C, F, I, L) ear (open white arrowheads).

Experiment was repeated three times, and representative images are shown. All images are lateral views, anterior to the left. Scale bars=40 µm for (A-B, D-E, G-H, J-K) and 100 µm for (C, F, I, L).

**Supplemental Figure 1. Exorh protein is expressed without a significant rhythm.** (A, B) Embryos were injected with (A) control or (B) exorh atg MO, fixed at 64 hpf, and processed for fluorescent whole mount immunostaining with the anti-bovine Rhodopsin antibody 4D2. (A) Control embryos have robust fluorescent signal in the pineal organ that is (B) severely reduced in Exorh depleted embryos. (C-C’) Embryos injected with beta-galactosidase mRNA have undetectable levels of immunoreactivity with the 4D2 antibody at 8 hpf. (D-D’) In contrast, embryos injected with exorh mRNA show strong antibody staining at 8 hpf. (E, F) Embryos were fixed in a circadian time course and the processed for 4D2
antibody staining. One-way Analysis of Variance (ANOVA) and Tukey’s analysis revealed no significant changes in pineal Exorh protein levels that followed a daily rhythm (n≥9 embryos per time point). However, a few time points were significantly different (p≤0.05) from each other including 72 and 108 hpf, 72 and 116 hpf, 76 and 116 hpf, and 80 and 116 hpf. (A-B, and E) are dorsal views, anterior to the top and (C-D’) are lateral views. (C’) and (D’) are higher magnification images of the regions boxed in (C) and (D), respectively. Scale bars=20 µm for (A-B, C’, D’, E) and 100 µm for (C, D).

Supplemental Figure 2. Comparison between larva processed for WISH with antisense and sense probes reveals low background staining. Embryos were raised in a 14:10 h L/D cycle and fixed and processed for WISH using aanat2 antisense or sense probe. Note that the sense probe produces no detectable signal, as it would recognize antisense mRNA, which should not be present. The brown regions are melanocytes in the skin, which have a natural pigment. All images are dorsal views, anterior to the top. For the samples in a L/D cycle, position within the photoperiod is indicated by ZT time and light conditions by the white (light period) and black (dark period) bars. Representative images are shown. Scale bar=30 µm.

References


Figure 1

**shh**
- 24 hpf
- WT: Panel A
- cyc: Panel B

**avpl**
- 46 hpf
- WT: Panel C
- cyc: Panel D

**avpl**
- 72 hpf
- WT: Panel E
- cyc: Panel F

Additional panels for comparison are shown in panels G, H, I, and J, highlighting different stages and conditions.
**Figure 2**

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Figure 3

WT

46 hpf

A

cyc

72 hpf

B

C

D

E

Exorh protein

46 hpf

72 hpf

Optical Density (Arbitrary Units)

WT  cyc

0  2  4  6  8  10  12  14  16

66
Figure 4

A

light/dark

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constant dark

WT

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Optical Density (Arbitrary Units)

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WT - light/dark

WT - dark/dark

cyc - dark/dark
Figure 5

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light/dark

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hpf 71.5 77.5 83.5 89.5 95.5 101.5 107.5 113.5 119.5

B

- WT - light/dark
- WT - constant light
- cyc - constant light

Optical Density (Arbitrary Units)

ZT 23.5 5.5 11.5 17.5 23.5 5.5 11.5 17.5 23.5

hpf 71.5 77.5 83.5 89.5 95.5 101.5 107.5 113.5 119.5

- WT - light/dark
- WT - constant dark
- cyc - constant dark

Optical Density (Arbitrary Units)

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hpf 71.5 77.5 83.5 89.5 95.5 101.5 107.5 113.5 119.5
Figure 7

**cryptochrome1b**

A: WT  
D: cyc

**cryptochrome3**

G: WT  
J: cyc

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Supplement Figure 2

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antisense probe

sense probe

light/dark
Chapter 3

Failure in closure of the anterior neural tube causes left isomerization of the zebrafish epithalamus

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* Corresponding author
Authors’ Contributions

PL designed the study, performed experiments of the parapineal phenotypes in flh mRNA injected embryos and oep$^{tz257}$ mutant embryos, performed experiments of the habenula phenotypes in flh mRNA injected embryos, helped analyzing the parapineal phenotypes in cdh2 mutants and performed experiments of the ctn expression pattern in cyc mutants. PL also wrote the first draft of the manuscript except for the abstract. CL and MHT performed experiments of the pineal phenotypes in sqt mutant embryos and flh mRNA injected embryos and the experiments of aanat2 rhythmic expression in flh mRNA injected embryo. SK performed the experiments of the parapineal phenotype in cdh2 mutants. AS performed the experiment of the pineal phenotype in oep$^{m134}$ and oep$^{tz257}$ mutants. JTG helped design and coordinated the study. JOL helped design and coordinated the study, wrote the first draft of the abstract and helped revise the manuscript. All authors helped draft the manuscript.
Abstract

Asymmetries between the left and right sides of the brain are present in many vertebrate and invertebrate species. These asymmetries are thought to be important for the efficient control of specialized brain functions. For instance, the left cerebral hemisphere in humans is largely responsible for language and tool use and the right for processing spatial information. Zebrafish and other lower vertebrates have prominent left-right asymmetries in their epithalami that have been associated with differential left and right eye use and navigational behavior. When midline tissues are absent, this asymmetry is disrupted: the first asymmetric expression of Nodal pathway genes in the left side of the pineal anlage becomes bilateral and subsequent sidedness of the parapineal organ and asymmetry of the habenula nuclei are randomized. In embryos that have an open neural tube, the pineal precursors never converge to the dorsal midline to form a single pineal organ. In contrast to embryos with midline defects, we found that the epithalamus becomes left isomerized when the forebrain neural tube does not close. Nodal pathway genes are expressed on both sides of the divided pineal organ, the normally left-sided parapineal organ develops on both sides, and both habenula nuclei have a “left” pattern of gene expression. In contrast, other aspects of pineal development, such as expression of photoreceptor, projection neuron, and rhythmic genes, are normal. These results suggest that there is a mechanism in normal embryos that prevents both sides from becoming left. This mechanism must persist in embryos with closed neural tubes and midline defects, but loses its function when the left and right sides of the dorsal brain are widely separated from one another. Loss of asymmetry has also been found in a subset of people with dyslexia, suggesting that failure of a similar mechanism might be part of the etiology of this learning disorder.
Introduction

Structural and functional asymmetries of brain have been described in humans and other vertebrates. These asymmetries are thought to be the result of the localization of specialized functions to one side or the other. One of the best understood examples in humans is the localization of language processing to the left hemisphere. This functional asymmetry has been linked to a morphological asymmetry in the planum temporale, a region in the temporal lobe of the cerebral cortex, that are detectable as early as the third trimester of fetal life and continue through adulthood (Preis et al., 1999). In normal adults, the left planum temporale has a larger volume and cellular differences such as a greater extent of myelination (Hugdahl, 2005; Steinmetz, 1996).

The zebrafish epithalamus has a several prominent left-right asymmetries that have also been linked to laterality in behavior. In wildtype (WT) embryos, the first apparent asymmetry is the expression of genes encoding components of the Nodal signaling pathway, *cyclops* (*cyc*), *lefty1* (*lft1*), and *pitx2* in the left side of the developing pineal complex (Concha et al., 2000; Liang et al., 2000; Sampath et al., 1998). Cells in this anlage will give rise to pineal gland, located at the dorsal midline of the brain, and the parapineal organ, located to the left of the pineal (Concha et al., 2003; Gamse et al., 2003). The parapineal regulates the asymmetry in the underlying habenular nuclei, which include higher neuropil density and expression of the potassium channel tetramerisation domain containing 12.1 (*kctd12.1*) gene on the left and higher *kctd12.2* expression on the right (Aizawa et al., 2007; Gamse et al., 2005; Gamse et al., 2003; Kuan et al., 2007a; Kuan et al., 2007b). The left and right habenula also develop different connections. Axons from the left habenula extend to the dorsal and ventral regions of the Interpenducular Nucleus (IPN) in the midbrain, while axons from the right habenula extend only to the ventral region (Gamse et al., 2005).
The habenula and IPN have both been implicated in the regulation of behavior, suggesting that the morphological asymmetries in the zebrafish brain may be important for behaviors that show left-right differences (Bianco and Wilson, 2009; Vallortigara et al., 1999). In many species of birds, frogs, and fish, including zebrafish, there is a tendency to use the left and right eyes differently. The right eye is used preferentially when an object is unfamiliar or complicated, and the left for familiar objects and scenes (Andrew et al., 2009; Bianco and Wilson, 2009; Miklosi and Andrew, 1999; Miklosi et al., 1997; Vallortigara et al., 1999). In zebrafish, there is strong evidence that this laterality in eye use is controlled by the asymmetries in the epithalamus and IPN. The frequent situs inversus (fsi) line of zebrafish has a high rate of situs inversus in the epithalamus and the viscera (Barth et al., 2005). Fish with reversed situ also had reversed eye use when viewing themselves in a mirror or when preparing to bite a target (Barth et al., 2005). When WT fish with reversed parapineal sidedness were placed into a tank with a mirror, they also had a delay in the onset of navigation around the tank and a decrease in the total distance traveled within a given time (Facchin et al., 2009).

There are many features shared between the pathways that establish asymmetry in the visceral organs and asymmetry in the zebrafish brain. Both asymmetries depend upon the function of cilia that create a leftward flow of fluid across the organizer during gastrulation and on the asymmetric expression of Nodal pathway genes on the left side (Cooke, 2004). In addition, midline tissue has a conserved role in maintaining asymmetry by preventing “left” signals from crossing over to the right side. In visceral asymmetry, midline floor plate and notochord form a physical barrier and also express Lefty, an inhibitor of the Nodal signaling pathway (Cooke, 2004). In the zebrafish brain, lefty1 is expressed throughout the left pineal anlage, not exclusively in the midline, suggesting it may not only function as a barrier. However, as in visceral asymmetry, when the notochord or prechordal plate, the mesodermal structures beneath the developing neural tube, is defective, the ventral midline of the brain is disrupted,
genes that are normally on the left side are expressed bilaterally (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). Interestingly, subsequent formation of asymmetric structures including the parapineal and habenular nuclei is still established, but the sidedness is randomized between normal and reversed situs (Gamse et al., 2002; Gamse et al., 2003).

We find that zebrafish embryos that have an open anterior neural tube have a brain asymmetry phenotype that is distinct from that of embryos with midline defects. Instead of developing randomized left-right asymmetry, both the left and right epithalamus exhibit the gene expression pattern of the left habenula, which can be described as “left isomerized”. When the neural tube does not close, the two groups of pineal precursors that originate in the left and right neural plate remain widely separated instead of fusing at the dorsal midline (Aquilina-Beck et al., 2007). We find that pineal cells maintain their identity despite their abnormal location. Both domains of pineal precursors express markers characteristic of pineal photoreceptors or projection neurons. Further, the gene serotonin N-acetyltransferase 2 (aanat2), which encodes an enzyme required for pineal melatonin biosynthesis, is expressed rhythmically. In contrast, both domains express the normally left-sided Nodal pathway genes, and both domains make a parapineal. The habenula nuclei are formed, but they are more widely spaced and both have gene expression consistent with a “left” identity. This left-isomerized phenotype suggests that there is a mechanism that normally prevents both sides from becoming left that is absent when the neural tube does not close.

**Methods**

**Zebrafish**

Zebrafish stocks were maintained at 28.5°C in a 14:10 light:dark cycle according to standard procedure (Westerfield, 2000). Stocks used were the WT strain Zebrafish Danio rerio (ZDR) (Aquatica...
Tropicals, Plant City, FL), foxd3:GFP (Eichele et al., 2005), Tg(foxd3:GFP)\textsuperscript{flg3} (Gilmour et al., 2002), n-cadherin (cdh2\textsuperscript{vu125}) (von der Hardt et al., 2007), oep\textsuperscript{otz257} (Brand et al., 1996), sqt\textsuperscript{cz35} (Feldman et al., 1998), and sqt\textsuperscript{cz35},cyc\textsuperscript{m294} (Feldman et al., 1998). Genotyping and phenotyping information on cdh2\textsuperscript{vu125} is published in von der Hardt et al., 2007. WT, transgenic, and mutant embryos were obtained through natural matings (Westerfield, 2000). oep\textsuperscript{otz257}, sqt\textsuperscript{cz35} and sqt\textsuperscript{cz35},cyc\textsuperscript{m294} embryos were raised at 33°C for the first 24hpf. sqt\textsuperscript{cz35},cyc\textsuperscript{m294} embryos with sqt phenotypes but without cyc\textsuperscript{m294} phenotypes were used as sqt\textsuperscript{cz35} mutants.

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

WISH was carried out as previously described (Thisse et al., 1993). Antisense RNA probes included: serotonin N-acetyl transferase (aanat2) (Gothilf et al., 1999), exorhodopsin (exorh) (Mano et al., 1999), orthodenticle homeobox 5 (otx5) (Gamse et al., 2002), paired-like homeodomain transcription factor 2 (pitx2) (Tsukui et al., 1999), crestin (Rubinstein et al., 2000), lefty1/antivin (lft1/atv) (Thisse and Thisse, 1999), floating head (flh) (Talbot et al., 1995), kctd12.2/lov (Gamse et al., 2003), kctd12.2/ron (Gamse et al., 2005), growth factor independent 1.2 (gfi1.2) (Dufourcq et al., 2004).

**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.

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(Invitrogen) was used at a dilution of 1:2000. Rabbit anti-GFP (Torrey Pines Biolabs) was used at a dilution of 1:500.

*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 with a 20X objective. Confocal images were collected on a Zeiss/Perkin Elmer spinning disk confocal microscope with a 40X oil-immersion objective and analyzed with Volocity software (Improvision).

*mRNA Injections*

Embryos from the ZDR and foxd3:GFP lines were injected with .63 pg or 5 pg of *lft1* mRNA or *GFP* mRNA at the one cell stage using a Harvard Apparatus PLI-90 nitrogen picoinjector.

**Results**

*Neural tube closure is not required for the differentiation of dorsal neural tube cells*

Our previous work demonstrated that Nodal signaling is required for closure of the anterior neural tube in zebrafish (Aquilina-Beck et al., 2007). In Nodal defective embryos, the dorsal neural tube is disrupted. The cells that normally converge at the dorsal midline are displaced laterally and fail to fuse to form a single tissue (Figure 1). For instance, in WT embryos the two regions of pineal precursors are located at the lateral edges of the neural plate at the onset of neurulation. As the neural tube folds, these precursors move closer together and ultimately form a single structure that spans the midline of the epithalamus. Despite the severely disrupted neural tube morphology of Nodal defective embryos, cells of the pineal gland differentiate to form specific cell types and begin their rhythmic function. For example mutants that lack expression of the zebrafish Nodal signal Sqt have an extremely variable phenotype, including neural tube closure defects phenotypes from normal to severe (Aquilina-Beck et al.,
2007). However, flh, a transcription factor for pinealocyte differentiation, is still expressed at high levels even in severely affected sqt mutants and in cyc;sqt double mutants, which have almost no Nodal signaling (Figure 1, Supplemental Tables 1 and 2) (Aquilina-Beck et al., 2007).

Injections of the Nodal inhibitor lft1 mRNA can phenocopy the phenotypes found in Nodal signaling mutants (Thisse and Thisse, 1999) (Supplemental Figure 1). Consistent with this, we found that embryos overexpressing lft1 have a high frequency of open neural tube defects (Figure 2M). In this study, lft1 mRNA injections were used to produce large numbers of Nodal deficient embryos for analysis. We found that the cells in embryos with divided pineal organs also initiated the expression of cell type specific genes and proteins. The center of the pineal organ is populated by photoreceptors that are responsible for detecting changes in environmental light conditions and entrain the pineal circadian clock (Cahill, 2002; Korf et al., 1998). These photoreceptors are surrounded by projection neurons that extend axons to the ventral brain (Wilson and Easter, 1991). In WT embryos, control injected embryos, and lft1 mRNA injected embryos with a single oval shaped pineal, the protein Exorh, a pineal photopigment, was expressed in the center domain of the pineal corresponding to the photoreceptor end segments (Figure 2G). Similarly, the exorh mRNA was found in an oval shaped domain that spanned across the dorsal midline (Figure 2A). In embryos with an open neural tube, both Exorh protein and exorh mRNA were expressed in either a pineal anlage that was elongated or divided into two widely spaced domains (Figure 2C, I).

The foxd3 gene is expressed in pineal projection neurons and in some pineal photoreceptors (Lein et al., 2007; Morris, 2009). In lft1 mRNA injected foxd3:GFP transgenic embryos, the GFP signal was detected in an oval shaped domain typical of a normal pineal anlage, an elongated domain or in two divided regions (Figure 2D-F). Overlaying of foxd3:GFP and Exorh immunostaining signal confirmed that the majority of the foxd3:GFP expressing area did not overlap with the Exorh expressing area,
suggesting that the two major cell types were being specified in both WT embryos and embryos with an open neural tube (Figure 2J-L).

One of the main functions of the pineal organ is the rhythmic biosynthesis of the hormone melatonin, which regulates daily rest activity rhythms (Cahill, 2002; Korf et al., 1998). In zebrafish, onset of rhythmic expression of the melatonin pathway gene \textit{aanat2} depends upon the activity of pineal photoreceptors (Gothilf et al., 1999; Ziv and Gothilf, 2006; Ziv et al., 2005). In WT embryos raised in a normal 14:10 light:dark cycle, \textit{aanat2} mRNA is expressed at low levels during the day and at high levels during the night, mirroring the nocturnal rise in melatonin levels (Figure 3)(Gothilf et al., 1999). We found that this rhythmic expression pattern of \textit{aanat2} was preserved in embryos with open neural tubes, suggesting that rhythmic function of the pineal has been initiated (Figure 3).

Neural crest cells are specified at the border between the cells that are going to give rise to the dorsal neural tube and the cells that are going to give rise to the epidermis of the skin. Thus, the morphology of the region that normally specifies the cranial neural crest is severely disrupted in embryos with neural tube defects, suggesting that differentiation of these cells might be affected. In WT embryos at the 6 somite stage, \textit{crestin} was highly expressed in two rows of presumed premigratory neural crest cells along the developing hindbrain, and during somitogenesis the expression areas gradually expend rostrally and caudally along the left and right borders of the developing neural tube (Rubinstein et al., 2000)(Figure 4A). We found that in \textit{sqt} mutants with divided pineal, the two \textit{crestin} expression domains still formed, including the concentrated groups of cells adjacent to rhombomeres 6 (Figure 4). Consistent with the fact that the neural crest derived cranial structures are severely disrupted in Nodal signaling mutants, the neural crest cells at later stages were present, but their arrangement was very different than in their WT siblings (Supplemental Figure 2) (Rubinstein et al., 2000).
The initiation of asymmetry in the epithalamus is disrupted when the neural tube is open

Left-sided expression of cyc, lft1 and pitx2 in the left side of the presumptive pineal organ during the late somite stages is the earliest known left-right asymmetry in the zebrafish brain (Concha et al., 2000; Liang et al., 2000). In sqt mutants with an oval shaped pineal and closed neural tube, both lft1 and pitx2 were most often expressed bilaterally, with a smaller proportion of embryos having normal left-sided expression (Figure 5). These two phenotypes likely reflect the variable severity in the defects caused by Nodal deficiency on the prechordal plate, which forms a midline barrier that prevents bilateral expression (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). In sqt mutants with a divided pineal, the expression of lft1 and pitx2 was nearly always bilateral (n=24/25) (Figure 5D, H, I).

Parapineal organs are formed on both sides of the divided epithalamus when the neural tube does not close

The parapineal organ forms to the left of the pineal organ in many teleost fish (Borg et al., 1983). The cells of the parapineal organ originate from both sides of the pineal, and migrate leftward out of the pineal domain shortly after the left-sided expression of Nodal pathway genes (Concha et al., 2003; Gamse et al., 2003). We found that when the pineal organ of lft1 mRNA injected embryos was oval shaped, more than 80% of the embryos had a single gfi1.2 staining left to the pineal organ (Figure 6A-C, G). In contrast, when the pineal was divided, two gfi1.2 expression domains were observed (n=19/27) (Figure 6D-F, G). Our results indicate that the defects in neural tube closure often leads to a divided pineal organ and bilaterally placed parapineal organs.

n-cadherin (cdh2nu125) mutants exhibit both fused and divided pineal phenotypes, similar to those observed in Nodal pathway deficient embryos
We decided to further analyze parapineal formation in another mutant that exhibits a neural tube closure defect, \( cdh2^{vul25} \). We previously reported that Nodal pathway mutants exhibit reductions in N-cadherin expression and trafficking (Aquilina-Beck et al., 2007). Additionally, \( n\text{-}cadherin \) mutants have neural tube closure defects comparable to embryos with disrupted Nodal signaling (Aquilina-Beck et al., 2007). As predicted, \( cdh2^{vul25} \) mutants display a range of pineal fusion phenotypes (Figure 7).

Furthermore, we also observed that two parapineal organs are formed in embryos with more severe pineal fusion defects (Figure 7G).

To characterize the pineal complex morphology in \( cdh2^{vul25} \) mutants, we utilized the \( foxd3\):GFP transgene, which is expressed in the pineal and parapineal organs (Figure 7A-C). In WT embryos, the pineal organ is fused and is flanked on the left side by a single parapineal organ (arrowhead) (Figure 7A). \( cdh2^{vul25} \) mutants, as in \( lft1 \)-overexpressing embryos, fused pineal organ while other develop with a divided pineal organ (Figure 7B, C). Notably, in embryos with divided pineal and two parapineal organs, the parapineal cells still send projections (open arrowhead) anteriorly, suggesting that these cells differentiate as neurons regardless of their location (Figure 7C).

It is difficult to distinguish between pineal and parapineal cells by looking only at the \( foxd3\):GFP transgene. Thus, we also use two-color \textit{in situ} hybridization to distinguish pineal and parapineal cells, which express \( flh \) and \( gfi1.2 \), respectively (Figure 7D-F). In WT siblings, all of the embryos had a fused pineal and a single parapineal organ (n=261), with the vast majority being left sided (Figure 7D, G and Table 1. A minority (29%, n=160) of \( cdh2^{vul25} \) mutant embryos formed a fused pineal, and in most (85%) of these individuals, the parapineal formed on the left side. In contrast, the majority of \( cdh2^{vul25} \) mutants exhibit a divided pineal phenotype (71%, n= 160). In \( cdh2^{vul25} \) mutants with a divided pineal phenotype, 42% of embryos had a single, left sided parapineal organ (Figure 7E), while 41% had two distinct bilaterally located parapineal organs (Figure 7F).
We noticed that the percentage of divided pineal embryos with left isomerized phenotypes in the \textit{cdh2}\textsubscript{vu125} mutant is lower than \textit{lft1} mRNA injected embryos. We hypothesized that the chance of left-isomerization increases with the increasing of the distance between the divided pineals. To test this hypothesis, we first categorized the \textit{cdh2}\textsubscript{vu125} mutant embryos based on the severity if their parapineal phenotypes. Then we measured the width of the pineal anlage, defined by the distance between the two most lateral points of the pineal anlage, regardless of the pineal phenotypes. We found that the width of the pineal anlage is correlated to the severity of the abnormal parapineal phenotype in \textit{cdh2}\textsubscript{vu125} mutation (Figure 8A). However, whether such correlation is also true in Nodal deficient embryos remains to be investigated.

We also noticed that the relative position of the parapineal to the pineal might not be random when the pineal is divided. To test the hypothesis that the parapineal is biased toward certain direction when the neural tube is open, we group the position of the parapineals into nine categories: overlapping with the pineal organ without directional bias; overlapping with the pineal organ but biased to the anterior, posterior, left or right; not overlapping with the pineal organ and biased to the anterior, posterior, left or right. Currently we only obtained a small sample size (n=6) and preliminary results indicate that when the divided pineal phenotype was caused by the \textit{cdh2}\textsubscript{vu125} mutation, the parapineals tend to locate on the anterior side of the pineal anlage (Figure 8 B).

\textit{Habenulae are left isomerized when the neural tube is open}

The parapineal is responsible for directing the habenula asymmetry. In normal embryos and embryos with reversed asymmetry, the parapineal is always associated with the habenula nucleus with the “left” pattern of high neuropil density, higher \textit{kctd12.1} expression, and lower \textit{kctd12.2} expression (Concha et al., 2003; Gamse et al., 2003). Further, when the parapineal was ablated, the habenula
developed a right sided pattern of gene expression on both sides, suggesting that it became right isomerized (Gamse et al., 2003).

Consistent with this, we found that embryos with an open neural tube, which have a parapineal on each side, often form habenular nuclei that are left isomerized (Figure 9). As in earlier studies, kctd12.1 was highly expressed in the left habenula (L > R) in more than 80% of WT embryos, with the majority of the remaining embryos having higher expression of kctd12.1 on the right (L < R) (Figure 9). The expression of kctd12.2 was higher in the right habenula in more than 80% of the WT embryos, although generally the difference between left and right was not as dramatic as for kctd12.1 (Figure 10). The rate of reversed or abnormal kctd12.1 expression was slightly increased in GFP mRNA injected control embryos, even though they had an oval shaped pineal (Figure 9E). This increase was also apparent in the lft1 mRNA injected embryos with an oval shaped pineal, suggesting that the process of injection can sometimes affect the pathways needed to establish the left-right axis of the brain (Figure 9E).

When the neural tube was open, both of the habenula nuclei had a “left” pattern of high kctd12.1 expression at a high frequency (56%; Figure 9D, G). In contrast, kctd12.2 expression level was either extremely low or undetectable in the majority of embryos (Figure 10D, E, F). In addition, when lft1 mRNA injection resulted in a divided pineal morphology, the average distance between the left and right habenulae was significantly increased (Figure 9H).

To address the concern that neither sqt mutation nor lft1 mRNA injection were completely eliminating Nodal signaling during neurogenesis, we took advantage of oep^{m257} mutant embryos, which have a null mutation in a gene that encodes a component of the Nodal receptor. 57% of oep^{m257} mutant embryos had an open neural tube (Figure 11). This was significantly higher than the 14% rate of neural tube closure in oep^{m134} mutants, which have a hypomorphic mutation (Figure 11E) (Gritsman et al., 1999;
Zhang et al., 1998). We found that when an oep\textsuperscript{12757} embryo had a fused pineal, indicated by the expression of otx5, there was always a single parapineal located either to the left (56%) or right (44%) of the pineal (Figure 12 A, D). In contrast, when the pineal was divided, the majority (86%) of the embryos had two bilaterally located parapineals (Figure 12 C, D). Interestingly, when the pineal was elongated, we most often detected a single, medially located parapineal (67%) (Figure 12 B, D).

Discussion

Communication between the left and right sides of the brain may be important for development of left right asymmetry

In this study we find that all three known left-right asymmetric structures in zebrafish epithalamus, including the pineal organ, parapineal organ, and habenulae nuclei are left-isomerized when the neural tube does not close due to defects in the Nodal signaling pathway (summarized in Figure 13). Several cell types that normally differentiate at the dorsal midline still express cell type specific genes and proteins including foxD3 (Figure 1, 2), exorh (Figure 2), aanat2 (Figure 3), crestin (Figure 4), thus there is no evidence that the cells of the dorsal neuroepithelium are mis-specified. Embryos that have an open neural tube due to lack of N-cadherin mediated cell adhesion also have bilateral formation of parapineal organs (Figure 7). There is no evidence that Nodal signaling is altered in \textit{n-cadherin} mutant embryos, suggesting that this phenotype is due to the morphological changes that occur when the neural tube does not close.

Previous studies have shown that embryos with ventral neural tube midline defects, such as lack of prechordal plate, are initially left-isomerized as they have bilateral expression of normally left-sided genes in the pineal anlage (Concha et al., 2000; Halpern et al., 2003). However, the subsequent formation of asymmetric structures in the brain of midline mutants is not left isomerized but has either
normal or reversed situs. Comparison of embryos with open neural tubes and those with midline defects and WT embryos suggests that there is a mechanism that normally prevents both sides of the brain from developing a “left” morphology (Figure 13).

This proposed mechanism must be active in the absence of a ventral neural tube midline, but fail when the left and right sides are separated from one another. Further, since we find that embryos with neural tube defects become left-isomerized, it seems logical that the role for this mechanism is to tell one side to become “right” or to prevent one side from becoming “left”. It could be an active signal causing right sided morphology, or a signal that somehow blocks the pathway that causes left morphology. The timing of the switch between left isomerism and randomized asymmetry in embryos with midline mutants suggests that this mechanism acts after the expression of Nodal genes in the pineal anlage and before the formation of the parapineal (Figure 13).

One possibility is that this mechanism is mediated by a secreted signaling molecule released from the left epithalamus and instructing the opposite side to become right/not left. Consequently, when the distance between left and right epithalamus is increased due to the open neural tube, the signal received by the right epithalamus is no longer sufficient to prevent it from developing a “left” identity.

Although we cannot rule out other pathways, Lft1 itself is a good candidate to be this “right/not left” signal as part of a reaction-diffusion interaction. In the reaction-diffusion model, pattern is set up in the embryo by a group of cells producing both a slowly diffusing morphogen and a faster diffusing inhibitor (Gierer and Meinhardt, 1972; Meinhardt, 2008; Turing, 1990). These different rates of diffusion set up areas of high morphogen activity, which adopt a specific cell fate, surrounded by areas that adopt a different cell fate. In zebrafish, Cyc, Sqt, and their inhibitor Lft1 have been proposed to act according to this reaction-diffusion model during the formation of the organizer (Chen and Schier, 2001, 2002; Gritsman et al., 2000; Schier and Talbot, 2001). In mice, Nodal and its inhibitor Lefty2 have been
proposed to follow this reaction-diffusion model in the formation of visceral left-right asymmetry (Chen and Schier, 2001, 2002; Meinhardt, 2008; Nakamura et al., 2006).

Based on our proposed reaction-diffusion model, in a developing WT brain, Cyc protein is produced on the left side of the presumptive pineal and consequently activates the transcription of \( lft1 \) (Liang et al., 2000). Lft1 protein is an antagonist of Nodal signal which inhibits the activity of Cyc protein (Meno et al., 1999). However, while Cyc protein can only diffuse a few cell diameters, Lft1 is capable of diffusing to a significantly longer distance (Chen and Schier, 2001, 2002). Eventually the more widely distributed Lft1 protein suppresses the Cyc activity in any areas with low Cyc protein concentration. Cyc signaling remains active only in the left pineal, where Cyc concentration is too high to be fully suppressed by Lft1.

This Cyc/Lft1 based reaction-diffusion model is consistent with previous findings that in embryos with ventral neural tube midline defects the epithalamus is initially left-isomerized (Concha et al., 2000; Halpern et al., 2003). However, the subsequent formation of asymmetric structures is not left isomerized but with randomized sidedness. When the midline structures are disrupted due to Nodal deficient, Cyc is produced on both sides of the pineal anlage and consequently the expression of Lft1 is also bilateral (Halpern et al., 2003; Liang et al., 2000). The increased Cyc level may lead to increased \( lft1 \) expression and Lft1 activity; however the negative-feedback mechanism between Lft1 and Cyc eventually leads to the Lft1 protein suppressing any Cyc expression area until only one randomly located area with the highest Cyc activity and left characteristics remains.

This Cyc/Lft1 based reaction-diffusion model can also explain the cause of the left-isomerization phenotype. When the neural tube is open and the left and right sides of the epithalamus, including the pineal anlage, are much further apart from one another, the distance between the two sides becomes too
far for the diffusing Lft1 protein to cross. The lack of communication between the two sides of the developing brain leads to each side independently keeps a high Cyc area that leads to left identity.

When MZoep<sup>m134</sup> mutants (which lack both maternal and zygotic oep mRNA) are injected with oep mRNA to overcome gastrulation defects, the parapineal is either on the left or on the right in 95% of the embryos (Gamse et al., 2002; Gamse et al., 2003). However, previous work and our studies of neural tube defects suggest that the oep<sup>m134</sup> mutation is a hypomorph (Gritsman et al., 1999; Zhang et al., 1998). Thus, the MZoep<sup>m134</sup> mutants that were examined may have had some remaining Oep activity. In this study, a null mutation, oep<sup>tz257</sup> (Brand et al., 1996) was used. Our results from the Zoep<sup>tz257</sup> embryos confirmed that when the Nodal signaling is completely lost, randomized asymmetry of parapineal can still be established as long as the neural tube is closed. The Zoep<sup>tz257</sup> results further confirmed that epithalamus asymmetry can be established when Nodal signaling is lost.

A possible alternative mechanism of the This Cyc/Lft1 based reaction-diffusion model is the contact-dependent mechanism such as cell adhesion or lateral inhibition are involved in generating left-right asymmetry in the zebrafish brain. For instance, Nodal signaling mutants have reduced levels of N-cadherin (Aquilina-Beck et al., 2007). In the absence of N-cadherin, many aspects of polarity in the neuroepithelium are disrupted, including the oriented intercalation of cells to form a neuroepithelium with a single cell layer and lack of spatially oriented cell divisions (Hong and Brewster, 2006; Zigman et al., 2011). Thus, the lack of N-cadherin may cause an overall lack of polarity in the neural tube that also affects left-right axis formation.

We also have to consider the possibility that the open neural tube phenotype and the left isomerization phenotype are independently caused by the Nodal defects. Our results from cdh2 mutant suggest that the left isomerization can be produced via a Nodal independent mechanism. However we cannot rule out the possibility that the cdh2 mutation actually causes certain abnormality in Nodal
signaling pathway and it are the Nodal defects, instead of the neural tube phenotype that directly lead to the left isomerization. In chicken embryos, blocking N-cadherin disrupts the asymmetrical expression pattern of several genes, but the expression of nodal and lefty is unchanged (Garcia-Castro et al., 2000).

Differentiation of dorsal cell fates in neuroepithelium persists in embryos with neural tube defects

Our results demonstrated that pineal and neural crest cell differentiation occurs even in embryos with severe open neural tube phenotypes. The dorsal neural tube is the source of many morphogens such as Bone Morphogenic Protein 4, which sets up the dorsal ventral axis of the neural tube, and Wnts, which are involved in specifying neural crest cells. Thus, it is somewhat surprising that differentiation of dorsal cell types is so normal. However, this finding is consistent with the fact that expression of the transcription factor Flh, which has been shown to be required for the differentiation both pineal photoreceptors and projection neurons, is expressed in mutants that lack Nodal signaling (Aquilina-Beck et al., 2007; Masai et al., 1997). It also fits with the developmental pathway of the pineal cells in normal embryos, as expression of flh begins in the pineal precursors during neural plate stage, before the neural tube starts to fold (Aquilina-Beck et al., 2007; Masai et al., 1997).

Our results also demonstrated that initiation of pineal rhythmic function occurs in embryos with open anterior neural tubes. In mammals, the pineal organ is not directly light sensitive. Environmental lighting conditions are detected by retinal ganglion cells that reset circadian rhythms within the suprachiasmatic nucleus (SCN) in the hypothalamus {Liu, 2007 #767}. The SCN controls rhythms in the pineal through a multisynaptic pathway {Korf, 1994 #18;Korf, 1998 #17}{Moore, 1996 #973}. In contrast, rhythms in the zebrafish pineal are likely started by the detection of a dark-to-light transition by pineal photoreceptors. Pineal rhythms initiate in embryos that lack eyes or an SCN, but not in embryos that lack expression of the light sensitive pineal protein Period2 (Kennedy et al., 2004; Noche et al.,
2011; Ziv and Gothilf, 2006; Ziv et al., 2005). Thus, our finding that rhythmic gene expression in the pineal is present suggests that the differentiated photoreceptors are functional in embryos with open anterior neural tubes.

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Figure Legends

Figure 1: *flh* is expressed in embryos with an open neural tube. The (A - D) *flh*:EGFP transgene and (E) endogenous *flh* mRNA are expressed similarly in the pineal precursors of (A, B, E, F) WT and (C, D, G, H) mutant embryos with a closed neural tube. Dorsal views of the epithalamus in (A - D) live or (E - H) fixed embryos, anterior to the top. Scale bar = 20 µm.

Figure 2: Projection neuron and photoreceptor differentiation occurs in the pineal glands of embryos with open neural tubes. (A - C) Expression of the photoreceptor specific gene *exorh* is also expressed in embryos with all three pineal phenotypes (n=21 oval shaped, n=2 elongated, n= 6 divided). *foxd3*:GFP transgenic embryos were used and the pineal phenotypes were identified by the GFP signal detected in pineal organs. (D - L) Double fluorescent images of embryos expressing the *foxd3*:GFP transgene and Exorh protein, detected by antibody staining, show that these markers 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 shaped, n=4 elongated, n=11 divided). (M) Percentage of lft1 mRNA injected fish expressing the foxd3:GFP transgene in a oval shaped pattern (closed anterior neural tube), or elongated or divided pattern (open neural tube). n >120 embryos analyzed. All embryos at 48 hpf, dorsal views, anterior to the top. Scale bar = 25 μm.

**Figure 3: 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 - C) GFP mRNA injected control embryos with oval shaped pineal glands. (D - F) lft1 mRNA injected embryos with divided pineal glands. 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≥10 for embryos with both oval shaped and divided pineal. Scale bar =25μm

**Figure 4: The neural crest gene crestin is expressed in embryos with an open neural tube.** sqt embryos with an open neural tube were identified by two-color WISH by first detecting flh in the pineal with a red precipitate (not shown). The crestin signal in the neural crest cells was then detected via a purple precipitate. (A - C) In WT embryos, crestin is expressed in a large region next to rhombomeres 6 (open arrowheads), and in two stripes of cells on the left right sides at the borders of the folding neuroepitheiulm. (B and C) In sqt embryos with an open neural tube, both regions of crestin are present, although they are further apart. The experiment was repeated three times and representative images are shown. Embryos were at the 8-10 somite stage. All images are dorsal views with anterior to the left. Scale bar = 50μm.
Figure 5: Left-sided gene expression in the developing pineal is disrupted when the neural tube does not close. (A) Left sided expression of lft1 and bilateral expression of flh in the left pineal anlage of a WT embryo. (B) WT expression pattern of lft1 and flh in a sqrt mutant with a closed neural tube. (C) Bilateral lft1 expression in a sqrt mutant with a closed neural tube. (D) Expression of lft1 and flh in both domains of a sqrt embryo with a divided pineal. (E) WT embryo with pitx2 on the left and flh on both sides of the developing pineal. (F) sqrt mutant with a WT pattern of pitx2 expression. (G) Bilateral pitx2 expression when the pineal is not divided. (H) sqrt mutant with bilateral pitx2 expression and a divided pineal. (I) Quantification of lft1 and pitx2 expression patterns in embryos with closed and open neural tubes. WT and ndr1 embryos that did not show lft1 or pitx2 gene expression because they were too young or too old were not included. The midline of the brain is marked with a dotted lines and open arrowheads. All embryos were fixed at 26 somites stage and are shown in dorsal views with anterior to the top. The experiment was repeated three times and representative images are shown. Scale bar = 50µm.

Figure 6: Both the left and right sides of the brain form a parapineal when the epithalamus is divided. foxD3:GFP homozygous embryos were injected with GFP mRNA or lft1 mRNA at the one to two cell stage. Injected embryos and their uninjected siblings were sorted by the pineal phenotype using fluorescent microscopy, fixed at 3 dpf, and then assayed for gfi1.2 in the parapineal (closed arrowheads) and flh (open arrowheads) in the pineal. (A - C) In a WT embryo, GFP mRNA injected embryo, and lft1 injected embryos with oval shaped pineals, the parapineal is located adjacent to left side of the pineal. (D - F) In the lft1 injected embryos with divided pineals (not shown, as pineal is out of the focal plane of the image), there is a region of gfi1.2 expression on both the left and right sides of the brain, suggesting
two parapineals have formed. The experiment was repeated four times and representative images are shown. \( n \geq 26 \) for each pineal phenotype. Dorsal views, anterior to the top. Scale bar = 50 µm.

**Figure 7: n-cadherin (\( cdh2^{vu125} \)) mutants exhibit both fused and divided pineal phenotypes.** (A - C) Confocal images showing immunofluorescence labeling of \( \text{Tg(foxd3:gfp)}^{\text{fg3}} \) positive embryos at 2 dpf. \( \text{foxd3:GFP} \) is expressed in the pineal and parapineal organs (arrowheads). The projections from the parapineal cells are denoted with open arrowheads. (D - F) Two color *in situ* hybridization showing parapineal cells expressing *gfi1.2* (blue) and pineal cells expressing *flh* (red) in 2 dpf embryos. The dashed lines represent the embryonic midline. (A, D) WT embryos have a fused pineal organ and a single left sided parapineal organ. (B, E) Some \( cdh2^{vu125} \) mutants have a divided pineal organ, but only a single parapineal organ. (C, F) Other \( cdh2^{vu125} \) mutants have a divided pineal organ and two bilaterally located parapineal organs. All images are dorsal views with representative images shown. All scale bars = 20 µm. (G) A graph depicting parapineal placement seen in either WT siblings (\( n=261 \)), \( cdh2^{vu125} \) mutants with fused pineal (\( n=46 \)) and \( cdh2^{vu125} \) mutants with divided pineal (\( n=114 \)).

**Figure 8: The frequency of left isomerization phenotype may be determined by the severity of the neural tube phenotype.** (A) The correlation between the width of the pineal anlage and the severity of the parapineal phenotype in \( cdh2 \) mutant embryos. The embryos were grouped based on the severity of the parapineal phenotype, then the width of the pineal anlage was measured, defined by the distance between the two most lateral points of the pineal, regardless of whether the pineal is oval shaped, elongated or divided. Each symbol on the graphs indicates the result from a single embryo and the short horizontal lines indicate the average of each group, with error bars indicating ±1 standard deviation. (B) The relative position of the parapineals to the pineal anlage. The graph is divided into two parts: left and
right. In each part the smaller circle in the center indicates the number of parapineals overlapping with the pineal without bias to any direction. The larger circle divided into four zones indicates the numbers of parapineals overlapping with the pineal but biased to certain direction. The area outside of the larger circle, also divided into four zones, indicates the numbers of parapineals not overlapping with the pineal.

**Figure 9: kctd12.1 expression in the habenulae becomes bilateral when the neural tube does not close.** 
*foxD3:GFP* embryos injected with *GFP* mRNA or *lft1* mRNA and uninjected siblings were sorted by the pineal phenotype, fixed at 3 dpf, and the assayed for expression of *kctd12.1* in the habenula nuclei. (A - C). When the pineal is oval shaped, *kctd12.1* expression pattern is higher on the left than on the right. (D - F) When the pineal is divided, the habenula can either have (D) high levels of *kctd12.1* in both the left and right sides, (E) higher expression on the left, or (F) higher expression on the right. The image in F is a composite, as the two habenula were in different focal planes. Closed arrowheads indicate the habenulae with higher level of *kctd12.1* expression, open arrowheads the habenulae with lower *kctd12.1* expression. (G) Distribution of the *kctd12.1* expression patterns in each group of embryos. The embryos categorized as “other” (<6%) had either a single *kctd12.1* expression domain near the midline or many small areas of *kctd12.1* expression. n ≥ 25 for each group. (H): The distance between the two habenulae measured at the points where they were closest to each other. n ≥ 13 for each group. Avg ± standard deviation. By single-factor ANOVA, P < 0.001. Tukey’s HSD test finds significant differences between the divided pineal group and all other groups beyond 0.01 confidence level. The experiment was repeated two times and representative images are shown. All images are dorsal views, anterior to the top. Scale bar = 50 µm.
Figure 10: *kctd12.2* expression is low in both habenulae when the epithalamus is divided. Embryos were processed as in Figure 8 except for they were instead assayed for *kctd12.1* expression. (A - C) *kctd12.2* expression is higher on the right in embryos with a oval shaped pineal and (D) expressed at low or undetectable levels in embryos with divided pineal organs. The low levels of *kctd12.2* expression were not due to technical difficulties, as WT embryos processed in parallel had normal levels of expression. Closed arrowheads indicate the habenulae with higher level of *kctd12.2* expression. Open arrowheads indicate the habenulae with lower expression. (F) the distribution of *kctd12.1* expression pattern in group of embryos. “other” includes embryos with either a single *kctd12.2* expression domain or many small areas of *kctd12.1* expression. n ≥ 17 for each group. The experiment was repeated three times and representative images are shown. All images are dorsal views, anterior to the top. Scale bar = 50 µm.

Figure 11: *oep*<sup>tz257</sup> mutant phenotypes include divided pineal. (A) WT siblings and (B) a minority of homozygous *oep*<sup>tz257</sup> mutants have an oval shaped pineal phenotype. (C, D) The majority of *oep*<sup>tz257</sup> mutants have an elongated or divided pineal phenotype, indicating the neural tube is open. (E) Comparison of number and percentage of WT, *oep*<sup>m134</sup>, and *oep*<sup>tz257</sup> embryos with each pineal phenotype. Embryos are between the 25 somite stage and 24 hpf. The experiment was repeated 2 times and representative images are shown. All images are dorsal views with anterior to the top. Scale bar = 50 µm.

Figure 12: *oep*<sup>tz257</sup> mutants exhibit both fused and divided pineal phenotypes. (A - C) Two color *in situ* hybridization shows parapineal cells expressing *gfi1.2* (blue) and pineal cells expressing *otx5* (red) in 3 dpf embryos. The dashed lines represent the embryonic midline. (A) WT embryos have an oval shaped pineal (p, closed arrowheads) organ and a single left sided parapineal organ (pp, open
arrowhwads). (B) Some oep<sup>ez257</sup> mutants have an elongated pineal organ with a single parapineal organ in the middle of the pineal complex. (C) Other oep<sup>ez257</sup> mutants have a divided pineal organ and two bilaterally located parapineal organs. All images are dorsal views with representative images shown. Scale bar = 50μm. (D) Graph depicting parapineal placement seen in WT siblings (n=20), oep<sup>ez257</sup> mutants with an oval shaped pineal (n=9), oep<sup>ez257</sup> mutants with an elongated pineal (n=7) and oep<sup>ez257</sup> mutants with divided pineal (n=6).

**Figure 13: Model for laterality in the zebrafish epithalamus.** In WT embryos, left-right asymmetry is first apparent in the left-sided expression of the Nodal pathway genes cyc, lft1, and pitx2 in the pineal anlage. Subsequently, the parapineal is formed on the left side of the brain, adjacent to the pineal anlage. The left sided position of the parapineal then causes differences between the underlying habenula nuclei, such as the more extensive expression of leftover in the left habenula. In embryos with midline defects, the expression of cyc, lft1, and pitx2 becomes bilateral. Despite this, the parapineal and habenula still develop left-right asymmetry, although some of the embryos have normal situs and some have situs inversus. Thus suggests that there is an inhibitory factor that prevents both sides from becoming left. In embryos with an open neural tube, all asymmetries in the epithalamus become left isomerized, suggesting that the inhibitory signal that prevents both sides from developing a “left” morphology is not functional.

**Supplemental Figure 1: Lefty1 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 lefty1 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 = 200μM

Supplemental Figure 2: neural crest gene crestin is expressed in embryos with an open neural tube at the 26 somite stage. (A) At the 26 somite stage, crestin is expressed in the premigrating and migrating neural crest in the WT siblings and in sqt mutants with cyclopic eye and (B) a oval shaped pineal (C) divided pineal. Open arrowheads labeled with r2, r4 and r6 indicate presumed rostral cranial neural crest cell migration streams, which originate from rhombomeres 2, 4 and 6, respectively. “e” indicates eye. The experiment was repeated three times and representative images are shown. n > 50 for WT siblings and n = 20 for sqt mutants. All images are dorsal views with anterior to the left. Scale bar = 50μm.

References


Figure 1

$flh$:GFP

$flh$ expression

A  WT  B  C  D  cyc;sqt

E  WT  F  sqt  G  sqt  H  cyc;sqt

oval:closed NT  oval:closed NT  elongated:open NT  divided:open NT

oval  oval  divided  divided
Figure 2

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<tr>
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<td>C</td>
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M: Percent of Embryos

- oval
- elongated
- divided
- other
Figure 3

A
control mRNA

B
lft1 mRNA

aanat2 expression

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<th>B 52 hpf</th>
<th>A 70 hpf</th>
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46 hpf
52 hpf
70 hpf
Figure 4

*crestin expression*

A  closed neural tube

B  open neural tube

C  open neural tube
Figure 5

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Figure 6

**gfi1.2/flh expression**

Uninjected control mRNA

- **A** uninjected oval pineal
- **B** control mRNA oval pineal
- **C** lft1 mRNA oval pineal
- **D** lft1 mRNA divided pineal
- **E** lft1 mRNA divided pineal
- **F** lft1 mRNA divided pineal

**G**

- Percentage of embryos not detected
- Injected mRNA: none, GFP, lft1, lft1
- Pineal phenotype: oval, divided

Legend:
- L
- L + R
- R
- not detected
Figure 7

Pineal complex phenotypes

<table>
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<td>B</td>
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<tr>
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Percentage of embryos

- WT siblings
- cdh2
- cdh2

G

- 2 bilaterally located parapineal organs
- right sided parapineal organ
- medially located parapineal organ
- left sided parapineal organ

Genotype: WT siblings
Pineal phenotype: oval

Genotype: cdh2
Pineal phenotype: oval

Genotype: cdh2
Pineal phenotype: divided
Figure 8

A

B

pineal width/distance (µm)

pineal phenotype:

parapineal phenotype:

oval

single, sided

elongated & divided single, on midline

elongated & divided single, sided

elongated & divided two

midline

anterior

posterior
Figure 9

**kctd12.1 expression**

**Oval pineal**

- A: GFP mRNA
- B: lft1 mRNA
- C: lft1 mRNA

**Divided pineal**

- D: lft1 mRNA
- E: lft1 mRNA
- F: lft1 mRNA

**Graphs:**

- **G:** Bar graph showing percentage of embryos with different phenotypes.
- **H:** Graph showing distance in microns.

**Legend:**

- L > R
- L = R
- L < R
- other

**Injections:**

- injected mRNA:
  - none
  - GFP
  - lft1
  - lft1

**Phenotypes:**

- pineal phenotype:
  - round
  - divided
**Figure 10**

$kctd12.2$ expression

A. control
B. $lft1$ mRNA
C. $lft1$ mRNA
D. $lft1$ mRNA

E. Percentage of embryos

- **injected mRNA:** none, GFP, $lft1$
- **pineal phenotype:** oval, divided

- L < R
- undetected
- L > R
- L = R
- other
Figure 11

**otx5 expression**

<table>
<thead>
<tr>
<th></th>
<th>Pineal Phenotype</th>
<th>oval</th>
<th>elongated</th>
<th>divided</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT sibs</td>
<td>37 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>oep&lt;sup&gt;tz257&lt;/sup&gt;</td>
<td>22 (84%)</td>
<td>2 (7%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td></td>
<td>WT sibs</td>
<td>54 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>oep&lt;sup&gt;tz257&lt;/sup&gt;</td>
<td>33 (41%)</td>
<td>7 (8%)</td>
<td>39 (49%)</td>
</tr>
</tbody>
</table>

A oval B oval C elongated D divided
Figure 12

gfi1.2/otx5 expression

pineal phenotype: oval
parapineal phenotype: single, left sided

Percentage of embryos

D

genotype: WT sibling oep<sup>oep<sup>-257</sup></sup> oep<sup>oep<sup>-257</sup></sup> divided
pineal phenotype: oval elongated divided
parapineal phenotype: two, bilaterally located

WT sibling oeptz257           oeptz257            oeptz257
oval               oval            elongated         divided

pp p

A B C

Figure 1

2 bilaterally located parapineal organs
right sided parapineal organ
medially located parapineal organ
left sided parapineal organ
**Figure 13**

**Wildtype**

- Left
- Right

**Midline defect**

- Left
- Right

**Open neural tube**

- Left
- Right

### Expression of Nodal pathway genes in the pineal

### Parapineal organ

### Expression of kctd12.1 in the habenula nuclei
Supplemental Figure 1

Phenotype

Percentage of Embryos control mRNA

WT Class I Class II Class III

E

control mRNA

lft1 mRNA

Phenotype

WT Class I Class II Class III

100 90 80 70 60 50 40 30 20 10 0
Supplemental Figure 2

crestin/flh expression

A
WT siblings
oval pineal

B
sqt
oval pineal

C
sqt
divided pineal
Chapter 4

The roles of a novel zebrafish ER pore component gene in the developing pineal organ and craniofacial structures

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Authors’ Contributions

PL designed the study, conducted Exorh immunostaining, ctn and dlx2 WISH, helped performing otx5 WISH, conducted image analysis, optimized the cartilage staining protocol and wrote the text. SB conducted otx5 WISH and cartilage staining, helped performing dlx2 WISH and took pictures of the otx5 and dlx2 WISH and cartilage staining experiments. JOL helped design and coordinate the study and helped revise the text.
Abstract

The zebrafish habenular complex is a structure with significant left-right asymmetries. Recently, the gene sec61a-like 1 (sec61al1) was identified in a screen for mutations that lead to habenular left-isomerization (Doll et al., 2011). Since the asymmetrically located parapineal is known to direct the habenular left-right asymmetry in zebrafish (Gamse et al., 2003), we hypothesized that the mutation, sec61al1c16, might lead to phenotypes related to pineal complex, composed of the pineal and parapineal organ. In this study, we found that the size of the pineal organ is not significantly changed in the sec61al1c16 mutant embryos. However, minor changes in the level of gene expression within the pineal organ may exist.

sec61al1c16 mutant embryos also exhibit other morphological defects. One of these is loss of lower jaw structures. We found that at as early as 72 hpf, all of the cranial neural crest (CNC) derived structures, including the mandibular arch, hyoid arch, all branchial arches and ventral neurocranium were either missing or severely underdeveloped in sec61al1c16 mutant embryos. Surprisingly, based on gene expression patterns we found no evidence of defects in the early CNC differentiation and migration. We conclude the jaw phenotype of sec61al1c16 mutant embryos is not caused by the early CNC differentiation and migration defects. Instead, we hypothesize that after initiating the CNC cell migration correctly, the migrating CNC cells fail to differentiate into functional chondrocyte and cartilage structures.
Introduction

sec61al1c163 is a novel zebrafish mutant discovered via forward genetic screen focusing on altered habenular asymmetry (Doll et al., 2011). The gene sec61al1 encodes a pore-forming protein. The protein plays a key role in the endoplasmic reticulum (ER) translocation channel complex by controlling the transportation of peptides and the integration of transmembrane proteins into the ER membrane (Doll et al., 2011; Rapoport, 2007). The c163 mutant allele of sec61al1 leads to the truncation of the Sec61al1 protein, deleting the ribosomal interacting domain and five out of the ten transmembrane domains, and is likely nonfunctional (Doll et al., 2011).

As described in Chapter 3, the zebrafish habenular complex is a structure with significant left-right asymmetric characteristics. These include larger size and higher neuropil density on the left side versus the right. Also, the expression pattern of several genes and the targets of axonal projection are different between the left and right habenula nuclei (Aizawa et al., 2007; Gamse et al., 2005; Gamse et al., 2003; Kuan et al., 2007a; Kuan et al., 2007b). In the sec61al1c16mutant embryos, both the left and right habenulae exhibit “left” properties including larger size, higher neuropil density and altered gene expression pattern. Interestingly, the parapineal, which normally locates on the left side of the pineal complex and direct the left-right asymmetry in habenulae, is not affected by the sec61al1c163 mutation (Doll et al., 2011). sec61al1c163 mutant embryos also have shorter, dorsally curved body and severely defective lower jaw phenotypes (Doll et al., 2011). The sec61al1c163 mutation is embryonic lethal and most of the mutant embryos die before 5 dpf (Doll et al., 2011).

We originally hypothesized that the size of the pineal complex is altered in the
sec61allc163 mutant based on the fact that the left-right asymmetry in the pineal complex directs the habenular left-right asymmetry (Gamse et al., 2003). We found no evidence of altered pineal complex size in the sec61allc163 mutant embryos by measuring the width and length of the expression area of two pineal complex specific genes. However our results suggest that the gene expression level within the pineal organ may be slightly higher in the sec61allc163 mutant embryos.

We hypothesized that the jaw defects observed in the sec61allc163 mutant embryos are likely to be caused by defects related to the differentiation or migration of the anterior neural crest. We found that the early differentiation and migration of cranial neural crest (CNC) cells is not affected by the sec61allc163 mutation by detecting the expression pattern of an early neural crest specific gene crestin (ctn) and a migrating CNC specific gene dlx2. We hypothesized that the jaw defects observed in the sec61allc163 mutant embryos are likely to be caused by defects related to the differentiation or migration of the anterior neural crest. It is known that in all vertebrates with jaw structures, cranial neural crest (CNC) contributes directly to most of the neurocranium (skull structures) as well as to the entire pharyngeal arch related mesodermal structures, including lower jaws, hyoid and gill structures (Knight and Schilling, 2006; Santagati and Rijli, 2003). The CNC cells from the midbrain level migrate between the eyes to contribute to the neurocranium while the CNC cells from rhombomere 2 to 7 migrate as three distinct streams into mandibular (stream 1), hyoid (stream 2), and five branchial (stream 3) arches (Figure 1) (Knight and Schilling, 2006). Newer evidences suggest that stream 1 also contributes to the ventral neurocranium (Cerny et al., 2004). We first examined the detail of the jaw phenotype of the sec61allc163
mutant embryos from 3 to 6 dpf and found that the entire CNC derived mesodermal craniofacial structure is poorly developed. Surprisingly, both premigrational neural crest and migrating CNC markers indicate that the early differentiation and migration of CNC cells is not significantly changed. We conclude the jaw phenotype of sec61al1c16 mutant is not caused by the early CNC differentiation and migration defects.

Methods

Zebrafish

Zebrafish husbandry was as described in chapter 3. Stocks included the WT strain Zebrafish Danio Rerio (ZDR) (Aquatica Tropicals, Plant City, FL), sec61al1c163 (Doll et al., 2011).

Whole mount in situ hybridization (WISH)

Single probe WISH was carried out as described in Chapter 3. Antisense RNA probes included: orthodenticle homeobox 5 (otx5) (Gamse et al., 2002), crestin (ctn) (Rubinstein et al., 2000), dlx2 (Akimenko et al., 1994).

Image analysis

All images were taken as described in Chapter 3. To semi-quantify the signal strength of the WISH results, ImageJ was used for the line scanning analysis. The images were converted to 8 bit greyscale images first, then the black and white of the images were inverted. Then a 242 pixels long horizontal line was placed over the otx5 expression area of an image, with the middle of the line over the arbitrary center of the presumptive
pineal organ. The data value of each of the pixels over the line were then taken by ImageJ and transferred to Microsoft Excel. The plot graphs were generated by averaging the data from 10 images of sec61al1^{c163} embryos or the WT siblings. The zero point of the Y axis is set at the average value of the leftmost 10 pixels from the WT siblings.

**Cartilage staining**

Cartilage staining protocol was modified based on a published protocol (Walker and Kimmel, 2007). In brief, the embryos were fixed by 4% paraformaldehyde at 4°C for at least 24 hours then dehydrated and stored in 100% methanol at -20°C until used. When preforming the staining, the embryos were moved to 50% ethanol for 10 minutes at room temperature. Then the ethanol was removed and replaced by a staining solution which contains 0.02% alcian blue, 60 mM MgCl₂, and 70% ethanol. The embryos were incubated in the staining solution overnight at room temperature.

After removing the stain solution, the embryos were washed once with water, and then the water was replaced by a bleaching solution which contains 1.5% H₂O₂ and 1% KOH. The tubes were then sat with lids open at room temperature for 20 min.

The embryos were cleared with successive changes of a solution of glycerol and KOH. After removing the bleaching solution, a clearing solution containing 20% glycerol and 0.25% KOH was added and the embryos were rocked at room temperature for 30 min to overnight. The cleaning solution was then replaced by the second clearing solution which contains 50% glycerol and 0.25% KOH and rocked at room temperature for another 2 h to overnight. The embryos were stored in a solution of 50% glycerol and 0.1% KOH at 4°C and photographed as described in Chapter 3.
sec61al1c163 genotyping

The sec61al1c163 mutant embryos younger than 3 dpf cannot be easily identified by morphology. To overcome this, when the embryos used in the WISH experiments were younger than 3 dpf, the embryos were then individually genotyped by PCR and RFLP.

DNA of each embryo was extracted by digesting the embryo with 30 µL of 50 mM NaOH at 95 °C for 20 minutes. The solution was then cooled to 4 °C and buffered by 3 µL of 1 M Tris, pH 8.0, and the supernatant was collected by centrifuge.

The embryo was then genotyped by PCR with the primer sequence:

F: 5’-ATGAAACCATTGAGGGTGAT-3’,
R: 5’-CCATGCTCTCTCACCTGAATGATGATCAGGAG-3’. PCR using the described primer pair generates a 242 bp product which with a Hinf I restriction site for the sec61al1c163 mutation but no Hinf I site for the WT.

The PCR product was then digested with Hinf I restriction enzyme at 37 °C for 3 hours to overnight and analyzed by electrophoresis in a 3% agarose gel.

Results

The pineal organ size of the sec61al1c163 mutant was not significantly changed

To determine the effects of the sec61al1c163 mutation on pineal organ size, we examined the expression of otx5, a pineal complex marker. We found no difference in either the length or width of the otx5 expressing area between the sec61al1c163 mutants and their wildtype siblings at 4 dpf (Figure 2A-D). However, examination of the digital
images suggested that the \textit{otx5} staining in the pineal of the \textit{sec61al1}^{cl63} embryos was often darker. To evaluate this observation, I analyzed the WISH signal strength along a horizontal line crossing the approximate center of the pineal on each of the WISH images. The edge (the left edge was arbitrarily used) of each sampling line, where generally low or no WISH signal, was used to normalize the background value so the data set from each image was comparable. Preliminary results supported my observation that \textit{sec61al1}^{cl63} embryos exhibited higher \textit{otx5} expression level in the middle region of the pineal organ (Figure 2E).

Pineal organ contains a group of photoreceptive cells that are able to entrain, or reset, this oscillator in response to photic input (Cahill, 1996). To examine whether the photoreceptive cells are affected by the \textit{sec61al1}^{cl63} mutation, we assayed the expression of \textit{exorh} in \textit{sec61al1}^{cl63} embryos by WISH and immunostaining. \textit{exorh} encodes a putative light-sensing, G-protein coupled receptor that is expressed in the photoreceptive cells of the pineal organ (Mano et al., 1999). We found no difference in either the length or width of the \textit{exorh} expressing area between the \textit{sec61al1}^{cl63} mutants and their wildtype siblings at 4 dpf (Figure 3). The results supported our conclusion from the \textit{otx5} results that the pineal size is not affected by the \textit{sec61al1}^{cl63} mutation.

\textit{The cartilage structures of lower jaw are defective in the sec61al1}^{cl63} \textit{mutant}

To understand the progress of the lower jaw phenotype in \textit{sec61al1}^{cl63} mutant more clearly, we conducted cartilage staining in the \textit{sec61al1}^{cl63} mutant embryos and their WT siblings at 3 to 6 dpf. We demonstrated that as early as at 3 dpf, in WT embryos the Meckel’s cartilage (future mandible) and ceratohyal cartilage (future hyoid) formed a
distinctive arch-shaped structure (Figure 4, closed arrowhead). However in the sec61all<sup>cl63</sup> embryos, the Meckel’s cartilage and ceratohyal cartilage were nearly completely lost (Figure 4). Other branchial arch derived structures are either poorly developed or severely misformed when compared with the WT (Figure 4, open arrowhead). We also carried out cartilage staining at later developmental stages. We found no sign of normal but delayed lower jaw development as late as at 6 dpf (Figure 4), suggesting that the jaw defects are not likely caused by delayed development in the sec61all<sup>cl63</sup> mutant.

The jaw phenotype of the sec61all<sup>cl63</sup> mutation is not caused by defects in early neural crest differentiation or migration.

We hypothesized that the jaw phenotype may be caused by defects in differentiation or migration of the CNC cells. To test this, we used WISH to detect the expression pattern of <i>dlx2</i>, a gene strongly expressed in the migrating CNC cells of stream 1, 2 and 3 (Knight et al., 2003). Surprisingly, we found no difference in <i>dlx2</i> expression pattern between the sec61all<sup>cl63</sup> mutants and their WT siblings at 28 hpf (Figure 5).

We also examined the expression pattern of <i>crestin</i> (<i>ctn</i>), an early neural crest marker (Rubinstein et al., 2000). Currently we have conducted <i>ctn</i> WISH on about 50 embryos from sec61all<sup>cl63</sup> heterozygous parents, which were expected to contain about one fourth of the sec61all<sup>cl63</sup> homozygous embryos. Our preliminary results revealed no significant difference among the examined embryos.
Discussion

Despite many other defects in development of the forebrain, we found no evidence of pineal size change in sec61all<sup>c163</sup> embryos. However, our preliminary results suggest that cell density of the pineal complex may be slightly higher in sec61all<sup>c163</sup> embryos. Our collaborators found that while the habenulae of the sec61all<sup>c163</sup> embryos are left-isomerized, parapineal, the structure known to direct the sidedness of habenula complex, is correctly located on the left side of the pineal organ. Our results support our collaborators’ conclusion that the pineal complex in sec61all<sup>c163</sup> mutant embryos is largely normal (Doll et al., 2011). Our results also support that the left isomerization of habenular complex in sec61all<sup>c163</sup> mutant embryos is likely not caused by defects in pineal complex (Doll et al., 2011).

Results from our jaw phenotype study suggest that the defect that leads to the jaw phenotype of sec61all<sup>c163</sup> mutants likely occurs between 28 to 72 hpf. The expression patterns of the pre-migrating neural crest gene ctn and the migrating pharyngeal arch gene dlx2 are both normal in sec61all<sup>c163</sup> embryos at as late as 28 hpf, suggesting early CNC defects are unlikely responsible for the jaw phenotype clearly detected at 3 dpf.

Detailed observation of changes on morphology and chondrocyte related gene activity in the sec61allc163 mutant embryos between 28 to 72 hpf may provide crucial answers to the role of sec61all gene. To achieve this, one strategy we have taken is to take advantage of another gene highly expressed in neural crest derived cells, sox10. We have crossed the sec61all<sup>c163</sup> mutation into a sox10:EGFP transgenic line. With the sec61all<sup>c163</sup>/sox10:EGFP line, tracing the migration of the CNC cells in live embryos will be possible.
One hypothesis of the mechanism of the cartilage defects in the sec61al1c163 embryos is that although the CNC cells start differentiation and migration correctly, the cells eventually fail to exhibit functional chondrocyte characteristics. It is possible that one or more genes required for chondrocyte function may be affected by the sec61al1c163 mutation. This is not completely unexpected, since Sec61al1 is responsible of peptides travelling through the ER, and chondrocyte is a secretion-heavy cell type that likely requires Sec61al1 activities to making cartilage matrix correctly. Alternatively, the morphogens regulating the development of cartilage, including endothelin-1 (edn1) (Kimmel et al., 2003; Miller et al., 2000; Walker et al., 2006; Walker et al., 2007) and hedgehog signaling pathways (Eberhart et al., 2006) may be affected by the sec61al1c163 mutation, and consequently the migrating CNC cells are not able to differentiate into functional chondrocyte.

While our current results suggest the CNC cell migration is normal at 28 hpf, we cannot rule out the possibility that CNC migration is disrupted in later stages. Cell movement also depends heavily on the arrangement of extracellular matrix and how the cells interact with the matrix (Ettinger and Doljanski, 1992; Oster et al., 1985). Therefore when the protein secretion ability of cells within the embryo is defective, it is possible that the mobility of the chondroblast precursors may be affected.

Acknowledgements

We thank Dr. Joshua T. Gamse and Caleb A. Doll for providing the sec61al1c163 mutant line of zebrafish. Dr. Thomas F. Schilling for providing the dlx2 probe construct and granting us the permission to reproduce the CNC migration figure. Dr. Marnie E.
Halpern for providing the *ctn* probe construct. This research is supported in part by grant NIH 1 RO1 HD054523 (to J.T.G and J.O.L.).

**Figure Legends**

**Figure 1. The migration and patterns of in the CNC.** Camera lucida drawing of the head of a zebrafish embryo at 24 hpf, lateral view, anterior to the left. Hindbrain rhombomeres (r2-r7) give rise to streams of CNC in each pharyngeal arch: stream 1 (red) forms the mandibular arch; stream 2 (green) forms the hyoid arch; and stream 3 (blue) forms the five branchial arches. Premandibular CNC cells migrate in more anterior streams (light green) between the eyes to form the neurocranium. Modified from Knight & Schilling, 2000.

**Figure 2. Pineal organ size is not significantly different between wildtype and sec61al1c163 mutant embryos.** (A, B) The expression of *otx5* in the pineal organ of a typical (A) WT embryo and a typical (B) *sec61al1c163* mutant embryo. (C, D) The average length and the width of *otx5* expressing area. N=7 for each group. All embryos are 4 dpf. All images are dorsal view, anterior to the top. (E) Line scan results of *otx5* signal strength from WT or *sec61al1c163* images. Each curve is composited of the average value from ten images. Scale bar = 50µm.

**Figure 3. The size of *exorh* expressing area is not significantly different between wildtype and sec61al1c163 mutant embryos.** (A, B) WISH results showing the expression of *exorh* in the pineal organ of a typical (A) WT embryo and a typical (B)
sec61al1c163 mutant embryo. (C, D) Immunostaining results showing the existence of Exorh protein in the pineal organ of a typical (C) WT embryo and a typical (D) sec61al1c163 mutant embryo. (E, F) The average (E) length and the (F) width of exorh expressing area. N=7 for each group. All embryos are 4 dpf. All images are dorsal view, anterior to the top. Scale bar = 50µm.

Figure 4. The jaw cartilage phenotypes of sec61al1c163 mutant embryos.

Figure 5. dlx2 is expressed normally in sec61al1c163 mutant embryos. Representative images of dlx2 expression pattern from (A) a sec61al1c163 mutant embryo and (B) a WT sibling at 28 hpf. Open arrowhead: stream 1 (mandibular arch) of the migrating CNC cells. Close arrowhead: stream 2 (hyoid arch) of the migrating CNC cells. Arrows: stream 3 (branchial arches) of the migrating CNC cells. All images are lateral view with anterior to the left. N >10 for each group. Scale bar = 50µm.

References


Figure 1

midbrain

r2  r3  r4  r5  r6  r7  hindbrain

ear

hyoid arch

branchial arches

mandibular arch

premandibular arch
Figure 2

A  WT

sec61all<sup>c163</sup>

B

C  Pineal Length

D  Pineal Width

E

P<sub>0.64</sub>

P<sub>0.41</sub>

Optical Density (Arbitrary Units)

Pineal Length

Pineal Width

OTX5
Figure 3

A  WT

exorh

B  sec61al1c163

C  Exorh

D

E  pineal length

F  pineal width

p=0.94

p=0.17

Length (micron)

Width (micron)
Figure 4

Ventral Views

WT  sec61all^{c163}

3 dpf

4 dpf

6 dpf

Lateral Views

WT  sec61all^{c163}
Figure 5

A

WT

B

sec61al1<sup>c163</sup>
Chapter 5

Discussion
In the studies described in this dissertation I made three major contributions focusing on the circadian and asymmetric properties of zebrafish epithalamus. First I demonstrated that the zebrafish Suprachiasmatic nucleus (SCN) is not required for establishing the circadian rhythms of the pineal organ [Chapter 2 and (Noche et al., 2011)]. This finding further supports the theory that the circadian rhythms in zebrafish are more decentralized when compared with the highly centralized mammalian circadian system. I then demonstrated that a closed neural tube is required for the establishment of epithalamus left-right (L/R) asymmetries (Chapter 3). This finding implies a previously unknown short-ranged mechanism that prevents one side of the epithalamus from acquiring left characteristics when the epithalamus L/R asymmetries are being established. I also contributed in characterizing a novel zebrafish mutation sec61al1c163, which was originally identified for affecting the habenular complex L/R asymmetries. In this ongoing study I demonstrated that the sec61al1c163 mutation only has minor effects on the pineal organ structure. I also found that the neural crest derived craniofacial structures in the sec61al1c163 mutant embryos are severely defective, likely caused by failure in chondrocyte differentiation (Chapter 4). In this chapter I will summarize my findings and discuss the implications of my contributions and future directions of investigation.

The role of SCN in lower vertebrate circadian rhythms

In mammals and birds, the SCN is a major regulator of circadian rhythms. However, the role of the SCN in lower vertebrates remains poorly understood. To further investigate the role of SCN in establishing the pineal rhythms in zebrafish, we took advantage of zebrafish cyclops (cyc) mutants which lack ventral brain structures including the presumed SCN. I discovered that the rhythmic expression pattern of aanat2 in the pineal organ can still be observed in cyc mutant larvae (Chapter 2). Such rhythmic expression patterns of aanat2 were observed no matter when the mutant larvae were kept
in normal light dark cycle or in constant light or constant dark environments. This finding demonstrates that the observed rhythms can be defined as true circadian rhythms instead of only a passive responses to periodic environmental changes. We also observed that when kept in constant environments for more than two days the amplitude of *aanat2* rhythms became lower in the mutant embryos than in the WT siblings. Thus, we concluded that while the zebrafish SCN is not required for establishing pineal circadian rhythms, the SCN may involve in maintaining the rhythms.

**The significance of the findings**

Our finding that the normally rhythmic expression of *aanat2* were remained in *cyc* larvae without the SCN structure (Chapter 2) provides one of the first pieces of evidence that the zebrafish SCN is not required for establishing the circadian pineal rhythms. Our results further support the hypothesis that the circadian rhythms in teleost pineal may be SCN independent (Cahill, 2002; Cahill et al., 1998).

In non-mammalian vertebrates, the whole circadian system can be present in an individual pineal cell. Pineal cells in zebrafish have been shown to detect input (photoreception), have a molecular oscillator, and produce circadian outputs (melatonin synthesis) (Cahill, 2002; Cahill et al., 1998; Hicks and Molday, 1986; Whitmore et al., 2000; Whitmore et al., 1998). Thus, it is not entirely unexpected that we found the zebrafish pineal organ had circadian rhythms that were independent from the SCN. However, we cannot rule out the possibility that, as in mammals, the zebrafish SCN has a role in regulating rhythms in other tissues (Burrill and Easter, 1994; Springer and Mednick, 1984).

**Future Directions on Analysis of Zebrafish SCN Function**
While our studies demonstrated that the zebrafish SCN is not required for initiating the pineal circadian rhythms in zebrafish, we did not answer the fundamental question: is there a mechanism that synchronizes the individual peripheral oscillators in zebrafish?

In order to test the hypothesis that the peripheral oscillators in zebrafish function independently and are not synchronized between each other, but entrained by environment cues directly, one possible strategy is to manipulate the circadian phase of a specific tissue, making the circadian phase of the affected tissue desynchronize with other parts of the individual. The treated individual would then be kept in constant environment. Whether or not the altered circadian phase can resynchronize with the other parts of the individual would be observed. One approach that had been applied on mammalian model systems is tissue transplantation. For example SCN transplantation has been done in mammals to introduce an alternative circadian phase to an individual (Lehman et al., 1987; Stephan and Zucker, 1972). However, the small body size makes precise tissue transplantation challenging for zebrafish. Currently, nearly all reported adult zebrafish transplantation experiments were achieved by injecting suspendable cells including hematopoietic cell and cancer cell lines (Mizgireuv and Revskoy, 2006; Traver et al., 2004; White et al., 2008).

An alternative strategy to introduce an alternative circadian phase in a specific tissue would be applying a light signal, possibly via optical fiber, directly on the surface of or into the target tissue. Eye, heart and liver may be tested individually with this method. This method is practical since it has been demonstrated that a one hour photic pulse is sufficient to initiate the pineal circadian rhythms in zebrafish kept in constant environment (Vuilleumier et al., 2006). This finding suggests that applying the light for the entire length of a normal light-dark cycle is not necessary. The protocol for anesthetizing and holding an adult zebrafish still for a relatively short period of time is readily available as a part of the well-established adult zebrafish surgery protocols (Kan et al., 2009; Sadler et al., 2007).
Another question unanswered in our study is whether SCN plays a role in maintaining or synchronizing the peripheral oscillators. One strategy to answer this question is to keep the SCN defective larvae in constant environments for a longer period of time, then observe if the circadian rhythms of different peripheral oscillators are lost or become desynchronized. However, the \( \text{cyc}^{m294} \) mutation used in this study is a lethal mutation and the mutant embryos die within several days post fertilization. Therefore it is impossible to use \( \text{cyc}^{m293} \) mutants to study the role of SCN on pineal circadian rhythms in juveniles or adults. Also, the mechanism leads to the decreased amplitude of \( \text{aanat2} \) expression rhythm in \( \text{cyc} \) embryos when the embryos were approaching 5dpf cannot be determined, since both the generally poor health condition and the loss of ventral brain could be responsible (Chapter 2, Figure 5).

To overcome this problem, selective ablation of SCN cells may be a useful tool for further understanding the role of SCN. One well-established method is laser-mediated ablation (Kohli and Elezzabi, 2008; Liu and Fetcho, 1999; Roeser and Baier, 2003). In this method, the target cells first have to be fluorescent labeled by using transgenic fish that express tissue-specific promoter-driven fluorescent protein. The labeled tissue can then be targeted by conventional fluorescent microscope and ablated by a laser source. The suitable type of laser and optimized ablation specification depends on many factors and will not be described here. While laser ablation is capable of achieving extremely high precision, it is labor intensive to ablate each embryo (Curado et al., 2007; Roeser and Baier, 2003). Laser ablation is also high cost and requires and custom-made equipment (Kohli and Elezzabi, 2008; Roeser and Baier, 2003).

Another approach would be the conditional genetic ablation strategy (Curado et al., 2007; Davison et al., 2007; Pisharath et al., 2007). In this method, a gene which encodes the bacterial Nitroreductase (NTR) enzyme is engineered to be driven by a target tissue specific promoter. This
construct is then used to generate the transgenic fish. When the transgenic fish is exposed to a prodrug Metronidazole (Mtz), NTR converts Mtz into a cytotoxic DNA cross-linking agent and consequently kills the NTR expressing cells. This method can be carried out in a laboratory capable of producing transgenic zebrafish and once the transgenic line is created, a large number of embryos with selectively ablated tissues will be available for analysis. However, the specificity of the promoter used to express the NTR limits the specificity of the ablation. For example, \textit{avpl}, the gene used as a SCN marker in Chapter 2, is expressed in SCN and many other ventral brain neurons (Balment et al., 2006). Therefore, using \textit{avpl} as the primer to drive NTR will lead to the ablation of all the \textit{avpl} expressing tissues instead of specific SCN ablation.

On the contrary, by laser ablation, it is possible to determine the location of the SCN anatomically and leave most of the \textit{avpl} expressing, non-SCN tissue undamaged while genetic ablation will destroy all the \textit{avpl} expressing tissue. Therefore, my evaluation is that laser ablation will be a better method when the availability of equipment is not a concern but genetic ablation may be a more practical approach when considering the cost of the experiments.

\textbf{The mechanism of establishing the vertebrate L/R epithalamus asymmetry}

The zebrafish epithalamus exhibits prominent L/R asymmetries that have been associated with side biased behaviors. Epithalamus asymmetry is disrupted when midline tissues are defective: the L/R asymmetrically expressed Nodal pathway genes become bilaterally expressed in the pineal organ, and subsequent asymmetric properties of the parapineal and habenula become randomized.

To test the hypothesis that the interaction between the left and right sides of the epithalamus is required for establishing the epithalamus L/R asymmetry, we examined epithalamus asymmetric characteristics in embryos with an open neural tube. I discovered that when the neural tube failed to
close, no matter if it was caused by inhibiting the Nodal activity (Chapter 3, Figure 6, 9 and 10), loss of Nodal receptor activity (Chapter 3, Figure 12) or loss of N-cadherin (Chapter 3, Figure 7), left-isomerization was observed. This indicates that a closed neural tube is required for the establishing of epithalamus left-right asymmetry, possibly via a previously unknown short ranged interaction between the left and right sides of the brain. However, since Nodal-defective embryos with a closed neural tube can only exhibit randomized sidedness in epithalamus (Gamse et al., 2002; Gamse et al., 2003), the closed neural tube is likely only sufficient for establishing asymmetries of the epithalamus but not for determining the sidedness.

**Proposed model**

Our results indicate that when the two sides of the dorsal neural tube are too far from each other, the L/R asymmetry establishing mechanism will not function normally to prevent one side of the epithalamus from gaining left identity. Thus, this L/R asymmetry establishing mechanism is likely a short-ranged signal that inhibits the establishment of the left identity. We proposed that a reaction-diffusion mechanism may be responsible for this L/R asymmetry establishing mechanism, similar to the well-studied mechanism that establishes visceral left-right asymmetry (Hamada et al., 2002; Schier, 2009). A most basic form of the reaction-diffusion model is composed of an active factor which stimulates both its own synthesis and the synthesis of an inhibitory factor (Chapter 1, Figure 2). The difference between the diffusion range of the two factors leads to high active signaling being restricted in a small area. In the adjacent area nearly all the activity of this active factor is inhibited (Hamada et al., 2002; Schier, 2009; Turing, 1990).

We propose that the reaction-diffusion mechanism responsible of the establishing of epithalamus asymmetry may be composed of Cyc as the active factor and Lft1 as the inhibitory factor. In a
developing WT zebrafish brain, Cyc protein, a Nodal signal molecule, is produced on the left side of the presumptive pineal (Liang et al., 2000). Lft1 protein functions as an antagonist of all Nodal signals including Cyc (Meno et al., 1999). However, while Cyc protein can only diffuse a few cell diameters, Lft1 is capable of diffusing to a significantly longer distance (Chen and Schier, 2001, 2002). This difference in the diffusion ranges between the active and inhibitory factors is the key characteristic for a reaction-diffusion mechanism to create different local activities at different locations (Schier, 2009; Turing, 1990). In the model we propose, Cyc activity is the subject of reaction-diffusion control and eventually becomes restricted in one side of the pineal organ in WT situation.

The evolutionary aspect of epithalamus L/R asymmetries

It is argued that the evolution of directional L/R asymmetric characteristics took an unconventional path (Palmer, 2004). In the proposed unconventional phenotype-precedes-genotype mode, sometimes called genetic assimilation, developmental plasticity creates novel phenotypes without heritable variation that affects their development. The heritability ultimately arises later by means of random mutations (Hall, 2001; Palmer, 2004). In contrast, in the classical neo-Darwinian genotype-precedes-phenotype mode, mutations initially generate different forms, which are then selected by natural selection (Palmer, 2004). The argument that the directional L/R asymmetric characteristics have evolved via unconventional approach is supported by reconstructed evolutionary history. For example, in a study on the evolutionary history of phallostethid fishes it is revealed that: 1. directional and random asymmetries characteristics arose from symmetry with similar chances; and 2. a significant amount of directional asymmetries arose from random asymmetries while only in one case random asymmetries arose from directional asymmetry (Parenti, 1996).
The structural and functional asymmetries in the brain are observed in a wide variety of vertebrates including fish, birds and mammals (Corballis, 2009). However it is still unclear whether the left-right brain asymmetries in different species reflect basic homology or independent parallel evolutionary histories (Bisazza et al., 1998). Based on the reaction-diffusion model supported by our results and other studies, the genetic assimilation is likely. The reaction-diffusion mechanism is capable of generating random asymmetric characteristics, a group of non-heritable traits, based only on the random distribution of certain signal molecules. Later, when a directional symmetry-breaking factor is introduced to affect the distribution of said signal molecules, the reaction-diffusion mechanism is also capable of amplifying this initial bias to the directional asymmetric characteristics.

A Nodal independent mechanism may contribute to the establishment of epithalamus L/R asymmetry

Our results from the \textit{oep}\textsuperscript{iz257} mutant suggest the existence of a random asymmetry mechanism independent from the proposed reaction-diffusion mechanism. The \textit{oep}\textsuperscript{iz257} is a null mutation of a Nodal receptor component (Brand et al., 1996), which should completely eliminate our proposed epithalamus L/R asymmetry establishing mechanism based on Cyc and Lft1 interaction. However, our studies revealed that random asymmetry phenotype can still form in the \textit{oep}\textsuperscript{iz257} mutant embryos as long as the neural tube is closed (Chapter 3, Figure 12). Thus, this result should not be confused with the random asymmetry explainable by the Cyc-Lft1 based reaction-diffusion model we proposed, in which remaining Nodal activity cannot be ruled out. While the detailed mechanism of this Nodal independent random symmetry is beyond the scope of this dissertation, similar phenomena have been observed in the establishing of visceral asymmetries. For example, in mutant mice lacking the expression of \textit{nodal} in the LPM, both the direction of heart looping and the rotation of the stomach become randomized (Lowe et al., 2001). In zebrafish, \textit{oep}\textsuperscript{iz257} mutant embryos exhibit randomized heart looping and pancreas location
(Yan et al., 1999). A generally accepted theory for such Nodal independent, random left-right asymmetry of visceral structures is that in order to pack all the necessary structures into a body with limited size, certain forms of asymmetry are physically unavoidable (Levin, 1997; Pansera, 1994). A similar explanation that there is simply no room for the parapineal to be located over the midline may be applied to the situation of epithalamus asymmetry. Consequently, the parapineal is physically pushed to one side of the pineal organ even when the sided molecular signal is lost.

**Future Directions on testing the Cyc/Lft1 reaction-diffusion model**

While our studies indicate the existence of a short-ranged mechanism that inhibits the establishing of left identity, currently there is no evidence regarding which signaling factors are responsible for this left-inhibiting mechanism. Testing the hypothesis that Lft1 is responsible may provide definitive results regarding the molecular base of this proposed mechanism. A lft1 defective model is likely to be useful in providing crucial information on whether the Cyc/Lft1 system is responsible of establishing the L/R asymmetry in epithalamus. If our hypothesis of the Cyc/Lft1 system is correct, when lft1 is defective, the distribution of Cyc activity will not be restricted by Lft1. Consequently, Cyc activity will likely be presented in both sides of the epithalamus to cause left-isomerization even when the neural tube is closed. This prediction is supported by a study focused on the effect of lft1 deficiency on zebrafish LPM. Researchers used morpholino injections to knockdown Lft1 protein level and found that the normally asymmetrically expressed gene of a Nodal signaling molecular, spaw, became bilaterally expressed in the LPM (Wang and Yost, 2008). Other than using morpholino or other antisense based techniques, recently two mutant lines, the lft1^{hg6} and lft1^{hg7} have been generated in a NIH laboratory (data retrieved from The Zebrafish Model Organism Database, [http://zfin.org/action/mutant/mutant-list?zdbID=ZDB-GENE-990630-10](http://zfin.org/action/mutant/mutant-list?zdbID=ZDB-GENE-990630-10)). However these mutant lines
are not yet available to the public. Assuming the \(lft1^{bg6}\) and \(lft1^{bg7}\) or other \(lft1\) mutant zebrafish lines become available in the future, the following steps can be taken to test our hypothesis:

Central hypothesis: Lft1 functions as an inhibitor of Nodal signaling, likely Cyc, during the establishment of the epithalamic asymmetries in zebrafish.

Aim 1: To examine the differentiation of the pineal organ in \(lft1\) mutant embryos.

Hypothesis: Pineal cell types differentiate normally in \(lft1\) mutant embryos.

Rationale: Before conducting any further study it is necessary to confirm that the tissue of interest exists in the model being used. Lefty is involved in regulating the Nodal- and Wnt-responsive genes in the organizer during gastrulation (Branford and Yost, 2002). Thus, Lefty deficient embryos may exhibit severe phenotypes due to gastrulation elated failures. In mouse, a \(lefty2\) (homologue of zebrafish \(lft1\)) null mutation caused the embryos to die between E8 to E11, before visceral or brain asymmetries could be analyzed (Meno et al., 1999). However current results in zebrafish suggest that \(lft1\) deficient phenotype may not be as severe. When \(lft1\) was knocked-down in zebrafish embryos, a significant amount of the Lft1 deficient embryos could survive to 30 hpf, with relatively intact neural tube structure (Agathon et al., 2001; Wang and Yost, 2008).

Further, assuming the \(lft1\) mutant zebrafish embryos can develop to the somitogenesis stage, \(flh\), the earliest detectable pineal gene should be expressed in the pineal anlage at the beginning of somitogenesis (Masai et al., 1997). On contrary, \(lft1\) is detected in the pineal anlage at about 15 to 25 somite stage (Liang et al., 2000; Wang and Yost, 2008). Thus, it is unlikely that \(lft1\) is directly required for the differentiation of the pineal cells once the embryos enter the somitogenesis stage. Since pineal L/R asymmetry is the
earliest step to establish other epithalamus asymmetries, we expect that certain forms of asymmetries can be examined if the pineal organ is developed.

Expected results: In WT zebrafish embryos, the expression of flh can be detected in the presumed pineal organ at as early as when the pineal precursors originate from two domains at the lateral edges of the neural plate (Masai et al., 1997). foxD3 is expressed in the projection neurons of the pineal complex (Lein et al., 2007; Morris, 2009) and exorh is expressed in the photoreceptive cells of the pineal complex (Mano et al., 1999). aanat2 is a rhythmically expressed gene involved in the biosynthesis of melatonin (Gothilf et al., 1999). We expect that in the lft1 mutant embryos, flh, foxD3, exorh and aanat2 should be expressed in the pineal anlage and the aanat2 expression should remain rhythmic.

Aim 2: Determine the effects of lft1 mutation on the asymmetrically expressed pineal genes.

Hypothesis: When Lft1 activity is lost, asymmetrically expressed pineal genes will become bilaterally expressed.

Rationale: We propose that Lft1 functions as an inhibitory factor to restrict the Nodal activity in one side of the epithalamus. Earlier researches have demonstrated that Lft1 functions as an inhibitor to regulate the establishment of visceral asymmetries. For example when lft1 was knocked-down, the expression of a Nodal signaling gene, spaw, became bilateral in the LPM (Wang and Yost, 2008). Since we propose that a similar mechanism is responsible for the establishing of the epithalamus asymmetry, we expect that Lft1 deficiency should also lead to the bilateral expression of Nodal related genes in the epithalamus should (Figure 1).
Expected results: In WT zebrafish embryos \( \text{cyc} \) and \( \text{pitx2} \) are expressed in the left side of the pineal anlage during late somitogenesis. We predict that the expression of \( \text{cyc} \) and \( \text{pitx2} \) should become bilaterally expressed in the pineal organ of the \( \text{lft1} \) mutant embryos.

Aim 3: To observe the effects of \( \text{lft1} \) mutation on the localization of the parapineal organ in \( \text{lft1} \) mutant embryos.

Hypothesis: If Lft1 activity is lost during the establishing of the epithalamus asymmetries, a random asymmetry will not be established by the inhibitory property of Lft1. Consequently the parapineal cells will not be directed to a specific direction to form a sided parapineal organ. Eventually, two parapineal organs will form, one on each side of the pineal organ. An alternative hypothesis is that the parapineal organ may fail to form and parapineal cells may become scattered in the pineal complex.

Rationale: Earlier studies demonstrated that in embryos with a pineal organ bilaterally expressing the normally sided genes, as long as the neural tube is closed, downstream asymmetries are still established (Gamse et al., 2002; Gamse et al., 2003). Based on our proposed central hypothesis, Lft1 activity is required for restricting the Cyc activity to a single side of the developing pineal complex. Thus, when Lft1 activity is lost, Cyc activity can exist on both sides of the pineal complex without being restricted to one side of the epithalamus (Figure 1). Consequently, parapineal organs will form on both sides. Alternatively, since Cyc activity may exist in the whole pineal complex when Lft1 activity is lost, scattered parapineal phenotype may be created because the parapineal cells originated from the pineal organ fail to migrate toward any specific direction.
Expected results: In WT embryos, the parapineal organ is formed by the cells originated from the anterior part of the pineal precursor (Concha et al., 2003). The parapineal organ is located on the left of the pineal organ and gfi1 is expressed in the parapineal organ (Dufourcq et al., 2004). Bilaterally expressed gfi1 is expected to be observed in the pineal complex of the lft1 mutant embryos, indicating the bilaterally located parapineal organs. An alternative possibility is that the parapineal cells may fail to migrate out from the anterior part of the epithalamus, where the cells originated, and remain scattered in the pineal complex.

Aim 4: To observe the gene expression pattern of the habenular complex in lft1 mutant embryos.

Hypothesis: The habenular complex becomes left-isomerized in lft1 mutant embryos.

Rationale: Since the localization of the parapineal directs the habenula nuclei to acquire a left identity (Gamse et al., 2003), bilaterally located parapineal organs should lead to the left-isomerization of the habenular complex. This prediction is similar to what has been described in Chapter 3 of this dissertation (Chapter 3, Figure 9, 10). Even if the parapineal cells become scattered, as alternatively predicted in Aim 3, the parapineal cells located on both sides of the pineal complex may still direct both sides of the habenular complex to acquire left identities.

Expected results: In WT embryos, kctd12.1 is mostly expressed in the left habenula and kctd12.2 in the right habenula. In the lft1 deficient embryos a left-isomerized habenular complex is expected. Consequently, kctd12.1 should be highly expressed on both sides of the habenular complex while no more than a low level of kctd12.2 should be detected.
Potential difficulties: If Lft1 is not responsible of establishing the epithalamus asymmetries, random asymmetries will still be observed in \textit{lft1} null mutant embryos. Since there is currently no other candidate gene, a genetic screening approach is needed. The traditional procedure is to generate random mutagenesis via radiation or chemical treatment (Westerfield, 1993), then screen for the desired phenotypes. The mutant genes will then be mapped by positional cloning (Bahary et al., 2004; Zhou and Zon, 2011). However instead of preforming the labor intensive and time consuming positional cloning, we may take advantage to many gene-trap methods that are being developed recently (Stanford et al., 2001). For example, a novel, transponson based “gene-breaking” method has been developed for zebrafish to provide the highly efficient and regulated mutagenesis, while the mutant loci can be easily cloned (Sivasubbu et al., 2007; Sivasubbu et al., 2006).

**Figure Legend:**

**Figure 1. A conceptual diagram of the interaction of an activator and an inhibitor to establish L/R asymmetry.** In WT epithalamus, the activator Cyc (the black lines) is originally expressed on one side of a structure over the midline (the blue lines). Cyc stimulates the expression of the inhibitor Lft1 (the red lines), which is capable of diffusing a distance greater than Cyc. Lft1 then inhibits the small amounts of the Cyc that “leaks” to the other side of the midline, consequently restricts the net activity of Cyc (the green lines) to a specific side. This initial pineal asymmetry later directs the localization of the parapineal organ to one side of the pineal complex. However when the Lft1 is missing, the activity of Cyc that diffuses to the
“wrong side” of the midline will not be inhibited. Later, due to the positive feedback mechanism of the Cyc activity, the expression of the Cyc is upregulated on the “wrong side”. Eventually, the activator will become highly expressed on both sides, leading to the bilateral localization of the parapineal.

References


Figure 1

**protein concentration**

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**net activity**

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**pineal complex phenotype**

<table>
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- **Cyc activity**
- **Lf1 activity**
- **net activity**
- **midline**