Defining the Role of the Mouse Jhy Gene in the Ependyma and Choroid Plexus

BY

HILMARIE MUÑIZ-TALAVERA
B.S., University of Puerto Rico-Mayagüez, 2010

THESIS

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Defense Committee:
Teresa V. Orenic, Chair
Jennifer V. Schmidt, Advisor
Peter Okkema
John Leonard
Angela Tyner, Biochemistry and Molecular Genetics
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HMT
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CP</td>
<td>choroid plexus</td>
</tr>
<tr>
<td>LV</td>
<td>lateral ventricle</td>
</tr>
<tr>
<td>MW</td>
<td>medial wall of the lateral ventricle</td>
</tr>
<tr>
<td>LW</td>
<td>lateral wall of the lateral ventricle</td>
</tr>
<tr>
<td>AJ</td>
<td>adherens junction</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>Cx</td>
<td>cerebral cortex</td>
</tr>
<tr>
<td>RG</td>
<td>radial glial cells</td>
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<tr>
<td>PCP</td>
<td>planar cell polarity</td>
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SUMMARY

The cerebrospinal fluid (CSF) is produced by the choroid plexus and is contained within the brain ventricular system (Damkier et al., 2013). CSF flows from the lateral ventricles to the third ventricle, where it then enters the fourth ventricle through the cerebral aqueduct (Brinker et al., 2014). The composition of the CSF is derived from passive filtration of plasma and membrane secretion and it has three main functions: 1) protect the brain by serving as a cushion against mechanical shock, 2) serve as a route for nutrient delivery and signaling factors, and 3) carry waste products and toxins away from the brain (Lun et al., 2015; Spector et al., 2015). CSF production and circulation should be carefully regulated to allow proper brain development (Gato et al., 2014; Mohammad Nabiuni, 2015). Increased CSF volume causes ventricular dilation and it can lead to the development of a neurological condition known as hydrocephalus. Congenital hydrocephalus is the most common form of the disease, which is present at birth and it affects 1-2/1000 children in the United States (Kahle et al., 2015). Hydrocephalus can result from abnormalities in the production, flow or absorption of the CSF, and if untreated it can lead to death.

The ventricles of the brain are lined by a monolayer of ciliated squamous epithelia known as the ependymal cells. The ependyma carry motile cilia (9+2), which coordinately beat to produce a laminar flow and promote CSF circulation throughout the ventricles (Spassky, 2013). The choroid plexus is composed of a monolayer of modified ependymal cells though these cells have developed a unique polarization of membrane associated proteins, along with other secretory properties that allow choroid plexus (CP) cells to function in CSF secretion (Damkier et al., 2013; Dziegielewska et al., 2001). Hydrocephalus can result from the disruption of ependymal cells and/or the choroid plexus by the loss of CSF flow
and CSF overproduction, respectively (Baas et al., 2006a; Banizs et al., 2005). Despite the undeniable importance of the ependyma and the CP in CSF homeostasis, the mechanisms and factors involved in their differentiation are largely unknown.

The work presented here aimed to further characterize the structure and function of the ependymal cells and the specialized ependymal cells of the CP. The \textit{Jhy}^{lacZ} mouse line carries an insertional mutation in the \textit{Jhy} gene (formerly 4931429I11Rik), and homozygous \textit{Jhy}^{lacZ/lacZ} mice develop a rapidly progressive juvenile hydrocephalus (Appelbe et al., 2013). Molecular analysis of the ependymal cells in \textit{Jhy}^{lacZ/lacZ} mice was performed using a cell-type specific marker approach to assess the expression of markers involved in vital cellular processes of these cells. \textit{Jhy}^{lacZ/lacZ} mice display abnormal ependymal cell differentiation with ventricular ependyma retaining an unorganized and multi-layered morphology, representative of immature ependymal cells. Morphological and molecular analysis of the ependyma demonstrated a delay rather than a block in differentiation. Additionally, \textit{Jhy}^{lacZ/lacZ} ependymal cells manifest disruptions in adherens junction formation. Ultrastructural analysis of postnatal \textit{Jhy}^{lacZ/lacZ} mice found abnormal organization of the motile cilia lining the lateral ventricles of the brain, structures believed to be required for proper CSF flow. \textit{Jhy}^{lacZ/lacZ} ependymal cells have defects in the polarized organization of the apically located cilia. The latter resulted in severely reduced motility, a likely cause for the development of hydrocephalus.

A second \textit{Jhy} mutant model (i.e. \textit{Jhy}^{lacZNeo}) was generated upon the availability of \textit{Jhy} targeted embryonic stem cells from the Knockout mouse consortium (KOMP). We sought to identify the role of \textit{Jhy} in the specialized ependymal cells of the choroid plexus in the \textit{Jhy}^{lacZNeo} mouse line. Our analysis indicates that \textit{Jhy}^{lacZNeo/lacZNeo} develop early onset hydrocephalus, with mice rarely surviving past 3 weeks of age. \textit{Jhy}^{lacZNeo/lacZNeo} CP also
SUMMARY (continued)
displays disruptions in adherens junction formation, with abnormal localization of the key adherens junction protein, E-cadherin. TEM analysis of the CP in Jhy\textsuperscript{lacZNeo/lacZNeo} demonstrated defects in ciliary ultrastructure, along with altered microvilli distribution. Together, this data identifies Jhy as a gene required for proper adherens junction formation and cilia ultrastructure, in the ependyma and the specialized ependyma of the choroid plexus.
I. GENERAL INTRODUCTION

1.1 Congenital anomalies of the nervous system: Congenital hydrocephalus

The human brain is one of the most fascinating and complex biological systems. The development of the brain is an intricate process requiring the contribution of genes and environmental inputs, and the disruption of any of these factors can impair proper brain development. Hydrocephalus is a neurological disorder characterized by the abnormal accumulation of cerebrospinal fluid (CSF) in the brain, and it results from disturbances in CSF production, flow or absorption (Thompson, 2009). CSF buildup causes an increase in intracranial pressure, which may lead to loss of hearing, loss of vision, developmental disabilities, and if untreated, it can lead to death. Infant hydrocephalus is the most common disease treated by pediatric neurosurgeons with 1-2/1000 children suffering from hydrocephalus in the United States (Simon et al., 2008; Kahle et al., 2015). Clinical presentation of hydrocephalus can occur at any age. Individuals presenting hydrocephalus signs around the time of birth are said to have congenital hydrocephalus, while those developing hydrocephalus during adulthood are diagnosed with acquired hydrocephalus (Thompson, 2009). Hydrocephalus may be classified into two categories: 1) communicating, where CSF can pass through the brain ventricles, and 2) non-communicating, where passage of CSF within the ventricles is obstructed. Approximately 60% of congenital hydrocephalus cases are associated with ventricular and subarachnoid obstruction due to hemorrhage; however, the factors contributing to the remaining cases of hydrocephalus remain unidentified (Kahle et al., 2015).
1.1.1 Treatments for hydrocephalus

Historically, the treatment for hydrocephalus involves the use of ventricular shunts. These catheters were designed to remove excess CSF from the ventricles and drain this fluid into either the peritoneal cavity (ventriculoperitoneal shunts), the right atrium (VA shunt) or the pleural cavity (ventriculopleural shunt) (Phan et al., 2016; Thompson, 2009). For years, ventricular shunts remained the standard treatment option though the high risk of failures associated with infections, blockage, and excess drainage generated an urgency to create alternate treatments (Piatt and Garton, 2008; Pople, 2002; Warf, 2014). In the recent years, patients displaying either communicating or non-communicating hydrocephalus most often undergo endoscopic third ventriculostomy (ETV) (Jones et al., 1990). In this procedure, an artificial opening is generated so the excess CSF may escape and enter the subarachnoid space where CSF absorption occurs. However, an alternative treatment for patients with communicating hydrocephalus has been implemented since the 2000s. Choroid plexus cauterization (CPC) was added to the ETV treatment with the working premise that cauterization of the choroid will result in the decrease of CSF production, which in turn will improve the efficacy of the treatment (Warf, 2005). Despite the improvement in surgical treatment for hydrocephalus, little progress has been made to generate novel therapeutic treatments to replace the need for surgery.

1.1.2 Understanding the pathology of congenital hydrocephalus in humans

The quest for understanding hydrocephalus dates back to the 5th century where the father of medicine, Hippocrates, was thought to be the first to describe and attempt to treat hydrocephalus (Aschoff et al., 1999). Due to the poor pathophysiological knowledge of hydrocephalus prior to the 19th century, most attempts to treat hydrocephalus resulted in
failures (Aschoff et al., 1999). Around 1825, the physiologist Magendie described the
circulation of CSF in the brain, and by 1914 Weed confirmed the arachnoid villi as the site
for CSF absorption (Lifshutz and Johnson, 2001). It was not until the 19th and 20th centuries
when the therapeutic attempts were far more successful given the striking advance in the
understanding of CSF homeostasis. However, centuries worth of effort have not been able
to clearly define the development and progression of this disease. It is estimated that
approximately 40% of congenital hydrocephalus cases in humans are genetic in origin
(Zhang et al., 2006). In humans, one genetic cause of congenital hydrocephalus has been
relatively well studied. A missense mutation in L1CAM gene has been associated with X-
linked hydrocephalus; nevertheless, the pathogenic mechanism of hydrocephalus in these
individuals remains unknown (Jouet et al., 1993; Jouet et al., 1993). X-linked hydrocephalus
patients display non-communicating hydrocephalus due to stenosis of the aqueduct of
Sylvius, which is the narrow canal that allows flow of CSF within the ventricles. The L1CAM
gene produces the cell adhesion molecule known as L1 protein, responsible for neuronal
migration, axonal growth, fasciculation, and synaptic plasticity (Weller and Gartner, 2001).
Itoh et al. hypothesized that hydrocephalus in L1 deficient patients may be due to
corticogenesis defects (Itoh and Fushiki, 2015). Corticogenesis, the process in which a
cerebral cortex is generated, is impaired in L1-KO mice; moreover, these mice display
thinned cortical layers, agenesis of the corpus callosum and severe ventricular dilation
(Itoh and Fushiki, 2015). In 2013, Al-Dosari et al. reported two Saudi families who had
multiple cases of massive communicating hydrocephalus in the family history. A truncation
mutation in the tight junction gene, MPDZ, was identified as the cause for the autosomal
recessive congenital hydrocephalus displayed by these families (Al-Dosari et al., 2013).
Additional cases of hydrocephalus in humans have been reported, with families displaying
either autosomal recessive (Haverkamp et al., 1999; Teebi and Naguib, 1988) or autosomal dominant hydrocephalus (Verhagen et al., 1998). Nevertheless, the genetic causes for hydrocephalus in these reported cases remains elusive.

1.2 Proposed mechanisms for CSF function, production and homeostasis

1.2.1 CSF function

CSF is contained within the brain ventricular cavities. In mice, the ventricular system is composed of four cavities, two lateral ventricles, one third ventricle and one fourth ventricle (Fig. 1). These cavities are interconnected with one another, with the lateral ventricles connecting to the third ventricle, and the latter connecting to the fourth through a narrow canal called the aqueduct of Sylvius. CSF is held in all four cavities and it travels freely within all of these compartments providing the support needed for proper brain development. CSF composition is derived from plasma filtrates and membrane secretion, and its primary composition is water, glucose, sodium chloride and proteins (Di Terlizzi and Platt, 2006). CSF provides mechanical support for the brain, as well as having crucial roles in nutrient circulation and removal of toxic molecules from the brain (Segal, 1993). Studies have shown that CSF may affect brain cell behavior through the active delivery of diffusible signals throughout the brain (Dziegielewska et al., 1980; Sawamoto et al., 2006; Segal, 1993) (Czosnyka et al., 2004). For example, a study done in chick embryos shows that embryonic CSF contains trophic factors that promote neuroepithelial cell proliferation and neurogenesis (Gato et al., 2005). Consistent with this function, gradients of the axon guiding molecule, Slit2, are delivered by CSF to promote neuronal guidance to the olfactory bulb (Sawamoto et al., 2006). Proper CSF homeostasis is important to maintain normal variations of intracranial pressure (ICP), and this is influenced by brain
blood flow (Di Terlizzi and Platt, 2006). Cerebral blood flow changes greatly in response to respiration and physical effort; consequently, CSF must be displaced to compensate for these changes and maintain a healthy ICP (Di Terlizzi and Platt, 2006; Owler et al., 2004). Altered CSF homeostasis affects the intra-cerebral transport of crucial components and may lead to a wide range of central nervous system conditions such as epilepsy, Alzheimer’s and hydrocephalus (Banizs et al., 2005; Czosnyka et al., 2004; Podell and Hadjiconstantinou, 1997; Shaw et al., 2009).
Figure 1. Mouse ventricular system at different developmental time points. The mouse ventricular system as it appears in mid-late embryos (left), early postnatal animals (top right) and adult (bottom right). Yellow, blue, and orange compartments highlight the lateral (Lv), third (III) and fourth (IV) ventricles, respectively. As development progresses, each ventricular compartment becomes significantly narrower as the cerebral cortex develops. The aqueduct of Sylvius (Aq, enclosed by orange arrows) connects the third and fourth ventricle and becomes visible around P0.5. Ad, adult; e, embryonic day; p, postnatal day. Figure adapted from (Ibanez-Tallon et al., 2004).
1.2.2 CSF production

In 1919, Walter Dandy performed experiments on dogs where he blocked the canal that connects both lateral ventricles (i.e. Foramen of Monro), and he deduced that CSF is produced intraventricularly given that blocking this canal resulted in lateral ventricle enlargement (Dandy, 1919). These studies were the first reported indications that the tissue responsible for CSF secretion was located within these ventricles. The current hypothesis is that CSF production and secretion is primarily done by the choroid plexus (CP) epithelium (Dandy, 1919) (Oldendorf and Davson, 1967; Rubin et al., 1966). It is estimated that approximately 50-70% of CSF is produced by the CP (Wright et al., 1977). CSF secretion rate in humans by the CP is predicted to be .35mL/minute, with the turnover of the entire CSF volume occurring approximately 3-4 times a day (Czosnyka et al., 2004; Ekstedt, 1978). The CP is located in all four ventricles of the brain and they project into these cavities as an extension of the ependymal cells that line the ventricular walls. It is believed that CPs in all four cavities has the capability to secrete CSF; however, a substantial amount of the CSF is produced in the lateral ventricles (Bering and Sato, 1963).

Given that the CP produces the bulk of the CSF, the majority of studies performed on CSF production and secretion have been done in the choroid. Interestingly, CSF secretion precedes CP formation, thus, it has long been speculated that other sources for CSF production must exist. Weiss and Gato considered the neuroepithelium as the source for embryonic CSF (eCSF). Their reports show that the neuroepithelium display secretory properties such as secretory vesicles, thus they proposed the neuroepithelium as an alternative source for CSF secretion (Gato et al., 1993; Weiss, 1934). It has been proposed that non-neuroepithelial inputs may also regulate CSF production. For example, studies have shown that reducing the hydrostatic pressure in the vascular system in chick embryo,
results in lower levels of eCSF and a decreased brain ventricle expansion (Desmond, 1991). These findings suggest that blood pressure acts as a regulator for CSF secretion.

The ependymal cells have been mostly associated with the flow needed to clear CSF from the ventricles (Spassky, 2013). Interestingly, CSF secretion by ventricular ependyma has been previously proposed; nevertheless, this subject remains largely debated and poorly studied. Pollay and Curl performed the single report on this matter where they used a perfusion system lined by ventricular ependyma to measure net water movement to determine the secretory activity of ventricular ependyma (Pollay and Curl, 1967). Their findings support that ventricular ependyma is able to contribute to approximately 30% of CSF secretion in rabbit (Pollay and Curl, 1967). While these studies are pending confirmation, is possible one day the ependyma might be recognized as the second major site for CSF production.

1.2.2.1 Mechanisms of CSF secretion by the choroid plexus

The CP consists of a monolayer of epithelial cells, along with a vast network of fenestrated capillaries that extends into the ventricles of the brain (Fig. 2). The fenestrated endothelium of the choroid allows the free passage of plasma filtrates, which are then selectively filtered by the epithelial cells through the respective transmembrane channel (Oreskovic and Klarica, 2010). The CP epithelium is highly polarized and allows the controlled transport of ions (e.g. Na⁺, K⁺, HCO₃⁻, Cl⁻) across the membrane, creating an osmotic gradient that drives the passage of water, thus generating what we know as CSF. The net osmotic gradient is a relatively complex process, but the general agreement is that the active influx and efflux of Na⁺, Cl⁻ and HCO₃⁻ is what initiates this process. Basolateral transporters mediate the Na⁺ and HCO₃⁻-dependent accumulation of Cl⁻ while apically the
Na⁺-K⁺ ATPase pumps Na⁺ out of the cell. This is concurrent with the exit of Cl⁻ and HCO₃⁻ ions from the CP cell. The changes in ion concentration drive the movement of water across the apical water channel Aquaporin 1 (AQP1), leading to increased levels of H₂O in the brain ventricles. Changes in the osmotic gradient and water transport regulate CP membrane secretion, and they are both needed for CSF production. For example, AQP1 null mice display reduced levels of CSF production and ICP, suggesting that the osmotic gradient is necessary, but without AQP1, CSF secretion is impaired (Oshio et al., 2005).
Figure 2. Choroid plexus structure and polarity. Schematic representation of the CP epithelial morphology (left). The choroidal epithelium is composed of a single layer of cuboidal cells enclosing a vast network of blood capillaries and mesenchymal (stromal) cells. Cilia in CP are present on the CSF-facing surface. The CP is a highly polarized epithelium with distinct apical and basolateral membrane domains that allows the unidirectional transport of molecules across the membrane for CSF secretion (right). This image was adapted from (Oreskovic and Klarica, 2010).
1.2.3 CSF flow and circulation

CSF is continuously being produced and its circulation is crucial to maintain normalized levels of ICP and CSF volume. Moreover, CSF flow ensures the delivery and removal of substances, as well as proper absorption of the CSF. The secretion and absorption of CSF occurs in two separate compartments of the brain; thus, fluid movement must occur at a similar rate to its production in order to maintain a healthy balance. Some have proposed that intracranial blood flow may facilitate CSF flow. Bering et al. showed that CSF movement is pulsatile, with cerebral blood flow pulsations affecting CSF circulation (Bering, 1959). CSF also moves in a directional manner thanks to the ciliary action of the ventricular ependyma. Each ependymal cell contains a bundle of motile cilia (9+2), with 9 outer microtubule doublets and 2 microtubule singlets in the middle (Takeda and Narita, 2012). The coordinated beating of ependymal cilia creates a laminar flow of CSF throughout the ventricles, and this process is often called ependymal flow. The classical view assumes that CSF flow is generated through motile ciliary mechanisms (Baas et al., 2006a; Delgehyr et al., 2015; Spassky, 2013; Tissir et al., 2010). Thus it is possible, that proper CSF flow is achieved with the collaboration of both mechanisms.

1.2.4 Mechanisms for CSF absorption
1.2.4.1 CSF absorption through the arachnoid villi

Proper circulation ensures the exit of CSF from the ventricular cavities where it enters the subarachnoid space for absorption (Fig. 3). For years, researchers believed that CSF was absorbed into the venous system only through the arachnoid granulations (McComb, 1983; Miyajima and Arai, 2015; Oreskovic and Klarica, 2010). The arachnoid granulations are formed by arachnoid membrane protrusion (arachnoid villi) extending
into the brain dural sinuses, and these structures function as a one-way valve that opens up to allow the passage of CSF when the fluid pressure increases (Di Terlizzi and Platt, 2006). Once CSF enters through the arachnoid space where blood vessels are held, it gets immediately absorbed into the venous system. The notion of CSF absorption through arachnoid villi dates back to the 1960s, when Davson proposed the arachnoid villi as the main site for CSF absorption. He performed experiments where he injected colored gelatin into the ventricles and found that the dye passed though the villi, where it then entered the venous system (Davson, 1966; Davson et al., 1973; Davson et al., 1970). The view of CSF absorption occurring only at the arachnoid villi has become debated as new theories for absorption emerge. Recent findings suggest that the attributed role of arachnoid villi in CSF absorption may be incorrect as they found that rats normally have no sinuses until P20, yet they do not develop hydrocephalus (Miyajima and Arai, 2015). Others have proposed that the arachnoid may serve as a site for the clearance of CSF proteins (e.g. waste products of neuronal metabolisms) and that rather they serve as a secondary pathway for CSF absorption in pathological conditions (Miyajima and Arai, 2015) (Mawera and Asala, 1996).

1.2.4.2 The role of lymphatic system in CSF drainage

Recent findings suggest that CSF absorption employs dual outflow mechanisms involving the arachnoid villi as well as the lymphatic system (Pollay, 2012; McComb, 1983). CSF drainage through the lymphatic system has been tested by the use of tracers, where CSF protein tracers were found to enter the nasal lymphatic system through the olfactory tract near the nasal cavity (Fig. 3). For example, rabbits injected with albumin dye into the CSF contained 5% of the tagged protein in the cervical nodes, and 26% was found in the blood stream (Courtice and Simmonds, 1951). Moreover, studies performed in sheep
estimate that approximately 40-48% of CSF gets absorbed by the lymphatic system (McComb, 1983) (Boulton et al., 1998). So far, ~50% of CSF absorption has been attributed to lymphatic drainage (Bradbury et al., 1981) (Boulton et al., 1997). Presumably, the remaining CSF is cleared through the arachnoid villi. Despite the progress made in the understanding of CSF absorption, this phenomenon remains largely debated even to this day.
Figure 3. CSF absorption occurs through the arachnoid granulations and the lymphatic system. Drainage of CSF by the lymphatic system occurs through the cribriform plate on the roof of the nasal cavity (left panel). The lymphatic vessels in the nasal mucosa absorb the incoming CSF and drain it into the bloodstream. Arachnoid granulations contain arachnoid villi (right panel, black arrows) that project out into the dural sinuses. These structures allow the passage of CSF from the subarachnoid space into the dural sinuses, where vessels located there allow CSF back into the blood. This figure was adapted from (Louveau et al., 2015).
1.3 Structure, function and pathology of neuroepithelial derivatives: Ependymal cells and the Choroid plexus

1.3.1 Ependymal cells anatomy and its function in CSF flow

Ependymal cells form a single uninterrupted layer of ciliated squamous epithelia lining the entire ventricular system (Sarnat, 1998). Ependymal cells are of glial origin, and the primary role assigned to them is in CSF circulation through ciliary mechanisms. Each ependymal cell contains approximately 50 cilia, and these cilia are all strictly polarized with respect to each other. This polarization allows the coordinated beating needed to generate a directional fluid flow (Spassky, 2013). Many studies have shown that ependymal ciliary beating is crucial for CSF flow, and their disruption results in hydrocephalus (Dominguez-Pinos et al., 2005; Ibanez-Tallon et al., 2004b; Sapiro et al., 2002). For example, mice deficient for the *Stumpy* gene develop ependymal cells with no cilia, and as a consequence these mice develop communicating hydrocephalus (Town et al., 2008). Presumably, the flow generated by ependymal cilia also contributes to debris clearance and dispersion of neural messengers needed for proper central nervous system development (Spassky, 2013).

1.3.1.1 Ependymal cells have functions other than CSF circulation

Adult neurogenesis occurs near the ventricles, in a discrete region of the lateral ventricle called the subventricular zone (SVZ). The SVZ is the main source of adult neural stem cells, and is the site from which undifferentiated neurons (neuroblasts) migrate to reach the olfactory bulb (Pignatelli and Belluzzi, 2010). This process is crucial for the development of the olfactory system in mice. An emerging body of evidence suggests that ventricular ependyma helps direct neuroblast migration (Paez-Gonzalez et al., 2011) (Gotz
and Stricker, 2006; Sawamoto et al., 2006). Sawamoto utilized chemorepulsive recombinant Slit2–alkaline phosphatase fusion protein to demonstrate that chemorepulsive gradients generated by ependymal flow were crucial for neuroblast migration (Sawamoto et al., 2006). The ankyrin G protein (Ank3) is a large adaptor molecule that binds to N-cadherin in the ependyma of the SVZ. Moreover, loss of Ank3 in mice results in disruption of the SVZ arrangement, along with impaired neuroblast migration (Paez-Gonzalez et al., 2011). A similar phenotype in SVZ cytoarchitecture and neuroblast migration was observed in p73 deficient mice; interestingly, these mice also display hydrocephalus (Gonzalez-Cano et al., 2015). Undoubtedly, ependymal cells are fundamental for vital processes during mammalian brain development, including CSF homeostasis and directed neuronal migration.

1.3.2 Ependymal cell differentiation

1.3.2.1 Radial glia to ependymal cell transition is needed for ependyma cell differentiation

Multiciliated ependymal cells originate from monociliated bipolar radial glia (RG) cells (Batiz et al., 2011b; Spassky et al., 2005). RG cells are born during E14-E16 and at this time these cells are the ones lining the ventricles (i.e. ventricular zone)(Spassky et al., 2005). During postnatal development the anatomy of the ventricular zone begins to undergo massive changes as RG cells detach from the ventricular surface and undergo their terminal differentiation, giving rise to neurons, oligodentrocytes, astrocytes and ependymal cells (Merkle et al., 2004). Around birth, when RG differentiation is triggered a subsets of these cells are retained at the ventricular wall and are destined to become adult ependyma (Spassky et al., 2005; Tramontin et al., 2003). During differentiation the subgroup intended to become ependymal cells become postmitotic and they lose the
ventricular processes extended by the RG cells. At this time, these cells undergo drastic morphological changes going from a monociliated columnar epithelium to multiciliated squamous epithelia (Fig. 4A). These changes in morphology are concomitant with the expression of ciliogenesis transcription factor, FoxJ1. FoxJ1 expression is upregulated around P0, coinciding with the timing of radial glial to ependymal cell transition. Moreover, mice deficient for FoxJ1 lack ependymal cells and astrocytes, suggesting that FoxJ1 is required for RG differentiation into ependysma and astrocytes (Jacquet et al., 2009).

In the lateral ventricle, ependyma differentiation is spatiotemporally regulated, with cells progressively acquiring mature characteristics in a caudal-rostral and ventral-dorsal gradient (Delgehyr et al., 2015) (Fig. 4B). In a coronal brain section, the lateral ventricle may be divided into two regions, the medial wall facing the striatum and the lateral wall facing the brain septum. Interestingly, differentiation appears to also follow a medial-to-lateral wall gradient, with medial wall cells displaying signs of differentiation prior to the lateral wall (Bruni, 1998). Mature ependyma are visible as early as P5, but ependymal differentiation continues until P21 (Batiz et al., 2011). Changes in the molecular profile are also evident during the RG to ependyma transition. Glutamate transporter protein (Glast) and intermediate filament protein (RC2) are expressed by RG cells and are often used as RG markers (Shibata et al., 1997) (Park et al., 2009). Upon ependyma differentiation, these markers become repressed and a new profile emerges with ependymal cells expressing the EF-hand calcium binding protein (s100β), glycoprotein (CD24) and intermediate filament protein (vimentin) (Liu and Zheng, 2007; Sarnat, 1998; Takano et al., 1996).
1.3.2.2 Transcription factors in ependymal cell differentiation

Over the last two decades, a series of reports have identified some of the factors needed for ependymal cell differentiation. Transcription factor, Six3, is required for neuroretinal specification embryonically; however, postnatally this protein is essential for ependymal cell maturation (Lavado and Oliver, 2011). Mice deficient for Six3 are unable to repress radial glial characteristics, and as a consequence ependymal cells display a mixture of ependyma and RG features, resulting in hydrocephalus. Additionally, transcription factors Vax1 and Sox2 are expressed in the ependyma, and their disruption leads to reduced numbers of mature ependymal cells, impaired neuroblast migration and hydrocephalus (Ferri et al., 2004; Soria et al., 2004). Despite the progress in the identification of factors involved in ependymal cell differentiation, the molecular mechanism through which these transcription factors facilitate radial glia to ependymal cell differentiation remains unknown.
Figure 4. Ependymal cell differentiation in mice. (A) As RG cells differentiate into ependymal cells drastic morphological changes are observed as they change from a columnar cell with one primary cilium into a squamous “flat-like” multiciliated cell. (B) Newly differentiated ependyma (red dots) undergo maturation in a caudal to rostral and ventral to dorsal gradient, with mature ependyma covering most of the ventricular surface by P15. C, caudal; D, dorsal. Mouse brain image was obtained from (Fliegauf et al., 2007).
1.3.2.3 Adherens junctions in ependymal cell morphology

Adherens junctions (AJs) play crucial roles during cell differentiation. Changes in assembly and disassembly of AJs are needed as cells experience morphological changes, or begin their migratory journey. AJs are mediated by cadherin-catenin cell adhesion, and these molecules play crucial roles during development especially in cell and tissue morphology (Gumbiner, 2005b). AJ disruption often leads to malignancies such as cell dedifferentiation, invasive growth and tumorigenesis (Behrens et al., 1993; Birchmeier et al., 1993). Cadherins are a single-pass transmembrane proteins known for its Ca²⁺ mediated cell-cell adhesion properties (Vestweber, 2015). Although many cadherin proteins have been discovered, a great deal of today’s knowledge comes from the study of E- and N-cadherin, the epithelial and neuronal cadherin, respectively (Harris and Tepass, 2010). Cadherin proteins mediate cell adhesion through their interaction with β–catenin and α–catenin, the latter connecting this complex to the actin cytoskeleton (Vestweber, 2015). AJs are highly dynamic structures, particularly during cell differentiation when they undergo cycles of endocytosis, degradation and recycling (Kowalczyk and Nanes, 2012).

Ependymal cells constitute a special kind of epithelial cell as they do not possess tight junctions, and their cell-to-cell contact is dependent solely on AJs. Proper formation and stabilization of AJ in these cells is crucial for their development and function. As RG begin their differentiation, AJs must disassemble to allow for cell dissociation and migration of RG derivatives (neurons) to the brain cortical layers. At this time, RG cells destined to become ependyma must reassemble their AJs to form the respective monolayer covering the ventricular walls. Afadin, an actin filament binding protein found at the AJ, is essential for ectoderm organization and mesoderm migration in embryogenesis (Ikeda et al., 1999). Moreover, Afadin conditional-KO mice lack AJs in radial glial and ependymal
cells, and ependymal cells dissociate from underlying subventricular wall, blocking the aqueduct and causing aqueduct stenosis and hydrocephalus (Yamamoto et al., 2013a). N-cadherin, the primary cadherin in ependymal cells, is crucial for ependymal cell stability (Antonio J Jiménez, 2014). Blocking of N-cadherin function in ependyma from adult bovine leads to abnormal cellular distribution of N-cadherin, resulting in ependymal denudation (Oliver et al., 2013).

1.3.2.4 Ciliogenesis and ependymal cell polarity during differentiation

Cilia are classified based on microtubule composition: 1) primary cilia (9+0), with nine outer microtubule doublet and no central pair and 2) motile cilia (9+2), with nine outer doublet microtubules and one pair of singlet microtubules in the center. Primary cilia are present in many tissues, functioning as a sensory antenna with critical roles in mechanosensory and intracellular signaling (Adams, 2010). On the contrary, motile cilia function in: 1) directional fluid flow to clear mucus and dirt in the trachea and lungs, and clearing of CSF from the ventricles, 2) transportation of eggs from the ovary to the uterus, and 3) sperm motility (Sapiro et al., 2002) (Halbert et al., 1997) (Stannard and O'Callaghan, 2006).

Development of ependymal cells requires the transition from a 9+0 monociliated RG into 9+2 multiciliated ependyma. During ependymal cell differentiation, a large number of centrioles migrate and anchor apically to eventually become the modified centrioles serving as a base for motile cilia (i.e. basal body) (Marshall, 2008). Basal bodies serve as a structural base of a cilium, and their correct positioning is crucial to properly build a cilium. For example, mice deficient for basal body docking protein, Chibby, display chronic airway infection due to defective lung ciliogenesis (Burke et al., 2014). Following basal body
docking, cilia elongation takes place with the resulting product of an ependymal cell with ~50 motile cilia, each approximately 8-15μm in length (Spassky, 2013). In ependyma, this process occurs concurrently with RG to ependyma morphological changes, starting at postnatal day 1 and ending at P21 (Delgehyr et al., 2015; Spassky et al., 2005).

Transcription factor FoxJ1, has been widely accepted as a gene required for the differentiation of radial glia into ependymal cells. FoxJ1−/− null mice are unable to properly localize basal bodies into the apical cell surface, suggesting a role for this gene in basal body docking and ependymal cell multiciliation (Jacquet et al., 2009). FoxJ1 expression is in turn regulated by transcription factor RFX3, which acts as a co-activator to induce FoxJ1 expression during ciliated cell differentiation (Didon et al., 2013). Transcription factor RFX3 has been tightly associated with ciliogenesis, and its expression has been observed in ependymal cells and the choroid plexus (Baas et al., 2006a; Choksi et al., 2014). For example, Rfx3-deficient mice display a significant loss of cilia in the choroid plexus (Baas et al., 2006a). Furthermore, ependymal cells deficient for Rfx3 also display a significant reduction of ciliary growth in ependymal cell cultures (El Zein et al., 2009). Ciliary defects have helped in the identification of genes involved in ependymal cilia formation and function. These genes may be categorized based on the observed ciliopathy, with defects in: 1) basal body docking (Dvl1, Fltp, Chibby) (Park et al., 2008) (Enjolras et al., 2012; Gegg et al., 2014), 2) cilia elongation (Stumpy, Ulk4) (Town et al., 2008; Vogel et al., 2012), and 3) axoneme structure and configuration (Hydin, Mdnah5, Hsf1) (Ibanez-Tallon et al., 2004b; Lechtreck et al., 2008b; Takaki et al., 2007). Although the identification of these factors is promising, it is likely that these are only a small representation of the genes involved in ependymal ciliogenesis.
1.3.2.5 Planar cell polarity controls cilia polarity in ependyma

Ependymal cells constitute a special kind of multiciliated cell as these cells display unipolar clustering of basal bodies. Before differentiation, ependymal cell basal bodies are randomly distributed across the apical surface. Upon differentiation these structures become asymmetrically localized and polarized through a process known as planar cell polarity (PCP). The non-canonical WNT/PCP pathway involves the WNT-mediated activation of transmembrane proteins Fizzled and Vangl, and cytoplasmic components (e.g. Dishevelled, Prickle) to direct the uniform polarization of epithelial cells along a two-dimensional plane (Gao, 2012) (Walck-Shannon and Hardin, 2014). The contribution of the PCP pathway to cell polarity has been extensively characterized in Drosophila; however, the discovery of this pathway in ependymal ciliogenesis is relatively recent (Park et al., 2006; Wallingford, 2006) (Kishimoto and Sawamoto, 2012a). Ependymal cilia polarity by PCP may be divided into 3 kinds of polarity: 1) translational polarity, 2) rotational polarity, and 3) tissue-wide polarity (Fig. 5). Translational polarity ensures that basal bodies affixed to the apical surface become asymmetrically localized, covering about 20% of the cell surface area (Boutin et al., 2014a). During rotational polarity, cilia lengthen and begin to beat, and they generate a fluid flow that helps direct basal body orientation (Guirao et al., 2010). Correct positioning or orientation of basal bodies is crucial for all cilia to beat in the same direction. Cilia are also oriented in respect to other cells along the tissue axis, and this process is referred to as, tissue-wide polarity.

1.3.2.5.1 Genes involved in ependyma PCP-mediated polarity

The PCP pathway is an intricate process that requires many factors to control the asymmetrical localization of proteins needed for polarity. Although it has taken years to
dissect this mechanism, much progress has been made in the identification of the players needed for PCP in ependymal cells. For example, triple knockout of the known PCP protein *Dishevelled* (i.e. Dvl1, 2, 3) in Dvl TKO<sup>GFP-Cre</sup> mice resulted in loss of translational, rotational and tissue-wide polarity in ependyma (Ohata et al., 2014a). Furthermore, non-muscle myosin II has been shown to aid in basal body distribution thus, facilitating translational polarity in ependyma (Hirota et al., 2010).

Some have suggested that PCP mediated polarity is regulated by RG primary cilia. Primary cilia in RG have been shown to be asymmetrically localized, and lack of primary cilia results in impaired positioning of multicilia bundles (Boutin et al., 2014a). Ohata et al. suggested that mechanosensory proteins Pkd1 and Pkd2 localized to RG primary cilia control Vangl2 asymmetrical localization, and their disruption leads to impaired establishment of PCP in RG and subsequently in ependyma (Ohata et al., 2015).

PCP regulation in the ependyma is multifaceted, as it has been shown to regulate cilia polarization as well as the apical docking of basal bodies. The working hypothesis is that PCP is involved in the regulation of apical actin network formation needed for basal body docking. Park et al. demonstrated that loss of the PCP protein *Dishevelled* in *Xenopus* embryos results in defective cilia growth in multiciliated cells (Park et al., 2008). *Dishevelled* deficient embryos were unable to affix basal bodies to the apical membrane and to properly polarize basal bodies in multiciliated cells. Mice deficient for atypical cadherin genes *Celsr2/Celsr3* display abnormal distribution of PCP components, Vangl2 and Fzd3; consequently, these mice display aberrant ependymal ciliogenesis (Tissir et al., 2010). Thus, PCP has been proven crucial for ependymal cell polarity and functionality, and its disruption often times leads to serious conditions such as hydrocephalus (Ohata et al., 2014; Tissir et al., 2010; Matsuo et al., 2013).
Figure 5. Cilia polarity is mediated by planar cell polarity mechanisms. (A) Schematic representation demonstrating the changes in basal body positioning and orientation during ependymal cell differentiation. Basal bodies attach to the actin cytoskeleton through their basal feet and this structure in turn controls cilia stroke directionality. Rotational polarity regulates the orientation of basal feet (red) during differentiation (I). Basal bodies (blue) form patches and are asymmetrically localized to one pole of the cell through translational polarity (II). Ependymal cell translational polarity is conserved across the tissue axis (tissue-wide polarity) (III).
1.3.3 Choroid plexus anatomy

The CP is a highly conserved structure across species and is deemed as the primary site for CSF secretion (Damkier et al., 2013) (Brocklehurst, 1979). The CP anatomy has been extensively studied from lower vertebrates to humans with most studies using light and electron microscopy (Tennyson and Pappas, 1964) (Millen and Rogers, 1956) (Carpenter, 1966) (Brocklehurst, 1979). In adults, the choroid lies as an extension of the ventricular ependyma with the appearance of a folded sheet-like tissue floating in the CSF inside the brain ventricles (Fig. 6A) (Sturrock, 1979). This tissue possesses a unique surface appearance with a convex shape cells corresponding to choroidal cells (Tamega et al., 2000). CP is present in all ventricular cavities in the brain, however, whether they are all functionally equal is still debated (Gomez and Potts, 1981).

The CP is composed of a single cell layer of cuboidal epithelial cells, and they are often referred to as modified ependyma. The CP is a highly vascularized epithelium with an extensive capillary network enclosed by the epithelial monolayer (Fig. 6). On the CSF-facing side (apical) the CP contains a brush border of cytoplasmic extensions known as microvilli (Fig. 6). These structures greatly enhance cell surface area and are particularly common in secretory and absorptive tissues. Electron microscopy analysis of the CP has identified two types of microvilli: 1) clavate, of bulbous shape and 2) filiform, of finger-like shape (Fig. 6A') (William J. Schultz, 1977). William proposed that clavate microvilli are indicative of active CSF secretion and are, therefore, the most predominant type in CP. Meanwhile, the filiform microvilli correspond to lower levels of CSF production and active fluid absorption. Vasopressin, a neurohormone that acts as a blood pressure regulator in kidney, can also signal in the CP. Rats treated with vasopressin caused an inversion in microvilli
distribution, where microvillar conformation changed from clavate to filiform (William J. Schultz, 1977). The striking overturn of microvilli from the high CSF secretion form (clavate) to the low CSF secretion conformation (filiform) suggest that vasopressin treatment may lead to a decrease in CSF secretion and/or increase in transchoroidal absorption (Faraci et al., 1994; William J. Schultz, 1977).

Microvilli extensions are visible as early as E11; however, their number greatly increases over time due to the increased load of CSF secretion in the CP (Sturrock, 1979). The CP epithelium forms one or two dozens of cilia in the apical surface (Narita and Takeda, 2015). An emerging body of literature suggests that CP cilia are primarily non-motile (9+0) (Inoue et al., 2015; Narita et al., 2010; Narita et al., 2012; Narita and Takeda, 2015; Nonami et al., 2013) (Swiderski et al., 2012). However, other studies report a more heterogeneous population of cilia; thus, an agreement on CP ciliary ultrastructure has not been reached.

CP polarization of epithelial transport proteins allows the movement of ions to generate the osmotic gradient needed for CSF production (Damkier et al., 2013). CP polarization has intrigued many scientists over the years, particularly because CP polarity appears to be inverse to other epithelial cells. For example, ion channels typically associated with the basolateral compartment (Na⁺K⁺ATPase, NKCC1, KCC4, and NHE1) are localized to the apical membrane in CP cells (Damkier et al., 2013; Marrs et al., 1993). Some have suggested that the fodrin-ankyrin cytoskeleton contributes to the unique polarization of Na⁺K⁺ATPase in CP; however, the precise mechanism in which CP achieves its unusual polarity remains unknown (Alper et al., 1994).
Figure 6. The anatomy of the choroid plexus. The CP forms a single layer of specialized ependymal cells (purple cells) that extends from the ventricular ependyma (pink cells) (A). Ultrastructural analysis of CP by TEM demonstrates the variations in microvilli population (A’). Some microvilli display a bulb-like shape called clavate, whereas others have a finger-like shape known as filiform (A’). Schematic diagram adapted from (Alexander de Lahunta, 2015)
1.3.4 Choroid plexus development and differentiation

The development of CP in all ventricular cavities does not occur concomitantly. The fourth ventricle CP is the first one to develop; this is followed by the emergence of CP in both lateral walls and lastly the CP in the third ventricle. In mice the CP begins to form around E12.5 and by E15 the folded sheets of the CP are found occupying all ventricular cavities in the brain (Sturrock, 1979). A series of developmental changes have been described during CP maturation. These have been categorized into 4 stages based on changes in cell morphology. During stage 1, CP cells are mainly a pseudostratified epithelium, while in stage 2, these cells change into a single columnar appearance. In stage 3, the cells become cuboidal with apical/central nuclei; however, the movement of the nuclei into a more basal position indicates that the cells have completed stage 4 in development (Lun et al., 2015). These morphological changes are accompanied with the formation of microvilli and cilia, as well as the establishment of tissue polarization.

Microvilli are present early during CP formation, but their number greatly increases over time; by E16 these structures have formed a brush-border covering the entire CP apical surface (Lun et al., 2015b; Sturrock, 1979). On the other hand, CP cilia are present as early as E15 and they also show a significant increase in their numbers. This phenomenon has been attributed to the noted increase in CSF secretion by the CP (Dziegielewska et al., 2001). Transcription factors E2f5, Rfx3 and FoxJ1 are known to regulate ciliogenesis in CP, and their disruption leads to dysfunctional CP cilia and hydrocephalus (Lindeman et al., 1998; Yu et al., 2008; Baas et al., 2006; Jacquet et al., 2009).

Over the last 3 decades, a great effort has been made to identify factors associated with CP development. Bone morphogenic proteins (BMPs) have been tightly associated
with this process, particularly, BMP4 which was shown to promote CP cell fate
determination in mice (Panchision et al., 2001; Hebert et al., 2002). Moreover, lack of BMP
signaling significantly delays CP growth (Fernandes et al., 2007). Transcription factors,
Hes1, Hes3 and Hes5 have also been implicated in lateral ventricle CP cell fate
determination (Imayoshi et al., 2008). Additionally, transcription factor Msx1 was also
identified as a regulator of choroid plexus patterning and development (Bach et al., 2003).
The exact mechanisms by which these factors control CP differentiation are unknown. Is
possible that CP cell determination may involve complex interactions between known
transcription factors involved in CP development. In support of this notion, transcription
factors Emx1 and Emx2 involved in neuroepithelial cell fate determination have been
shown to control the levels of master regulator of CP development, Otx2 (Johansson et al.,
2013) (von Frowein et al., 2006).

1.3.5 Diverse functions of the choroid plexus in development

One of the main functions attributed to CP is in CSF production, and it is a known
fact that the atypical polarity of the CP facilitates this process (refer to section 1.2.3.1)
(Damkier et al., 2013) (Lun et al., 2015a; Lun et al., 2015). More recently, it was suggested
that CP cilia have a role in CSF secretion (Narita et al., 2010; Narita et al., 2012; Narita and
Takeda, 2015) (Banizs et al., 2005). The mouse gene Tg737, encodes ciliary protein Polaris
involved in Intraflagellar Transport (IFT), a process that facilitates the movement of
proteins across the ciliary axoneme (Taulman et al., 2001; Pazour et al., 2000; Banizs et al.,
2005; Scholey, 2003). Polaris was found to mediate the localization of primary cilia
signaling protein, Pkd1, involved in WNT signaling, and presumed to regulate pathways
involved in cilia-regulated CSF secretion (Kim et al., 1999; Wodarczyk et al., 2009).
Interestingly, *Tg737* deficient mice (*Tg737*orpk) display abnormal CP cilia, and aberrant Pkd1 localization, with mice displaying hydrocephalus (Banizs et al., 2005).

**1.3.5.1 The choroid plexus functions as a blood-CSF barrier**

The CP participates in the exchange of nutrients and proteins needed for proper brain development. The diffusion of molecules is observed throughout the CP fenestrated capillaries; thus, the CP has devised a mechanism to regulate the transport of these factors into the brain. The choroidal epithelium contains apical tight junctions and basolateral adherens junctions. Together these structures form a physical barrier known as the blood-CSF barrier (Fig. 7) (Liddelow, 2015). Proper formation and localization of these junctions prevents paracellular diffusion, allowing the passage of molecules only through the CP cell. The blood-CSF barrier has been associated with Alzheimer’s, and is believed to have a role in the progression of this disease (Krzyzanowska and Carro, 2012) (Serot et al., 2003) Amyloid β-oligomers associated with Alzheimer’s disease affect CP cell morphology, disrupting tight junctions and barrier integrity; consequently leading to blood-CSF barrier leakage (Brkic et al., 2015).
Figure 7. The choroid plexus acts as a blood-CSF barrier. Molecules (i.e. Na⁺, Cl⁻; glucose and proteins) pass freely through the fenestrated capillaries into the choroid plexus stroma. Apical tight junctions and basolateral adherens junctions prevent the paracellular passage of these molecules acting as a barrier, thus promoting only transchoroidal transport of molecules. Figure adapted from (Szmydynger-Chodobska et al., 2007)
1.4 The mouse *Jhy* gene is involved in CSF homeostasis and hydrocephalus

The generation of genetically engineered mice has greatly enhanced our understanding of congenital hydrocephalus, yet little is known about the genetic mechanisms that underlie this condition (Vogel et al., 2012). Our lab has generated a juvenile hydrocephalus transgenic mouse line here referred to as the *Jhy*$_{lacZ}$ mouse line (Appelbe et al., 2013). The transgene insertion was mapped to chromosome 9, between intron 4 and exon 5 of the mouse gene *Jhy*. The gene was named after the phenotype of juvenile hydrocephalus observed in these mice. The integration of the transgene occurred at the beginning of exon 5, removing its splice acceptor site along with some exon 5 coding sequence. The transgene insertion caused the reduction of *Jhy* expression levels to 41% of wild type in homozygous (*Jhy*$_{lacZ}$/*lacZ*) animals. *Jhy*$_{lacZ}$/+ are phenotypically normal; however, *Jhy*$_{lacZ}$/lacZ mice develop communicating juvenile hydrocephalus, suggesting a loss of function phenotype. Ventricular dilation in *Jhy*$_{lacZ}$/lacZ is observed as early as P1.5 and mice die around 4-8 weeks of age.

The mouse *Jhy* gene contains 9 exons, and is predicted to encode a protein of approximately 770aa with a molecular weight of ~87KDa. The JHY protein sequence contains no known functional domains, and no functions of this gene have ever been reported. Through RT-PCR we identified *Jhy* expression in brain, lung, spleen and testis, where with the exception of brain, the tissues analyzed appeared morphologically normal. The *Jhy* gene is conserved among vertebrates. The mouse cDNA is 72% identical to the human, 82% to the rat, and 30% to the chicken.

A detailed analysis of tissues associated with the pathology of hydrocephalus was performed in the *Jhy*$_{lacZ}$ mouse line. TEM analysis demonstrated abnormal patterning of ependymal motile cilia ultrastructure. *Jhy*$_{lacZ}$/lacZ animals display an apparent loss of
microtubule central pair (singlet), a structure needed for motile cilia motility and tightly associated with the development of hydrocephalus (Baas et al., 2006; Swiderski et al., 2012; Lechtreck et al., 2008; Ibanez-Tallon et al., 2004; Banizs et al., 2005). In 2012, Ivliev et al. performed a transcriptome analysis of ciliated tissues and found \textit{Jhy} human orthologue, \textit{C11orf63}, to be localized to human lung and oviduct (Ivliev et al., 2012). This data provides some evidence to further supports the role of \textit{Jhy} in ciliated cells.

1.5 Purpose of this study

A stable equilibrium between CSF production, flow and absorption is fundamental in the development of the central nervous system. As described above, impaired CSF homeostasis results in many neurological conditions, among them hydrocephalus. Despite the high prevalence of hydrocephalus in children, very little progress has been made to understand the genetic mechanisms that cause this condition. Our lab has previously generated a mouse model for hydrocephalus (\textit{JhylacZ}) with mutations in the mouse \textit{Jhy} gene, and \textit{JhylacZ/lacZ} animals display abnormally patterned ependymal cilia. The purpose of this study is to further characterize the role of \textit{Jhy} in ependymal cells and confirm its role in the etiology of hydrocephalus. Our investigation demonstrated that \textit{Jhy} is required for proper ependymal cell development, with ependymal cells displaying abnormal adherens junction formation, and impaired ciliary motility. Interestingly, similar defects were also observed in the modified ependyma of the CSF-producing tissue, the choroid plexus. Thus, \textit{Jhy} function appears to be conserved between ependymal cells as well as the specialized ependyma of the choroid plexus.
II. MATERIALS AND METHODS

2.1 Animal maintenance

Animals were housed in a modified barrier facility with a 12hr light/dark cycle and fed 7012 Teklad LM-485 mouse diet. Mice were sacrificed using carbon dioxide followed by cervical dislocation as directed by the Office of Animal Care and Institutional Biosafety (OACIB). The generation of the JhylacZ mouse line has been previously described (Appelbe et al., 2013). The JhylacZ mouse line was maintained by intercrossing JhylacZ/+ animals to obtain JhylacZ/lacZ offspring. The JhylacZNeo mouse line was generated using ES targeted cells purchased from the Knockout Mouse Project (KOMP) repository (https://www.komp.org/geneinfo.php?geneid=10807). Correctly targeted ES cells were implanted into mouse blastocysts in collaboration with the UIC Transgenic Production Facility. Chimeric mice were crossed to C57BL/6J mice to obtain F1 generation mice uniformly heterozygous for the targeted allele. Crosses between heterozygotes (JhylacZNeo/+) were performed to obtain an offspring with the expected mendelian ratio. JhylacZNeo/+ littermates from both lines were used as controls.

2.2 Mouse tail prep and DNA extraction

A small fragment of a mouse tail (i.e. 0.5cm) was placed in a microcentrifuge tube with a tight cap. Tails were submerged in digestion buffer (50mM Tris-HCL pH 8.0, 100mM EDTA pH 8.0, 0.5% SDS, 500μg/mL Proteinase K, complete volume with diH2O) and incubated overnight at 55°C. Digested tails were then treated with phenol/chloroform/isoamyl alcohol (25:24:1), and tubes were then inverted multiple times. Samples were centrifuged at 12500 rpm for 3 minutes, and upper aqueous phase was placed into a new tube. Extracts were then treated with chloroform/isoamyl alcohol
(24:1), centrifuged, and upper aqueous phase was once again transferred into a new tube.

The extracted sample was treated with 50μl of 7.5M NH4OAc and 1mL of cold 200 proof ethanol. The sample was inverted for approximately 20 times. Pellets were transferred into new tubes containing cold 70% ethanol and spun at max speed for 10 minutes. Residual ethanol was removed using a pipette tip and pellets were air-dried for 10 minutes. diH2O (200μl) was used to re-dissolve the DNA pellet. Samples were incubated in a 55°C water-bath for no more than 1 hour to resuspend the DNA.

2.2.1 Genotyping PCR

2.2.1.1 PCR reagents for master mix:

16.75μL molecular grade, nuclease-free diH2O
6μL red juice (60% sucrose, 1mM cresol red, in dH2O)
3μL 10X PCR buffer (with 100mM Tris-HCl pH 8.3, 500mM KCl, 15mM MgCl2 and 0.01% gelatin)
1μL of 10mM dNTP
0.5μL 10 μM forward primer
0.5μL 10 μM reverse primer
0.25μL Taq polymerase enzyme
2μL of template DNA

2.2.1.2 PCR conditions for JhylacZ mouse line genotyping

WT allele primers:
(OL1080) 5’-CCAAGAGGTCAGTCTTCCGATGCA-3’
(OL1478) 5’-CGTAGATGGGCTGCGGCTGATAC-3’

JhylacZ allele primers:
(OL834) 5’-CCCGTTTTTCGCCGATTTGGCTACATGACA-3’
(OL1322) 5’-GCGTTCTGGCGCTGTAAAATTGG-3’
The PCR was performed in an Eppendorf Mastercycler (Hauppauge, NY) using the following conditions: 95°C 5 minutes, 95°C 30 seconds, 63°C 15 seconds and 72°C 35 seconds, for a total of 35 cycles.

2.2.1.3 PCR conditions for JhylacZ/Neo mouse line genotyping

WT allele primers:
(OL2269) 5’-CCACTGAACTCCAAGCCCAGC-3’
(OL1524) 5’-TCCAGTGGGATCATATCGGAGGT-3’

JhylacZ/Neo allele primers:
(OL2269) 5’-CCACTGAACTCCAAGCCCAGC-3’
(OL2267) 5’-TCCTACATAGTTGGCAGTGTTTGGG-3’

The following conditions were used for the genotyping PCR: 95°C 5 minutes, 95°C 30 seconds, 61°C 30 seconds and 72°C 30 seconds, for a total of 35 cycles.

2.3 Tissue preparation: fixation, dehydration and paraffin embedding

Mouse brains at P5, P10 and P14 were dissected and fixed in Bouin’s fixative for 18-24 hours at room temperature. Brains were first washed in tap water followed by washes in 70% ethanol with saturated lithium carbonate (Sigma-Aldrich, St. Louis, MO). Mouse lung, kidneys, testis and oviduct were fixed in 4%PFA overnight at 4°C. Tissues were then dehydrated in a graded series of ethanol washes (30%, 50%, 70%, 95% and 100%), followed by xylene washes for 60 and 90 minutes at room temperature. Specimens were incubated in paraffin for 60 and 90 minutes at 58°C. The tissues were paraffin embedded and sectioned at 8-10μm using a microtome (Leica Microsystems RM2125, Bannockburn, IL). Prior to histological analysis the slides were washed in xylene and rehydrated using a series of ethanol washes.
2.3.1 Tissue cryoembedding

Mouse tissues were dissected in cold 1xPBS, transferred into fresh 4%PFA and incubated overnight at 4°C. Tissues were then washed in cold 1xPBS and prepared for cryoprotection. Specimens were first incubated in cold 15% sucrose in 1xPBS for 30 minutes and then passed to 30% sucrose overnight or until the tissue sinks. Cryopreserved tissue was then incubated in OCT media at 4°C overnight. A freezing station was prepared using a dry ice block. Peel-A-Way molds with fresh OCT were used to embed tissues until the media completely freezes. Embedding molds were then covered with aluminum foil, placed in Ziploc bags and stored at -80°C for long-term storage.

2.4 Histology and tissue staining protocols

2.4.1 Immunofluorescence (IF)

2.4.1.1 IF on paraffin sections

Mouse tissues (brain, testis, kidney and lung) from E19-P14 were dissected and paraffin embedded as described in section 1.3. Sections 8-10μm thick were prepared from the paraffin embedded tissue. Slides were de-waxed by performing three consecutive washes in xylene for 5 minutes each. Tissue rehydration was performed using a series of ethanol washes (100%, 95%, 70%), and a final wash in 1x PBS. Heat antigen retrieval (0.3% Sodium Citrate, 0.05%, Tween-20 pH 6.0) was performed followed by tissue permeabilization (0.5% Triton X-100 in PBS) for 15 minutes at room temperature. Slides were blocked in blocking solution (5% goat/donkey serum, 1%BSA, 0.75% Glycine, 0.5% Tween 20X in PBS) for 1 hour at room temperature, and incubated in primary antibody overnight at 4°C in a humidifying chamber. Primary antibodies against the following antigens were used: human/mouse FOXJ1 (mouse, 1/500; 14-9965, eBiosciences, Inc.),
mouse Glast (guinea pig, 1:1000; AB1783, Millipore), mouse Acα-Tub (mouse, 1:1000; T6793, Sigma-Aldrich), mouse Vimentin (rabbit, 1/500; ab92547, Abcam), cow S100β (rabbit, 1/50; Z-0311, DAKO), human N-cadherin (rabbit, 1/50; sc-7939, Santa Cruz), rat AQP1 (rabbit, 1/200, Alpha Diagnostics International, AQP11-A), F-actin staining was achieved using Alexa Fluor 488-phalloidin (1/50, Invitrogen, A12379).

The sections were incubated in secondary antibody for 2 hours at room temperature using the following secondary antibodies: Alexa Fluor 488 goat anti-mouse (1/250, Invitrogen), Cy3-conjugated goat anti-mouse (1/250, Jackson ImmunoResearch), Alexa Fluor 647 donkey anti-rabbit (1/250, Jackson ImmunoResearch), FITC conjugated goat anti-rabbit (1/250, Jackson ImmunoResearch). Slides were DAPI stained following the steps mentioned in section 1.4.1.4 (D1306; Life Technologies, Grand Island, NY.). Slides were then mounted using vectashield mounting medium (H1000; Vector Laboratories, Burlingame, CA). Images were taken using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

2.4.1.2 IF on cryosections

Brains at P0.5, P5, P6 and adult testis were dissected and fixed in 4% PFA overnight at 4°C, and embedded as specified in section 1.3.1. Cryosections of 10-15μm thickness were used for IF analysis. Slides were air-dried for 30min at room temperature to improve tissue adherence to the slide. Tissue was fixed for a second time with 4%PFA for 10 minutes at 4°C and washed using 1xPBS. Permeabilization was performed using 0.2% Triton X-100 in 1xPBS for 10 minutes at room temperature. Blocking of sections was done using 5% normal serum (goat/donkey) in 1xPBS for 1 hour at room temperature. Primary antibody in 50% blocking solution and 50%1xPBS and was added to the slides, followed by
overnight incubation at 4°C in a rocking platform. The following primary antibodies were used: mouse monoclonal β-catenin (1/250, BD Transduction Laboratories, 610153), mouse monoclonal E-cadherin (1/50, BD transduction laboratories, 610181) and mouse AE2 (rabbit, 1/100, kindly provided to us by Dr. Seth L. Alper, Harvard Medical School). Slides were incubated in secondary antibodies as specified in section 1.4.1.1. All washing steps were performed using 1xPBS. Sections were DAPI stained, coverslipped and mounted using Vectashield mounting medium.

2.4.1.3 Whole mount IF

Medial and lateral ventricular walls from P10 mice were isolated in cold PBS and further sectioned using a vibratome (Leica V1000S, Buffalo Grove, IL). Tissue was fixed in 4% PFA in PBS overnight at 4°C, followed by blocking with 2.5%BSA, 0.2% Triton x-100 in PBS for 4 hours at room temperature. The following primary antibodies were used: N-cadherin (rabbit, 1/50; sc-7939, Santa Cruz), γ-tubulin (mouse 1/250; ab11316, Abcam). Samples were incubated with primary antibody for 24-48 hours at 4°C in a rocking platform. Secondary antibody incubation was performed at 4°C for 48 hours using Alexa Fluor 488 goat anti-mouse (1/250, Invitrogen) and Alexa Fluor 647 donkey anti-rabbit (1/250, Jackson ImmunoResearch). Isolated tissues were placed flatly on a glass slide, mounted and imaged using an Andor WD Spinning Disk confocal microscope system (Yokagawa CSU-W1).

2.4.1.4 DAPI staining

DAPI was purchased from (D1306; Life Technologies, Grand Island, NY.) and a stock solution of 5mg/mL was prepared. DAPI solution I was prepared using 10μL of DAPI stock
(5mg/mL) and added to 990μL of dH₂O. A working solution was used to stain sections using 20μL of solution I and 980μL of 1xPBS. The working solution was added to tissues/cells and incubated at room temperature for 5 minutes. Washes were done using 1xPBS and slides were then mounted and coverslipped for imaging.

2.4.2 Immunohistochemistry

Bouin’s fixed and paraffin embedded sections were deparaffinized and rehydrated as specified in section 1.4.1.1. Heat antigen retrieval (0.3% sodium citrate, 0.05%, Tween-20 pH 6.0) was performed and tissues were washed with TNT buffer solution (0.1M Tris-Hcl, pH 7.5, 0.15M NaCl, and 0.05% Tween 20). Quenching of endogenous peroxides was done by using 3% H₂O₂ in methanol for 10 minutes. Slides were blocked with 5% normal goat serum in TNT buffer for 1 hour at room temperature. Overnight incubation of primary antibody in 1% BSA was performed at 4°C using antibody α- human TTR (Proteintech, 1189-1-AP, 1:100). Secondary goat α-rabbit biotinylated antibody was added in 50% block (goat serum in TNT):50% PBS for 1 hour at room temperature. ABC reagent (Vector laboratories, PK-6100) was added to the slides for 30 minutes at room temperature, followed by 5 minutes in DAB substrate (Vector laboratories, SK-4100) for final signal detection. Hematoxylin was used as a counterstain. Images were taken on a Zeiss Axiovert 200M deconvolution microscope equipped with an Axiocam 105 color camera (Zeiss, Göttingen, Germany).

2.4.3 Hematoxylin and Eosin staining (H&E)

Mouse brains at E19, P0.5, P5, P10 and P14 were dissected and fixed in Bouin’s fixative for 18-24 hours at room temperature. Brains were first washed in tap water
followed by washes in 70% ethanol with saturated lithium carbonate (Sigma-Aldrich, St.
Louis, MO). Tissues were dehydrated in a graded series of ethanol washes, embedded in
paraffin and sectioned into 8-10μm sections using a microtome (Leica Microsystems
RM2125, Bannockburn, IL). Brain sections were stained using harris hematoxylin and
alcoholic eosin Y solution (Sigma-Aldrich, St. Louis, MO). Slides were deparaffinized using
xylene washes, rehydrated in ethanol washes (100%, 95%, 70%, 30%), and lastly washed
in dH₂O. Hematoxylin incubation time varied depending on the freshness of solution and
the tissue being tested. The incubation time ranged from 10 seconds to 1 minute. Slides
were washed in running tap water for 5 minutes. Sections were washed in 0.25% HCL/acid
alcohol for 2 seconds, followed by washes in running tap water. 1.36% Lithium carbonate
was used to wash slides for 5 seconds and this was washed off with tap water. Eosin
solution was added to slides with times ranging from 5 seconds to 1 minute depending on
the freshness of the solution. Eosin was washed off using a 95% ethanol wash. Slides were
subsequently dehydrated in a series of ethanol washes (95%, 100%), washed in xylene and
mounted using Cytoseal XYL (Fisher Scientific, Pittsburgh, PA). Imaging was performed
using a Leica MZFLIII dissecting microscope (Wetzlar, Germany).

2.4.4 Embryo and newborn skull decalcification

E19 embryos and P0.5 newborn heads were collected and their skin was removed.
Isolated heads containing skull and brain tissues were submerged into 10%EDTA in dH₂O
pH-7.4 overnight at 4°C. Once the skull was decalcified, the tissues were paraffin embedded
as specified in section 1.3.
2.5 Scanning electron microscopy

Brains collected at P5 and P10 were fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1M sodium cacodylate buffer pH 7.4) for 7 days at 4°C. Brain ventricles where split and fixed in Karnovsky's for 4 more days. Brains were washed in 0.1M cacodylate buffer and dehydrated through series of ethanol washes. Dehydrated tissue was immersed in pure hexamethyldisilazane (HMDS) (EMS, Hatfield, PA) twice for 15 minutes at room temperature. Samples were covered in fresh HMDS and left under the hood until solution completely evaporates. Specimens were mounted and sputter coated with gold/palladium using a Polaron E5100 (Polaron Instruments, East Grinstead, West Sussex, UK). Images were taken using a variable pressure SEM Hitachi S-3000N (Hitachi High Technology Corp., Tokyo, Japan).

2.6 Transmission electron microscopy (TEM)

Mouse choroid plexus was dissected at P5 and fixed in fresh fixative containing 2.5% glutaraldehyde, 4% paraformaldehyde and 0.1M sodium cacodylate buffer, pH 7.4 for 3 days at 4°C. Tissue was then washed using 0.1M sodium cacodylate buffer and processing was done in collaboration with the Advanced Electron Microscopy Facility at the University of Chicago. Specimens were postfixed using 1% osmium tetroxide in 0.1M sodium cacodylate buffer, rinsed with maleate buffer (pH 5.1), and changed to 1% uranyl acetate in maleate buffer. Tissue was dehydrated following a series of ethanol washes. Dehydrated tissue was infiltrated first in a 2:1 and in 1:1 propylene oxide:spurr resin, followed by polymerization of spurr resin containing the tissue. Ultrathin (90-150nm) sections were cut using the Leica EM UC6 ultramicrotome. Post-staining of sections was performed using 2% uranyl acetate in 70% methanol and lead citrate in dH₂O. Images were taken under
150kV on a FEI Tecnai Spirit transmission electron microscope (Hillsboro, OR, USA) and examined using the Galtan CCD DigitalMicrograph software.

2.6.1 Rotational polarity analysis

Previously generated TEM micrographs of P5 ependymal cilia were reexamined to determine basal foot orientation (micrographs used for this analysis were generated by Brian Bollman) (Appelbe et al., 2013). The right-most basal body in each image was chosen to act as the reference. Using Fiji software (http://fiji.sc/Fiji), a line was drawn along the center of the basal body, terminating at the tip of the basal foot. This line was copied without varying the angle to each other basal body in the image. Independent lines were then drawn along the central length of each of the non-reference basal bodies in the cluster. If all basal feet were perfectly aligned, each pair of lines should be completely overlapping and the angle between them should be zero. If the lines were not fully overlapping, the Fiji angle tool was used to calculate the angle between them. To represent the full range of rotation from the reference, angles located to the right from a predominantly vertical reference line or above a predominantly horizontal reference line were labeled as positive, while those to the left or below the reference were labeled with as negative.

2.7 cAMP quantification by ELISA

Choroid plexus isolated from Jhy+/+ and JhylacZNeo/lacZNeo were collected and immediately frozen in liquid nitrogen. Tissue processing and cyclic AMP was quantified following the procedures specified by the cyclic AMP EIA Kit (Cayman Chemical, MI, USA).
2.8 Recording and analysis of ependymal-generated flow

*In vivo* ciliary flow recordings were performed in P10 mouse brains. Freshly isolated lateral ventricles were dissected in cold DMEM supplemented with 25mM HEPES, followed by further sectioning using a vibratome (Leica V1000S, Buffalo Grove, IL) for a final thickness of 500μm. Sections were maintained in DMEM containing 25mM HEPES at room temperature during the recoding period, which occurred immediately after euthanasia and tissue isolation. FluoSpheres Carboxylate–Modified microspheres (0.5μm yellow-green; F8813, Invitrogen) were used to track flow generated by ependymal cilia. Live images were recorded at 300 frames per second using an Ultima In Vivo multiphoton laser scanning microscope (Prairie Technologies) with a 10X immersion objective and an optical zoom of 80X. Imaris software (7.7 Ultimate Collection) was used to analyze the tracked fluorescent particles and calculate the average speed of the beads.
III. THE MOUSE JHY GENE REGULATES EPENDYMAL CELL DIFFERENTIATION AND CILIogenesis

3.1 Abstract
During the first postnatal week of mouse development, radial glia lining the ventricles of the brain differentiate into ependymal cells, undergoing a morphological change from pseudostratified cuboidal cells to a flattened monolayer. Proper ependymal development is crucial to forming the brain tissue:CSF barrier, and to the establishment of ciliary CSF flow, but the mechanisms that regulate this differentiation event are poorly understood. The JhylacZ mouse line carries an insertional mutation in the Jhy gene (formerly 4931429I11Rik), and homozygous JhylacZ/lacZ mice develop a rapidly progressive juvenile hydrocephalus, with defects in ependymal cilia morphology and ultrastructure. Here we show that JhylacZ/lacZ mice also display abnormal ependymal cell differentiation. Ventricular ependyma in JhylacZ/lacZ mice retain an unorganized and multi-layered morphology, representative of immature ependymal cells, and show altered expression of differentiation markers. Most JhylacZ/lacZ ependymal cells do eventually acquire mature ependymal characteristics, suggesting a delay, rather than a block, in the differentiation process, but ciliogenesis remains perturbed. JhylacZ/lacZ ependymal cells also manifest disruptions in adherens junction formation, with altered N-cadherin localization, and have defects in the polarized organization of the apically located motile cilia. Functional studies showed that cilia of JhylacZ/lacZ mice have severely reduced motility, a likely cause for the development of hydrocephalus. JHY is therefore a crucial component of the ependymal differentiation process, with roles in ependymal cell morphogenesis and ciliary organization and function.
3.2 Introduction

The ependyma is a monolayer of multiciliated epithelial cells that lines the ventricles of the vertebrate brain (Garcia-Verdugo et al., 2002). Ependymal cells serve as a protective barrier between the cerebrospinal fluid (CSF) and the brain tissue, and they are believed to contribute to CSF flow through the ventricular system by the coordinated beating of their apical cilia (Bruni, 1998a; Bruni et al., 1985; Del Bigio, 2010). The ependyma produces a small percentage of the CSF (the majority of the CSF is secreted by the choroid plexus), and provides metabolic support to developing neural stem cells (Coskun et al., 2008; Kuo et al., 2006). Mouse models with loss of ependymal ciliary motility often develop hydrocephalus, a pathologic increase in ventricular CSF volume, presumably because ciliary stasis reduces both CSF flow and its absorption (Jacquet et al., 2011; Lavado and Oliver, 2011a; Peng et al., 2013; Tissir et al., 2010a). Mutations in the Hydin gene, for example, cause the production of ependymal cilia that are structurally normal, but cannot beat due to microtubule defects (Lechtreck et al., 2008a; Lechtreck and Witman, 2007). Hydin mutant animals develop outwardly visible hydrocephalus within the first postnatal week, and die by 7 weeks of age (Davy and Robinson, 2003).

Ependymal cells are postmitotic cells that develop from radial glia, a precursor that also gives rise to neurons, astrocytes, and oligodendrocytes (Kuo et al., 2006; Rodriguez et al., 2012; Spassky et al., 2005; Tramontin et al., 2003a). The terms maturation and differentiation are often used interchangeably to refer to the transition from a radial glial cell to a multiciliated ependymal cell. The Gene Ontology consortium defines differentiation as “the process whereby a relatively unspecialized cell acquires specialized features of a specific cell type”, and maturation as “a developmental process, independent of morphogenetic (shape) change, that is required for a cell to attain its fully functional
state”. As the ependymal transition involves clear changes in cell morphology, we will use the term differentiation to describe this process (Ashburner et al., 2000). The transition from radial glia to ependymal cells occurs during the first postnatal week in the mouse, with the differentiation process moving across the ventricular surface in a caudo-rostral/ventro-dorsal/latero-medial gradient (Bruni, 1998a; Spassky et al., 2005). As radial glia differentiate to ependyma, they undergo a morphological change from pseudostratified cuboidal cells to a monolayer of flattened multiciliated cells. The factors that regulate the transition from radial glial cells to mature ependyma are not well characterized, nor are many of the steps in motile ciliogenesis. FOXJ1 is one protein required for ependymal differentiation and cilia formation, and is thought to be at the top of a transcriptional hierarchy controlling these processes. Mice lacking FoxJ1 have immature ependymal cells that lack cilia, and these animals die in the early postnatal period from multiple abnormalities including hydrocephalus (Brody et al., 2000; Jacquet et al., 2009a; Stubbs et al., 2008a). The homeobox gene Six3 is known to repress radial glial properties, and its disruption results in ventricular walls lined with cells that display a mixture of radial glial and ependymal characteristics (Lavado and Oliver, 2011a).

As ependymal cells differentiate, they undergo massive replication of centrioles to generate the basal bodies required for multiciliogenesis. Basal bodies arise centrally within the cell, but migrate to one pole through processes controlled by the planar cell polarity (PCP) pathway. This basal body localization, termed translational polarity, is uniform in direction across the ventricular surface. Each basal body gives rise to a single cilium, with roughly 40 cilia formed per ependymal cell (O’Callaghan et al., 2012). Motile cilia are composed of microtubule polymers in a 9+2 arrangement, with 9 outer doublets surrounding a central pair of singlet microtubules. The orientation of the central pair
determines the direction of motility of the cilia, and must be coordinated across the tissue surface for proper CSF flow. Ciliogenesis also progresses in a caudo-rostral gradient/ventro-dorsal/latero-medial gradient as the ependymal cells differentiate, with caudoventral regions of the lateral ventricles carrying abundant cilia by postnatal day 5 (P5) (Appelbe et al., 2013; Spassky et al., 2005). Ependymal differentiation and ciliogenesis is completed throughout the ventricular system by P21 (Batiz et al., 2011a; Rubenstein and Rakic). Ciliary abnormalities in humans and animal models range from a complete lack of cilia (Jacquet et al., 2009a; Taulman et al., 2001a), ciliary malformation (Lattke et al., 2012; Tissir et al., 2010a), disruption of the axonemal structure (Castleman et al., 2009a; Lechtreck et al., 2008a), or the loss of ciliary motility, with or without morphological abnormalities (Ibanez-Tallon et al., 2002). Loss of motility can result from altered microtubule organization (Clare et al., 2014a), defects in structural components such as radial spokes or dynein arms (Ibanez-Tallon et al., 2004a; Zhou et al., 2012), or changes in cilia polarity that impact the direction of beating (Mirzadeh et al., 2010; Ohata et al., 2014b; Ying et al., 2014).

We reported previously that a loss of function mutation for the mouse Jhy gene (JhylacZ) causes congenital hydrocephalus that manifests by P1.5, and leads to death by 6-8 weeks of age (Appelbe et al., 2013). Homozygous JhylacZ/lacZ mice develop fewer and shorter ependymal cilia, and these cilia show loss of the central pair of ciliary microtubules. These data indicated that Jhy is required for proper ependymal ciliogenesis, and might have a broader role in regulating ependymal cell differentiation. Little is known about the Jhy gene, which was unstudied prior to our mapping of the JhylacZ integration. The Jhy sequence is conserved across vertebrates, but it has no identifiable paralogs, nor does it contain any recognizable functional domains. As much effort has not yielded an antibody that is
competent for immunofluorescence, the cellular localization of the JHY protein remains unknown. Here we show that in addition to its role in ciliary morphogenesis, Jhy is required for proper ependymal cell differentiation from radial glia. Ependymal cells of Jhy^{lacZ/lacZ} mice are delayed in their differentiation, and they display abnormal adherens junctions, with mislocalization of N-cadherin and β-catenin proteins. Jhy^{lacZ/lacZ} ependyma also have defects in ciliary translational and rotational polarity, aspects of epithelial organization regulated by the planar cell polarity pathway. Jhy therefore plays a role in multiple steps during the differentiation and functional specialization of ependymal cells.

3.3 Results

3.3.1 Ventricular ependymal cells are morphologically abnormal in Jhy^{lacZ/lacZ} mice

As ependymal differentiation and ciliogenesis are coupled, we considered that the morphological defects observed in the cilia of Jhy^{lacZ/lacZ} mice might be a result of altered ependymal cell differentiation. To assess the differentiation of Jhy^{lacZ/lacZ} radial glial cells to ependyma, we examined the expression patterns of cellular markers spanning the transition period (Abouhamed et al., 2009; Baas et al., 2006b; Jacquet et al., 2011; Lavado and Oliver, 2011a). The gradient of ependymal differentiation means that the cells of the medial wall are noticeably advanced compared to cells of the lateral wall during the first postnatal week (Kuo et al., 2006; Spassky et al., 2005). The early onset of Jhy^{lacZ/lacZ} hydrocephalus, and increasing accumulation of CSF, causes progressive damage to brain tissues. To be able to observe ependymal differentiation as early as possible during postnatal development, while brain deformation from the hydrocephalus remains limited, our studies were performed primarily on earlier-maturing medial wall tissues from the lateral ventricles. When it has been possible to analyze lateral wall tissues, i.e. once lateral
ependymal differentiation has begun but before significant hydrocephalic changes occur, these data are presented as well.

The ependymal cell marker S100β is an EF-hand calcium binding protein expressed in a variety of brain cell types, including all ventricular ependyma (Didier et al., 1986). S100β protein was localized by immunofluorescence (IF) to examine the ependymal cell morphology of Jhy+/+ and JhylacZ/lacZ animals (Fig. 8A,B). S100β expression in medial wall ependymal cells of Jhy+/+ mice at P5 highlights the flattened appearance characteristic of mature ependymal cells (Fig. 8A, arrowhead). In JhylacZ/lacZ mice, however, medial wall ependyma have a pseudostratified morphology, representative of immature ependyma (Fig. 8B, arrowhead). At this age, the later-maturing lateral wall of both Jhy+/+ and JhylacZ/lacZ ventricles shows a pseudostratified layer of immature ependymal cells, indicating that differentiation likely has not yet begun (Fig. 8A,B). To more clearly analyze ependymal cell morphology, histological analysis was performed on both the later maturing dorsal, and earlier maturing ventral, regions of the lateral ventricle at P5 (Fig. 8C-F). In coronal sections of Jhy+/+ brains, the medial wall displays a single layer of flattened ependymal cells both dorsally (Fig. 8C) and ventrally (Fig. 8E), indicating that morphological differentiation is complete by P5 in these animals. In JhylacZ/lacZ brains, however, dorsal cells display a pseudostratified appearance resembling immature ependyma (Fig. 8D), while ventral cells have begun to take on a more mature morphology (Fig. 8F). These results suggest that ependymal cell differentiation is altered in the medial ventricular walls of JhylacZ/lacZ mice, particularly in the dorsal region. The lateral wall cells appear immature by histological analysis in both dorsal and ventral regions of both Jhy+/+ and JhylacZ/lacZ at this stage, similar to what was seen with S100β (Fig. 9A-D).
Figure 8. Delayed differentiation of medial ventricular wall ependymal cells in Jhy<sup>lacZ/lacZ</sup> mice. (A, B) IF detection of S-100β in P5 coronal sections from Jhy<sup>+/+</sup> (A) and Jhy<sup>lacZ/lacZ</sup> (B) brains. White arrowheads denote S-100β expression in medial wall ependymal cells. Jhy<sup>+/+</sup> medial wall cells display a flattened single cell layered ependyma, while Jhy<sup>lacZ/lacZ</sup> medial wall cells retain a pseudostratified immature appearance. (C) Schematic of lateral ventricle showing regions designated as dorsal and ventral. (D-G) H&E staining of sections of P5 Jhy<sup>+/+</sup> (D, E) and Jhy<sup>lacZ/lacZ</sup> (F, G) lateral ventricle medial ependymal walls. Jhy<sup>+/+</sup> ependyma has a mature appearance in both dorsal and ventral regions (D, E), while Jhy<sup>lacZ/lacZ</sup> remains pseudostratified dorsally, but is largely mature ventrally (F, G). MW, medial wall; LW, lateral wall; LV, lateral ventricle; D, dorsal; V, ventral; L, lateral. Scale bars: 50μm (A-B); 20μm (D-G).
Figure 9. Subtle changes in lateral wall ependymal cell morphology with aberrant N-cadherin localization in JhylacZ/lacZ mice. H&E staining of P5 lateral walls in both Jhy+/+ (A, C) and JhylacZ/lacZ (B, D) animals show immature cuboidal ependyma in both dorsal (A, B) and ventral (C, D) regions of the lateral wall. Lateral wall sections were used for IF for Vimentin (pink) and Glast (green) in Jhy+/+ (E, G) and JhylacZ/lacZ (F, H). In both Jhy+/+ (E, G) and JhylacZ/lacZ (F, H), both dorsal (E, F) and ventral (G, H) cells were Glast(-)Vimentin(+). N-cadherin IF (green) in P10 brain shows normal apicolateral localization in Jhy+/+ (I, inset), while JhylacZ/lacZ lateral wall ependyma display abnormal basolateral N-cadherin localization (J, inset). CP, choroid plexus; MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bars: 50μm (A-D); 20μm (E-H); 20μm (I-J).
3.3.2 $JhylacZ/lacZ$ mice show delayed ependymal cell differentiation

To better track the delayed ependymal differentiation process in $JhylacZ/lacZ$ mice, a time course of histology was performed. Sections were prepared from $Jhy^{+/+}$ and $JhylacZ/lacZ$ animals at P5, P10 and P14, and examined for medial wall ependymal differentiation. It was not possible to extend the time course beyond P14, as the overall morphology of $JhylacZ/lacZ$ brains became significantly altered by the hydrocephalus. As expected, $Jhy^{+/+}$ brains display a monolayer of mature ependymal cells both dorsally and ventrally by P5, which persists at P10 and P14 (Fig. 10A,C,E, insets B,D,F). (Note the choroid plexus tissue that lies between the closely opposed medial and lateral walls of $Jhy^{+/+}$ mice at P10 and P14.) In $JhylacZ/lacZ$ mice, a small region of the ventral-most cells appears to be mature at P5 (Fig. 10G, bracket), but the cells are increasingly immature at more dorsal regions (inset H). At P10, the region of mature cells has lengthened dorsally (Fig. 10I, bracket, inset J), moving as a wave-like transition towards the roof of the ventricle. At P14, the mature ventral region has further lengthened (Fig. 10K, bracket, inset L), but the most dorsal cells remain immature at all ages examined (Fig. 10K, inset M). $JhylacZ/lacZ$ ependymal cell differentiation is therefore delayed at least 9 days beyond the time point at which the last $Jhy^{+/+}$ dorsal cells are mature. These results indicate that $JhylacZ/lacZ$ medial wall ependymal cells do continue to differentiate in the expected pattern, although it is not known if the process is ever fully completed. Lateral wall ependymal cells, in both $Jhy^{+/+}$ and $JhylacZ/lacZ$ mice, appear largely mature at P10, at least by morphological criteria.
Figure 10. Ventromedial ependymal cells progressively acquire mature ependymal characteristics. H&E staining of coronal sections of P5 (A, G), P10 (C, I) and P14 (E, K) 
Jhy+/+ and JhylacZ/lacZ lateral ventricles. Jhy+/+ medial wall ependymal cells at P5 (A, inset B), P10 (C, inset D) and P14 (E, inset F) display mature characteristics in both dorsal and ventral regions. The boxed region in each large image shows the region of the inset, the dotted line in F indicates the medial ependymal cell layer. JhylacZ/lacZ dorsal cells remain immature at P5 (G, inset H), P10 (I), and P14 (K, inset M). JhylacZ/lacZ ventral cells progressively acquire a mature appearance (inset J, L), with the mature (bracketed) region advancing dorsally (G, I, K). CP, choroid plexus; MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bar for all images is 100μm.
3.3.3 \( Jhy^{lacZ/lacZ} \) ependymal cells retain expression of an immature radial glial marker

The delay in radial glial to ependymal differentiation was further characterized by IF using stage-specific markers. Glast is a glutamate transporter that is highly expressed in radial glia, but downregulated upon ependymal differentiation (Kuo et al., 2006; Lavado and Oliver, 2011a; Shibata et al., 1997a). The intermediate filament protein Vimentin is a marker of mature ependymal cells, and acetylated \( \alpha \)-Tubulin (Ac\( \alpha \)-Tub) can be used to visualize the ependymal cilia (Lavado and Oliver, 2011a; Tissir et al., 2010a). These markers were used to analyze \( Jhy^{+/+} \) (Fig. 11A,E) and \( Jhy^{lacZ/lacZ} \) (Fig. 11I,M) medial ventricular wall at P10. \( Jhy^{+/+} \) dorsal (Fig. 11A-D) and ventral (Fig. 11E-H) cells are Glast(-)Vimentin(+)Ac\( \alpha \)-Tub(+), indicative of fully differentiated ependymal cells. In \( Jhy^{lacZ/lacZ} \) mice, however, some dorsal cells retain expression of the radial glial marker Glast, while they also express the ependymal-specific markers Vimentin and Ac\( \alpha \)-Tub (Fig. 11I-L). \( Jhy^{lacZ/lacZ} \) ventral cells are Glast(-)Vimentin(+)Ac\( \alpha \)-Tub(+), confirming the histological data that shows these cells are mature (Fig. 11M-P). These data indicate that ventral cells, which appear morphologically differentiated at P10, are also differentiated on the basis of their gene expression. Dorsal cells, however, which remain morphologically immature, retain the expression of an immature marker (Fig. 11I-L). The Glast(+) regions of the \( Jhy^{lacZ/lacZ} \) medial wall ependyma appeared to be too few in number to account for the near complete failure of differentiation by morphological criteria. The failure to silence Glast is therefore unlikely to be the primary cause of the differentiation delay, but rather one consequence of a broader defect in ependymal differentiation. We also analyzed the expression of differentiation markers in the later-maturing lateral ependymal wall. Lateral wall of both \( Jhy^{+/+} \) and \( Jhy^{lacZ/lacZ} \) mice have ependymal cells that are Glast(-)Vimentin(+), in both dorsal and ventral regions (Fig. 9E-H). At this developmental stage it appears that the
lateral walls in $Jhy^{+/+}$ and $Jhy^{lacZ/lacZ}$ mice are equivalent in their differentiation status based on gene expression criteria.
Figure 11. *Jhy*<sup>lacZ/lacZ</sup> mice exhibit delayed radial glial to ependymal cell differentiation. IF analysis of P10 lateral ventricle coronal sections from *Jhy*<sup>+/+</sup> (A, E) and *Jhy*<sup>lacZ/lacZ</sup> (I, M) mice for expression of Vimentin (pink), Glast (green) and Accα-Tub (orange) in dorsal (A-D, I-L) and ventral (E-H, M-P) brain regions. In *Jhy*<sup>+/+</sup>, medial wall dorsal and ventral cells express the mature ependymal markers Vimentin (A, B, E, F) and Accα-Tub (A, D, E, H), but are negative for the radial glial marker Glast (A, C, E, G). In *Jhy*<sup>lacZ/lacZ</sup> brains, some dorsal cells remain positive for the immature marker Glast (I, K), while also expressing Vimentin and Accα-Tub (I, J, L). *Jhy*<sup>lacZ/lacZ</sup> ventral cells express only Vimentin and Accα-Tub (M-P). The dotted line indicates the medial wall ependymal cells in (C, G, O). MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bar for all images is 50μm.
3.3.4 FOXJ1 expression is unaltered in Jhy^{lacZ/lacZ} mice

The transcription factor FOXJ1 is upregulated as radial glia transition to ependymal cells, and is required for proper differentiation and ciliogenesis (Yu et al., 2008a). Altered expression of FOXJ1 might underlie the defects in Jhy^{lacZ/lacZ} ependymal differentiation, with the retention of Glast expression a consequence of an altered developmental program. FOXJ1 expression was examined by IF in P5 lateral ventricle medial wall (Fig. 12). In Jhy^{+/+} mice, ependymal cells are positive for FOXJ1 in both dorsal (Fig. 12A-C) and ventral (Fig. 12D-F) regions. Vimentin was used as a marker for mature ependymal cells, and colocalizes with FOXJ1 in all cells. Jhy^{lacZ/lacZ} ependymal cells are also positive for FOXJ1, in both immature-appearing dorsal (Fig. 12G-I), and mature-appearing ventral (Fig. 12J-L), regions of the medial wall. These results indicate that while P5 Jhy^{lacZ/lacZ} ependymal cells retain an immature morphology, and continue to express radial glial markers, they do activate the ependymal differentiation protein FOXJ1. The cause of the differentiation delay in Jhy^{lacZ/lacZ} ependyma appears to lie downstream of FOXJ1, but upstream of Glast.
**Figure 12. Delayed Jhy^{lacZ/lacZ} ependymal cells express FOXJ1.** IF analysis of P5 lateral ventricle medial wall sections from Jhy^{+/+} (A-F) and Jhy^{lacZ/lacZ} (G-L) mice for expression of Vimentin (pink) and FOXJ1 (green). Jhy^{+/+} dorsal (A-C) and ventral (D-F) cells express the mature ependymal markers Vimentin and FOXJ1. In Jhy^{lacZ/lacZ} brains, both immature-appearing dorsal (G-I) and mature ventral (J-L) cells express Vimentin and FOXJ1. The boxed region in each large image shows the region of the inset. CP, choroid plexus; MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bar for all images is 50μm.
3.3.5 N-cadherin is mislocalized from Jhy^{lacZ/lacZ} adherens junctions

The integrity of the radial glial cell layer, and the later ependyma, is maintained by junctional complexes that bind cells at their adjacent membranes, and tether these membranes to the internal cytoskeleton (Gumbiner, 2005a; Harris and Tepass, 2010a; Shindo et al., 2008). Adherens junctions (AJs) are one form of junctional complex, and in addition to their structural roles AJs function as signaling centers through interactions with cellular proteins such as the α- and β-catenins (Bertocchi et al., 2012; McEwen et al., 2012; Meng and Takeichi, 2009). The AJ complex is defined by the presence of one of several cadherins, transmembrane proteins that interact with the homologous domains of cadherins on adjacent cells, and N-cadherin is the primary AJ protein expressed in the developing brain (Halbleib and Nelson, 2006; Rodriguez et al., 2012). The N-cadherin dimer is bound intracellularly by P120 and β-catenin, and then by α-catenin, which links the complex to the actin filaments of the cytoskeleton, either alone or through the protein Vinculin (Gumbiner, 2005a). AJs are strictly required in ependymal cells, and alterations in the expression or localization of cadherins, or other AJ-associated proteins, cause disruptions in the ependymal cell layer (Rodriguez et al., 2012).

We investigated the structural integrity of AJs in Jhy^{lacZ/lacZ} mice by localization of their components. In Jhy^{+/+} dorsal and ventral ependymal cells at P10, N-cadherin expression is localized to the most apical part of the lateral cell membranes, referred to as the apicolateral cell border, the site of the AJs (Fig. 13A,E). In Jhy^{lacZ/lacZ} dorsal cells, however, N-cadherin localization is disrupted, with protein found throughout the basal and lateral membranes (Fig. 13B). N-cadherin in these cells is also retained intracellularly, in large aggregates or inclusions (Fig. 13B). N-cadherin localization is disrupted in the mature-appearing ventral Jhy^{lacZ/lacZ} cells as well, though less significantly so, with smaller
amounts of basolateral signal and more protein localizing to AJs (Fig. 13F). N-cadherin mislocalization was not restricted to the medial ventricular wall, as basolateral accumulation of the protein is also found in the lateral wall of Jhy\textsuperscript{lacZ/lacZ} ventricles at P10 (Fig. 9I-J). These data indicate that the ventromedial Jhy\textsuperscript{lacZ/lacZ} cells, which are delayed but do eventually appear morphologically mature, are still abnormal by other parameters. The same appears to be true for the lateral wall ependyma of Jhy\textsuperscript{lacZ/lacZ} animals.

Cadherin levels at the AJs are regulated by balancing membrane transport with endocytosis, recycling and degradation, and these structures undergo dynamic reorganization as cells differentiate (Kowalczyk and Nanes, 2012a). We asked whether the N-cadherin mislocalization of Jhy\textsuperscript{lacZ/lacZ} animals was first apparent in ependymal cells. Radial glial cells also carry AJs, and these were analyzed in Jhy\textsuperscript{+/+} and Jhy\textsuperscript{lacZ/lacZ} mice at P1, the onset of ependymal differentiation. All radial glial cells, in Jhy\textsuperscript{+/+} ventricle and in both dorsal and ventral regions of the Jhy\textsuperscript{lacZ/lacZ} medial wall, show proper apicolateral localization of N-cadherin (Fig. 14). This data provides a temporal reference for the onset of N-cadherin mislocalization, suggesting the impairment arises with the beginning of ependymal differentiation.
3.3.6 β-catenin localization to AJs is also disrupted in JhylacZ/lacZ mice

The mislocalization of N-cadherin in JhylacZ/lacZ ependyma prompted us to examine the distribution of β-catenin, a core AJ component that mediates adhesion as well as signaling (Heuberger and Birchmeier, 2010; Zhang et al., 2013). β-catenin is an effector of the WNT pathway, but cytoplasmic β-catenin is usually ubiquitinated and degraded. Activation of WNT signaling stabilizes β-catenin, allowing it to translocate to the nucleus where it functions in transcriptional activation (Chilov et al., 2011; Clevers and Nusse, 2012a). β-catenin binding to N-cadherin sequesters it at the AJ, preventing both its degradation and its nuclear translocation. In Jhy+/+ ependyma at P10, β-catenin is concentrated at the apicolateral AJs, with only minor signal at the basolateral membranes (Fig. 13C,G). In JhylacZ/lacZ ependyma, β-catenin is still found at the AJs, but increased protein is also present throughout the basolateral membrane (Fig. 13D,H). The disruption of JhylacZ/lacZ AJ components is therefore not limited to N-cadherin localization, but involves the β-catenin signaling molecule as well.
Figure 13. Abnormal N-cadherin and β-catenin localization in JhylacZ/lacZ ependymal cells. In P10 Jhy+/+ medial wall ependymal cells, N-cadherin (A, E) and β-catenin (C, G) expression in dorsal (A, C) and ventral (E, G) regions localize to the adherens junctions at the apicolateral cell borders. In JhylacZ/lacZ ependyma, dorsal cells showed mislocalization of N-cadherin and β-catenin (B, D), with both proteins found throughout the basolateral cell membrane (inset in B, D). Some dorsal cells also contained large cytoplasmic inclusions that were positive for N-cadherin (inset in B). Some JhylacZ/lacZ ventral cells display proper apicolateral N-cadherin localization (inset in F, asterisk), while other cells retain N-cadherin throughout the lateral walls (inset in F, arrowhead). All ventral cells in JhylacZ/lacZ showed mislocalization of β-catenin throughout the basolateral membranes (H). DAPI is depicted in blue, the boxed region in each large image shows the region of the inset. CP, choroid plexus; MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bars: 20μm (A, B, E, F); 10μm (C, D, G, H).
Figure 14. Jhy<sup>lacZ</sup>/lacZ radial glia progenitors show normal N-cadherin localization. N-cadherin (green) IF in P0.5 medial wall of Jhy<sup>+/+</sup> (A, C) and Jhy<sup>lacZ/lacZ</sup> (B, D). Jhy<sup>+/+</sup> dorsal (A) and ventral (C) ependyma display normal apicolateral N-cadherin localization. Jhy<sup>lacZ/lacZ</sup> dorsal (B) and ventral (D) ependyma also show N-cadherin localized to the expected apicolateral position. CP, choroid plexus; MW, medial wall; LW, lateral wall; LV, lateral ventricle. Scale bar for all images is 50μm.
3.3.7 Dorsal and ventral ependymal cilia are abnormal in JhylacZ/lacZ mice

Delayed ependymal cell differentiation could underlie the abnormal ciliogenesis of JhylacZ/lacZ animals (Appelbe et al., 2013). If the delayed JhylacZ/lacZ ependymal cells do not initiate ciliogenesis within a certain developmental window, proper ciliary morphogenesis and patterning may be impossible. In this case, we might expect the ciliary defects to be more severe in the dorsal region of the ventricle, where the ependymal delay is most pronounced, and less so in the ventral region. Our previous electron microscopy studies did not compare different regions of the ventricle, so scanning electron microscopy (SEM) was carried out focusing specifically on dorsal and ventral regions of the medial wall. In P10 Jhy+/+ mice, both dorsal and ventral ependymal cells carry abundant long cilia with a consistent directional orientation (Fig. 15A,B). The cilia of JhylacZ/lacZ mice are less abundant than in Jhy+/+, as we have shown previously, and individual ciliary bundles are disorganized and randomly oriented (Fig. 15C,D). While both dorsal and ventral JhylacZ/lacZ cilia are abnormal, extensive imaging suggests a more severe effect in dorsal cells. Specifically, in dorsal ependyma the density of cilia appears lesser with some cells that carry no cilia at all (Fig. 15C). These may be the Glast-expressing immature ependymal cells that were identified by IF, which never progress to ciliogenesis.
Figure 15. Ependymal cells in $Jhy^{lacZ/lacZ}$ mice have abnormal cilia. Scanning electron microscopy of lateral ventricle medial wall at P10 in $Jhy^{+/+}$ (A, B) and $Jhy^{lacZ/lacZ}$ (C, D). $Jhy^{+/+}$ dorsal (A) and ventral (B) ependymal cells carry apical tufts of elongated cilia. In $Jhy^{lacZ/lacZ}$ mice, both dorsal (C) and ventral (D) cells have fewer and shorter cilia, with areas where cells have no cilia (C, asterisk). Dorsal cells were typically more severely affected than were ventral cells in $Jhy^{lacZ/lacZ}$. Scale bar for all images is 20μm.
3.3.8 Impaired ciliary-generated flow in \(Jhy^{lacZ/lacZ}\) mice

The morphologically abnormal ependymal cilia of \(Jhy^{lacZ/lacZ}\) mice, and particularly the loss of the central microtubule pair, suggest that these cilia will not exhibit normal movement. To examine the flow produced by \(Jhy^{lacZ/lacZ}\) cilia, we performed high-speed video imaging of ventricular wall sections. Ventricular tissues were removed from \(Jhy^{+/+}\) and \(Jhy^{lacZ/lacZ}\) P10 animals, and ependymal vibratome sections quickly prepared. Fluorescent microbeads were applied to one end of the section under microscopic imaging and bead speed and directionality were recorded. In \(Jhy^{+/+}\) samples, beads moved quickly across the ventricular surface, with strong linear vectors and an average speed of 114 \(\mu\text{m}/\text{second}\) (Fig. 16A,C). \(Jhy^{lacZ/lacZ}\) cilia were able to generate only minimal flow, with beads displaying irregular short tracks, and moving at speeds averaging 16 \(\mu\text{m}/\text{second}\) (Fig. 16B,C). The morphologically abnormal \(Jhy^{lacZ/lacZ}\) cilia therefore cannot generate significant flow, likely causing or contributing to the hydrocephalus of \(Jhy^{lacZ/lacZ}\) mice.
Figure 16. Impaired cilia-generated flow in Jhy\textsuperscript{lacZ/lacZ} mice. (A, B) High-speed video imaging of fluorescent bead movement on Jhy\textsuperscript{+/+} (A) and Jhy\textsuperscript{lacZ/lacZ} (B) ventricular wall explants allowed speed and directionality of ciliary flow to be calculated. Vectors indicating bead tracks from Jhy\textsuperscript{+/+} mice were long and strongly directional (A), while vectors from Jhy\textsuperscript{lacZ/lacZ} mice were much shorter with disorganized movement and poor directionality (B). (C) Graphical representation of average bead speed in Jhy\textsuperscript{+/+} (black bar) and Jhy\textsuperscript{lacZ/lacZ} (grey bar) explants shows the greatly reduced flow generated by Jhy\textsuperscript{lacZ/lacZ} cilia. Red circles represent fluorescent beads; **** denotes p≤0.0001. Scale bar: 15 μm (A-B).
3.3.9 Compromised translational and rotational polarity in \textit{JhylacZ/lacZ} ependyma

The noncanonical WNT/planar cell polarity (PCP) signaling pathway directs the unified polarization of epithelial cells across a tissue surface (Wallingford, 2010). PCP pathway factors localize asymmetrically within individual cells, and then act to translate that asymmetry into a reorganization of cell polarity. PCP mechanisms underlie numerous events during embryonic development, including the orientation of the motile cilia of the ependyma (Kishimoto and Sawamoto, 2012). During ciliogenesis, PCP controls the asymmetric clustering of the basal bodies to one end of each ependymal cell (translational polarity), the maintenance of this polarity across a tissue surface (tissue polarity), and the unidirectional orientation of individual basal bodies within each ciliary bundle (rotational polarity) (Boutin et al., 2014b; Hirota et al., 2010a; Ohata et al., 2014).

To evaluate the translational polarity of \textit{JhylacZ/lacZ} ependymal cilia, whole mount IF was performed on P10 ventricular walls. An antibody against γ-tubulin was used to label the ciliary basal bodies, and an antibody against N-cadherin was used to highlight the cell membranes. \textit{Jhy}\textsuperscript{+/+} medial and lateral wall tissues show the presence of elongated ependymal cells expressing abundant N-cadherin at their lateral borders (Fig. 17A,C). Although ciliogenesis is not complete at P10, medial wall ependymal cells of \textit{Jhy}\textsuperscript{+/+} mice already display an asymmetrical clustering of basal bodies towards one end of most cells, and this was largely maintained across the ependymal surface (Fig. 17A). Lateral wall cells are larger in size than medial wall cells, and display the typical “rosette” appearance of ependymal cells organized around a central neural stem cell (Fig. 17C) (Harding et al., 2014). These cells have inconsistent basal body polarization, perhaps reflecting the stage of their differentiation process (Fig. 17C). In \textit{JhylacZ/lacZ} tissues, both medial and lateral wall ependymal cells have membranes that appeared thickened, with a more irregular shape
than those of $Jhy^{+/+}$ cells (Fig. 17B, D). This likely reflects the broadened distribution of N-cadherin protein already described (Fig. 17, 9). $Jhy^{lacZ/lacZ}$ lateral wall cells also appear smaller in size and rounder in shape than the elongated cells seen in $Jhy^{+/+}$ lateral walls, and show fewer rosette structures (Fig. 17C,D). In $Jhy^{lacZ/lacZ}$ medial and lateral walls, the basal bodies are found across the apical cell surface with little polarization, often located centrally or dispersed across the cell (Fig. 17B,D). The loss of translational polarity means that tissue polarity, an extension of translational polarity, cannot be assessed.

To investigate the maintenance of rotational polarity in $Jhy^{lacZ/lacZ}$ ependymal cells, lateral ventricle TEM sections generated previously were reexamined for orientation of the ciliary basal feet (Appelbe et al., 2013). The basal foot is an anchoring structure found at the base of each cilia, which determines the direction of cilia motion. Basal foot rotation reflects the orientation of the microtubule central pair, also an indicator of the direction of motility, but exactly how these two structures are linked is unknown. During early ciliogenesis, cilia arise without rotational polarity, and basal feet point in random directions. As the planar cell polarity pathway begins to organize each ciliary cluster, individual basal bodies rotate to align all basal feet within the cluster. Although the brains examined in these experiments were P5, younger than the tissues examined for translational polarity, $Jhy^{+/+}$ animals already showed significant polarization of the basal feet (Fig. 18A,C). Within each cilia cluster, the majority of basal feet were aligned along a consistent direction (Fig. 18A), and the angle of deviation from a randomly selected reference basal foot was small (Fig. 18C). In equivalent sections of $Jhy^{lacZ/lacZ}$ mice, however, basal feet remained randomly positioned (Fig. 18B), with a far greater range of deviation across a single cluster (Fig. 18D). These data indicate a disruption of the PCP signals controlling multiple aspects of cell polarity in $Jhy^{lacZ/lacZ}$ ependymal cells.
Figure 17. Translational polarity is disrupted in Jhy<sup>lacZ/lacZ</sup> ependymal cells. Whole mount IF of P10 medial (A, B) and lateral (C, D) wall of lateral ventricle ependyma. N-cadherin (red) delineates the cell boundaries, while γ-tubulin (green) marks basal body patches on the apical cell surface. Medial wall ependymal cells in Jhy<sup>+/+</sup> display basal body polarization to one end of the cell, while lateral wall basal bodies are only partly polarized (A, C). In Jhy<sup>lacZ/lacZ</sup> medial wall, basal body patches are located centrally within the cells, and display little evidence of polarization (B). In Jhy<sup>lacZ/lacZ</sup> lateral walls, some cells display a degree of polarization, while others have centrally located basal bodies (D). Both the medial and lateral walls of Jhy<sup>lacZ/lacZ</sup> ependyma display morphological changes as well, with thickened cell membranes that reflect the altered distribution of N-cadherin (B, D). Jhy<sup>lacZ/lacZ</sup> lateral wall cells are also much smaller and rounder in shape than their Jhy<sup>+/+</sup> counterparts (D). Scale bar for all images is 10μm.
Figure 18. Impaired rotational polarity in $Jhy^{lacZ/lacZ}$ mice. (A,B) TEM images of P5 ependyma from $Jhy^{+/+}$ (A) and $Jhy^{lacZ/lacZ}$ (B) mice. The rightmost basal foot was considered the reference (white asterisk) in each image, and a line (red) was drawn from the outer edge of the basal body through the center of the basal foot. This line was copied to other basal bodies in the cluster, then a second line (yellow) was drawn indicating the actual orientation of each basal foot, and the angle of offset between each line pair was calculated. Even at P5, rotational polarity is well established in $Jhy^{+/+}$ (A), with only small angles of deviation (C). In $Jhy^{lacZ/lacZ}$, however, basal foot alignment is far more variable (B,D). (C,D) Histogram showing the rotational angles of basal feet in $Jhy^{+/+}$ (C) (n=68) and $Jhy^{lacZ/lacZ}$ (D) ependyma (n=49). Scale bar: 0.5 μm.
3.4 Discussion

Mice lacking the JHY protein product of the Jhy gene (JhylacZ/lacZ) develop juvenile hydrocephalus as early as postnatal day 1.5, and rarely survive past 6 weeks of age (Appelbe et al., 2013). These mice have sparser and shorter ependymal cilia, and most lack the central pair of microtubules that is required for motility. We have now determined that these abnormal cilia are nearly immotile, and incapable of generating significant fluid flow (Fig. 16). We show that Jhy plays a role in regulating ependymal cell differentiation in the mouse, and also controls the generation, patterning and organization of motile cilia. The Jhy gene is also implicated in the development and function of cilia in other species, and is under the control of the ciliogenesis program. The human Jhy ortholog (c11orf63) was identified in a proteomic study of ciliated tissues, with expression in epithelia of the lung and oviduct (Ivliev et al., 2012). Choksi et al found that the zebrafish Jhy ortholog (C10h11orf63) is upregulated in transgenic zebrafish overexpressing the ciliogenesis regulator Foxj1 (Choksi et al., 2014).

The ciliary defects of JhylacZ/lacZ mice led us to more closely examine ependymal cells during the postnatal differentiation period. We find that JhylacZ/lacZ medial wall ependymal cells are delayed in their differentiation by at least 9 days when compared to Jhy+/+ mice. Jhy+/+ cells have acquired the monolayer typical of mature ependyma by P5, while JhylacZ/lacZ cells are not fully mature even by P14. This phenotype is most severe in the dorsal regions of the lateral ventricles, which are the last cells to differentiate in wild type animals. Some JhylacZ/lacZ ependymal cells also retain expression of the radial glial marker Glast, beyond the time at which this gene is silenced in Jhy+/+ cells. JhylacZ/lacZ cells do activate the ependymal marker FOXJ1, however, indicating that they are capable of initiating the differentiation
process. The coexpression of Glast and FOXJ1 in some cells suggests they are stalled in a transitional state between immature radial glia and mature ependyma. Detailed SEM imaging showed that while both ventrally- and dorsally-located ependymal cells make abnormal cilia in JhylacZ/lacZ mice, the more significantly delayed dorsal cells more often lack cilia entirely (Fig. 15C). These may represent the cells that coexpress Glast and FOXJ1 and remain stalled in the differentiation process. How are delayed ependymal differentiation and abnormal ciliogenesis correlated? For a process that is highly temporally regulated, it is likely there is a window in which differentiating ependymal cells are competent for ciliogenesis. Beyond such time, the activators and/or components required for ciliogenesis may be unavailable. The increasing differentiation delay from ventral to dorsal ependyma might therefore cause a progressive defect in ciliary number and morphology.

JhylacZ/lacZ ependymal cells show altered localization of the adherens junction proteins N-cadherin and β-catenin, with reduced protein at the apicolateral adherens junctions, and more distributed throughout the basolateral membranes (Fig. 13B,F). The most immature dorsal cells often have intracellular inclusions of N-cadherin, suggesting a defect in the process of AJ remodeling that accompanies many developmental transitions. The pattern seen in JhylacZ/lacZ ependymal cells could result from reduced N-cadherin transport to the apicolateral AJs, increased removal from the AJs, and/or an inability recycle the protein. The presence of normal N-cadherin localization in JhylacZ/lacZ radial glial cells indicates a defect that begins at the onset of ependymal differentiation (Fig. 14). Despite the AJ alterations, JhylacZ/lacZ ependymal cells remain a discrete epithelial monolayer and do not detach from the underlying tissue, i.e. there is no ependymal denudation as is seen in some models of cadherin depletion (Guerra et al., 2015; Oliver et al., 2013a;
The JhylacZ/lacZ phenotype therefore manifests less severely than a complete loss of cadherin function.

In addition to their structural role, AJs have signaling functions that are mediated through interactions with a diverse array of proteins (Derycke and Bracke, 2004; McEwen et al., 2012; Teng et al., 2005). N-cadherin binding of β-catenin retains this protein at the AJs, preventing its translocation and downregulating the WNT response (Kam and Quaranta, 2009). AJ localization of β-catenin also prevents its degradation, providing a pool of inactive but quickly releasable protein if WNT signaling is activated. N-cadherin and β-catenin do not first associate at the AJ, but form a complex in the cytoplasm prior to their transport to the membrane (Nakamura et al., 2008). It is not surprising then that β-catenin is also mislocalized in JhylacZ/lacZ ependymal cells (Fig. 13D,H). Altered recycling and/or redelivery of AJ components to the plasma membrane may underlie the mislocalization of both N-cadherin and β-catenin.

The direction of ciliary motion is determined by the orientation of the basal bodies that organize each cilium, and the positioning of the central microtubule pair. The translational and rotational polarity of the basal bodies are under the control of the WNT/PCP pathway, which shares components with canonical WNT signaling (Guirao et al., 2010a; Hirota et al., 2010a; Kishimoto and Sawamoto, 2012). JhylacZ/lacZ animals show a loss of translational polarity of the ependymal cilia, with basal bodies remaining dispersed across the apical cell surface (Fig. 17B,D). The lack of a consistent basal foot orientation in JhylacZ/lacZ ciliary bundles indicates loss of rotational polarity in these animals as well, which may be correlated with the missing central pairs (Fig. 18B,D). There is precedent for defects in PCP signaling altering ependymal cilia number, morphology and polarity, and
these defects often result in hydrocephalus (Boutin et al., 2014b; Ohata et al., 2014b; Tissier et al., 2010).

In this and earlier work we have shown that inactivation of Jhy causes defects in 1) ependymal cell differentiation, 2) ciliary length and number, 3) microtubule organization, 4) polarity of ependymal cilia, and 5) N-cadherin transport and AJ establishment. What function(s) might Jhy have that its loss causes such varied effects? We propose that most aspects of the JhylacZ/lacZ phenotype can be attributed to the failure to establish N-cadherin complexes at adherens junctions in differentiating ependymal cells. AJs contribute structure and tensile strength to an epithelial cell layer, and constrain the shape and movement of cells through organization of the cytoskeleton (Ladoux et al., 2015). Remodeling of AJs accompanies morphological changes during development, including those of the neuroepithelium as it differentiates to radial glia, and then to neurons and ependyma (Etienne-Manneville, 2011; Ladoux et al., 2015; Stocker and Chenn, 2015). Defects in AJ structure in JhylacZ/lacZ may affect the ability of radial glia to transform from cuboidal cells to the flattened ependymal shape, and/or to undergo the movements necessary to refine into a single cell layer. This would be reflected as altered tissue morphology in histological cross-sections, and altered cell shape in whole mount analyses, as we have demonstrated.

The signaling functions of AJs intersect numerous cell differentiation cascades, including the WNT, Epidermal Growth Factor (EGF), and TGF-β pathways, and changes in AJ structure have been found to alter developmental signaling (Hoschuetzky et al., 1994; Rappl et al., 2008). N-cadherin recruitment of β-catenin to the plasma membrane decreases the signaling-competent pool of this transactivator, and cells with reduced levels of N-cadherin show increased LEF-mediated transcriptional activation through β-catenin
Changes in N-cadherin/β-catenin localization at JhylacZ/lacZ AJs could function through two possible mechanisms to affect WNT signaling pathways. Reduction in the levels of N-cadherin at AJs might result in less β-catenin recruited to these sites, with a concomitant increase in signaling-competent β-catenin in the cytoplasm that can potentiate WNT-driven effects. Alternatively, ectopically localized N-cadherin in the basolateral membranes could titrate activated β-catenin and reduce its nuclear function. To our knowledge canonical WNT signaling thru β-catenin has not been characterized in ependymal cells, so the functions of this pathway, or the potential ligands involved, are unknown. Discriminating between these possibilities can be accomplished by the construction of a WNT-responsive signaling system in ependymal cells, in which JHY and N-cadherin levels can be manipulated.

Lastly, altered interactions between cadherins and components of the PCP pathway may underlie the defects in polarity seen in JhylacZ/lacZ ependymal cells. The PCP pathway has been suggested to control cytoskeletal reorganization that regulates basal body positioning and ciliogenesis during ependymal cell differentiation (Ohata et al., 2015a; Tissir et al., 2010). For example, loss of Celsr2, a PCP component, perturbs the actin cytoskeleton network, impairing proper basal body arrangement (Boutin et al., 2014). Recent work has shown direct interactions between cell adhesion molecules and PCP proteins, for example the core PCP protein Vangl2 binds to N-cadherin in developing neurons (Nagaoka et al., 2014). Both deletion and overexpression of Vangl2 cause adhesion defects in these cells, with altered distribution of N-cadherin and β-catenin, suggesting dosage of Vangl2/N-cadherin is critical (Lindqvist et al., 2010). There is cross-talk between the canonical (β-catenin) and noncanonical (PCP) WNT pathways that converge at the Aj. β-catenin and Vangl2 bind to the same domain of N-cadherin, and these
interactions are mutually exclusive. β-catenin binding stabilizes N-cadherin at the membrane, while Vangl2 binding promotes its removal, so these factors may compete to control N-cadherin localization and abundance (Nagaoka et al., 2014). We propose that the JHY protein acts during ependymal cell differentiation to accomplish N-cadherin targeting to, or stabilization at, the apicolateral membrane. Loss of this function not only upsets the distribution of N-cadherin, but also impacts the many developmental signaling pathways with AJ components.

It is less clear how loss of JHY could result in the lack of the microtubule central pair; particularly since the factors that position the central pair are not known. It is possible that this defect is a consequence of the overall delay in ciliary development. Alternatively, it may be that the PCP pathway has an unacknowledged role in microtubule positioning that is affected in JhylacZ/lacZ ependyma. Basal foot rotation parallels the orientation of the central pair (Guirao et al., 2010), suggesting coordinated processes may position these two structures. The loss of rotational polarity and the central pair in JhylacZ/lacZ animals may reflect a broader inability to polarize individual cilia in the absence of Jhy.
IV. STRUCTURAL DEFECTS IN ADHERENS JUNCTIONS AND THE CILIARY AXONEME OF THE CHOROID PLEXUS IN JHY DEFICIENT MICE

4.1 Abstract

Juvenile hydrocephalus results from abnormalities in the production, flow or absorption of cerebrospinal fluid (CSF) within the ventricles of the brain. The choroid plexus (CP) consist of a single layer of epithelial cells and is regarded as the principal source for CSF in the brain. CP form a continuous layer with the ventricular ependyma, and are often referred to as modified ependyma. CSF overproduction by CP dysfunction is among the leading causes for hydrocephalus in humans. Despite the importance of CP in CSF homeostasis, this tissue remains relatively understudied. Disruption of the mouse gene Jhy results in abnormalities in ependymal cell differentiation, adherens junction formation, ciliary patterning and motility, altogether contributing to the resulting hydrocephalus in these mice. In this report, we sought to identify the role of mouse gene Jhy in the specialized ependymal cells of the choroid plexus in the gene-targeted mouse line (Jhy\textsuperscript{lacZNeo}). Our analysis indicates that Jhy\textsuperscript{lacZNeo/lacZNeo} develop early onset hydrocephalus, with mice rarely surviving past 3 weeks of age. Jhy\textsuperscript{lacZNeo/lacZNeo} CP also displays disruptions in adherens junction formation, with abnormal localization of key adherens junction protein, E-cadherin. Ultrastructural analysis of the CP in Jhy\textsuperscript{lacZNeo/lacZNeo} demonstrated structural defects in ciliary ultrastructures, along with altered microvilli distribution. Together, this data identifies Jhy as a gene required for proper adherens junction formation and cilia ultrastructure, in the ependyma and the specialized ependyma of the choroid plexus.
4.2 Introduction

Congenital hydrocephalus is one of the most common human birth defects with an estimated prevalence of 1 in 1000 live births (Schurr et al., 1953; Zhang et al., 2006). Hydrocephalus results from the buildup of CSF in the brain ventricles. CSF is produced by the CP, a single layer of specialized ependyma localized to the lateral, third, and fourth ventricles of the brain. The CP is composed of a network of capillaries enclosed by a single layer of epithelial cells that extends from the ependymal cells lining the brain ventricles, acting as a blood-CSF barrier (Liddelow, 2015). This feature limits the passage of solutes through the highly regulated transport of molecules across each choroidal cell. The choroid is a highly polarized epithelium with expression domains of membrane transport proteins on either the apical or basolateral membrane of the cell. This polarization allows the unidirectional passage of ions (e.g. Na+, Cl-, K+, HCO3-) into the ventricular cavity, creating an osmotic gradient that drives the passage of H2O, through the water channel known as Aquaporin1 (AQP1) (Christensen et al., 2013). Changes in the osmotic gradient and water transport regulates CP membrane secretion, and they are both needed for CSF production. For example, AQP1 null mice display reduced levels of CSF production and ICP, which suggests that the osmotic gradient is necessary, but without AQP1, CSF secretion is impaired (Oshio et al., 2005)(Johansson et al., 2005). The integrity and polarization of the CP is dependent on tight junctions, located apically, and adherens junction complexes, localized basolaterally. Numerous studies have demonstrated that tight junction complexes are critical for the blood-CSF barrier property of the CP (Engelhardt and Sorokin, 2009; Kratzer et al., 2012). Although suspected to have similar roles in the CP barrier, few studies have explored the roles of adherens junctions in this tissue (Liddelow et al., 2013). Compromised CP polarity and integrity allows the leakage of potentially harmful
metabolites into the CSF cavity, and this could ultimately lead to impaired CSF homeostasis and hydrocephalus (Lindeman et al., 1998) (Banizs et al., 2007) (Liddelow, 2015). For example, humans patients with hydrocephalus often display increased protein concentrations in CSF, and this pathology is associated with permeability disturbances of the blood-CSF barrier (Seyfert et al., 2004; Seyfert and Faulstich, 2003).

CP cell fate specification begins approximately at embryonic day 9.5 (E9.5), but it peaks around E11-12 (Dziegielewksa et al., 2001; Sturrock, 1979). A series of reports suggest that CP emergence in all ventricular cavities does not occur concurrently. At E9.5 the CP in the fourth ventricle begins to develop, and by E14 the choroid extends into the fourth ventricle. At E12.5, while the 4th ventricle CP development is still ongoing, the choroid in the lateral ventricle begins to emerge, followed by the third ventricle CP (Dziegielewksa et al., 2001; Johansson et al., 2013). Finally CP is fully formed in all cavities by E14. Little is known about the process of maturation of the CP. A study performed in human fetuses established that CP development in the lateral ventricles can be divided into 4 stages based on nuclei positioning and changes in cell morphology: 1) tissue contains pseudostratified cells and centrally located nuclei (7th week); 2) cells are now columnar with apical nuclei (9th week); 3) mostly cuboidal cells with central to apical nuclei (17th week); and 4) mature cuboidal cells with basal/central nuclei (29th week) (Dziegielewksa et al., 2001; Shuangshoti and Netsky, 1966) (Damkier et al., 2013) (Lun et al., 2015). As CP development progresses, cells develop apical membrane protrusions known as microvilli. Many believe that the expansion of microvilli reflect an increase in the secretory capability of the CP (Dziegielewksa et al., 2001; William J. Schultz, 1977).

Despite the vital role the CP has in development, this tissue remains largely understudied. Little is known about the factors needed to regulate CP development. The
few studies addressing this issue, suggest that CP specification requires neural cell fate repression. Reciprocal inhibition between Hes genes (Hes1, 2 and 5) and Neurogenin 2 plays a role in specifying CP epithelium versus other neuronal cells (i.e. Cajal-Retzius cells), respectively (Imayoshi et al., 2008). Neuroepithelial transcription factors, Emx1 and Emx2, downregulate the expression of the transcription factor and CP master regulator, Otx2, and their misregulation interferes with CP differentiation (Johansson et al., 2013; von Frowein et al., 2006).

In mice, CP ciliogenesis occurs after the CP primordia emerge during organogenesis (E11), with occasional hair-like organelles (i.e. cilia) present on the tissue surface (Sturrock, 1979; Nonami et al., 2013). Ultrastructural analysis of the mouse CP demonstrated a significant increase in cilia number by E12, with cilia bundles fully formed by E15 (Sturrock, 1979). Immature CP cells display fewer cilia, but this number increases over time (Dziegielewska et al., 2001) (Sturrock, 1979).

There are two major types of cilia: 1) primary and 2) motile cilia. Primary cilia are often referred to as (9+0), contain nine outer microtubules pairs with no microtubule singlets or central pair. On the other hand, motile cilia, or (9+2) cilia, have nine outer microtubule pairs on their periphery, as well as a singlet pair in the center. Little is known about ciliogenesis in the CP; though some believe that CP cells display mostly cilia bundles with a 9+0 axonemal configuration, constituting a unique category where primary cilia form bundles (Narita and Takeda, 2015). Interestingly, cilia bundle formation is an arrangement mostly seen in motile cilia (Takeda and Narita, 2012). This notion prompted the investigation of CP cilia motility. Nonami demonstrated that CP cilia exhibit rotational motility, similar to that observed in nodal cilia; however, the biological role for this apparent ciliary beating remains unknown as it is not able to produce a directional fluid
flow (Narita and Takeda, 2015; Nonami et al., 2013). Cilia are increasingly being thought of as important regulators of brain homeostasis (Christensen et al., 2007; Narita and Takeda, 2015). An emerging body of literature suggests that CSF secretion is mediated by the primary cilia on the CP, where these structures are believed to regulate the intracellular levels of cAMP, which in turn control the levels and chloride and CSF secretion (Narita et al., 2010; Satir and Christensen, 2007; Banizs et al., 2005).

Our lab has previously demonstrated that loss of the mouse gene Jhy in the transgenic mouse line JhylacZ results in juvenile hydrocephalus. The generation of these mice resulted from the integration of a lacZ transgene into the Jhy gene. This integration occurred in the beginning of Jhy exon 5, causing the removal of 513bp, of which 425bp are intronic sequence. The remaining deleted base pairs correspond to exon 5 coding sequence, removing exon 5 splice acceptor site, and resulting in the inability to make a functional JHY protein. Mice homozygous for the transgene integration display ventricular dilation by P1.5, with most hydrocephalic mice dying within 6-8 weeks of age (Appelbe et al., 2013). Ultrastructural analysis of ependymal motile cilia (9+2) revealed abnormal cilia patterning where the microtubule central pair was absent in JhylacZ/lacZ brains. Further analysis of ependymal cells in JhylacZ/lacZ demonstrated delayed ependymal cell differentiation with abnormal adherens junction formation and impaired ciliary motility (Muniz-Talavera and Schmidt, submitted).

A second mouse line was generated using gene targeted embryonic stem (ES) cells, where a new Jhy-targeted allele was developed to further evaluate the role of Jhy in development. Jhy targeted ES cells were purchased from the Knockout Mouse Project (KOMP) and mice carrying the modified allele are referred here as JhylacZNeo. This allele served as a corroboration of the role of Jhy in hydrocephalus. In this report we show that
*Jh*<sup>lacZNeo/lacZNeo</sup> mice develop hydrocephalus, with ventricular dilation as early as P0.5. Although ependymal cilia defects may indeed contribute to the development of hydrocephalus in *Jh*<sup>lacZ/lacZ</sup> animals, the onset of hydrocephalus in both *Jh*<sup>lacZ/lacZ</sup> and *Jh*<sup>lacZNeo/lacZNeo</sup> precedes the formation of motile cilia in ependyma. It is possible that defects in the modified ependymal cells of the CP may be the initiating factor for hydrocephalus, while the later-developing abnormalities in the ependymal motile cilia greatly worsens the fluid buildup. Thus, the CP became the focus of our studies. Here we show that CP in *Jh*<sup>lacZNeo/lacZNeo</sup> display abnormal localization of the adherens junction protein E-cadherin, along with atypical axoneme ultrastructure in CP cilia. Therefore, we propose that hydrocephalus in *Jh*<sup>lacZNeo/lacZNeo</sup> animals originates from abnormalities in CP development, involving adhesion defects and abnormal ciliary microtubule patterning in CP, with hydrocephalus worsening due to impaired ependymal-generated CSF flow. Thus, the *Jhy*<sup>lacZNeo</sup> mouse line serves as a suitable tool to study CP function in development.

### 4.3 Results

#### 4.3.1 *Jh*<sup>lacZNeo/lacZNeo</sup> mice show progressive juvenile hydrocephalus

Targeted ES cells contained a targeted allele carrying a promoterless *lacZ* reporter gene with a transcriptional and termination signals, a β-actin-*Neo* selection marker, and a floxed exon 3 (flanked by loxP sites) (Fig. 19). The resulting transcript from this allele should produce an RNA molecule with exon 1 and 2 fused to *lacZ* only, thus resulting in a truncated transcript. The modified ES cells were generated in the C57BL/6 background and microinjected into albino C57BL/6 pseudo-pregnant females with the help of the Transgenic Production Facility at the University of Illinois at Chicago. Mice homozygous for this integration (*Jh*<sup>lacZNeo/lacZNeo</sup>) display progressive juvenile hydrocephalus as previously
seen in the $Jhy^{lacZ}$ mouse line. Heterozygous ($Jhy^{+/lacZNeo}$) animals display no signs of hydrocephalus (data not shown). $Jhy^{lacZNeo/lacZNeo}$ mice display prominent doming of the skull by postnatal day 10 (P10); though, visible outward signs of hydrocephalus in $Jhy^{lacZNeo/lacZNeo}$ animals are evident as early as P5 (Fig. 20A, data not shown). This is approximately 4 days earlier than in $Jhy^{lacZ/lacZ}$ animals, which display doming of the skull at 2 weeks of age. Histological examination of control $Jhy^{+/+}$ (Fig. 20B) and mutant $Jhy^{lacZNeo/lacZNeo}$ (Fig. 20C) brains at P5 confirmed that $Jhy^{lacZNeo/lacZNeo}$ ventricles are significantly enlarged compared to $Jhy^{+/+}$ littermates. Additionally, we also observe severe neuronal cell death in the corpus callosum around the lateral ventricles in P5 $Jhy^{lacZNeo/lacZNeo}$ brains, similarly to $Jhy^{lacZ/lacZ}$ animals (Fig. 20C, asterisk) (Appelbe et al., 2013). $Jhy^{lacZNeo/lacZNeo}$ mice rarely survive past 1 month, with 72.5% of hydrocephalic pups dying by 3 weeks of age (Fig. 20D). $Jhy^{lacZ/lacZ}$ mice died by 4-8 weeks of age, thus $Jhy^{lacZNeo/lacZNeo}$ mice experienced a more rapid progression of the disease.
Figure 19. Schematic diagram for the generation of the Jhy\textsuperscript{lacZNeo} allele. The targeting vector carried a promoterless lacZ reporter with a fused splice acceptor site (En2SA), a translation initiation sequence (IRES) and a polyadenylation sequence (pA). Neomycin fused to β-actin promoter served as a selection marker. The vector was introduced into the Jhy genomic sequence through homologous recombination to generate the Jhy\textsuperscript{lacZNeo} allele.
Figure 20. Progressive juvenile hydrocephalus observed in the Jhy$^{lacZNeo/lacZNeo}$ mouse line. (A) Jhy$^{+/+}$ (left) and Jhy$^{lacZNeo/lacZNeo}$ littermates (right). (B-C) H&E staining on P5 brains of Jhy$^{+/+}$ (B) and Jhy$^{lacZNeo/lacZNeo}$ (C). Severe neuronal loss in the corpus callosum in Jhy$^{lacZNeo/lacZNeo}$ (C, asterisk). Jhy$^{+/+}$ animals (triangles) survive past 12 weeks of age, as expected. However, > 70% of Jhy$^{lacZNeo/lacZNeo}$ die by 3 weeks of age (squares) (D). (D) Jhy$^{+/+}$ n=60 and Jhy$^{lacZNeo/lacZNeo}$ n=40. LV, lateral ventricles; 3V, third ventricle; CX, cerebral cortex.
4.3.2 Analysis of hydrocephalus progression in JhylacZNeo/lacZNeo mice

In order to determine the timing of onset of hydrocephalus in JhylacZNeo/lacZNeo mice, we performed a histopathological examination of brains at P0.5. Our analysis showed that at P0.5, JhylacZNeo/lacZNeo brains display ventricular enlargement when compared to the control Jhy+/+ (Fig. 21A,B). We then wondered whether hydrocephalus was present prior to birth, thus we performed H&E staining using E19 brains. At this time, JhylacZNeo/lacZNeo ventricles showed no size differences compared to Jhy+/+ ventricles (Fig. 21C,E).

Early onset congenital hydrocephalus has been previously associated with abnormalities in the CP (Baas et al., 2006a; Lindeman et al., 1998). The CP develops embryonically, but the morphological changes observed during its differentiation take place early postnatally. Coincidently, studies performed in rats and sheep demonstrate a significant increase in CSF secretion postnatally; presumably, the role of CP in CSF secretion increases at this time (Johanson, 1974; Keep and Jones, 1990; Keep et al., 1986). Given this information, we hypothesized that CP dysfunction may be the underlying cause for hydrocephalus in JhylacZNeo/lacZNeo mice. Consequently, CP morphology was assayed by H&E staining of P5 Jhy+/+ and JhylacZNeo/lacZNeo. We chose P5 as the time point for most of our CP analysis, with the aim of identifying changes that may cause hydrocephalus rather than changes secondary to the progression of the disease. Gross anatomical examination of P5 CP did not reveal any significant differences in tissue size or cell morphology between control Jhy+/+ (Fig. 21D) and JhylacZNeo/lacZNeo (Fig. 21F). Nevertheless, congenital hydrocephalus associated with CP dysfunction has been reported even in the absence of obvious morphological changes (Banizs et al., 2005; Lindeman et al., 1998; Zhang et al., 2006).
Figure 21. Early onset hydrocephalus in \textit{Jhy}^{lacZNeo/lacZNeo}. (A-B) H&E of coronal brain sections at P0.5 from \textit{Jhy}^{+/+} (A) and \textit{Jhy}^{lacZNeo/lacZNeo} (B). \textit{Jhy}^{lacZNeo/lacZNeo} brain displays ventricular enlargement at this stage (B). (C-F) H&E staining on E19 coronal brain (C, E) section and P0.5 CP (D, F) from \textit{Jhy}^{+/+} and \textit{Jhy}^{lacZNeo/lacZNeo}. No apparent hydrocephalus can be detected at E19 in \textit{Jhy}^{lacZNeo/lacZNeo} (E). CP morphology appears normal in both \textit{Jhy}^{+/+} (D) and \textit{Jhy}^{lacZNeo/lacZNeo} animals (F). LV, lateral ventricle; CP, choroid plexus. Scale bar: 100μm (A and B); 10μm (D and F)
4.3.3 Normal apical and basolateral polarity in \textit{Jhy}^{lacZNeo/lacZNeo} CP

The unidirectional transport of ions across the CP membrane needed for CSF secretion is carefully controlled by the polarized localization of transport proteins in the CP epithelium (Brown et al., 2004). The CP water channel, AQP1, displays a polarized localization to the apical membrane compartment (Boassa et al., 2006; Owler et al., 2010). Additionally, actin filament (F-actin) network, found in the apical microvilli projections, also display a strong polarized expression. AQP1 and FITC-Phallodin were used to assess apical polarity of the CP in \textit{Jhy}^{+/+} and \textit{Jhy}^{lacZNeo/lacZNeo} (Fig. 22). As expected, both markers were restricted to the apical border of CP cells in \textit{Jhy}^{+/+} (Fig. 22A,C). This was also the case for \textit{Jhy}^{lacZNeo/lacZNeo} CP (Fig. 22B,D). The basolateral membrane was also evaluated using the basolateral transporter Anion Exchange type 2 (AE2) (Lindsey et al., 1990). AE2 immunoreactivity was mainly observed in the basolateral domain in both \textit{Jhy}^{+/+} (Fig. 22E) and \textit{Jhy}^{lacZNeo/lacZNeo} CP (Fig. 22F). These results suggest that the cellular distribution of plasma membrane proteins to their respective domain remains unaffected in \textit{Jhy}^{lacZNeo/lacZNeo} mice.
Figure 22. *Jhy*_{lacZNeo/lacZNeo} CP display normal apical and basolateral polarity. IF analysis of P5 CP coronal sections from *Jhy*^{+/+} (A, C, E) and *Jhy*_{lacZNeo/lacZNeo} (B, D, F). Apical markers Phalloidin (A, B, green) and AQP1 (C-D, green) predominantly localized to the apical region of the CP in *Jhy*^{+/+} (A, C) and *Jhy*_{lacZNeo/lacZNeo} (B, D). Basolateral marker AE2 (red) displays normal expression in *Jhy*^{+/+} (E) as well as in *Jhy*_{lacZNeo/lacZNeo} mice (F). DAPI is depicted in blue in all images. Scale bars: 10μm (A, B, E, F) and 50μm (C and D).
4.3.4 \textit{JhylacZNeo/lacZNeo} exhibit abnormal E-cadherin expression

CP cells are of epithelial origin, and as such, they display adherens junctions in the basolateral membrane (Christensen et al., 2013). In CP, the basolateral adhesion molecule E-cadherin links to the actin cytoskeleton through direct association with \( \alpha \)-catenin and \( \beta \)-catenin (Christensen et al., 2013b; Nelson, 2008; Tietz and Engelhardt, 2015). E-cadherin binds to \( \beta \)-catenin, through the C-terminus. \( \beta \)-catenin binds to \( \alpha \)-catenin, the latter connecting the complex to the actin cytoskeleton (Harris and Tepass, 2010). Adherens junctions mediate cell-to-cell contacts, and promote cell maturity and plasticity (Tietz and Engelhardt, 2015; Vestweber, 2015). Several studies have addressed the role of these junctional complexes in CP barrier integrity; unfortunately, few studies have focused on the maturation of cell-to-cell junctions and the targeting of adherens junction proteins in the CP. We investigated the structural integrity of adherens junctions by analyzing the localization of two key components, E-cadherin and \( \beta \)-catenin.

In P5 \textit{Jhy}\textsuperscript{+/+} CP cells, E-cadherin localized to the basal membrane of the cell (Fig. 23A). E-cadherin expression in \textit{JhylacZNeo/lacZNeo} appeared broader, with some cells expressing E-cadherin across the basolateral membrane (Fig. 23B, asterisk), while other cells display an aberrant accumulation of E-cadherin in the CP stroma (Fig. 23B, arrowhead). Given the direct association between E-cadherin and \( \beta \)-catenin, we wondered whether \( \beta \)-catenin would also present any abnormalities in its localization. Interestingly, the polarized expression of \( \beta \)-catenin in \textit{Jhy}\textsuperscript{+/+} was not identical to that of E-cadherin since its expression was not restricted to the basal end compartments, but in fact was present throughout the entire basolateral membrane Fig. 23A,C). This expression pattern difference was previously documented; though, how \( \beta \)-catenin gets recruited into the lateral walls where no E-cadherin is present remains unknown. Moreover, \textit{JhylacZNeo/lacZNeo} CP cells
display normal basolateral expression of β-catenin (Fig. 23C,D). Therefore, $Jhy$ disruption in $Jhy^{lacZNeo/lacZNeo}$ CP appears to uniquely affect E-cadherin localization. Whether β-catenin and E-cadherin employ separate mechanisms for membrane localization is presently unknown.
Figure 23. Abnormal expression of the adherens junction protein E-cadherin in Jhy\textsubscript{lacZNeo/lacZNeo}. (A-B) E-cadherin (green) IF expression on P5 coronal brain section. Normal basal expression of E-cadherin observed in Jhy\textsuperscript{+/+} (A). In Jhy\textsubscript{lacZNeo/lacZNeo}, some cells express E-cadherin basolaterally (B, asterisk), while others appear to have mislocalized E-cadherin to the CP stroma (B, arrowhead). (C-D) β-catenin IF on P5 coronal brain sections in Jhy\textsuperscript{+/+} and Jhy\textsubscript{lacZNeo/lacZNeo}. Both Jhy\textsuperscript{+/+} (C) and Jhy\textsubscript{lacZNeo/lacZNeo} (D) display normal basolateral expression of β-catenin. DAPI depicted in blue. Scale bar for all images is 10μm.
4.3.5 *Jhy*\textasciitilde*lacZNeo/lacZNeo* CP display abnormal microvilli distribution

Although no gross morphological abnormalities were observed in CP by H&E, we used electron microscopy to analyze the CP and achieve a more detailed morphological characterization of this tissue. First, we assessed CP ultrastructure by scanning electron microscopy (SEM) in P5 *Jhy^+/^* and *Jhy*\textasciitilde*lacZNeo/lacZNeo* animals (Fig. 24). Low magnification SEM images in *Jhy^+/^* mice display a homogeneous population of dome-shape cells, corresponding to CP cells (Fig. 24A). CP from *Jhy*\textasciitilde*lacZNeo/lacZNeo* displayed a normal appearance and was virtually indistinguishable from *Jhy^+/^* (Fig. 24B). At higher magnification, individual cell boundaries and ciliary tufts in *Jhy^+/^* were difficult to discriminate due to the extensive number of microvilli (Fig. 24C). The cell boundaries in *Jhy*\textasciitilde*lacZNeo/lacZNeo* were far more discernable, suggesting a difference in the microvilli projections between *Jhy^+/^* and *Jhy*\textasciitilde*lacZNeo/lacZNeo* (Fig. 24D).

Previous studies have demonstrated that CP epithelium displays two distinct forms of microvilli. The more predominant one called the clavate, of bulbous shape, followed by the filliform, a less abundant form with a finger-like shape (William J. Schultz, 1977). *Jhy^+/^* CP cells carried primarily clavate shape microvilli, with some filliform microvilli scattered among them (Fig. 24C). However, in *Jhy*\textasciitilde*lacZNeo/lacZNeo* the predominant form of microvilli was filliform, with few cells displaying clavate microvilli (Fig. 24D). This observation was further confirmed by transmission electron microscopy (TEM) where cells in *Jhy^+/^* displayed predominantly clavate, with some filiform microvilli present per cell (Fig 24E). Unlike, *Jhy^+/^* where both forms of microvilli are present in every cell, the CP in *Jhy*\textasciitilde*lacZNeo/lacZNeo* contain cells with either only filiform or clavate microvilli (Fig. 24F).
Figure 24. EM in Jhy<sup>lacZNeo/lacZNeo</sup> CP reveals differences in microvilli morphology. (A-D) SEM in P5 Jhy<sup>+/+</sup> (A, C) and Jhy<sup>lacZNeo/lacZNeo</sup> (B, D). Jhy<sup>+/+</sup> displays primarily clavate microvilli and with some filliform (C, white arrows). In Jhy<sup>lacZNeo/lacZNeo</sup>, this distribution is reversed with cells mostly displaying filliform and few cells displaying clavate microvilli (D, white arrows). TEM micrographs of CP at P5 in Jhy<sup>+/+</sup> (E) and Jhy<sup>lacZNeo/lacZNeo</sup> (F). Microvilli in Jhy<sup>+/+</sup> display filliform and clavate shape (E, black arrows). Meanwhile, Jhy<sup>lacZNeo/lacZNeo</sup> microvilli is mostly filliform (F, black arrows). C, cilia; Fl, filiform; Cl, clavate.

Scale bars: 1μm (E and F). Scale bars: 50μm (A and B); 5μm (C and D); 1μm (E and F).
4.3.6 Atypical ciliary axoneme configurations observed in Jhy+/+ and JhylacZNeo/lacZNeo CP

The loss of the microtubule central pair and the reduced motility of JhylacZ/lacZ ependymal motile cilia, prompted us to examine CP cilia ultrastructure. Although some believe cilia in CP to be immotile, studies have shown transient motility acquisition postnatally (Nonami et al., 2013). Nevertheless, CP cilia motility is predicted to have minimal contribution to overall CSF flow (Nonami et al., 2013). The ultrastructure of CP cilia remains a debated subject, as it has been shown to display varying microtubule patterns of 9+0, 9+2 and 9+1 (Narita et al., 2012; Swiderski et al. 2012). Moreover, the factors needed and steps undertaken in CP cilia development remain unknown. A detailed analysis of CP cilia by TEM was performed in hopes of shedding some light on this matter, as well as addressing whether Jhy has a role in CP cilia ultrastructure as it does in ependymal cilia (Appelbe et al., 2013).

To our surprise, TEM analysis in Jhy+/+ CP cilia displayed a larger than expected array of microtubule patterning (Fig. 25A). A new nomenclature system was designed to categorize our findings. Cilia were assorted based on the axoneme ultrastructure using the following nomenclature (n+#, s/d/t), where (n) corresponds to the number of outer microtubule doublets, and (#) corresponds to the number of microtubules found in the center of the axoneme. Microtubules located in the center were categorized using letter (s) for microtubule singlet, (d) for microtubule doublets and (t) was used for microtubule triplets, which are arrangements observed normally in basal bodies. As expected, the predominant ciliary axoneme configuration observed in Jhy+/+ displayed a 9+0 ultrastructure, corresponding to 62.5% of cilia (Fig. 25A, C). Surprisingly, the second most frequent configuration with 21.7% occurrence were cilia with a 9+1d ultrastructure, with an apparent microtubule doublet in the center (Fig. 25A, C). This configuration was
previously reported, though at much lower frequency (Narita et al., 2012). In a much lower incidence than expected, we also observed 9+2s cilia with the central pair singlet observed in the center. Unexpectedly, we saw 6 other cilia configurations not previously reported in wild-type CP (Fig. 25C).

In Jh\textsubscript{lacZNeo/lacZNeo}, the two most predominant arrangements were 9+0 and 9+1d, and they were equally abundant (35.6\%) (Fig. 25B, C). Cilia ultrastructure determination in Jh\textsubscript{lacZNeo/lacZNeo} was far less conserved. Jh\textsubscript{lacZNeo/lacZNeo} displayed 13 different cilia ultrastructures, of which 7 were unique to mutant CP. We found many variations of CP cilia with the following ultrastructure: 8+0, 7+0, 6+0, 5+0 and 2+0, altogether corresponding to 12.6\% of the cilia reported here (Fig. 25C). The remaining 16.2\% of cilia displayed other ultrastructural configurations ranging from 10-12 outer microtubule doublets on the periphery, to combined microtubule doublets and singlets in the middle (Fig. 25C). Such striking variability has only been observed in tracheal cilia in human patients presenting chronic respiratory tract infections and sperm flagella abnormalities (Plesec et al., 2008). Nevertheless, the cause for the ciliary abnormalities observed in these individuals remains unknown. Altogether this data suggests that CP cilia ultrastructure can vary greatly in microtubule number and pattern. It is worth mentioning that variability in CP cilia ultrastructure was previously observed; nevertheless, the alternate configurations were fewer than those reported here (Narita et al., 2012; Nonami et al., 2013; Swiderski et al., 2012).
Figure 25. Ultrastructural analysis of CP cilia in $Jhy^{+/+}$ and $Jhy^{lacZNeo/lacZNeo}$ mice. (A-B) TEM micrographs of P5 CP in $Jhy^{+/+}$ (A) and $Jhy^{lacZNeo/lacZNeo}$ (B). (C) Ciliary ultrastructures observed in $Jhy^{+/+}$ (black bars) and $Jhy^{lacZNeo/lacZNeo}$ (grey bars). (C) $Jhy^{+/+}$ n=96 and $Jhy^{lacZNeo/lacZNeo}$ n=87. Scale bars: 200nm (A and B).
4.4 Discussion

Despite the undeniable importance attributed to CP in CSF secretion and brain homeostasis, the mechanisms of CSF production by the choroid remains relatively understudied. CP cilia are believed to function as a chemosensor to regulate CSF production. For example, cilia in the CP have been associated with the regulation of cAMP-regulated chloride transport, which in turn regulates CSF production (Narita et al., 2010). The role of CP cilia as a signaling platform was further supported by the detection of the calcium-mediated signaling receptor Polycystin-1 in CP cilia (Distefano et al., 2009). Moreover, disruption of Polycystin-1 localization to the cilia leads to hydrocephalus (Wodarczyk et al., 2009). Several groups have reported that CP cilia disruption leads to enhanced fluid movement into the brain ventricles resulting in communicating hydrocephalus; thus reinforcing a role of cilia in CSF regulation (Banizs et al., 2005; Narita et al., 2010). To further investigate and confirm the biological role of Jhy, we used the gene-targeted mouse line JhylacZNeo. Homozygous JhylacZNeo/lacZNeo mice developed an accelerated form of congenital hydrocephalus with CP cilia displaying axoneme abnormalities in JhylacZNeo/lacZNeo. Additionally, we show that Jhy is implicated in the localization of the adherens junction protein, E-cadherin. We have previously shown that lack of Jhy in ventricular ependyma leads to disruptions in adherens junction formation and aberrant motile cilia ultrastructure, implying that JHY function is conserved between the ependymal cells and the CP (Appelbe et al., 2013; Muniz-Talavera and Schmidt, submitted).

4.4.1 Rapid progression of hydrocephalus in the JhylacZNeo/lacZNeo mouse line

Disruption of the mouse gene Jhy in the transgenic mouse line JhylacZ (i.e. JhylacZ/lacZ) results in ependymal motile cilia defects, leading to communicating hydrocephalus with
ventricular enlargement observed at P1.5, and mice dying by 6 weeks of age (Appelbe et al., 2013). Targeted deletion of Jhy in JhylacZNeo/lacZNeo recapitulates this phenotype, however, with a more accelerated progression of the disease. We find that 73.5% of JhylacZNeo/lacZNeo mice die by the third week of age causing a shift in the survival rate curve by 3 weeks (Fig. 20D). Ventricular dilation in JhylacZNeo/lacZNeo is present as early as P0.5; however, there are no visible signs of hydrocephalus embryonically (Fig. 21). Thus, JhylacZNeo displays a more accelerated form of hydrocephalus.

These lines were generated using mice with different genetic backgrounds. Differences in genetic background can have a large effect on experimental studies, as strain-associated modifiers can greatly influence the obtained results (Vogel et al., 2012). The JhylacZ line was produced in the FVB/N background, while the JhylacZNeo was generated in the C57BL/6 background, with the latter being known as a line prone to hydrocephalus (Sundberg JP, 1991). Therefore, we believe the accelerated hydrocephalus in JhylacZNeo may be a result of the differences in genetic background between these lines. This will be tested experimentally by crossing the JhylacZNeo allele to FVB/N background mice and explore this possibility.

Nonetheless, we still needed to address the etiology of hydrocephalus in Jhy deficient mice. The onset of hydrocephalus in both JhylacZ/lacZ and JhylacZNeo/lacZNeo precedes the formation of ependymal motile cilia; thus ciliary defects in ependyma may contribute to the pathology rather than cause it. In mice, the CP arises by mid-gestation (E11-12) (Liddelow, 2015). Although CP is able to secrete CSF during embryonic stages, studies performed in rat have correlated CP maturation postnatally with a sudden increase in CSF production (Pentschew and Garro, 1966). This prompted us to consider the CP, which is
tightly involved in CSF secretion and is functionally active during early postnatal
development.

4.4.2 CP characterization in \( Jh^{lacZNeo/lacZNeo} \) revealed abnormalities in E-cadherin localization

It is possible that CP dysfunction may be responsible for the early onset hydrocephalus observed in \( Jh^{lacZNeo/lacZNeo} \) mice. To address this possibility, we performed a detailed morphological and molecular analysis of this tissue. CP morphology appears intact in both \( Jh^{+/+} \) and \( Jh^{lacZNeo/lacZNeo} \). CP dysfunction may manifest even in the absence of morphological defects as seen by Wodarczyk, where conditional inactivation of membrane the receptor Polycystin-1 results in morphologically normal CP cells, but impaired ciliary signaling and hydrocephalus (Wodarczyk et al., 2009; Swiderski et al., 2012; Banizs et al., 2005).

CSF formation by the CP is an active metabolic process that requires the strict polarization and distribution of membrane proteins needed to generate the osmotic gradient changes that drive CSF secretion. The mechanisms of membrane protein sorting in CP remain unknown. Some have suggested targeting mechanisms directed by G-proteins, and selective retention and stabilization by ankyrin/adducins to the spectrin/actin-based membrane cytoskeleton (Alper et al., 1994). We find that \( Jh^{lacZNeo/lacZNeo} \) displayed normal expression of apical markers AQP1 and Phalloidin, and basolateral marker, AE2 (Fig. 22). Adducins, the alternative to ankyrins in CP, have been associated with stabilizing the AE2 a basolateral domain in the CP (Christensen et al., 2013). Moreover, the fodrin-ankyrin cytoskeleton in the CP is associated with the establishment and stabilization of apical membrane components (Alper et al., 1994). Normal polarization of apical and basolateral
components in \textit{JhylacZNeo/lacZNeo} suggest that \textit{Jhy} may not have a function in polarity mediated by anchoring proteins and cytoskeleton mechanisms.

Adherens junctions participate in the establishment of apical-basal polarity, and they play critical roles in the integrity, permeability and polarity of the choroid (Szmydynger-Chodobska et al., 2007; Marrs et al., 1993). In \textit{JhylacZNeo/lacZNeo}, adherens junction protein E-cadherin is abnormally accumulated in the basolateral membrane and stroma of the CP (Fig. 23). E-cadherin mislocalization to the CP stroma has been previously observed in choroid plexus papilloma, though histological analysis of CP found no obvious signs of CP tumorigenesis in \textit{JhylacZNeo/lacZNeo} (Dominique Figarella-Branger, 1995; Nentwig et al., 2012). The expression of the cadherin-binding partner, \(\beta\)-catenin, remained unaffected despite the obvious mislocalization of E-cadherin. A discrepancy between the regulation and cellular distribution of known binding partners in the cadherin-catenin complex has been previously reported. For example, \(\beta\)-catenin expression in a human CP carcinoma cell line (CPC-2) localized at the adherens junction, while E-cadherin displayed a different cellular distribution (Szmydynger-Chodobska et al., 2007). Moreover, \textit{slc4a10 KO} mice display normal E-cadherin localization, but aberrant expression of the binding partner, \(\alpha\)-catenin (Christensen et al., 2013). It is possible that these proteins may not be regulated in parallel with their associating partners. \(\beta\)-catenin may display a different subcellular localization possibly by being targeted along with other basolateral membrane proteins (e.g. \(\alpha\)-catenin) and this might occur through E-cadherin-independent mechanisms.

Despite the aberrant accumulation of E-cadherin in the CP stroma, these cells form a relatively normal epithelial monolayer. This suggests that the molecules of E-cadherin retained at the cell boundaries in \textit{JhylacZNeo/lacZNeo} are sufficient to maintain cell-to-cell
junctions. The CP stroma is enriched with immune cells and is primarily associated with the brain immunological response though some believe that the stromal cell transcriptome may have indirect effects on the CP epithelium by interacting with basal membrane receptors (Kivisakk et al., 2003; Marques and Sousa, 2015). However, whether stromal cells mediate adherens junction maintenance and/or establishment is presently unknown. At this time, the factors needed for E-cadherin membrane targeting and stability in the CP remain unidentified. N-cadherin, the most abundant cadherin in ependymal cells is mislocalized in $Jhy^{lacZ/lacZ}$ mice (Muniz-Talavera and Schmidt, submitted). The striking similarities in the phenotypes in cadherin mislocalization suggest that $Jhy$ may facilitate the recycling and/or delivering of cadherin molecules (i.e. E- and N-cadherin) to the adherens junctions. Moreover, this function may be conserved between the ependymal cells and the CP. Further investigation is needed to uncover the mechanisms that drive E-cadherin membrane targeting in the CP, as well as to define the exact role of $Jhy$ in this pathway.

### 4.4.3 Roles for $Jhy$ in CP ultrastructure and cilia

CSF secretion is a complex process that employs various components and/or mechanisms to regulate this system. Microvilli can effectively regulate CSF secretion by the CP. Clavate microvilli and CSF production has a directly proportional relationship. On the other hand, filliform microvilli are indirectly proportional to CSF secretion. SEM and TEM analysis in $Jhy^{lacZNeo/lacZNeo}$ CP demonstrated a reverse in the population of microvilli, with filiform microvilli being more predominant than the normally abundant clavate microvilli (Fig. 24). Given the role of microvilli in CSF secretion, the choroid might undergo structural changes in response to the excess CSF in the ventricles. Schultz et al. demonstrated that vasopressin-mediated transchoroidal absorption is possible, with vasopressin treated rats
experiencing an increase of vacuolization of apical cytoplasm, and changes in microvilli from clavate to filliform (William J. Schultz, 1977). Thus, we believe that the changes in microvilli population observed in JhylacZNeo/lacZNeo CP is the result of CP cells employing their absorptive capabilities in response to hydrocephalus.

Previous studies in CP cilia demonstrated ultrastructure variability, with microtubule arrangements including 9+0, 9+1 and 9+2 (Narita et al., 2010; Swiderski et al., 2012; Narita et al., 2012; Nonami et al., 2013). TEM analysis in Jhy+/+ CP demonstrated that cilia patterning is highly variable even in normal conditions, with 9+0 cilia being the most abundant one (Fig. 25C). This was in agreement with observations made by Narita et al. where cilia bundles in CP resemble primary cilia (Narita et al., 2012; Narita and Takeda, 2015). To our surprise, 9+1d cilia, with 9 outer microtubule doublets on the outer ring and 1 doublet in the center, were the second most abundant cilia in Jhy+/+, corresponding to 21% of the cilia (Fig. 25A, C). During neonatal stages, the CP displays 9+1d cilia these, however, are predicted to be less frequent than the second most common cilia configuration (i.e. 9+2s) (Narita et al., 2012). Additionally, 7 other cilia arrangements were reported, among them the 9+2s, which were found far less frequently than anticipated. These results suggest that the population of CP cilia is far more heterogeneous than previously appreciated. TEM in JhylacZNeo/lacZNeo CP yielded far more cilia structural variations than Jhy+/+. Contrary to Jhy+/+, 9+0 and 9+1d cilia were found in equal abundance. Ultrastructure ratios of 9+0 and 9+1d cilia shifted from 3:1 to 1:1 in JhylacZNeo/lacZNeo. Among the other 11 atypical axoneme structures found in JhylacZNeo/lacZNeo, 7 of these were unique to this genotype. The heterogeneity observed in CP cilia configuration suggests that this process is loosely regulated; however, loss of Jhy exacerbates the limited regulation employed in this pathway.
Previous work in our laboratory identified *Jhy* as a crucial component in ependymal motile cilia structure and function (Appelbe et al., 2013; Muniz-Talavera, submitted). In this report, we show a large array of microtubule patterning in *Jhy*+/+ CP cilia (Fig. 25). Ependymal motile cilia structure is highly conserved across eukaryotic species; however, the same cannot be said about CP cilia, which display a widespread disarray of the axoneme (Ishijima et al., 2002; Lechtreck et al., 2008b; Lechtreck et al., 2013). This suggests that CP cilia ultrastructure is not as tightly regulated as it is in motile cilia, this is likely due to their minimal contribution in CSF flow (Narita et al., 2012). Presumably, the pressure to keep a specific axoneme arrangement may have been lost in this tissue; as a consequence, cilia configuration may be dispensable for these structures to function as a signaling/sensory organelle. Nevertheless, lack of *Jhy* results in loss of whatever little regulation this process may have. This suggests that *Jhy* may have conserved functions in regulating ciliary axoneme ultrastructure in the ependymal motile cilia and the choroid plexus cilia (Appelbe et al., 2013) (Muniz-Talavera and Schmidt, submitted). Mutations in four ciliogenesis associated proteins (i.e. BBS 1, 2, 4 and 6), result in ultrastructural defects in cilia of the ependyma and choroid plexus (Swiderski et al., 2012). These findings support the existence of key ciliogenesis factors shared between these two tissues.

In this and previous studies we have shown that *Jhy* disruption leads to hydrocephalus with animals displaying abnormalities in 1) E-cadherin localization to adherens junctions, and 2) microtubule ultrastructure organization. We propose that *Jhy* mediates the targeting and/or stabilization of E-cadherin to the adherens junctions in the CP. It is possible that the phenotypes observed in *Jhy*lacZNeo/lacZNeo result from failure to properly localize E-cadherin at the adherens junction site. Adherens junctions are known to regulate the actin cytoskeleton by 1) directly linking to the actin network through actin...
associating proteins (i.e. Catenins, Vinculin, MyosinIV), and 2) by serving as a site for actin filament assembly through cadherin mediated mechanisms (Niessen et al., 2011) (Higgs and Pollard, 2001; Kovacs et al., 2002; Ratheesh and Yap, 2012). The focal adhesion protein, Vinculin, is found at the adherens junction and it has been shown to mediate cadherin-based cell-cell adhesion, and it has been shown to coordinate and control cell adhesion signaling (Weiss et al., 1998) (Carisey and Ballestrem, 2011) (Watabe-Uchida et al., 1998). It is possible that the adherens junction defects may be the underlying cause for the ciliary microtubule patterning defects in Jhy^lacZNeo/lacZNeo. A recent study suggested that ciliogenesis shares a common factor to the linkage proteins present at the adherens junction (i.e. Vinculin) (Antoniades et al., 2014). In this study, Vinculin was shown to link basal bodies to the actin cytoskeleton, and its disruption leads to ciliogenesis defects. Basal bodies serve as a template for cilia growth and ultrastructure, and compromised docking and/or stability can result in the instability of the microtubules which may lead to abnormally patterned cilia (Clare et al., 2014b; Sanders et al., 2015; Stephen et al., 2013). It is possible that excess E-cadherin in Jhy^lacZNeo/lacZNeo may recruit Vinculin to the membrane, disrupting the balance of Vinculin needed to establish the linkage of basal bodies to the actin cytoskeleton. Improper linkage of basal bodies may compromise their stability, resulting in abnormally patterned cilia. Nonetheless, we cannot rule out the possibility that the function of JHY in AJ formation and ciliary microtubule patterning may be independent from one another with JHY serving dual functions in the CP.

Our data shows striking phenotype similarities in AJ formation and ciliary microtubule patterning between the ependyma of Jhy^lacZ/lacZ and the CP in Jhy^lacZNeo/lacZNeo. The involvement of common factors for ependyma and CP differentiation is possible due to the common developmental origin shared between these two tissues. During early central
nervous system development, the dorsal telencephalon can be divided into three regions along the medial-lateral axis. The most lateral regions gives rise to neurons and glial cells (e.g. ependymal cells), whereas the medial region subdivides giving rise to the choroid plexus and Cajal-Retzius cells (Imayoshi et al., 2008) (Liddelow, 2015). The neuroepithelial cells that form the telencephalon begin to develop into ependymal cells around E10 (Bruni, 1998). The formation of the CP occurs when the ependymal neuroepithelium contacts the mesodermal germ layers, enclosing connective tissue along with blood vessels (Sarnat, 1998). The protrusion of the CP becomes visible by E12.5 (Imayoshi et al., 2008). Recent work has shown that mutations in mouse proteins polycystin-1, Polaris and RFX3, result in abnormalities in the differentiation of ependymal cells and choroid plexus cells in mice (Baas et al., 2006a; Banizs et al., 2005; Wodarczyk et al., 2009). Together, these studies support the hypothesis that these tissues may share common mechanisms for their differentiation. The present study will serve as a gateway to uncover the functional and ultrastructural commonalities shared between ependymal cells and the modified ependyma of the choroid plexus.
V. GENERAL DISCUSSION

Previous work performed in our laboratory identified the mouse gene \( Jhy \) as a gene involved in CSF homeostasis. The disruption of \( Jhy \) through random transgene integration resulted in juvenile hydrocephalus in the \( Jhy^{lacZ} \) mouse line (Appelbe et al., 2013). Hydrocephalus was only manifested in a homozygous background \((Jhy^{lacZ/lacZ})\), suggesting a loss of function phenotype. Phenotypic characterization of \( Jhy^{lacZ/lacZ} \) mice uncovered defects in motile cilia where the ependymal cilia were missing the central pair that confers ciliary motility (Appelbe et al., 2013). This work suggests that defects in ependymal cells may be the underlying cause for hydrocephalus in these mice. The work presented here aimed to further characterize the ependymal cells in hopes of defining the role of \( Jhy \) in the development and functionality of these cells. A cell type-specific marker approach was used to explore the functional status of ependymal cells in the \( Jhy^{lacZ} \) mouse line. A second approach taken to assess the role of \( Jhy \) in development encompassed a detailed phenotypic and molecular characterization of the choroid plexus using the newly generated gene targeted mouse line \( Jhy^{lacZ/Neo} \). This second mouse line served two purposes: 1) confirm that \( Jhy \) disruption indeed causes hydrocephalus, and 2) further characterize the role of \( Jhy \) in the brain.

5.1 Loss of \( Jhy \) in \( Jhy^{lacZ/lacZ} \) animals results in abnormal ependymal cell differentiation

The ependymal motile cilia defects prompted us to evaluate in more detail the ependymal cells in the \( Jhy^{lacZ} \) mouse line. Histological analysis in \( Jhy^{lacZ/lacZ} \) brains detected abnormalities in the ependymal cells, with cells displaying immature morphology (Fig. 8).
These cells progressively acquire mature ependymal characteristics suggesting a delay rather than a block in ependymal cell differentiation (Fig. 10). Nonetheless, differentiation in JhylacZ/lacZ is at least 9 days delayed, with ependymal cells still displaying immature characteristics at P14 (Fig. 10). Ependymal cell differentiation is a spatiotemporally regulated process, with differentiation occurring from P0 to P21, and progressing in a caudal-rostral, ventral-dorsal, and medial-lateral gradient (Merkle et al., 2004; Tramontin et al., 2003). Immature ependymal cells may have a strict window of opportunity to undergo all the necessary changes to become a functionally mature ependyma, and Jhy may facilitate this process through unknown mechanisms at this time (Spassky et al., 2005; Tramontin et al., 2003). Consequently, loss of Jhy leads to a large number of immature cells retained across the ventricular wall.

5.2 Jhy acts downstream of FoxJ1

The molecular mechanisms employed during ependymal cell differentiation remain largely understudied. The hypothesis is that subgroups of monociliated radial glial cells are retained along the lateral ventricles to undergo terminal differentiation into ependymal cells (Merkle et al., 2004; Spassky et al., 2005; Tramontin et al., 2003). The expression of transcription factor FoxJ1, was shown to be upregulated at birth (P0) coinciding with the onset of ependymal cell differentiation. Moreover, FoxJ1 was shown to be required for the radial glia to ependymal cell differentiation, with FoxJ1/-/- mice failing to produce ependymal cells (Jacquet et al., 2009). FoxJ1 immunofluorescence (IF) in ependymal cells demonstrated that immature cells in JhylacZ/lacZ are able to express FoxJ1 (Fig. 12). This suggests that ependymal cell differentiation programming is initiated in JhylacZ/lacZ animals; however, some cells are clearly encountering difficulties in the transition from a radial glial
to an ependymal cell by morphological criteria. It is possible that a factor downstream of FoxJ1 involved in the transition between immature radial glia and mature ependyma is now unable to function properly, causing the delay in differentiation. Interestingly, zebrafish Jhy, C10h11orf63, was upregulated upon FoxJ1 overexpression (Choksi et al., 2014). Altogether, these findings suggest that Jhy may act downstream of FoxJ1 to mediate ependymal cell differentiation.

One crucial aspect of ependymal cell differentiation is ciliogenesis. A microarray analysis identified over 100 genes upregulated by FoxJ1, and some of those include cilia associated proteins such as: 1) dynein arm component (Dnah9), 2) cilia central pair apparatus (Spag6), and 3) radial spokes proteins (Rshl2, Rshl3) (Stubbs et al., 2008; Yu et al., 2008; Kiselak et al., 2010). JhylacZ/lacZ ependymal cells displayed randomly oriented ciliary bundles, while others were completely devoid of cilia (Fig. 15). Moreover, JhylacZ/lacZ displayed aberrant ependymal cilia ultrastructure, where the central pair apparatus is missing and cells display greatly reduced ependymal flow (Fig. 16) (Appelbe et al., 2013). These findings further support the possibility that FoxJ1 may indeed recruit Jhy to facilitate multiple aspects of ependymal cell differentiation.

5.3 Jhy regulates the localization of adherens junction components in polarized epithelia

The cells of the ventricular ependyma and the CP are both of epithelial origin, and as such, they both display adherens junctions (AJs) (Oliver et al., 2013; Christensen et al., 2013; Vestweber, 2015; Gumbiner, 2005). The core components of the AJs involve the association of classical cadherins (i.e. N-, E-cadherin) with various members of the catenin
family of proteins (i.e. α-, β- catenin, p120) (Niessen and Gottardi, 2008). Catenin molecules in turn facilitate the connection of the AJ complex to the actin cytoskeleton. The localization of AJs in ependymal cells occurs in the apicolateral region, while in CP these junctions are localized to the basal membrane. Loss of Jhy resulted in the abnormal localization of N-cadherin and E-cadherin in JhylacZ/lacZ ependymal cells and JhylacZNeo/lacZNeo CP, respectively (Fig. 13, 23). These findings suggest that Jhy may facilitate the transport, targeting and/or recycling of cadherin molecules at the AJs in both the ependyma and the CP.

Little is known about the factors regulating AJ complex assembly and targeting in polarized epithelial cells. AJs are highly dynamic structures, though some believe that the targeting and recycling of AJ components is what regulates their membrane localization. For example, cadherin proteins are targeted to the membrane as a complex with β- catenin, and this occurs as a two-step process, where the complex is first targeted to the recycling endosomes, and from there, they are delivered to the AJ site at the plasma membrane (Chen et al., 1999). Studies have shown that the cadherin-catenin complex exits the trans-Golgi network and is transported to the recycling endosomes via Rab11 association with the exocytosis machinery (Lock and Stow, 2005; Kawauchi, 2011; Desclozeaux et al., 2008). Once in the endosome, the complex is targeted to the AJs through the scaffolding protein Pals1, and is then delivered to the plasma membrane through Sec6/8 exocytosis complex mechanisms (Wang et al., 2007; Grindstaff et al., 1998). Although the exact mechanisms by which Jhy facilitates AJ formation is not known, it is possible its role may lie in the regulation of the factors involved in the targeting and/or recycling pathway(s). Jhy disruption may lead to the inability to properly regulate the localization of cadherin molecules at the AJ, thus resulting in the phenotypes observed in the JhylacZ/lacZ ependyma
and in Jhy<sup>lacZNeo/lacZNeo</sup> CP. The striking similarity in the cadherin mislocalization phenotypes between ependymal cells and the cells of the CP, leads us to believe that Jhy may have conserved roles in the recycling and/or delivery of AJ components in ependymal cells as well as the choroid plexus. Further analysis is needed to determine the molecular mechanisms employed by Jhy during AJ formation.

5.4 Loss of Jhy results in β-catenin mislocalization in ependymal cells, but not in CP cells

Although the role of Jhy in cadherin protein localization may be conserved between the ependyma and the CP, the same cannot be said about the cadherin-associating partner, β-catenin. Loss of Jhy in ependymal cells in Jhy<sup>lacZ/lacZ</sup> resulted in the abnormal localization of β-catenin throughout the basolateral membrane, rather than its designated region, the apicolateral cell border (Fig. 13). Despite the mislocalization of E-cadherin in Jhy<sup>lacZNeo/lacZNeo</sup>, β-catenin expression appears unaffected in the CP (Fig. 23). Contrary to ependymal cells which express both N-cadherin and β-catenin only in the apicolateral region, the CP displays β-catenin expression extending throughout the entire lateral membrane rather than localizing only to the basal membrane where E-cadherin is expressed. Chen et al. suggested that E-cadherin and β-catenin are assembled and targeted together as a unit in MCDK cells (Chen et al., 1999). It is possible that in CP, E-cadherin and β-catenin employ separate mechanisms to localize to their respective membrane compartments, with only the cadherin targeting pathway being affected by the loss of Jhy. Alternatively, E-cadherin and β-catenin may be normally targeted together as a complex though there might be an alternate mechanism to localize β-catenin in response to failures in the main pathway.
β-catenin serves as a signaling component for the WNT/β-catenin pathway, which is known to regulate cell proliferation, migration, and fate specification (Clevers and Nusse, 2012). During WNT/β-catenin signaling activation, β-catenin must dissociate from the AJ and translocate into the nucleus to exert its signaling function. β-catenin dissociation and stabilization is crucial for proper WNT response; thus, it is possible that the abnormal accumulation of β-catenin throughout the basolateral membrane of JhylacZ/lacZ ependymal cells may prevent β-catenin from dissociating, consequently impairing the WNT response. Alternatively, excess β-catenin may alter cycling between cytoplasm and membrane resulting in a smaller pool of β-catenin in the cytoplasm. It is worth mentioning that our antibody recognized only the N-cadherin bound form of β-catenin. Further analysis using an antibody against the cytoplasmic (free) form of β-catenin is needed to detect any changes in cytoplasmic or nuclear localization of this protein, which will in turn provide information on the implications of losing Jhy expression in the WNT/β-catenin signaling pathway.

5.5 **Adherens junction defects may be the underlying cause for the impaired ciliogenesis in the ependyma**

The non-canonical WNT/planar cell polarity (PCP) pathway is known to direct ependymal cell polarization and it controls cilia growth, organization, and polarization by regulating the base of the cilium (i.e. basal body) (Boutin et al., 2014; Gegg et al., 2014; Park et al., 2008; Tissir et al., 2010; Ohata et al., 2014). Recent findings suggest that this pathway may be regulated by interactions between AJ proteins and PCP components. Crosstalk between the canonical WNT/β-catenin and non-canonical/PCP pathway, involving β-catenin (canonical) and Vangl2 (non-canonical) has been previously reported (Pinzon-Daza
et al., 2014; Nagaoka et al., 2014). Nagaoka et al. demonstrated that β-catenin and Vangl2 bind to the same intracellular region of N-cadherin, and they mediate N-cadherin stabilization or internalization, respectively (Nagaoka et al., 2014).

Immature ependymal cells in JhylacZ/lacZ displayed randomly oriented ciliary bundles, while others were completely devoid of cilia (Fig. 15). Ependymal cells in JhylacZ/lacZ also display abnormal ependymal cilia polarity, suggesting PCP defects in these cells (Fig. 17, 18). It is possible that defects in the AJ may in turn disrupt the interactions needed between AJ proteins and PCP components for proper cilia growth and polarity. PCP signaling has been shown mediate basal body docking through the regulation of the apical actin network formation (Boutin et al., 2014a; Park et al., 2008). Apical positioning of basal bodies is critical for cilia growth and organization (Park et al., 2008; Tissir et al., 2010). For example, loss of PCP proteins Celsr2 and Dishevelled, results in impaired actin cytoskeleton organization and defective basal body positioning (Boutin et al., 2014; Park et al., 2008). Moreover, loss of Dishevelled (Dvl) expression in Dvl TKO^{GFAP-Cre} mice results in abnormal ependymal cell ciliogenesis (Ohata et al., 2014). Conceivably, Jhy may regulate cilia formation through PCP-mediated mechanisms, and its disruption leads to impaired ciliogenesis. The abnormal levels and/or distribution of N-cadherin upon Jhy disruption may result in an unstable balance between the interaction of N-cadherin with Vangl2 and β-catenin, which could ultimately lead to aberrant PCP signaling (Fig. 26). The PCP gene Prickle also mediates the internalization of Vangl2, and excess Prickle disrupts the asymmetrical distribution of PCP components and planar cell polarity (Cho et al., 2015; Nagaoka et al., 2014). It is also possible that excess β-catenin bound at the AJ may maintain N-cadherin molecules sequestered at the membrane, preventing its interaction and
internalization with Vangl2, which may ultimately lead to the loss of PCP signaling. Jhy may act as a mediator between AJs and the PCP pathway to facilitate ciliogenesis. Apical docking of basal bodies through PCP is facilitated by the exocytosis complex component Sec8, and loss of Dvl results in disordered distribution of Sec8 vesicles in the apical membrane, along with impaired docking of basal bodies (Park et al., 2008). Further analysis of Sec8 vesicles localization in JhylacZ/lacZ ependymal cells may help determine whether the ciliogenesis defects observed in these cells result from impaired delivery of basal bodies through PCP-mediated mechanisms.

5.6 Ciliary microtubule patterning defects may be a result of improper basal body positioning

Abnormal ciliary microtubule patterning is present in both the ependyma and the CP. In JhylacZ/lacZ ependymal cilia the central pair apparatus was missing, whereas cilia in the CP displayed a large array of abnormally patterned cilia (Fig. 25C) (Appelbe et al., 2013). The 9 outer doublet microtubules in the cilium are assembled as a continuation from the basal bodies, the latter serving as a base/template for cilia development (Yasunaga et al., 2011; Satir, 2007; Satir and Christensen, 2007; Szymanska and Johnson, 2012). The outer microtubule doublets in turn fix and stabilize the motile cilia central pair apparatus through a multi-unit protein structure known as a radial spoke (Lechtreck et al., 2013; Satir and Christensen, 2007). Moreover, the microtubule central pair extends from a distal basal body structure called the transition zone (McKean et al., 2003). This suggests that ciliary microtubule organization and stability is dependent on the correct positioning and stabilization of basal bodies. Indeed, defective basal body formation, docking and stability results in impaired ciliogenesis (Pearson et al., 2009) (Gegg et al., 2014) (Burke et al.,
A second formal possibility may involve other factors associated with the AJs. For example, Vinculin is an actin linking protein that facilitates the association of the AJ complex with the actin cytoskeleton (Weiss et al., 1998; Carisey and Ballestrem, 2011; Watabe-Uchida et al., 1998). Recent studies suggest that Vinculin is also responsible for linking basal bodies to the cortical actin cytoskeleton (Antoniades et al., 2014). Presumably, a stable balance must exist between the Vinculin needed either at the AJ or at the basal bodies to allow the dual function of this protein in these cells. Thus, it is possible that a defect in AJs may disrupt the basal body-cytoskeleton linkage function of Vinculin by disrupting the balance and keeping Vinculin sequestered at the AJ. This disruption may in turn lead to basal body instability and ciliary ultrastructural abnormalities.

Lastly, microtubule assembly is dependent on the ciliary trafficking machinery known as the intraflagellar transport (IFT), and its function is dependent on proper docking into the basal body (Kobayashi and Dynlacht, 2011) (Taschner et al., 2012). Distal structures in the basal bodies (i.e. transition fibers) help dock the basal body to the membrane and form the transition zone, which acts as a “ciliary gate” that controls the trafficking in and out of the cilium (Deane et al., 2001; Szymanska and Johnson, 2012).
elegans, disrupting basal body associated components (i.e. KIAA0556, BBS7, BBS8) and/or transition zone associated genes (i.e. nphp-2, arl-3) results in abnormal ciliary microtubule patterning (Warburton-Pitt et al., 2014) (Blacque et al., 2004) (Sanders et al., 2015). It is possible that abnormal anchoring of basal bodies resulting from defective AJ and/or PCP signaling may compromise the transition zone, affecting the entry or exit of ciliary cargo and the cilium ultrastructure. Alternatively, it is also possible that JHY regulates microtubule patterning through mechanisms independent of cadherin and/or PCP signaling. Future studies are needed to dissect the molecular mechanisms of action of Jhy, as well as to provide more insight into developmental commonalities between the ependyma and the specialized ependyma of the choroid plexus.
**Figure 26. Proposed model for Jhy function in ependymal cell development.** Adherens junctions form a protein complex composed of cadherin molecules (i.e. N- and E-) and catenins proteins (i.e. α- and β) (Gumbiner, 2005). The cadherin-catenin complex may in turn bind to the actin cytoskeleton with or without the help of the adhesion protein Vinculin. We propose that JHY functions during ependymal cell differentiation to target and/or stabilize cadherin proteins (i.e. E- and N-cadherin) at the adherens junction sites. Proper establishment of cadherin molecules at the adherens junctions is crucial for cadherin-based signaling. Loss of JHY results in the abnormal distribution of the adherens junction components and may ultimately lead to the disruption of crucial developmental signaling pathways controlling ependymal cell polarity and ciliogenesis (e.g. PCP pathway) (Nagaoka et al., 2014; Clare et al., 2014; Ohata et al., 2014; Gegg et al., 2014).
APPENDIX A. Enhancer trapping of a gene expressed in the interdigital mesenchyme

A.1 Purpose and objective

Congenital limb malformations, specifically in digit specification and patterning occur in approximately 1/500 births (Linder et al., 2009). Although these deficiencies are not life threatening, they are considered a disability and are, thus, a subject of interest in research. Most limb malformations are a result of genetic abnormalities (Wilkie, 2003). Hence, the identification of novel genes in limb development is essential for the understanding of the complex mechanisms that regulate limb patterning. Our lab has previously generated a transgenic mouse line (Tg37), which carries a lacZ reporter gene that randomly integrated into the genome functioning as an enhancer trap. LacZ displayed an expression pattern localized to the interdigital mesenchyme (IDM). The IDM is a signaling center for bone morphogenic proteins (BMPs), which are known to regulate digit specification (Benazet and Zeller, 2009; Zeller et al., 2009). Studies have shown that interdigit (ID) regions help determine each digit identity. For example, digit 2 and 3 are specified by the expression of genes in the ID2 and ID3 (Suzuki1, 2013). In chick, the removal of ID2 and ID3 resulted in the transformation of digit 2 and 3 into digit 1 and 2, respectively (Dahn and Fallon, 2000). During the later stages of digit formation, the IDM undergoes programmed cell death, and this process is critical for proper digit sculpting (Chimal-Monroy et al., 2003; Mori et al., 1995).

lacZ expression was first detected in the Tg37 forelimb IDM as early as E11.5, and this expression became significantly stronger by E12.5. A decrease in lacZ expression is observed by E13.5, coinciding with IDM apoptosis. The timing of lacZ expression is concurrent with the onset for mouse digit determination and formation (Zeller et al., 2009). In 1998, Holmes et al. performed in situ hybridization against Slit2, an axon-guiding gene,
and found it to be expressed in the developing forelimb around E10.5, with strong IDM expression at E12.5. *Slit2* expression in the IDM begins to dissipate by E13.5 while digit sculpting occurs through apoptosis of the IDM (Holmes et al., 1998).

In order to identify the endogenous IDM-expressing gene in *Tg37*, and localize the enhancer driving its expression, we decided to evaluate the expression profile of known genes found within 4 megabase pairs (Mb) Mb from the transgene integration. Among the genes annotated within this 4 Mbp window, *Slit2* was located approximately 2 Mbp from the transgene. Given the striking similarity between the expression reported by Holmes and our *lacZ* expression data, we decided to perform *in situ* hybridization against *Slit2* in order to do the following: (1) confirm *Slit2* expression in the developing limb, and (2) evaluate whether *Slit2* expression profile was altered in the *Tg37* mouse line (Fig. 27).

A.2 Materials and methods

A.2.1 Probe generation for *in situ* hybridization

A.2.1.1 Ligation of DNA probe into pBKSII vector

DNA from wild type FVB/N mice was amplified using the following primers:

**Slit2**

(SLIT2for) 5’-CCGGAATTCCCTCTCTGATGAAGAAGAATCTC-3’
(SLIT2rev) 5’-CCGGAATTCAGACAAGTCTTTATTATCATAATAA-3’

**Robo1**

(OL2025) 5’-CCGGAATTCTGCGGTGCTGAGATCCGCTACGCTAAG-3’
(OL2026) 5’-CCGGAATTCTGCGGTGCTGAGATCCGCTACGCTAAG-3’

The “CCGGAATTTC” sequence corresponding to the recognition site for digestion enzyme *EcoRI* was added to the 5’ end of each primer to allow for direct cloning of the probe into the pBKSII plasmid. The PCR product was digested with *EcoRI* to clone the product into the vector. The digestion and dephosphorylation of the pBKSII vector was
performed using *EcoRI* and 1μL of alkaline phosphatase, and the digest was incubated at 37°C for 1 hour. The ligation reaction was carried out using 1.5μL of T4 DNA ligase, 1.5μL of ligase buffer, 3μL of vector, and 9μL of insert, and this was incubated at 15°C overnight. The ligation reaction was added into competent cells using standard lab protocol (200μL of transformation with 20μL X-gal), and colonies were selected to isolate DNA plasmid using the QIAprep Spin Miniprep Kit according to manufacturer’s instructions. *EcoRI* digestions were performed to ensure insertion was correct, and restriction enzymes *SmaI* and *BamHI* were used to determine the direction of the PCR insertion. Plasmids were sent for sequencing using primers for the T7 and T3 promoter sequences, and those containing the correct insertion were saved in an 80% glycerol stock and stored at -80°C. Following sequencing, the saved aliquot from the previous mini prep were used to perform a maxi prep using the Qiagen Plasmid Maxi Kit according to manufacturer’s instructions. The obtained product was resuspended in 200μL of dH2O.

**A.2.1.2 Plasmid purification**

Plasmid linearization was performed using 11μg of pBKSII plasmid and this was cut using the following digestion enzymes: *EcoRV*, *SmaI* and *NotI*. The linearized plasmid was cut out using a clean scalpel. Gel fragments were spun in wizard columns for 3 minutes at top speed. The columns were pulled out and spun once again for 3 minutes at top speed, by then the gel had filtrated into the tube. 1/10 volumes of sodium acetate, 2μl of glycogen, and 2 times volume of 100% ethanol were added into the tube, and the tube was then centrifuged at top speed for 20 minutes at 4°C. The supernatant was removed and the pellet was washed with 500μL of 70% ethanol and air dried. Once dry, the pellet was resuspended in 10μL of DEPC-H2O.
2.1.3 Generating DIG labeled RNA probes for in situ

A transcription reaction was set up using 1μL of 5X transcription buffer, 2μL of 100nM DTT, 0.5μL of 40μg/mL RiboLock RNase inhibitor, 2μL of 10X DIG labeling kit, 1μg of linearized DNA, and DEPC-H₂O was added for a final volume of 18μL. 2μL of RNA polymerase T3 and T7 were added to the reaction, and this was incubated for 2 hours at 37°C. Adding 2μL of 0.5M EDTA stopped the reaction. Residual DNA was removed by adding 2U DNase I (RNAse-free), followed by incubation for 15 minutes at 37°C, and 2μL of 0.2M EDTA was added to stop the reaction. Probes were purified using the G-50 Sephadex columns for radiolabeled RNA purification (Roche, No. 11 274 015 001), and RNA quantification was performed by using a nanodrop spectrophotometer.

A2.2 Whole-mount in situ hybridization

A2.2.1 Embryo preparation

E11.5, E12.5 and E13.5 embryos were dissected in DEPC-treated PBS and fixed in fresh 4% PFA pH7.5-DEPC-PBS overnight at 4°C. Embryos were then washed in cold DEPC-PBST. Fixed embryos were dehydrated in methanol/DEPC-PBST (25%, 50%, 75%, and 100%) and stored in 100% methanol (MeOH) at -20°C for long-term storage. Before hybridization, embryos were rehydrated through a series of methanol washes. Embryos were bleached in 4:1 MeOH/H₂O₂ at room temperature for 1 hour. Protein degradation was carried out using 10μg/mL proteinase K for 15 minutes at room temperature, followed by washes in DEPC-PBST with 2mg/mL glycine and rinses in DEPC-PBST. Tissues were fixed in 4% PFA, 0.2% glutaraldehyde in DEPC-PBST for 20 minutes.
A.2.2.2 Hybridization

Embryos were washed with 50% pre-hybridization solution (50% formamide, 5X SSC, 50μg/mL yeast t-RNA, 50μg/mL heparin, 1% DEPC-SDS) and 50% DEPC-PBST, followed by a wash in 100% pre-hybridization solution at room temperature. This solution was replaced with 900μL of fresh pre-hybridization solution and incubated for at least 4 hours, but preferably overnight at 65°C. The probe was denatured by heating for 5 minutes at 95°C and placed on ice for 2 minutes. Fresh hybridization solution with 1μg/mL of probe was added to the embryos and incubated in a rotator overnight at 70°C.

A.2.2.3 Post-hybridization

Hybridization mixture was removed and embryos were washed with pre-warmed solution I (50% formamide, 20X SSC and 10% SDS) for 5 minutes at 65°C. This was followed with washes in 75% solI: 25% 2xSSC, 50% solI: 50% 2xSSC, 25% solI: 75% 2xSSC, all at 65°C. Embryos were then washed with 2x SSC with 0.5% CHAPS at 65°C for 30 minutes, followed by washes in DEPC-PBST at room temperature. Pre-blocking was performed using 10% normal goat serum (heat inactivated), 2% BSA in TBTX (50mM Tris pH 7.5, 150mM NaCl, 0.1% Triton X-100) for 3 hours at room temperature. The embryos were incubated with preabsorbed antibody overnight at 4°C.

A.2.2.4 Preabsorption of antibody

Embryo powder was prepared by homogenizing E12.5 embryos in PBS. Four volumes of ice-cold acetone was added to the mixture and incubated for 30 minutes on ice. The mixture was centrifuged at 10,000 x g for 30 minutes, followed by the removal of the supernatant. The pellet was washed with ice-cold acetone and centrifuged once again. The
resulting pellet was spread and grinded until a fine powder was created. The powder was incubated at 4°C for long-term storage. Preabsorption of the antibody was done by adding 3mg of embryo powder into 0.5mL TBTX and heating 70°C for 30 minutes. This was cooled on ice and 5μl goat serum along with 1μL of antibody were added, and this was shaken for 2-3 hours at 4°C.

A.2.2.5 Detection

Embryos were washed with TBTX with 0.1% BSA 6 times for 1 hour at room temperature, followed by one more wash overnight at 4°C. Embryos were washed two more times with TBTX for 30 minutes and twice with NTMT (100mM Tris pH9.5, 10mM NaCl, 50mM MgCl₂, 0.1% Tween-20) for 30 minutes at room temperature. 34μL of 50mg/mL NBT and 35μL of 50mg/mL BCIP in NTMT were added to the embryos and was allowed to rock for 20 minutes in the dark. Embryos were washed in PBSTX (1xPBS, 0.1% Triton X-100) until desired staining level is achieved.

A.3 Results and discussion

Forelimbs (Fig. 27A-D) and hindlimbs (Fig. 27I-L) from FVB/N mice used for in situ hybridization analysis to determine the identity of Slit2 expression in the IDM. Slit2 sense probes were used as a negative control to determine probe specificity (Fig. 27A, C, E, G, I, K, M, O). Antisense probes against Slit2 detected strong IDM expression at E12.5 in the forelimb (Fig. 27B) while Slit2 expression in hindlimb was present but was much weaker (Fig. 27J). Slit2 expression in the hindlimb IDM peaks around E13.5 (Fig. 27L). By E13.5, Slit2 expression in the forelimb has greatly decreased due to the massive apoptosis ongoing in the IDM (Fig. 27D) (Diaz-Mendoza et al., 2013). Presumably, Slit2 expression in the hind
leg will also decrease around E14.5 once apoptosis begins. This delay in expression may be explained by the lag in differentiation between forelimb and hindlimb, where forelimb develops approximately a day faster than their counterpart (Zeller et al., 2009). The *Slit2* gene was found over 2 Mb away from transgene insertion site. Nevertheless, the precise recapitulation in expression of this gene by *in situ* and *lacZ* leads us to believe that an interdigit enhancer for the *Slit2* gene was trapped by the transgene in the *Tg37* mouse line. The *Slit2* expression profile in *Tg37* was virtually undistinguishable from that of FVB/N in the forelimb (Fig. 27F, H) and the hindlimb buds (Fig. 27N, P). This suggests that the transgene acts as an enhancer trap, recapitulating gene expression without affecting its function. This notion was further supported as no developmental defects have been observed in *Tg37* mice.

In the brain, Slit2 acts as a chemorepellent protein that helps direct axonal growth cones during central nervous system development (Holmes et al., 1998). Assuming this function is conserved in the limbs, it is possible *Slit2* may facilitate digit innervation by guiding axons to the digits and preventing axon innervation at the IDM. The transient expression of *Slit2* in the IDM supports this role, as presumably, Slit2 protein expression will be needed in the tissue adjacent to the developing limbs only during the digit formation process. Once the digit has been sculpted and properly innervated *Slit2* will no longer be required.

Slit2 protein acts as a ligand; therefore, a receptor must be present to promote the Slit2 repellent action. The *Robo1* gene is known to regulate axonal growth through *Slit2-Robo1* mediated mechanisms (Vargesson et al., 2001). Thus, we performed *Robo1 in situ* hybridization assays to characterize *Robo1* expression in the developing limb. Sense probes were used as a negative control to verify probe specificity in the forelimb (Fig. 28A, C, E, G).
and hindlimb buds (Fig. 28I, K, M, O). At E12.5, Robo1 expression in the FVB/N forelimb localized along the peri-digital region (Fig. 28B). By E13.5, this expression became more defined and localized along all digits (Fig. 28D). Forelimb and hindlimb Robo1 expression at E12.5 (Fig. 28B, J) and E13.5 (Fig. 28D, L) display very similar profiles, with the exception of E13.5 hindlimb which had higher Robo1 expression distally in all digits (Fig. 28L).

Furthermore, Robo1 expression in the forelimbs (Fig. 28F, H) and hindlimbs (Fig. 28N, P) of Tg37 mice appeared unaffected at all evaluated time points. The complimentary expression observed between Slit2 and Robo1 during limb development, precisely during digit specification, may suggest that these molecules work together to promote digit innervation in mice. Although further confirmation is needed, is tempting to speculate that these two proteins may interact in the limbs in a similar manner as they do in brain.
Figure 27. *Slit2* expression was detected in the IDM of the developing forelimb and hindlimb in FVB/N and *Tg37* mice. Fore- and hindlimbs were treated with *Slit2* sense probes as a negative control (A, C, E, G, I, K, M, O). Anti-sense probes were used to define *Slit2* expression domains in the developing limbs (B, D, F, H, J, L, N, P). *Slit2* expression was recorded in FVB/N forelimb (A-D) and hindlimb (I-L), and in *Tg37* forelimb (E-H) and hindlimb (M-P). At E12.5 *Slit2* was highly expressed in the IDM of the forelimb (B, F), and this expression decreased by E13.5 when apoptosis takes place (D, H). *Slit2* begins to express in the hindlimb by E12.5 (J, N) becoming stronger by E13.5 (L, P). No differences in *Slit2* expression were reported between FVB/N and *Tg37* mice.
**Figure 28. Robo1 expression was complimentary to Slit2 in the developing limb.**

Fore- and hindlimbs were treated with Robo1 sense probes as a negative control (A, C, E, G, I, K, M, O). Anti-sense probes were used to define Robo1 expression domains in the developing limbs (B, D, F, H, J, L, N, P). Robo1 expression in FVB/N forelimb (A-D) and hindlimb was recorded (I-L). Digit expression of Robo1 was first visible at E12.5 in both fore- and hindlimbs (B, J), but the pattern becomes stronger and more refined by E13.5 (D, L). Expression of Robo1 in Tg37 forelimb (F, H) and hindlimb (N, P) was virtually identical to FVB/N in all stages evaluated.
APPENDIX B. Exploring the roles of \emph{Jhy} in the rostral migratory stream and neuronal migration

\section*{B.1 Purpose and objective}

The olfactory system is composed of the olfactory epithelium, derived from the olfactory placode, and the olfactory bulb (OB) (Helen B. Treloar, 2010). In rodents, the OB projects out in the most rostral part of the brain residing close to the nasal cavities. For years, the development of the OB has intrigued scientists, particularly due to the extensive neurogenesis happening in this tissue (Helen B. Treloar, 2010). During the phenotypic characterization of the \emph{JhylacZNeo} mouse line described in chapter IV, we observed that the OB in homozygous mutant animals (\emph{JhylacZNeo/lacZneo}) was smaller in size (Fig. 29). The aim of this work was to further characterize the olfactory bulb phenotype observed in \emph{JhylacZNeo/lacZneo} mice and determine whether \emph{Jhy} has a role in olfactory bulb development.

\section*{B.2 Materials and methods}

\subsection*{B.2.1 Nissl staining}

The cytoplasm of neuroblasts was stained using Nissl staining in P5 mouse brains. Specimens were treated with Bouin’s fixative overnight at 4\(^\circ\)C and paraffin embedding was carried out as described in section 2.3. Sagittal sections (10\(\mu\)m thick) were deparaffinized in xylene, hydrated in a series of ethanol washes (100\%, 95\%, and 70\%) and rinsed first in tap and then dH\textsubscript{2}O. Sections were incubated in warm 0.1\% cresyl violet solution (0.1g cresyl violet acetate, 100mL dH\textsubscript{2}O, 0.3mL of glacial acetic acid) for 4½ minutes at 37\(^\circ\)C. Slides were rinsed in running dH\textsubscript{2}O, followed by treatment in 95\% ethanol for 3 minutes for staining enhancement. Sections were dehydrated in 100\% ethanol, cleared with xylene
and mounted using Cytoseal XYL (Fisher Scientific, Pittsburgh, PA). Imaging was performed using a Leica MZFLIII dissecting microscope (Wetzlar, Germany).

B.2.2 Immunohistochemistry for apoptosis marker cleaved caspase 3

P5 brains were Bouin’s fixed and paraffin embedded as specified in section 2.3. Tissue was then deparaffinized and rehydrated following the steps indicated in section 2.4.1.1. Heat antigen retrieval (0.3% sodium citrate, 0.05%, Tween-20 pH 6.0) was performed and tissues were washed with TNT buffer solution (0.1M Tris-Hcl, pH 7.5, 0.15M NaCl, and 0.05% Tween 20). Quenching of endogenous peroxides was done by using 3% H₂O₂ in methanol for 10 minutes. Slides were blocked with 5% normal goat serum in TNT buffer for 1 hour at room temperature. Overnight incubation of primary antibody in 1% BSA was performed at 4°C using antibody against rabbit cleaved-caspase 3 (Asp175) (1:100, #9661, Cell Signaling). Secondary goat α-rabbit biotinylated antibody was added in 50% block: 50% PBS for 1 hour at room temperature. ABC reagent (Vector laboratories, PK-6100) was added to the slides for 30 minutes at room temperature followed by 5 minutes in DAB substrate (Vector laboratories, SK-4100) for final signal detection. Hematoxylin was used as counterstaining. Images were taken on a Zeiss Axiovert 200M deconvolution microscope equipped with Axiocam 105 color camera (Zeiss, Göttingen, Germany).

B.3 Results and discussion

During the dissection of P5 JhylacZNeo/lacZNeo brains we uncovered an unexpected olfactory phenotype in these mice. Unlike Jhy+/+, which displayed large bulbous projections corresponding to the OB, JhylacZNeo/lacZNeo had significantly smaller bulbs (Fig. 29, bracket).
The OB is a complex multi-layered structure, where the outermost layer is the glomerular layer, followed by the mitral and granular layer, respectively. Coronal cross sections from OB in Jhy\(^{+/+}\) displayed all three layers by P5 (Fig. 30A, inset). To our surprise, the OB in Jhy\(^{lacZNeo/lacZNeo}\) was missing the cell in the granular layer (Fig. 30B, inset).

The precursors that give rise to the OB layers arise from the subventricular zone (SVZ), a discrete region located in the lateral wall of the lateral ventricle which harbors immature neurons (neuroblasts) (Peretto et al., 1999). During early postnatal stages, the neuroblasts stored in the SVZ begin to proliferate and migrate towards the olfactory bulb, and once they reach this region, they populate the center of this structure (i.e. granular layer). At this time, these cells begin their maturation process becoming the interneurons constituting the granular layer. The process in which neuroblasts migrate towards the olfactory bulb is highly organized, forming a tubular structure called the rostral migratory stream (RMS). The RMS is composed primarily of the migrating neuroblasts, which organize themselves into chains and are surrounded by glial cells, forming the glial “tube” (Sun et al., 2010). In mice, the initial formation of the RMS is at E15-E17; however, neuroblast migration peaks at the time of birth and continues in adulthood though in a much lower rate (Sun et al., 2010). Given the apparent lack of cells in the granular layer, we wondered whether the RMS was able to form in the Jhy\(^{lacZNeo/lacZNeo}\) brain. At P5, Jhy\(^{+/+}\) animals displayed a sigmoidal dense structure with cells extending from the SVZ to the center of the OB all indicative of an intact RMS (Fig. 31A). In Jhy\(^{lacZNeo/lacZNeo}\), RMS is present, but it appears to be widened (Fig. 31B). Moreover, the RMS in Jhy\(^{lacZNeo/lacZNeo}\) displays clear gaps within the RMS structure (Fig. 31B). The absence of cells in the OB as well as in the RMS suggests that neuroblast migration may be impaired in Jhy\(^{lacZNeo/lacZNeo}\) mice. We wondered whether these gaps were a result of programmed cell death. Thus, we performed
an immunohistochemistry analysis using the apoptosis marker cleaved-caspase 3 (CC3) in P5 $Jhy^{+/+}$ and $Jhy^{lacZNeo/lacZNeo}$ brains, and quantified cells positive for CC3 within the olfactory bulb and the RMS (Fig. 31C). Preliminary data found a total of 14 cells positive for CC3 in $Jhy^{+/+}$, while approximately 32 cells per section were CC3 positive in $Jhy^{lacZNeo/lacZNeo}$ (Fig. 31C). This is close to a two-fold increase in apoptosis in $Jhy^{lacZNeo/lacZNeo}$, however, this data is preliminary and is pending confirmation. Nevertheless, is possible that increased levels of apoptosis may explain the gaps observed along the RMS and the OB in $Jhy^{lacZNeo/lacZNeo}$ animals. Motile cilia in ependymal cell regulate the migration of neuroblasts from the SVZ into the OB by contributing of the gradient establishment of the neuronal guiding molecule, Slit2 (Gotz and Stricker, 2006). This ependymal function is mediated by the CSF flow generated by the beating of ependymal cilia, and disruption of this directional flow results in impaired neuroblast migration in mice (Boutin et al., 2014) (Gotz and Stricker, 2006) (Ibanez-Tallon et al., 2004) (Banizs et al., 2005). It is possible that the loss of cells in OB may be a result of impaired neuroblast migration due to the loss of ependymal cell flow in $Jhy^{lacZNeo/lacZNeo}$ mice.
Figure 29. Abnormal olfactory bulb size in \textit{Jhy}^{lacZNeo/lacZNeo}. Dorsal view of P5 mouse brains from \textit{Jhy}^{+/+} (left) and \textit{Jhy}^{lacZNeo/lacZNeo} (right). Olfactory bulb (black bracket) in \textit{Jhy}^{lacZNeo/lacZNeo} appears smaller when compared to \textit{Jhy}^{+/+}. 
Figure 30. Disrupted olfactory bulb layers in *Jhy*<sup>lacZNeo/lacZNeo</sup> mice. Nissl staining on coronal sections of the olfactory bulb in P5 *Jhy*<sup>+/+</sup> (A) and *Jhy*<sup>lacZNeo/lacZNeo</sup> (B). Olfactory bulb in *Jhy*<sup>+/+</sup> displays all 4 layers of the olfactory epithelium (A) while cells in the ependymal/subependymal layer and the granular layer are missing in *Jhy*<sup>lacZNeo/lacZNeo</sup> (B, inset). GL, granular layer; ML, mitral layer; GIL, glomerular layer.
Figure 31. Structural abnormalities in the \textit{Jhy}^{laczNeo/laczNeo} RMS. Composite of P5 nissl stained sagittal brain sections in \textit{Jhy}^{+/+} (A) and \textit{Jhy}^{laczNeo/laczNeo} (B). The RMS in \textit{Jhy}^{+/+} has a fine sigmoidal structure characteristic of a normal RMS (A). However, the RMS in \textit{Jhy}^{laczNeo/laczNeo} appears broader and contains regions with cells missing along the structure (B). Quantification of cells positive for the apoptosis marker cleaved-caspase 3 (CC3) in immunohistochemistry (C).
APPENDIX C. Defining the role of Jhy in testis development and spermatogenesis

C.1 Purpose and objective

Spermatogenesis is the process in which a mature sperm is produced. This process takes place in the seminiferous tubules of the testis, and it occurs in wave cycles divided into 12 stages (Oakberg, 1956). The wave cycle method ensures the continuous supply of sperm in sexually mature males. During the last stages of spermatogenesis, spermiogenesis occurs where the round spermatids convert into developing spermatids by excreting their cytoplasm, condensing their chromatin, and lastly, developing a sperm flagella. Spermatogenesis defects are typically reflected by reduced fertility (Diemer and Desjardins, 1999; Ronfani et al., 2001). Approximately 15% of couples globally are affected by infertility where male infertility is responsible for 20-30% of these cases (Agarwal et al., 2015). The high prevalence of this issue has generated a great need to understand the process of spermatogenesis in hope of providing alternative treatment options for the millions of couples affected by this globally. RT-PCR data suggests that Jhy is highly expressed in the testis. Moreover, histological analysis demonstrated that JhylacZ/lacZ mice display fewer mature sperm cells in the seminiferous tubules, and these males are subfertile (Appelbe et al., 2013). The purpose of this study was to further characterize the role of Jhy in testis through (1) morphological examination of mature sperm and (2) by defining the JHY protein expression profile in this tissue.

C.2 Material and methods

C.2.1 Testes immunofluorescence

Testes isolated from adult mice were fixed in 4% PFA overnight at 4°C and washed using 1x PBS. Specimens were cryopreserved, cryoembedded, and sectioned as specified in
section 2.3.1. Immunofluorescence was carried out as specified in section 2.4.1.2. Blocking buffer (5% normal goat serum in 1x PBS) was added to the slides for 1 hour at room temperature. Primary antibody against human JHY (rabbit, 1:10; Sigma-Aldrich, HPA039612) was added to the slides for overnight incubation at 4°C. Slides were treated using secondary antibody FITC conjugated goat anti-rabbit (1/250, Jackson ImmunoResearch) for 2 hours at room temperature. Sections were DAPI stained (D1306; Life Technologies, Grand Island, NY.) and mounted using vectashield mounting medium (H1000; Vector Laboratories, Burlingame, CA). Images were taken using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

C.2.2 Testis histology

Adult mouse testis were isolated and fixed in 4% PFA in PBS overnight at 4°C. The tissue was then washed with PBS and prepped for paraffin embedding following the steps described in section 2.3. Testis sections were then stained using Hematoxylin and Eosin staining following the protocol in section 2.3.4. Stained tissue was imaged using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

C.2.3 Mouse sperm isolation protocol and immunofluorescence

Adult mouse cauda epididymis (over 6 weeks of age) was dissected and cut in pieces in 1x PBS. Using a 27½ gauge needle, the isolated epididymis was pierced and placed into a 37°C incubator for 10 minutes to allow sperm to swim out. Protease inhibitors were added into PBS extraction solution (1/200, cat# 539134-1SET, Millipore). A small drop of the extracted sperm solution was placed on a poly-L-lysine coated slide creating 1cm spots and allowing the spots to air-dry. Sperm were fixed using 4% PFA solution for 15 minutes.
Permeabilization was done using 0.25% Triton X-100 for 10 minutes, followed by blocking in 3% BSA in 1x PBSA for 30 minutes at room temperature. The primary antibody, mouse Acetylated α-tubulin (mouse, 1:1000; T6793, Sigma-Aldrich) was incubated for 1-2 hours at room temperature and washed off using 1x PBS. Slides were DAPI stained as previously specified in section 2.4.1.4, mounted and imaged using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

C.2.4 Whole mount lacZ staining of testis

Isolated adult mouse testes were dissected in 1x PBS and fixed in 4% PFA for 90 minutes on ice. Tissue was washed in (2mM MgCl₂, 0.01% deoxycholic acid, 0.02% NP-40 in 1x PBS) for 30 minutes at room temperature. Specimens were then stained at 37°C using 1mg/ml X-gal in DMF, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% deoxycholic acid, 0.02% NP-40. The time of incubation in staining solution varied depending on desired signal intensity, ranging from 1-4 hours to overnight. To stop the staining reaction the tissue was washed multiple times in 1x PBS. Jhy⁺/⁺ testes were lacZ negative and were used as a negative control. Stained tissues were then prepared for cryopreservation, cryoembedding, and cryosectioning. Hematoxylin and Eosin was used as counterstain method. Finally, the slides were mounted and coverslipped and imaged using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

C.3 Results and discussion

Histopathological examination of testis showed reduced numbers of mature sperm in seminiferous tubules from JhylacZ/lacZ adult testis (Fig. 32 A, B, black asterisk). Given this
observation we decided to evaluate the morphology of sperm isolated from adult Jhy+/+ and JhylacZ/lacZ animals. As expected, gross morphological examination of sperm in Jhy+/+ showed sperm with long, straight tails, with morphologically normal sperm heads and mid piece (Fig. 32C). On the other hand, sperm in JhylacZ/lacZ displayed abnormal coiled tails (Fig. 32D, red arrows). Coiled tails are associated with weak sperm beating (Escalier, 2006). Presumably, these sperm abnormalities (i.e. coiled tail) prevent the sperm from reaching the ovum and fertilizing the egg.

RT-PCR data obtained in the lab suggest that Jhy is highly expressed in testis (Appelbe et al., 2013). Given this information, we proceeded to define JHY expression localization in testis by immunofluorescence. For this assay we used a commercially available antibody for the human JHY orthologue, C11ORF63 (Sigma) (Ivliev et al., 2012). The C11ORF63 antibody recognizes an epitope that shares 87% identity to mouse protein sequence and was, therefore, used to probe for mouse JHY protein in testis. Nevertheless, the findings obtained using this antibody should be interpreted cautiously as cross-reacting products have been detected on Western blots. Immunofluorescence on Jhy+/+ testis indicated that JHY was present in the cytoplasm of round spermatids during stage II of spermatogenesis (Fig. 33A, white arrow in A'). As spermatogenesis continues, JHY expression is visible in the cytoplasm of the developing spermatids at stage IV (Fig. 33B, white arrow in B'). Around stage V and VI, JHY expression is primarily at the tip of the developing spermatids, where the acrosome is localized (Fig. 33C, white arrow in C'). No JHY expression was detected in progenitor cell spermatogonia or primary spermatocytes which are the cells undergoing mitosis and meiosis respectively. The restricted expression of JHY in post-meiotic cells (i.e. round and developing spermatids) suggests that JHY may act once cell division is over, to facilitate the differentiation of these cells during
spermiogenesis. Further analysis is needed to confirm this hypothesis and establish the exact role of $Jhy$ in spermatogenesis.
Figure 32. Reduced sperm in the lumen of seminiferous tubules and abnormal coiled-tail sperm observed in $Jhy^{lacZ/lacZ}$. (A-B) H&E staining of adult testis from $Jhy^{+/+}$ (A) and $Jhy^{lacZ/lacZ}$ (B). Mature epididymal sperm was collected from $Jhy^{+/+}$ (C) and $Jhy^{lacZ/lacZ}$ (D). Sperm in $Jhy^{+/+}$ exhibited elongated straight tails (C), while sperm in $Jhy^{lacZ/lacZ}$ displayed coiled-tails (D, red arrows). Black arrowheads point to red blood cells (RBC) found in the media.
Figure 33. JHY expression profile during spermatogenesis. Adult testis sections were stained for JHY (green) in Jhy+/+ animals (A-C, A’C’). Spermatogenesis in the seminiferous tubules occurs in cycles divided into 12 stages. During stage II, JHY is expressed in the cytoplasm of round spermatids (A, A’). This expression profile is retained in elongating spermatids during stage IV (B, B’). By stage V-VI, JHY expression at the tip of sperm head, where the sperm acrosomal structures are normally localized (C, C’). DAPI is depicted in blue in all panels. RS, round spermatids; ES, elongating spermatids.
APPENDIX D. Ependymal cell characterization in the \textit{Jhy}^{lacZNeo} mouse line

D.1 Purpose and objective

\textit{Jhy}^{lacZ/LacZ} animals display abnormal ependymal cell morphology and differentiation. Histological and molecular characterization of ependymal cell in \textit{Jhy}^{lacZNeo} animals was performed to characterize the ependyma in this newly generated mouse line, and confirm the role of \textit{Jhy} in ependymal cell differentiation.

D.2 Materials and methods

D.2.1. Histology of the ependyma

Mouse brains at P5 were dissected and fixed in Bouin’s fixative for 18-24 hours at room temperature. Brains were first washed in tap water followed by washes in 70% ethanol with saturated lithium carbonate (Sigma-Aldrich, St. Louis, MO). Tissues were dehydrated in a graded series of ethanol washes, embedded in paraffin and sectioned into 8-10μm sections using a microtome (Leica Microsystems RM2125, Bannockburn, IL). Brain sections were stained using harris hematoxylin and alcoholic eosin Y solution following the steps described in section 2.4.3 (Sigma-Aldrich, St. Louis, MO). Images were taken using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

D.2.2 Ependymal cell immunofluorescence

Mouse brains were dissected and Bouin’s fixed for 18hrs at room temperature. P5 brains were Bouin’s fixed and paraffin embedded as specified in section 2.3. Tissue was then deparaffinized and rehydrated following the steps indicated in section 2.4.1.1. Heat antigen retrieval (0.3% sodium citrate, 0.05%, Tween-20 pH 6.0) was performed, followed by blocking in 5% goat/donkey serum, 1%BSA, 0.75% Glycine, 0.5% Tween 20X in PBS for
1 hour at room temperature. Slides were incubated in primary antibody overnight at 4°C in a humidifying chamber. The following primary antibodies were used: mouse Acetylated α-tubulin (mouse, 1:1000; T6793, Sigma-Aldrich), mouse Glast (guinea pig, 1:1000; AB1783, Millipore), mouse Vimentin (rabbit, 1/500; ab92547, Abcam),

The sections were incubated in secondary antibody for 2 hours at room temperature and slides were DAPI stained following the steps mentioned in section 1.4.1.4 (D1306; Life Technologies, Grand Island, NY.). Slides were then mounted using vectashield mounting medium (H1000; Vector Laboratories, Burlingame, CA). Images were taken using an inverted fluorescent microscope (Carl Zeiss Axiovert 200M, Göttingen, Germany).

D.3 Results and discussion

Ependymal cells in JhylacZ/LacZ animals display a delay in their differentiation by morphological criteria (Fig. 8). This prompted us to examine the ependymal cells in the Jhy-targeted mouse line, JhylacZNeo. H&E staining of ependymal cells was performed in P5 Jhy+/+, JhylacZNeo/+, and JhylacZNeo/lacZNeo (Fig. 34). Ependymal cells located dorsally in the medial wall in Jhy+/+ (Fig. 34A) and JhylacZNeo/+ (Fig. 34B) were arranged in a monolayer of flattened cells, which is the characteristic arrangement of a mature ependymal. On the contrary, dorsomedial cells in JhylacZNeo/lacZNeo displayed a pseudostratified appearance with cuboidal cells, resembling immature ependyma (Fig. 34C). Ventromedial cell in Jhy+/+ (Fig. 34D), JhylacZNeo/+ (Fig. 34E) and JhylacZNeo/lacZNeo (Fig. 34F) all displayed a monolayer of flattened mature ependymal cells. The presence of mature ependymal cells ventrally in JhylacZNeo/lacZNeo brains suggests that ependymal cell differentiation is delayed, rather than blocked, in this animals. We then wondered if this delay in differentiation was also observed in the molecular profile of these cells. For this we performed an
immunofluorescence assay to label the ependymal progenitor (i.e. radial glia) using the radial glia marker Glast (Kuo et al., 2006; Lavado and Oliver, 2011) (Fig. 35). Radial glia differentiation into ependymal cells can be traced molecularly, by the repression of Glast and the expression of mature ependymal marker Vimentin, and the ependymal cilia marker, acetylated α-Tubulin (Acα-Tub) (Lavado and Oliver, 2011; Tissir et al., 2010). Ventromedial and dorsomedial ependymal cells in Jhy+/+ were Glast(-) Vimentin(+) Acα-Tub (+) suggesting that these cells were in a mature stage (Fig. 35A, C). A similar profile was observed in ventromedial cells in JhylacZNeo/lacZNeo which were also Glast(-) Vimentin(+) Acα-Tub (+); however, some dorsomedial cells in JhylacZNeo/lacZNeo retained Glast expression suggesting that these cells were still immature (Fig. 35 B, inset and D). Not surprisingly, this data shows great resemblance to the ependymal phenotype observed in JhylacZ/LacZ (Fig. 8, 10). Altogether this confirms that Jhy is indeed a crucial component for multiple aspects of proper ependymal cell differentiation by morphology and gene expression criteria.
Figure 34. Delayed ependymal cell differentiation in JhylacZNeo/LacZNeo mice. P5 mouse brains were H&E stained for Jhy<sup>+/+</sup> (A, D), JhylacZNeo<sup>+/+</sup> (B, E) and JhylacZNeo/lacZNeo (C, F). Medial wall ependymal cells dorsally and ventrally display a flattened and mature appearance in both Jhy<sup>+/+</sup> and JhylacZNeo<sup>+/+</sup> (A, B, D, E). Ventromedial ependymal cells in JhylacZNeo/lacZNeo also appeared mature, while dorsomedial cells retain pseudostratified immature characteristics (C, F). LV, lateral ventricles.
Figure 35. Some dorsomedial ependymal cells in $JhylacZNeo/lacZNeo$ retained the radial glia marker, Glast. Immunofluorescence of P5 lateral ventricles in $Jhy^{+/+}$ (A, C) and $JhylacZNeo/lacZNeo$ (B, D). Dorsomedial and ventromedial cells in $Jhy^{+/+}$ repress the immature marker, Glast (green), and express ependymal cell markers, Vimentin (pink) and Acetylated α-Tubulin (orange) (A, C). Some dorsomedial ependymal cells in $JhylacZNeo/lacZNeo$ retain the Glast expression (B, inset), while ventromedial cells display only mature ependymal cell markers (D). MW, medial wall.
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EDUCATION

University of Illinois at Chicago, Chicago, IL  
Doctorate of Philosophy in Biological Sciences  
Area of specialization: Mammalian development, genetics and microscopy  
• Thesis: Understanding the role of the mouse Jhy gene in ependymal cells and the specialized ependyma of the choroid plexus

University of Puerto Rico-Mayagüez, Mayagüez, PR  
Bachelor of Science in Biology, Magna cum laude  
Distinction in Biology, Honors College in the College of Arts and Sciences  
Undergraduate researcher with the Sloan Foundation Undergraduate Research Program

RESEARCH EXPERIENCE

Investigated the role of the mouse Jhy gene in ependymal cells and the specialized ependyma of the choroid plexus  
University of Illinois at Chicago, Chicago, IL  
Advisor: Dr. Jennifer V. Schmidt, PhD  
2010-present

My doctoral work focuses on understanding the role of Jhy in the etiology of hydrocephalus using the transgenic mouse line Jhylacz. Though histological and confocal microscopy analysis, I discovered that Jhylacz/lacz brains contained abnormally differentiated ependymal cells, which are the cells responsible for cerebrospinal fluid (CSF) clearance. I performed electron microscopy and ex-vivo imaging of brain ependyma and confirmed that Jhy disruption leads to abnormal cilia patterning and motility defects. Confocal microscopy and mammalian cell culture have been used to assess the functionality of the CSF producing tissue known as the choroid plexus.

Reconstructing the ancestry of humans in the Caribbean through the analysis of maternal lineages  
University of Puerto Rico-Mayagüez, Mayagüez, PR  
Advisor: Dr. Juan Martinez-Cruzado, PhD  
2007 – 2009

This population genetics study was focused on determining the Amerindian ancestry in the Caribbean using mtDNA sequencing. DNA samples obtained from participants were sequenced and analyzed using hallmark mitochondrial mutations to help define the ethnic descent of participants.

Analyzed the effects of estrogen on the expression of clock genes in brain and peripheral tissue of female rats undergoing jet lag  
University of Illinois Urbana-Champaign, Champaign, IL  
Advisor: Dr. Megan M. Mahoney, PhD  
2009

The aim of this study was to determine the role of estrogen in clock gene rhythms in the suprachiasmatic nucleus (SCN), the site of the master clock, following disruption of circadian rhythms. I performed qRT-PCR analysis in estrogen treated female rats to measure the effects of circadian gene, Per2, prior to and post jetlag. Results obtained in this study uncovered a regulatory effect of time of day, rather than estrogen, over Per2 mRNA expression levels.
Identifying interactions between RNA silencing and inflammatory pathways in antiviral response
University of Pennsylvania, Philadelphia, PA  Advisor: Dr. Kathleen Sullivan, MD/PhD  2008

The objective of this work was to identify proteins involved in the crosstalk between the RNA silencing and the inflammatory response pathways in response to Influenza virus. Co-immunoprecipitation of mammalian cell proteins was performed in virus-infected cells and the results demonstrated an interaction between RNA silencing proteins, Argonautes, with a transcription factor involved in the inflammatory response pathway, GCF2.

Genetic analysis of map distances between the White\textsuperscript{O} gene and the differential segment of the sex chromosomes of the crustacean Artemia franciscana.
University of Puerto Rico-Mayagüez, PR  Advisor: Dr. Richard D. Squire, PhD  2007

The purpose of this investigation was to determine the inheritance of an eye color mutation White\textsuperscript{O}, found in the crustacean Artemia franciscana. I performed a detailed analysis of breeding pattern and genetic testcrosses. Results obtained in this study revealed that the White\textsuperscript{O} gene is sex linked, and the white-eye phenotype resulted from a recessive mutation located in the sex chromosome.

AWARDS AND GRANTS

ASCB Minorities Affairs Committee Travel Award, American Society for Cell Biology Annual Meeting 2015
University Excellence in Teaching Award, University of Illinois at Chicago, Vertebrate Embryology 2015
FASEB Marc Travel Award, 27th Annual GSA Mouse Molecular Genetics Meeting 2014
Abraham Lincoln Graduate Fellowship, University of Illinois at Chicago 2010-2011
Sloan Foundation Undergraduate Research Program, University of Puerto Rico- Mayagüez 2007-2009
Outstanding Summer Student Researcher, University of Illinois Urbana-Champaign 2009
Scholarship for Academic Competitiveness, University of Puerto Rico- Mayagüez 2008

PEER-REVIEWED PUBLICATIONS


SKILLS AND STRENGTHS

• 5 years of experience in handling laboratory mice and rats.
• Experienced in antibody techniques for tissue sections and whole mount immunostaining analysis.
• Experience in histological assays, animal tissue sectioning using paraffin, cryostat, microtome and Hematoxylin/Eosin staining.
DNA and RNA isolation/purification, plasmid sub-cloning, restriction enzyme digestion, PCR and qRT-PCR.

Genome sequencing and annotation, NCBI (BLAST), Geneious database.

Protein isolation and quantification, SDS-PAGE, Western Blot, co-Immunoprecipitation analysis.

ELISA (enzyme-linked immunosorbent assay) and statistical microarray data analysis.

Live tissue extraction and handling for ex vivo analysis of cilia movement using a Prairie Multiphoton Laser Scanning Microscope.

Knowledgeable in mammalian cell culture handling, aseptic techniques, cell line maintenance, counting/viability.

Computer: Proficient in Macintosh and Windows operating systems, Microsoft Office, EndNote, Adobe Photoshop and Blackboard.

Microscopy experience


Experienced in Imaris image processing software.

TEACHING EXPERIENCE

Visiting lecturer at the University of Illinois at Chicago Spring 2014, Spring 2015
(Guest Lecturer in Vertebrate Embryology)

Teaching Assistant at the University of Illinois at Chicago Fall 2011-Fall 2015
(Cell Biology Laboratory, Vertebrate Embryology, Mendelian and Molecular Genetics, Genetics Laboratory)

CONFERENCE PRESENTATIONS

Oral Presentations

1. Muniz-Talavera H and Schmidt JV. March 2013. Jhy disruption: a new way of getting a big head. STEM networking meeting for graduate students, University of Illinois at Chicago, Chicago IL

2. Muniz-Talavera H and Mahoney MM. July 2009. The effects of estrogen on the expression of clock genes in brain and peripheral tissue of female rats undergoing jet lag. Summer Research Opportunity Program (SROP) Conference, University of Michigan, Ann Arbor, MI


Poster Presentations


2. Muniz-Talavera H and Schmidt JV. October 2014. The mouse Jhy gene plays a role in ependymal cell differentiation. 27th Annual Mouse Molecular Genetics Conference, Pacific Grove, CA

3. Muniz-Talavera H and Schmidt JV. June 2014. The mouse Jhy gene plays a role in ependymal cell differentiation. 1st Annual MBRB Retreat, University of Illinois at Chicago, Chicago IL


MENTORSHIP EXPERIENCE
- Mentored undergraduate and graduate students in research
- Trained and oversaw the employment of undergraduate work-study laboratory assistants

LEADERSHIP AND EXTRACURRICULAR EXPERIENCE
Science writer at online magazine Bitesize Bio 2015-present
Private tutoring for Science and Spanish at WyzAnt 2015-present
Organized and directed graduate student career panels and workshops at UIC 2014, 2015
Vice-president of the Biology Graduate Student Association at UIC 2014-2015
President of the Biology Graduate Student Association at UIC 2013-2014
Volunteer for the Chicago Expanding Your Horizons 2013 Conference for women in science 2013
Science fair judge for Chicago public elementary schools 2012, 2013

LANGUAGES
Spanish- native language
English- fluent in speaking, reading and writing
American Sign Language (ASL)- beginner level

PROFESSIONAL MEMBERSHIPS
American Society for Cell Biology
Society for Developmental Biology
American Society for Biochemistry and Molecular Biology