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PRIMARY CILIA IN HUMAN NEURON DIFFERENTIATION

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Cover illustration: Made by the author. Immunohistochemistry of a human forebrain cortical organoid section. Primary cilia are displayed in green, neurite networks in red, and nuclei in blue.

Primary cilia in human neuron differentiation

Thesis for Doctoral Degree (Ph.D.)

By

Andrea Coschiera

The thesis will be defended in public at Lecture Hall 9Q Månen, Alfred Nobels Allé 8, Huddinge, on Monday the 3rd of June 2024, at 9:30.

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"We see with the eyes, but we see with the brain as well.
And seeing with the brain is often called imagination."

- Oliver Sacks

Popular science summary of the thesis

Imagine neurons composing the brain as houses we live in. In terms of size, the brain is much bigger and can represent our whole planet. How do we communicate with our neighbors and friends living abroad? Cells, of course, have the answer and build their own rooftop antennae!

Our body is made up of building blocks known as proteins. Every cell contains the same basic language alphabet (DNA) that, once deciphered, produces proteins. However, different body tissues slightly modify this translation based on their specific needs. Communication is a fundamental feature of every living organism, and it is the way we obtain most types of information to increase our knowledge and develop as individuals.

So, what are cilia? Cilia (plural of cilium) are single or multiple hair-like appendages extending from the cell surface. For example, sperm use their cilium (also called flagellum) to move, while other cells use multiple cilia to direct the circulation of biological fluids. Most human cells extend cilia, but here we are exclusively interested in one lesser-known type expressed as a single unit per cell, called the primary cilium. Primary cilia are involved in cell-to-cell communication and, within the brain, in nervous system development. Like TV or radio antennae, cilia can catch signals from the outside and send that information to the cell to affect its behavior and response. A wide range of signals in our body represents that information, including light, odors, hormones, and more generally, proteins. By now, you have probably noticed that cilia act as call centers to keep updating the cells on what happens in the outside world. When a problem occurs in cilia (which can cause a disease class called ciliopathy), cells lose their GPS navigator, which may cause malfunction of several body organs.

Because of the presence of synapses, the neuronal smartphones more efficient and cooler than outdated antennae, primary cilia have been overlooked for a very long time, and their function underestimated. But, as often happens in sports, these “underdogs” of cellular organelles quickly moved “from zero to hero” as soon as their real roles were revealed. Cilia were found essential to regulate several aspects of neuronal growth characteristics before the establishment of synapses, helping neurons develop their “limbs” (axons and dendrites) and directing their journey toward their final workplaces during brain formation. However, a precise and accurate knowledge of the tasks of cilia is still missing, including a model

where it is possible to follow every step of human neuron development from birth to complete maturation.

In **Paper I**, we present an advanced human neuronal cell culture tool where neurons become adults in just a week and extend cilia at every single stage of development. In **Paper II**, we use that tool to implement the current knowledge on why, when, and how cilia are needed. We found cilia to be necessary for directing neuronal growth (axon development), like a construction site supervisor, and to orchestrate it by setting the right tempo, like a metronome, after neuronal birth. Finally, in **Paper III**, we use the same tool again to provide a survey of DNA codes used by the brain to translate DNA in neurons, thereby to better understand the development of neurons and the brain.

In conclusion, we have demonstrated the significance of cilia during human neurodevelopment in precise manner, and we have provided instructions akin to the Rosetta Stone for deciphering the complexities of the human system. Based on recent studies, globally, about three hundred million people suffer from neurodevelopmental disorders. Our discoveries strengthen and will facilitate the understanding of how cilia dysfunction is involved in common diseases like autism, schizophrenia, and dyslexia, with the final goal of developing effective therapies to successfully fight against them in the near future.

Abstract

Primary (non-motile) cilia have long been overlooked, considered vestigial organelles lacking specific functions. In humans, all cell types except sperm, ependymal and bronchial epithelial cells, and oviduct cells extend a single primary cilium. However, it was not until the 1990s that cilia garnered significant interest in biomedical research. Cilia serve as cellular sensors for various stimuli, including mechanical, light, osmotic, and chemoreceptive cues. Their ability to detect, integrate, and transmit external information to the cell is essential for cell signaling, development, and tissue homeostasis.

Among ciliary signaling pathways, the WNT pathway is evolutionarily conserved and regulates crucial aspects of organ patterning, cell proliferation, migration, and fate determination during embryogenesis. Not surprisingly, genetic mutations in WNT pathway-related proteins have been linked to several diseases and different types of cancer.

Alterations in cilia function may lead to the onset of a heterogeneous group of genetic conditions called ciliopathies. Given that cilia are nearly ubiquitous organelles, ciliopathies may affect multiple organs, including the brain. So far, dysfunction of cilia has also been implicated in a spectrum of complex neurodevelopmental disorders (NDDs) such as autism, schizophrenia, and dyslexia.

In **Paper I**, we established an advanced human neuronal ciliated cell model using Lund human mesencephalic (LUHMES) cells. These cells can differentiate into mature neurons in just about a week of culture, and they exhibit ciliation throughout the proliferative, differentiation, and fully differentiated phases. This *in vitro* cell model shared a high gene expression profile similarity with *in vivo* neuronal transcriptomes from humans. LUHMES neuronal cilia were shown to functionally transduce the well-known ciliary Sonic hedgehog (SHH) signaling pathway by regulating its target gene expression.

In **Paper II** we used the LUHMES cell model to explore the role of cilia during human neuron differentiation. Based on a dynamic differentiation ciliation pattern, we identified the functional ciliary time window and found cilia to be relevant in promoting axon outgrowth and branching. Furthermore, we generated a stable LUHMES mutant model for the ciliogenic transcription factor RFX2 and performed transient knockdowns (KDs) of essential ciliary genes *IFT88* and *IFT172*. LUHMES

RFX2 -/-, *IFT88* KD and *IFT172* KD neurons were not as efficient as WT counterparts in promoting the same aspects of neuron differentiation. Altered *RFX2* -/- cilia showed deregulation of the ciliary WNT signaling pathway and subsequent cytoskeleton rearrangement required for proper neuron differentiation.

In **Paper III**, we provided an extensive compendium of how neuron differentiation is molecularly regulated. By analyzing enhancer RNAs (eRNAs) using the native elongating transcript-cap analysis of gene expression (NET-CAGE) method, we revealed nearly triple the number of new LUHMES enhancers important for neuron differentiation, as compared to what was previously known. Our analysis also showed enrichment of active enhancers among certain NDDs, including neuropsychiatric disorders. We employed capture Hi-C (HiCap) to locate target genes through promoter-enhancer interaction. Interestingly, the binding motifs of ciliogenic RFX transcription factors and the transcriptional complex activated by WNT signaling were enriched in both promoter and enhancer sequences.

Altogether, we elucidated strong implications of cilia in neuron differentiation at the cellular level unveiling a critical ciliary time window, and at the molecular level, describing the regulatory machinery promoting neuron differentiation. We speculate that cilia play an essential role in neuron maturation and brain formation, underpinning the biogenesis of aspects of certain neurodevelopmental conditions when their function is disrupted.

List of scientific papers

I. Lauter G.*, Coschiera A.*, Yoshihara M., Sugiaman-Trapman D., Ezer S., Sethurathinam S., Katayama S., Kere J., Swoboda P. **Differentiation of ciliated human midbrain-derived LUHMES neurons**. *Journal of Cell Science*. 2020 Nov 9;133(21):jcs249789.

II. Coschiera A.*, Yoshihara M.*, Lauter G., Ezer S., Pucci M., Li H., Kavšek A., Riedel C. G., Kere J., Swoboda P. **Primary cilia promote the differentiation of human neurons through the WNT signaling pathway**. *BMC Biology*. 2024 Feb 27;22:48.

III. Yoshihara M.*, Coschiera A.*, Bachmann J.*, Pucci M., Li H., Bhagat S., Murakawa Y., Weltner J., Jouhilahti E.-M., Swoboda, P., Sahlén P., Kere J. **Transcriptional enhancers in human neuronal differentiation provide clues to neuropsychiatric disorders.** *Manuscript*.

*Equal contribution - joint first authorship

Scientific paper not included in the thesis

Coschiera A., Watts M. E., Kere J., Tammimies K., Swoboda P. **Human LUHMES and NES cells as models for studying primary cilia in neurons**. *Methods in Cell Biology*. 2023 Jan 28;176:27-41.

Contents

List of abbreviations

AKT	Protein kinase B
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
ATF	Activating transcription factor
BBS	Bardet-Biedl syndrome
bp	Base pairs
CAGE	Cap analysis of gene expression
cAMP	Cyclic adenosine monophosphate
CK1α	Casein kinase 1 alpha
CP	Cortical plate
CRISPRa	CRISPR activation
DBD	DNA binding domain
dCas9	Dead Cas9
DNA	Deoxyribonucleic acid
DVL	Dishevelled
eRNA	Enhancer RNA
FZD	Frizzled
GPCR	G protein-coupled receptor
GSK-3β	Glycogen synthase kinase 3 beta
GWAS	Genome-wide association studies
H3K27ac	Histone 3 lysine 27 acetylation
H3K4me	Histone 3 lysine 4 methylation
Hi-C	High-throughput chromosome conformation capture
HiCap	Capture Hi-C
HN	Human neurons

hTERT RPE-1	Human telomerase reverse transcriptase - retinal pigment epithelial cell-1
IFT	Intraflagellar transport
INPP5E	Inositol polyphosphate-5-phosphatase E
JNK	Rac1/c-Jun kinase
KD	Knockdown
LRP	Low-density lipoprotein receptor-related protein
LSCM	Laser scanning confocal microscopes
LUHMES	Lund human mesencephalic (cell line)
MAP	Microtubule-associated protein
MESC2.10	Human mesencephalic cell line
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin
NDD	Neurodevelopmental disorder
NES	Neuroepithelial stem cell
NET-CAGE	Native elongating transcript-cap analysis of gene expression
NPC	Neural progenitor cell
NPHP	Nephronophthisis
PCP	Planar cell polarity
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
qRT-PCR	Quantitative reverse transcription PCR
RFX	Regulatory factor X
RNA	Ribonucleic acid
ROCK	Rho-associated kinase
ROR	Tyrosine kinase-like orphan receptor
SAG	Smoothened agonist
SDCM	Spinning disk confocal microscope

SHH	Sonic hedgehog
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STRT	Single-cell tagged reverse transcription
SVZ	Subventricular zone
TCF/LEF	T-cell factor/lymphoid enhancing factor
TEM	Transmission electron microscopy
TF	Transcription factor
TFE	Transcript far end
TGF- β	Transforming growth factor-beta
TSS	Transcription start site
VZ	Ventricular zone
WB	Western blot
WNT	Wingless/integrated

1 Introduction

Neurodevelopmental disorders (NDDs) encompass a broad group of neuropsychiatric conditions that predominantly manifest during childhood, characterized by intellectual, linguistic, and/or behavioral disturbances. The complex interplay between genetic and environmental factors complicates our understanding of the neurobiology underlying NDDs.

Given that NDDs often involve brain malformations, the observation of neurological features in certain ciliopathies prompted an in-depth investigation into cilia function and structure in other brain-related non-ciliopathy disorders. Among these, dyslexia stands out as one of the most genetically well-defined NDDs. Dyslexia candidate genes were initially identified as important in controlling neuron migration, axonal and dendritic guidance (Kere, 2011), processes in which cilia were later discovered to play an essential role. Moreover, the presence of certain NDD candidate proteins has been detected at the cilium (Massinen et al., 2011; Chandrasekar et al., 2013), and their gene expression was shown to be regulated by the family of ciliogenic RFX transcription factors (Tammimies et al., 2016). Genetic variations examined through genome-wide association studies (GWAS) have confirmed an overrepresentation of ciliary genes in NDDs, supporting a causal relationship between cilia and neuron maturation defects associated with NDDs (Karalis et al., 2022).

1.1 NEURODEVELOPMENT

1.1.1 Neurogenesis

Neurogenesis is the process by which all neurons of the nervous system originate (see **Figure 1**). It is mainly active during embryogenesis, following the bending of the ectodermal neural plate into the neural tube and the formation of the three primary vesicles (forebrain, midbrain, hindbrain). The ventricular zone of the neural tube consists of highly proliferative multipotent immature cells (neural stem cells) that undergo asymmetric cell divisions, generating another stem cell and a new specialized cell based on environmental stimuli (see **Figure 2A**). The main function of neurogenesis is, therefore, to produce, on a large scale, the entire neuronal (and glial) population composing the adult brain.

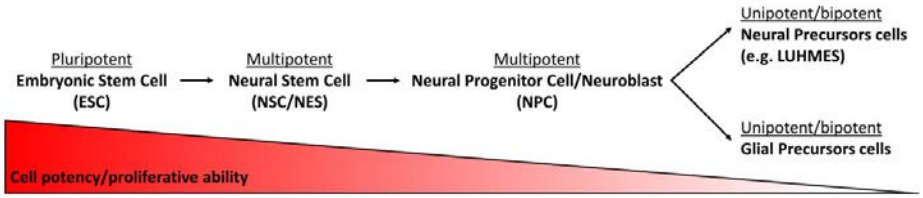

Figure 1: *Schematic representation of neurogenesis progression from pluripotent to unipotent LUHMES-like neuronal precursor cells.*

1.1.2 Neuron migration

Neurogenesis is followed by neuron migration. Specialized cells derived from asymmetrical neural stem cell divisions progressively lose their potency and proliferative abilities until they become postmitotic neuroblasts. Neuroblasts are polarized, undifferentiated cells of neuronal lineage that begin to leave the proliferative ventricular zone to reach their final destination in the cortical plate, completing the formation of the brain cortex. Neuron migration is defined as 'saltatory' due to the intermittent and non-steady cell movement, occurring either radially or tangentially to the ventricular zone based on the parallel or perpendicular transit of the cell, respectively. To achieve this, neuroblasts extend immature neurite-like projections to crawl along a supporting physical scaffold represented by radial glial cells: the leading process marks the direction of migration and will develop into future dendrites, while the trailing process is a short rear process that aids migration and will develop into the future axon.

1.1.3 Neuron differentiation

Upon reaching their final location, neuroblasts continue the differentiation process into neurons that began after becoming postmitotic cells. Neuron differentiation is staged based on morphological and functional maturation. After permanently exiting the cell cycle, newly born neurons begin to protrude a highly dynamic and specialized actin filament (F-actin)-based structure called the growth cone, along with neuron polarization and axon formation/specification processes (stage 1). The growth cone is important for guiding and supporting the initial uniform extension of two opposite and distinct neuron processes (stage 2 or the bipolar stage).

Subsequently, a single neurite (future axon) outgrows the opposite process (future dendrites), breaking the previously established bipolar symmetry (stage 3). Once the axon is determined and further developed (axon branching/arborization), neurons begin to form and refine dendrites and dendritic spines, ultimately leading to the final formation of functional synapses (stages 4 and 5) (Meka et al., 2020) (see **Figure 2B**).

All the anatomical changes described above during neurodevelopmental steps require massive and continuous cytoskeleton rearrangement, particularly of F-actin and microtubule elements, which actively interact. Although stage 2 bipolar neurites may appear morphologically similar, they already exhibit profound structural and molecular differences (Lasser et al., 2018). The microtubules invading the growth cone of the future axon display the same plus-end distal orientation towards the cell body, collected in parallel bundles further stabilized by microtubule-associated proteins (MAPs) and post-translational modifications (acetylation, detyrosination), consistent with the axon function as the main neurite and neuronal transporter. In contrast, the conformation of microtubules in future dendrites is more unstable and dynamic, with enrichment of mixed plus/minus-end oriented microtubules (mostly deacetylated and tyrosinated) and expression of different MAPs and unmodified microtubule domains (Baas et al., 2016). These differences contribute to the elongation and outgrowth of the future axon compared to the opposite neurite. Not surprisingly, alterations in cytoskeleton-related proteins may severely affect neuron differentiation and underlie neurodevelopmental disorders (Lasser et al., 2018).

A combination of intrinsic and extrinsic factors regulates the breakage of bipolar symmetry of stage 2 neurons. Among intrinsic mechanisms, the centrosome (microtubule-organizing center of the cell; MTOC) and the colocalizing Golgi apparatus play a critical role. While both organelles can nucleate microtubules, the Golgi is also relevant for sorting the cargo transported along neurites, specifying the structure of the future axon. Moreover, future axon outgrowth is determined by centrosome-mediated F-actin flow (Meka et al., 2020) and growth cone F-actin dynamics, which, in turn, interact with stable microtubules, leading to neurite elongation (Zhao et al., 2017). Extrinsic factors also play an essential role in promoting the transition from neuronal stage 2 to stage 3. Specifically, the future axon is guided by families of attractor (netrins) and repulsor (semaphorins) molecules. However, a clear understanding of this complex mechanism and the contribution of all components is still under debate.

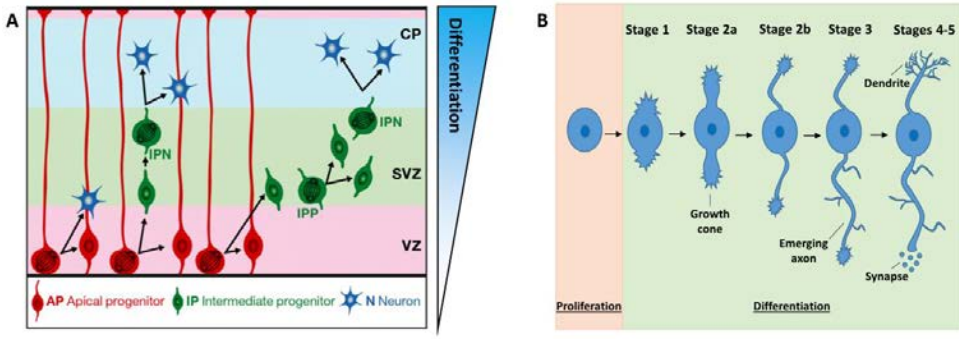

Figure 2: ***(A)*** *Overview of the neuron differentiation "journey" from proliferative progenitors in the ventricular (VZ) and subventricular zones (SVZ) to differentiating/differentiated neurons in the cortical plate (CP) (adapted from Postel et al., 2019).* ***(B)*** *Progressive differentiation stages of postmitotic neurons (from Paper II, Figure 1A).*

1.2 PRIMARY CILIA

1.2.1 Structure and function

Cilia can be grouped into two types: primary (sensory) cilia and motile cilia. Primary cilia are non-motile hair-like appendages protruding from cell surfaces as a single unit. They act as cellular antennae mainly involved in signal transduction and cell-to-cell communication, detecting molecules from the immediate extracellular environment, and transmitting signals outside the ciliary compartment (see **Figure 3**).

Cilia arise from a modified mother centriole (basal body), which is also a component of the centrosome. The cilia core structure, called axoneme, is composed of bundles of microtubule doublets displaying the common 9+0 organization. Cilia are anchored to the cell in the ciliary pocket due to plasma membrane invagination harboring the transition zone with Y-shaped connecting fibers. This domain represents the ciliary gate controlling the entry/exit of ciliary proteins and, consequently, ciliary trafficking and composition. Another essential ciliary component for trafficking and homeostasis is the Bardet-Biedl syndrome (BBS) protein complex, which recognizes cargo and transmembrane signaling proteins. Thus, the BBSome, together with the transition zone, accurately selects the cargo transported by the intraflagellar transport (IFT) protein machinery along the axoneme from the ciliary base to the tip (IFT-B including kinesin motor

proteins, in the anterograde direction) and from the tip to the base (IFT-A including dynein motor proteins, in the retrograde direction). As a result, the ciliary membrane composition makes the ciliary compartment extremely distinct and functionally specialized compared to the contiguous plasma membrane (Mukhopadhyay et al., 2017).

The biogenesis of primary cilia is controlled by the regulatory factor X (RFX) family of transcription factors (TFs), which also interplay and interact with the motile ciliogenesis regulator FOXJ1. RFX TFs possess an evolutionarily conserved DNA binding domain (DBD) that recognizes specific genomic sequences called X-box motifs. In humans, eight RFX TFs have been characterized, and notably, RFX1-4 and RFX7 are expressed in brain and spinal cord tissues (Sugiaman-Trapman et al., 2018). A more detailed description of their specific and redundant functions is still lacking.

Cilia serve as a major signaling hub of the cell, harboring a wide variety of signaling molecules that bind to external cues and activate signaling cascades, amplifying, and transducing information to affect cellular gene expression in response to stimuli. Many signaling molecules functioning at and localizing to the cilium are connected to crucial pathways involved in the regulation of developmental and homeostasis processes, including the wingless/integrated (WNT), Sonic hedgehog (SHH), Notch, G protein-coupled receptor (GPCR), mammalian target of rapamycin (mTOR), transforming growth factor beta (TGF-β), and Hippo pathways (Wheway et al., 2018).

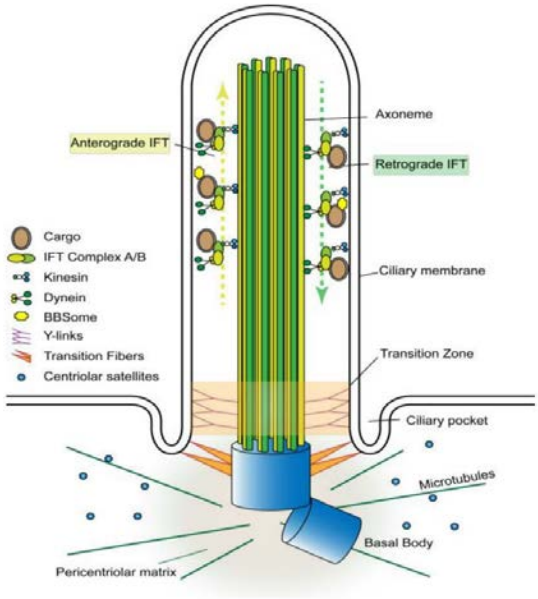

Figure 3: *Simplified representation of cilium structure consisting of centriolar basal body, transition zone and microtubule doublets-based axoneme. The IFT machinery with kinesin motor proteins moves the cargo in anterograde direction to the ciliary tip (IFT-B) and retrograde direction to the base with dynein motor proteins (IFT-A) (Lai and Jiang, 2020).*

1.2.2 Cilia-mediated WNT signaling pathway

WNT signaling is highly evolutionarily conserved with critical roles during embryonic development, affecting cell proliferation, migration, and differentiation. It is characterized by one canonical pathway, dependent on the main mediator β-catenin, and two non-canonical pathways, β-catenin independent: the WNT/planar cell polarity (PCP) pathway, important for regulating cytoskeleton remodeling and cell morphology, and the WNT/Ca2+ pathway, important for regulating intracellular calcium levels (Arredondo et al., 2020). All pathways are activated by the binding between transmembrane Frizzled (FZD) receptors of the G protein-coupled receptors (GPCRs) family and different Wnt protein ligands, totaling nineteen Wnt proteins identified in humans (Willert and Nusse, 2012).

β-catenin, besides being the main activator of the canonical WNT pathway (see **Figure 4A**), also plays a crucial role in stabilizing the cytoskeleton and intercellular tight junctions. In the absence of Wnt ligands, the β-catenin destruction complex

is assembled and activated. It includes four main components: the adenomatous polyposis coli (APC) protein, which marks β-catenin for degradation; the two kinases glycogen synthase 3 beta (GSK-3β) and casein 1 alpha (CK1α), which phosphorylate β-catenin; and the Axin scaffold protein, which assembles the complex. Once β-catenin is phosphorylated and inactivated, it is targeted to the ubiquitin-proteasome system for degradation. In the presence of canonical ligands, Wnt binds to the FZD receptor and co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6), causing the recruitment of the destruction complex to the plasma membrane and its inactivation by the Dishevelled (DVL) protein. As a result, active non-phosphorylated β-catenin can accumulate in the cytoplasm and translocate to the nucleus. β-catenin cannot directly bind to DNA; therefore, it acts as a coactivator for the transcription factor families T-cell factor/lymphoid enhancing factor (TCF/LEF) to induce cellular responses via gene expression regulation (Colozza and Koo, 2021).

The non-canonical PCP WNT pathway is β-catenin independent and uses other co-receptors than LRP5/6, such as receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/2) (see **Figure 4B-C**). Following non-canonical stimuli, DVL is recruited to start the signaling cascade of Rho-associated kinase (ROCK) and Rac1/c-Jun kinase (JNK) pathways, which are major regulators of cytoskeleton and cell morphology. In particular, Rac1/JNK signaling leads to the activation of the activating protein-1 (AP-1) transcription factors complex, including subunits Fos, Jun, and activating transcription factor (ATF) (van Amerongen, 2012).

Considering that both canonical and non-canonical WNT pathways are simultaneously active, cells can modulate their activity by increasing or decreasing the expression of specific endogenous WNTs, assembling cilia, or both. Cilia have been found to be important for finely tuning this balance by decreasing canonical activation in favor of the non-canonical one (Lee, 2020). Thus, several ciliary protein complexes, localizing at the transition zone and basal body, including nephronophthisis (NPHP) components, interact with the proteasome to promote DVL and β-catenin ubiquitin-mediated degradation. However, a clear understanding of the actual role of cilia in regulating this process is still lacking, partly due to controversial findings obtained in different *in vitro* and *in vivo* models where canonical activation does not always appear to be affected when cilia are mutated (Anvarian et al., 2019).

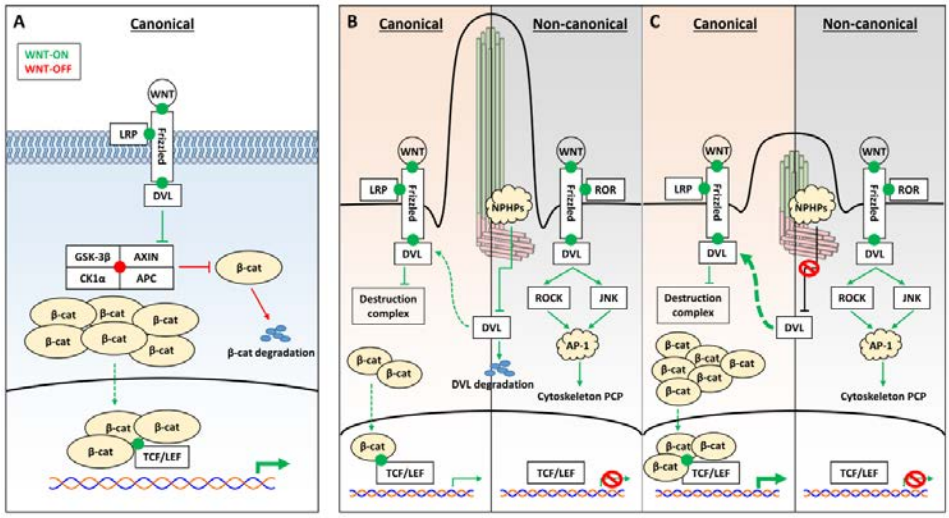

Figure 4: *The primary cilium finely tunes the WNT signaling pathway to promote a switch in balance from canonical to non-canonical activation.* ***(A)*** *Illustration of the canonical WNT/β-catenin signaling cascade in a non-ciliated cell in the presence (green) or absence (red) of a WNT ligand.* ***(B)*** *In the presence of functional cilia, the ciliary nephronophthisis (NPHP) protein complex partially mediates DVL degradation, reducing its inhibition on the destruction complex activity and leading to β-catenin cytoplasmic accumulation. As a result, canonical WNT activation is diminished in favor of non-canonical PCP pathway activity, leading to cytoskeleton remodeling.* ***(C)*** *When cilia are mutated, DVL proteins are not degraded and are recruited to the plasma membrane, where they fully inactivate the destruction complex, resembling cytoplasmic β-catenin accumulation and consequent canonical WNT activation of non-ciliated cells. Therefore, the balance between canonical and non-canonical activation is no longer affected, potentially impairing PCP-mediated cytoskeleton rearrangement (adapted from Paper II, Supplementary Figure S7).*

1.3 CILIA IN NEURODEVELOPMENT

1.3.1 Cilia in neurogenesis

The ventricular zone of the folded neural tube is composed of layers of stem cells that project cilia into the neural tube lumen, which is rich in morphogens such as WNT and SHH. Morphogen distribution follows non-uniform concentration

gradients, and their detection by cilia is critical to instruct the cell and govern the spatiotemporal patterning and tissue development (Lepanto et al., 2016).

Although primary cilia are non-motile organelles, their formation and presence are extremely dynamic and transient in highly proliferative cell types due to the double function of centrioles. Centrioles can either represent the ciliary basal body or, as part of the centrosome, produce mitotic spindle microtubules during cell divisions. Thus, cilia can only be assembled during the G0/G1 phases before being disassembled during the S/G2 phases preceding mitosis. However, the cilium does not get completely disassembled, remaining as a residual ciliary membrane on the mother centriole. The cell inheriting the membrane remnant will more likely maintain stem cell proliferative ability, being more conveniently re-extended to be quickly operative, unlike the sister cell, which will undergo the differentiation process and detach from the neuroepithelium (Paridaen et al., 2013).

Further evidence of the importance of cilia in neurogenesis regulation has been shown following the conditional cilia ablation from the cell, resulting in increased proliferation activity and a reserve of progenitor population at the expense of neuron production, in connection to microcephaly phenotypes (Wilson et al., 2012; Snedeker et al., 2017).

1.3.2 Cilia in neuron migration

The specialized cell generated from the asymmetrical stem cell division leaves the apical ventricular surface in a process called delamination and completely exits the cell cycle. The newborn neuron then extends a short rear trailing process and a leading process, which, together with the growth cone, guide the neuron migration from the ventricular zone to the final cortical area. Cilia and centrosomes direct this journey in a two-step process (see **Figure 5A**). Initially, the cilium is formed but remains non-extended in the cytoplasm, and further extension of the leading process microtubules occurs. The cilium and centrosome move forward within a swelling in the neurite (centrokinesis), where the cilium starts being extended from the plasma membrane. Then, the cytoskeletal microtubules of the initial segment of the neurite retract, pulling the soma toward the cilium-centrosome unit (nucleokinesis), which once again is not extended, completing the first cycle of "saltatory" neuron migration (Matsumoto et al., 2019).

This cilium-dependent process has been investigated in mouse models and found to be consistent in all migrating neurons regardless of the developmental stage (embryo, post-natal, adult) and type of migration (radial, tangential). At the molecular level, it involves the ciliary cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway (see **Figure 5B**). cAMP, a common signaling cascade second messenger, is produced and accumulates in the ciliary compartment before its translocation to the centrosome, where it is detected in transient hotspots during the nucleokinesis phase. PKA completes the pathway downstream and specifically localizes at the centrosome, where it interacts and gets activated by cAMP. Therefore, the cilium and centrosome function as a single signaling unit, where the cilium acts as the producer and the centrosome acts as the effector (Stoufflet et al., 2020).

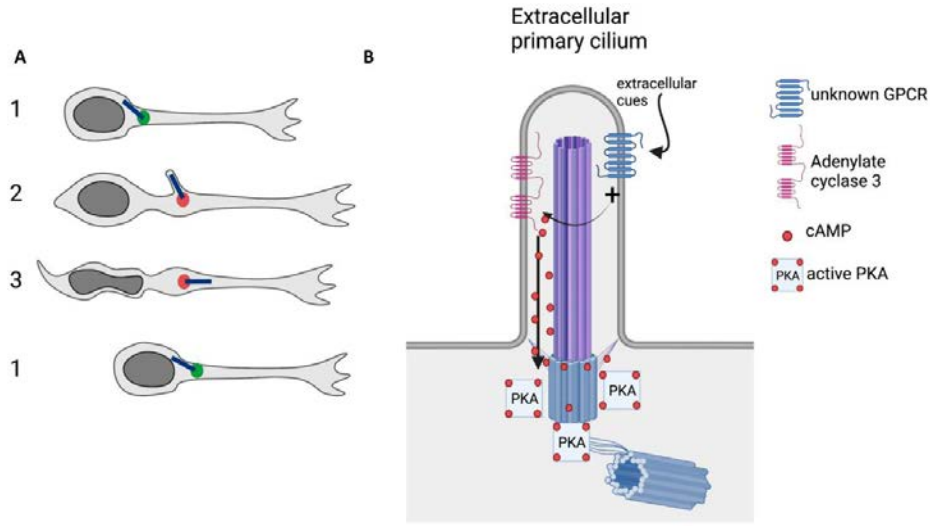

Figure 5: ***(A)*** *Cilium/centrosome dynamics during cyclic saltatory neuron migration. The cilium extends from the plasma membrane only during centrokinesis (phase 2) and not during the extension of the leading process (phase 1) or nucleokinesis (phase 3).* ***(B)*** *Molecular details of the ciliary cAMP/PKA signaling pathway. cAMP molecules are produced in the cilium and translocate to the centrosome, where hotspots (red dot) are detected during the nucleokinesis phase (Stoufflet and Caillé, 2022).*

1.3.3 Cilia in neuron differentiation

Although neurons need to migrate to the proper cortical brain area before differentiating, in reality, neuron migration and differentiation are intertwined and synchronized events. As described in the previous chapter, neuron migration orientation and direction rely on leading and trailing processes, which represent the future dendrites and future axon, respectively. Therefore, these distinct molecular structures are established before or during the initial phases of migration, in a process called neuron polarization. Cytoskeleton organization and rearrangement to determine neuron polarization and axon specification/axonogenesis are mainly regulated by the centrosome (MTOC) and the Golgi apparatus (Meka et al., 2020; Meka et al., 2022), before the cilium is assembled on top of it to direct neuron migration.

Once the neuron reaches its final cortical destination, it needs to mature and functionally connect to neighboring neurons. Guo et al. (2019) provided insight into how cilia play a critical role in the development of axon tracts, wiring, and connecting the brain in mice models. Molecularly, the protein complex consisting of ciliary Arl13b and inositol polyphosphate-5-phosphatase E (INPP5E) is essential for ciliary function. Arl13b regulates the localization of ciliary signaling receptors, while INPP5E controls the composition of the ciliary membrane, including receptor trafficking and activation of the downstream phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling cascade. Mice with mutations in either Arl13b or Inpp5e, resulting in impaired cilia structure and function, display abnormal PI3K/AKT pathway activation, along with reduced axon branching, disrupted growth cone architecture with imbalanced filopodia-lamellipodia morphology, and altered axon orientation. Consequently, defects in axon pathfinding may result in agenesis or hypoplasia of brain areas, leading to disrupted neuronal circuits and severe brain malformation phenotypes (Liu et al., 2021).

Cilia have also been found to be essential during the final steps of neuronal circuit formation. After developing the axon, cilia and ciliary signaling play a key role in refining dendritic connectivity and synapse formation through WNT/β-catenin pathway regulation (Kumamoto et al., 2012) and increasing neuronal excitability and synaptic transmission (Tereshko et al., 2021).

1.3.4 Ciliopathies and neurodevelopmental disorders

Malfunctions or abnormalities in ciliary structure or function can give rise to a group of diseases known as ciliopathies. These genetic disorders are primarily inherited in an autosomal recessive manner. Due to the nearly ubiquitous presence of cilia throughout the body's cells and tissues, ciliopathies can manifest in a wide range of conditions with pleiotropic effects, where one gene affects multiple traits (Mitchison and Valente, 2017; Reiter and Leroux, 2017). This means that multiple organs and systems can be simultaneously affected, including brain-related symptoms, leading to the development of various ciliopathies such as nephronophthisis, Bardet-Biedl, Joubert, and Meckel-Gruber syndromes. Severe neurological phenotypes observed in some ciliopathies have prompted research on cilia functions and defects in certain neurodevelopmental disorders not categorized as ciliopathies, but where proteins expressed or functioning at the cilium are involved when mutated, although their function remains poorly understood (Karalis et al., 2022).

Notably, cilia defects have been increasingly linked not only to the manifestation of neurodevelopmental conditions, including dyslexia (Massinen et al., 2011; Chandrasekar et al., 2013; Kere, 2014; Bieder et al., 2020), autism spectrum disorders, schizophrenia, and bipolar disorder (Reiter and Leroux, 2017; Lauter et al., 2018; Thomas et al., 2019), but also to neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases (Ki et al., 2021).

1.4 GENE REGULATION OF THE NONCODING HUMAN GENOME

1.4.1 Promoters and enhancers

Over 98% of the human genome is composed of noncoding sequences, including intronic regions (Perenthaler et al., 2019). Nonetheless, numerous noncoding sequences are transcribed into noncoding RNAs without ever being translated into proteins. The function of noncoding RNAs is still largely unknown, and there may be many more than those currently known. Most (gene) regulatory elements reside in the noncoding genome and can act either in *cis*, as gene-proximal promoters and gene-distal enhancers, or in *trans*, for example as transcription factors, to regulate gene expression (Signor and Nuzhdin, 2018) (see **Figure 6**). Furthermore, the vast majority of genetic variations, such as single nucleotide polymorphisms (SNPs), occur in noncoding regions and may be associated with

diseases (Zhang and Lupski, 2015), including in the brain, where enhancers are activated in a developmental, cell-type-specific manner, and their dysregulation is linked to neuropsychiatric disorders (Carullo and Day, 2019).

Promoters are short consensus DNA sequences bound by RNA polymerase and transcription factors to initiate the transcription of genes into mRNAs. Promoters are usually located right upstream of their target gene coding region, and their sequences are about 100-1000 base pairs (bp) long. Regulatory elements are located in the core promoter region, composed of sequences that help RNA polymerase bind and recognize the transcription start site (TSS), such as the highly conserved TATA box situated about 25 bp from the TSS (Putta and Mitra, 2010). To allow the protein transcription machinery to bind DNA, the chromatin around promoters must be loosely packed and accessible. Thus, histone modifications vary during gene transcription, with histone 3 lysine 4 trimethylation (H3K4me3) particularly important for transcription activation (Sharifi-Zarchi et al., 2017).

Enhancers are short DNA sequences that can boost gene transcription by hundred-fold or more. While functionally they are less well studied than promoters, structurally they are quite similar, harboring regulatory sequences. They also spatially differ since they do not need to be in proximity to their target genes but can be located even hundreds of thousands of bp upstream or downstream of the TSS (Panigrahi and O´Malley, 2021). Enhancers are typically bidirectionally transcribed to produce enhancer RNAs (eRNAs), and unlike promoters, their orientation can be inverted without decreasing their regulatory activity. The chromatin around enhancers is also open, with histone 3 lysine 4 monomethylation (H3K4me1) and histone 3 lysine 27 acetylation (H3K27ac) modifications. Uniquely, enhancer sequences and their distinct chromatin modifications are associated with cell and tissue-specific gene regulation (Sartorelli and Lauberth, 2020).

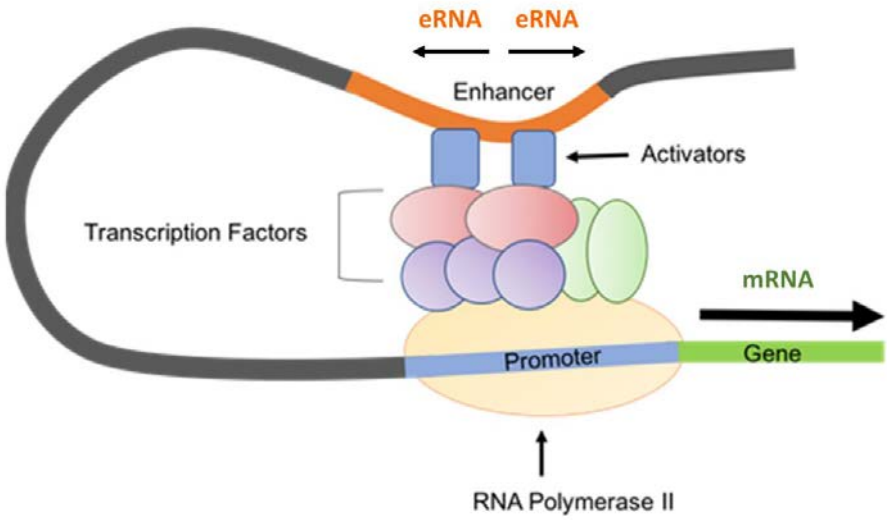

Figure 6: *Simplified illustration of gene expression regulation (adapted from www.addgene.org).*

2 Research aims

The overall aim of this thesis is to further our understanding of the role of human neuronal primary cilia during neuron differentiation in connection to defects observed in certain complex neurodevelopmental disorders and to investigate the molecular mechanisms that govern this process.

- The specific aim of Paper I is to establish an innovative human neuronal ciliated model (LUHMES) where it is possible to study cilia in proliferating, differentiating, and fully differentiated neurons.

- The specific aim of Paper II is to use the LUHMES cell model to precisely resolve the effective ciliary time window promoting neuron differentiation and to investigate how cilia and ciliary signaling impact this process.

- The specific aim of Paper III is to provide a catalog of molecular signatures of enhancer-promoter interactions for regulating LUHMES neuron differentiation, also providing clues on the etiology of neuropsychiatric disorders like schizophrenia and Parkinson's disease.

3 Methodological considerations

3.1 CELL CULTURES

The history of cell cultures is relatively recent. It was only at the end of the 19th century that cells began to be perceived as functional units capable of living autonomously. This understanding led to the establishment in 1951 of the first immortalized cell line, the popular HeLa cancer cells, which are still used as an *in vitro* model today. Nowadays, advanced genome engineering technologies have resulted in more than 4000 cell lines available from the American Type Culture Collection (ATCC), although there are not many non-malignant human neuronal cell lines currently available (see **Table 1**). In all three Papers I, II and III, we utilized Lund human mesencephalic (LUHMES) neuron precursor cells (ATCC). LUHMES cells are a subclone of the human mesencephalic-derived cell line MESC2.10, which originated from ventral mesencephalic tissue of an 8-week-old female fetus (Lotharius et al., 2005) and was immortalized through the retroviral LINX-v-myc vector approach (Hoshimaru et al., 1996; Lotharius et al., 2002). LUHMES cells were subsequently characterized morphologically, immunocytologically, and electrophysiologically by Scholz et al. (2011). As a model system, LUHMES cells offer unique advantages compared to other human neuronal cell lines. Immortalizing LUHMES neuron precursors overcame the common problem of primary cultures in creating a starting large neuronal population, and LUHMES cells were found to be much more sensitive and responsive to exogenous compounds than MESC2.10 cells, resembling primary cell behavior (Fountaine et al., 2008; Schilknecht et al., 2009). Moreover, thanks to the conditional control of the *v-myc* transgene via tetracycline, LUHMES cells differentiate into fully functional neurons within only a week, representing an unparalleled model to study cilia biology during both neuron proliferation and every step of the entire differentiation process. Thus, the LUHMES model allowed us to resolve and identify the dynamic ciliary time window that critically affects neuron maturation (see Paper II).

In Paper III, due to the challenge of efficiently transfecting LUHMES cells, human telomerase reverse transcriptase (hTERT)-immortalized retinal pigment epithelial-1 (RPE-1) cells (ATCC) were used to validate the LUHMES promoter-enhancer interactions identified by capture Hi-C (HiCap). hTERT RPE-1 cells are a commonly used human ciliated cell line, as ciliogenesis can be induced upon serum starvation (Pitaval et al., 2010).

Name and reference	Type of cell line	Advantages	Disadvantages
Neural Progenitor Cells (NPCs) (ATCC)	Primary	Biological relevance. Proliferate and differentiate. Can derive from iPSCs of patients.	Complete differentiation > 1 month.
Neuroepithelial Stem (NES) cells (Falk et al., 2012)	Primary	Biological relevance. Proliferate and differentiate. Can derive from iPSCs of patients.	Complete differentiation > 1 month.
Human Neurons (HN) (ScienCell Research Laboratories)	Primary	Biological relevance.	Do not proliferate. No long-term cultures.
MESC2.10 (Lotharius et al., 2002)	Immortalized	Proliferate and differentiate in a week.	Unstable dopaminergic phenotype. Low sensitivity to exogenous compounds.
LUHMES (Lotharius et al., 2005; Scholz et al., 2011; this thesis)	Immortalized	Proliferate and differentiate in a week. Stable dopaminergic phenotype. High sensitivity to exogenous compounds.	Synaptic integration studies may require longer maturation and/or exposure to other factors.

Table 1: *Common non-malignant human neuronal cell lines available.*

3.2 GENETIC MANIPULATION OF LUHMES CELLS

3.2.1 CRISPR/Cas9 gene knockout

In Paper II, to investigate cilia function during neuron differentiation, we generated a stable ciliary mutant model for the ciliogenic transcription factor RFX2 using CRISPR/Cas9 technology (see **Figure 7**). Among the eight RFX transcription factors in humans, RFX2 is highly expressed in brain tissue and plays a critical role in ciliogenesis. The CRISPR/Cas9 construct was designed with specific guide RNAs targeting our region of interest, and the Cas9 enzyme fused with GFP was used for visualization. Although LUHMES neurons are known to be difficult to transfect, we achieved sufficiently good transfection efficiency using both lipid-based transfection and electroporation methods. Our CRISPR/Cas9 system was designed to delete nucleotides (22 bp in *allele a*, 43 bp in *allele b*), resulting in a premature translational stop codon upstream of the DNA binding domain (DBD) coding region. Disruption of the DBD is essential to impair the function of RFX2 as a transcription factor and prevent its regulation of ciliary target genes. The resulting sequence deletions were verified by Sanger sequencing, mRNA expression levels were assessed by quantitative reverse transcription PCR (qRT-PCR), and protein expression was evaluated by Western blot (WB) analysis, confirming a significant reduction of mRNAs and complete absence of the final protein product.

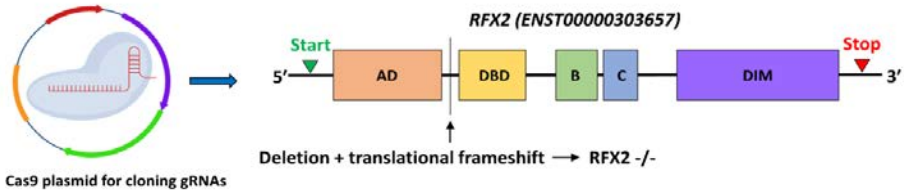

Figure 7: *Genetic engineering of RFX2 gene by CRISPR/Cas9 (adapted from Paper II, Figure 3A).*

3.2.2 siRNA-mediated gene silencing

In Paper II, to confirm that the observed neuron differentiation defects in *RFX2* -/- neurons were indeed caused by cilia alterations, we employed an additional independent method to knock down the expression of *IFT88* and *IFT172*, two ciliary genes essential for cilia assembly and function. One of the most traditional

techniques for reducing the expression of a specific gene is using small interfering RNAs (siRNAs). These small molecules, typically around 20-25 nucleotides in length, are complementary to mRNA sequences of a specific target gene, leading to mRNA cleavage and transient downregulation of its expression. To maximize the effects, we pooled four different single siRNAs specifically designed for each gene before transfecting LUHMES cells with lipofectamine. As a control, LUHMES cells were also transfected with non-specific scrambled siRNA sequences conjugated with fluorophores (Alexa Fluor 488) to assess transfection efficiency, and the relative mRNA expression levels were quantified by qRT-PCR.

3.3 IMMUNOCYTOCHEMISTRY AND CONFOCAL MICROSCOPY

Most of the results from Papers I and II originated from the immunocytochemistry method. This technique relies on specific antibody recognition and binding to the antigen and can be either direct, when the primary antibody is conjugated with a fluorophore, or indirect, when the secondary antibody is conjugated and binds to the unlabeled primary. Although the direct method allows the simultaneous use of primary antibodies isolated from the same animal host, the indirect method (used in Papers I and II) is more commonly employed due to its high sensitivity, flexibility, and cost-effectiveness. In the indirect method, multiple fluorescent secondary antibodies bind to each single primary antibody, and they are conveniently obtainable.

Primary cilia size varies widely depending on the cell type, ranging from 1 μm in chondrocytes to up to 30 μm in kidney epithelial cells (Saggese et al., 2012). LUHMES cells, in particular, extend short cilia (~2-5 μm long) on their surface (Coschiera et al., 2023), which presents a challenge for their detection and structural studies. However, these cilia are present throughout the entire process of neuron differentiation (as discussed in Paper I), making LUHMES cells a convenient model for studying neuronal cilia function.

Differently from live cell imaging, immunocytochemistry is performed on fixed cells, limiting our analysis to a snapshot of the current protein localization and biological situation of the cell. This approach may potentially overlook important aspects of highly dynamic processes such as neuron differentiation and primary cilium assembly/disassembly. Nonetheless, only a few samples are sufficient to analyze a large population of neurons, which is necessary to accurately identify

the ciliary contribution to promoting neuron differentiation (as discussed in Paper II).

Given the small size of primary cilia, fluorescent detection typically necessitates the use of confocal microscopy. Unlike widefield microscopy, which collects light from all in-focus and out-of-focus planes, laser scanning confocal microscopes (LSCM) utilize a pinhole in front of the light source and one before the detector to target the specimen at a single point, thereby shielding the detected light from out-of-focus planes. This setup drastically improves contrast and signal-to-noise ratio (Shaw, 2006). Since the majority of my experimental work involved a large number of cells, all immunocytochemistry images were acquired using a spinning disk confocal microscope (SDCM). Unlike LSCM, which scans point by point, SDCM utilizes a disk with hundreds of pinholes rotating at high speed, enabling faster imaging of specimens, and helping to reduce the occurrence of photodamage and photobleaching.

3.4 MODULATION OF CILIARY SIGNALING PATHWAYS

LUHMES cells have been demonstrated to serve as a valid model for drug treatment, particularly in neurotoxicity assays and drug screening (Smirnova et al., 2016; Beliakov et al., 2023). Primary cilia are involved in the transduction of a wide number of signaling pathways (Wheway et al., 2018). In Paper I, to demonstrate that cilia of LUHMES cells are not only extended but also functional in their primary role as signal transducers, we artificially manipulated the ciliary SHH pathway using both an agonist (Smoothened agonist; SAG) and an antagonist (cyclopamine). Besides being one of the most well-characterized ciliary signaling pathways, the SHH pathway is conveniently tested because most cells share common target genes expressed to regulate the pathway in a negative or positive feedback loop manner (*PTCH1* and *GLI1*, respectively) (Carballo et al., 2018). In Paper II, we modulated the WNT pathway due to its relevance in regulating neurogenesis, neuron differentiation, and development (Inestrosa and Varela-Nallar, 2015; Arredondo et al., 2020). Firstly, we used a pathway antagonist (Wnt-C59) to observe at both cellular and molecular levels how the absence of WNT activation impairs aspects of axon differentiation and negatively regulates the expression of key genes involved in cytoskeleton remodeling. Then, we tested an extracellular WNT agonist (Wnt3a) to determine how altered *RFX2* -/- cilia differently respond

to environmental WNTs compared to WT cilia, affecting downstream target gene expression.

3.5 TRANSCRIPTOME ANALYSIS BY RNA-SEQUENCING

3.5.1 STRT and CAGE

Single-cell tagged reverse transcription (STRT) (Islam et al., 2011) and cap analysis of gene expression (CAGE) (Kawaji et al., 2014) are RNA-sequencing (RNA-seq) methods to capture the transcript far 5´ ends (TFEs) to quantify the transcript expression at the TSS level. Both methods follow similar protocols for retrotranscribing RNA into cDNA, using either oligo-dT primers to specifically detect the poly-a tail of mRNAs (STRT) or random primers to capture all RNAs (CAGE). While in CAGE, the 5´ caps are trapped using biotinylation and streptavidin-coated magnetic beads, in STRT, cDNA templates are first tagged with unique barcodes and then pulled down using the same biotin-streptavidin system. In comparison to 3´ UTR tag RNA-seq, both STRT and CAGE are also able to identify essential promoter elements contained within the 5´ UTR (Mittanck and Rottwein, 1997), providing relevant insights into gene regulation dynamics.

In Paper I we used STRT (see **Figure 8A**) to compare the gene expression time course profile of LUHMES WT with single-cell RNA-seq data from fetal human midbrain (La Manno et al., 2016). In Paper II we used STRT to compare the transcriptomics analysis of LUHMES WT with the mutant LUHMES *RFX2* -/- background. In Paper III, CAGE (see **Figure 8B**) was used to obtain information on transcribed promoters and integrate them with transcribed enhancer data from NET-CAGE.

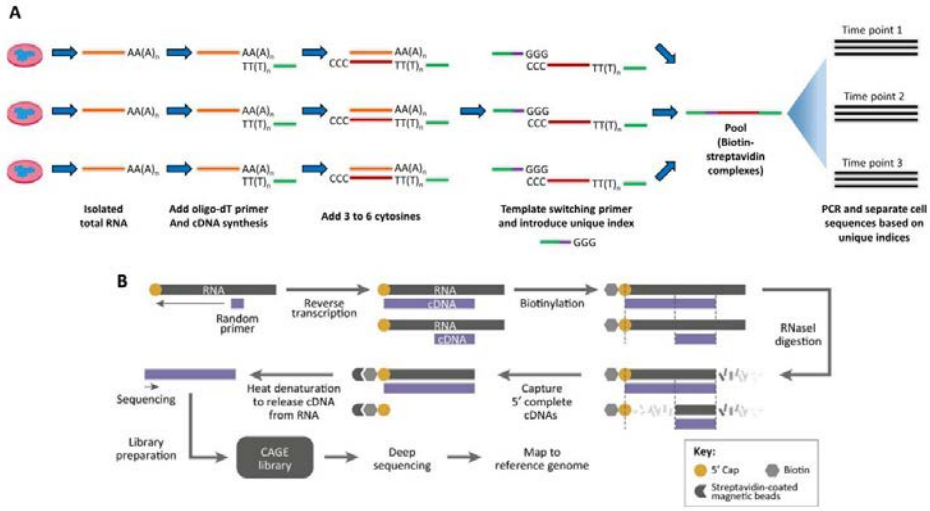

Figure 8: *Workflow illustrating STRT **(A)** and CAGE **(B)** RNA-seq protocols for library preparation (adapted from Murakawa et al., 2016).*

3.5.2 NET-CAGE

Native elongating transcript-cap analysis of gene expression (NET-CAGE) was a method developed by Hirabayashi et al. (2019) to characterize *cis*-regulatory elements, particularly enhancer sequences, in cells and tissues (see **Figure 9**). eRNAs are very unstable transcripts with a short half-life; therefore, they must be purified while being transcribed by RNA polymerase II in the nucleus, rather than from the highly processed cytoplasmic total RNA. Thus, NET-CAGE resulted in approximately three times greater sensitivity than CAGE for mapping enhancer loci. Enhancers were also found to be transcribed in a more cell type-specific manner than promoters, making their analysis essential for understanding molecular mechanisms behind the regulation of biological processes such as neuron differentiation.

In Paper III, LUHMES cells were harvested and lysed to separate the cytoplasmic fraction (containing total RNA) from the nuclear fraction, while blocking transcription activity with α-amanitin. The nuclei were further lysed to separate the soluble nucleoplasmic fraction from the insoluble chromatin fraction. The chromatin fraction was treated with DNase, and the remaining nascent RNAs were isolated and purified.

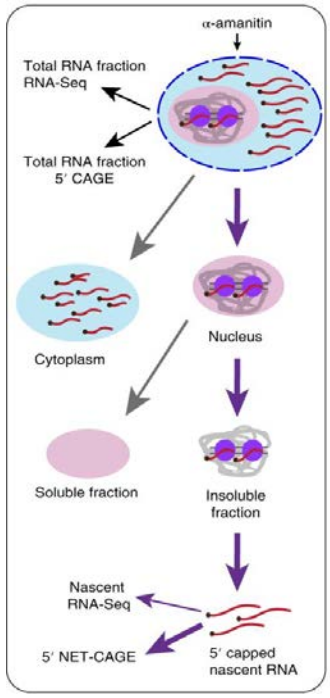

Figure 9: *Overview of NET-CAGE nascent RNAs isolation protocol (Hirabayashi et al., 2019).*

3.6 PROMOTER-ENHANCER INTERACTIONS ANALYSIS

3.6.1 HiCap

Chromosome conformation capture (3C) was developed to investigate physical chromatin interactions that bring distal enhancers in close spatial proximity to promoters to regulate gene expression (Lieberman-Aiden et al., 2009) (see **Figure 10**). Although several techniques exist to map enhancers genome-wide, locating enhancer target genes remains challenging. Sahlén et al. (2015) combined 3C high-throughput with sequencing methodologies (Hi-C) and captured only interactions of regions of interest (HiCap), thereby enhancing sensitivity and achieving the detection of enhancer-promoter interactions with close to single-enhancer resolution.

In Paper III, we used HiCap in combination with data from eRNAs (NET-CAGE) and mRNAs (CAGE) to gain insight into how neuron differentiation is molecularly

regulated at different stages: the beginning (day 1 of differentiation; d1), the middle (d3), and the end of the process (maturation) (d6).

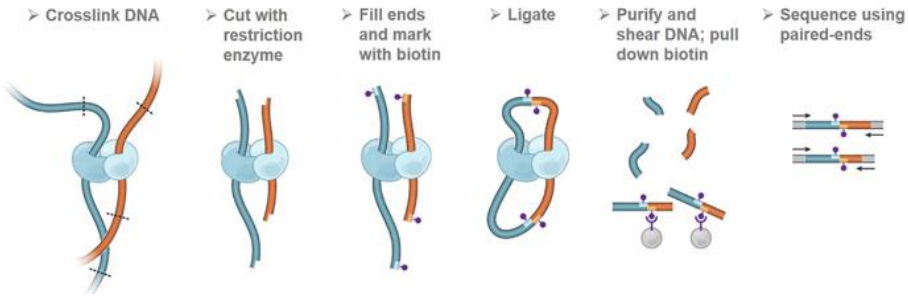

Figure 10*: Overview of Hi-C protocol (Lieberman-Aiden et al., 2009).*

3.6.2 Validation of promoter-enhancer interactions by CRISPRa

To validate genome-wide chromatin interactions detected by HiCap, in Paper III, we performed CRISPR-mediated gene activation (CRISPRa) on hTERT RPE-1 cells. CRISPRa was employed to activate the transcription of target genes and consisted of a plasmid encoding a catalytically inactivated Cas9 protein (dead Cas9; dCas9) ribonucleoprotein fused to a series of activation domains (Weltner et al., 2018). dCas9 was transfected into cells along with guide cassettes assembled by PCR (455 bp), containing the specific sequence of either promoters or enhancers, to guide the dCas9 to the selected target gene. The promoter-enhancer interaction, mapped by HiCap to regulate specific gene expression, was considered validated if the CRISPRa system increased the target mRNA expression when both promoter and enhancer guide cassettes were used, in comparison to either promoter or enhancer sequences by themselves. Moreover, no effect was expected if the specific enhancer sequence was used together with a nonspecific promoter sequence as a negative control.

3.7 ETHICAL CONSIDERATIONS

LUHMES and hTERT RPE-1 cells are immortalized lines purchased from ATCC without, therefore, any ethical concern regarding the use of human tissues.

4 Results

4.1 Paper I: LUHMES-derived neurons as model to study human neuronal cilia

The LUHMES cell line has been previously characterized and established as a robust research model, where neuron precursors differentiate into functional mature neurons within a week, making it ideal for studying neurodevelopmental processes. Due to the lack of established human neuronal models that are ciliated, we further characterized LUHMES cells, focusing on primary cilia biology.

We quantified the efficiency with which neurons become completely post-mitotic after 24 h of tetracycline exposure to shut down *v-myc* transgene expression during differentiation. Employing a number of fluorescent markers directed to centrosome and cilium structural components, we demonstrated that LUHMES cells are ciliated during proliferation (d0) and during all phase of differentiation (d1-6). As the final proof of cilia presence beyond question, we performed transmission electron microscopy (TEM), displaying the typical basal body 9+0 triplet microtubules and the axoneme 9+0 doublet arrangements.

To validate LUHMES cells as a model for studying neuronal cilia, it is important not only to demonstrate their presence but also to assess their function as signal transducers. We exposed LUHMES cells to an agonist (SAG) or antagonist (cyclopamine) of the known ciliary signaling pathway SHH and detected mRNA levels of specific SHH target genes, showing significant upregulation and downregulation, respectively, compared to control samples.

To further enhance the appeal of the LUHMES cell model as a novel *in vitro* research tool for investigating neuronal cilia, we compared its coding transcriptome analysis with the coding transcriptome of *in vivo* human fetal midbrain cells. This highlighted two important aspects: clear separate expression profiles between proliferating neuron precursors (d0) and differentiating neurons (d2-6), and strong expression profile overlap between the expected *in vitro* LUHMES neurotransmitter phenotype and the corresponding *in vivo* midbrain neuronal population type. Moreover, molecular analyses of neuron differentiation showed a significant enrichment of upregulated genes involved in axon and neuron development, along with increased RFX transcription factor binding motif activity (RFX2-3) and consequent upregulation of ciliary genes.

Altogether, we have established a convenient and reliable human neuronal model to study primary cilia in proliferating, differentiating, and differentiated, mature neurons in connection with the biological etiology of neurodevelopmental and neurodegenerative disorders. LUHMES neurons extend functional cilia and represent a valuable *in vitro* model resembling the gene expression profile and behavior of *in vivo* midbrain neurons.

4.2 Paper II: Cilia-mediated switch from canonical to non-canonical WNT signaling promotes axon differentiation of human neurons

After establishing a ciliated human neuronal cell model, we further investigated how cilia and ciliary signaling contribute to promoting human neuron differentiation of LUHMES neurons. Considering that LUHMES cells uniquely complete their maturation in just a week, we were able to follow and analyze the ciliation pattern at every step of neuron differentiation. We found that the percentage of ciliated cells started rising after the cell cycle exit (upon culture medium change), peaked on day 3 of differentiation (approximately 70% of ciliated cells), and steadily decreased alongside the completion of the maturation process (d6). Within the neuronal population, cilia were mostly extended on stages 2-3 neurons, promoting the neuron bipolar symmetry break and outgrowth of the emerging axon. These observations illustrated a critical ciliary time window where cilia effectively function to affect neurodevelopment.

To accurately estimate the impact of cilia on neuron differentiation, we mutated the brain-relevant ciliogenic transcription factor RFX2. This mutation led to structural anomalies (*RFX2* -/- cilia were significantly longer than WT cilia) and functional abnormalities. Thus, *RFX2* -/- altered cilia were notably less efficient than WT cilia in promoting axon outgrowth. Additionally, we analyzed another critical neuron differentiation process, axon branching/arborization. Ciliated WT neurons displayed more branched axons than their non-ciliated counterparts, whereas no anatomical difference was observed between the axons of ciliated and non-ciliated *RFX2* -/- neurons.

To strengthen the causal connection between cilia alterations and neuron differentiation defects, we performed siRNA-mediated gene silencing of two other well-known ciliary genes, *IFT88* and *IFT172*. Downregulation of gene expressions resulted in fewer and shorter cilia, and, similarly to *RFX2* -/- neurons, in a reduced number of ciliated neurons capable of outgrowing the axon and reaching differentiation stage 3 compared to ciliated control neurons. Interestingly, a different trend to *RFX2* -/- neurons was observed when axon branches were quantified. Lack of IFT88 or IFT172 translated into axons of ciliated neurons being significantly less branched than control ones.

After extensively studying LUHMES neurons at the cellular level, we analyzed their gene expression signatures in time course experiments covering the entire differentiation process. The differentiation of *RFX2* -/- neurons consistently

resulted in delays at each step (d1-6) together with downregulation of genes involved in axon and neurodevelopment compared to WT neurons. Interestingly, within this cluster of downregulated genes, a significant enrichment of binding motif sequences specific for transcription factor families belonging to the ciliary WNT signaling pathway, and specifically to the non-canonical branch, was found.

WNT signaling is a known and established pathway for regulating neurogenesis, differentiation, and neurodevelopment, and its activation is modulated by primary cilia. To determine the impact of WNT signaling on LUHMES neuron differentiation, we treated the cells with a drug (Wnt-C59) that inhibits the activation of the pathway by acting on endogenous secreted WNT ligands. In WT neurons, lack of WNT activation resulted in fewer neurons capable of progressing from stage 2 to stage 3 and outgrowing their axons, regardless of the presence of cilia. Altered *RFX2* -/- cilia, as described above, already impaired axon outgrowth, and Wnt-C59 treatment did not show any cumulative effect in that regard. Inhibition of the WNT pathway also led to deregulation of axon branching and pruning processes in WT neurons. Again, the antagonist treatment did not further deregulate axon branching in *RFX2* -/- neurons compared to control samples. The effects of WNT signaling were also analyzed at the molecular level. Treatment with Wnt-C59 resulted in significant downregulation of genes essential for cytoskeleton remodeling, including some non-canonical WNT target genes, similar to the basal expression level found in the RFX2 -/- background, thereby causally connecting cilia alteration to the morphological and differentiation defects observed.

To understand how cilia modulate the WNT pathway and, consequently, affect aspects of neuron differentiation, we examined the cellular expression pattern of the active form of β-catenin, the main mediator of the canonical WNT pathway. We found that cilia of WT neurons reduced β-catenin nuclear translocation and canonical WNT activation compared to non-ciliated neurons, while *RFX2* -/- ciliated neurons displayed nuclear accumulation as strong as non-ciliated ones.

Altogether, we characterized ciliation throughout the entire human neuron differentiation process, identifying a functional ciliary time window. We provided further insight into the roles of cilia during neuron differentiation and their essential contribution post neurogenesis to modulate the WNT signaling pathway in favor of non-canonical (β-catenin-independent) activation to promote cytoskeleton remodeling supporting the anatomical changes during neurodevelopment.

4.3 Paper III: Transcriptional landscape of human neurodevelopment

In both previous papers, we extensively characterized the human neuron differentiation process at the cellular level, focusing on cilia function and identifying a functional ciliary time window. In Paper III, we investigated the molecular regulatory machinery governing the neurodevelopment of LUHMES neurons.

To observe how the regulatory landscape dynamically changes during neuron differentiation, we performed CAGE and NET-CAGE analyses of three stages of LUHMES neuron differentiation: d1, d3, and d6. Through NET-CAGE analysis, we identified 47.350 active and dynamically regulated enhancers, of which 31.057 were newly discovered. Upregulated enhancers at d3 and d6 were enriched in genes related to axon development.

To uncover the most relevant transcription factors regulating neuron differentiation, we analyzed enhancer-associated binding motifs. Consistent with previous findings in Paper II, we found that upregulated enhancers at d3 were enriched with binding motifs of transcription factors related to non-canonical WNT pathway activation, while at d6, there was significant involvement of ciliogenic RFX transcription factors, confirming the essential role of cilia and ciliary signaling during the process.

Considering that ciliary defects contribute, at least in part, to the pathogenesis of several neurodevelopmental disorders, we assessed the enrichment of enhancer SNPs, expressed in LUHMES neurons, related to neuropsychiatric conditions using GWAS. We found overrepresentations associated, among others, with Parkinson's disease, schizophrenia, and bipolar disorders, consistent with the midbrain-derived dopaminergic origin of LUHMES neurons.

Only a small percentage of enhancers regulate the closest gene. Using targeted chromatin conformation capture HiCap, we identified LUHMES enhancer target genes through enhancer-promoter interactions, finding that differentially expressed enhancers were more likely to interact with differentially expressed genes.

Altogether, our work nearly tripled the number of known LUHMES neuronal transcriptional enhancers. Consistent with the ciliary time window found in Paper II, cilia-related activity increased after d1 of differentiation, highlighting their relevant function in regulating the process. Moreover, new enhancers overlapped

with GWAS hits for certain neuropsychiatric disorders. As enhancer activity is tissue-specific, their discovery and study become a powerful tool to better understand certain aspects of the pathogenesis of complex neuropsychiatric conditions.

5 Discussion and future perspectives

Our research advanced the current knowledge of how the human nervous system develops. Establishing the ciliated human neuronal LUHMES cell model, we were able to extensively investigate primary cilia and ciliary signaling at the cellular level during the whole neuron differentiation process and to molecularly uncover the different regulatory element dynamics between neural precursor cells, differentiating and fully mature neurons.

Cilia were mostly studied in either non-human or non-neuronal models. With the LUHMES cell model, we could provide the scientific community with a tool where it is possible to study human neuronal cilia *in vitro* at a similar qualitative level as *in vivo*. To better understand how cilia contribute to promoting neuron differentiation, we generated neurons with altered cilia function: a mutant model for the ciliogenic RFX2 TF, and two KD lines for the essential ciliary genes *IFT88* and *IFT172*. We found that functional cilia were needed in the early time window of differentiation to promote the axon development process, specifically aiding in the outgrowth of the opposite neurite and the projection of branches. To aid this critical cell cytoskeleton remodeling, we showed that cilia are implicated in the regulation of the canonical WNT signaling pathway main mediator β-catenin, reducing its nuclear activation. With advanced sequencing techniques, we mapped the transcriptional enhancer landscape of human neuron differentiation and identified genomic loci affected by SNPs in connection to relevant neuropsychiatric disorders like Parkinson's and schizophrenia.

Cilia biogenesis is dynamically regulated during cell cycle progression (Plotnikova et al., 2009). After adding the differentiation medium, LUHMES cells further proliferate for an additional 24 h before starting to differentiate and the neuronal population always consists of ciliated and non-ciliated neurons throughout the whole process. This showed that LUHMES cells become post-mitotic in a non-synchronous way based on the cell cycle phase they are involved in when the culture condition changes. After successfully testing cilia function of LUHMES neurons by exogenous activation of the ciliary SHH pathway, we accurately characterized neuronal ciliation during the maturation process. We found that ciliation gradually increased and peaked at d3 of differentiation (~70% of ciliated cells), before decreasing during the later phases of the maturation process. We speculate that cilia and ciliary signaling become relevant after the first steps of neuron differentiation (polarization, axonogenesis, initial neurite elongation) mainly

driven by the centrosome (MTOC) (Meka et al., 2020; Meka et al., 2022). This suggests that cilia play a critical role in regulating the subsequent differentiation steps (axon outgrowth/branching, migration), before being disassembled in concomitance with the establishment of synapses, the "final" cell signaling tool. In accordance, we observed a predominant cilia distribution on differentiation stages 2 and 3 neurons, when axon outgrowth and further development occur after breaking the bipolar symmetry of neurites.

To improve this correlation between cilia and axon outgrowth we mutated the ciliogenic TF RFX2 and knocked down the expression of two essential ciliary structure genes, *IFT88* and *IFT172*. Among the eight human RFX TFs, LUHMES neurons highly express *RFX1-3*, *RFX5*, and *RFX7*, whereas *RFX5* is not connected to ciliogenesis (Choksi et al., 2014). On the other hand, *RFX2* is widely expressed in brain tissues (Sugiaman-Trapman et al., 2018), in LUHMES neurons it is the most expressed, and it has been only implicated in either ciliogenesis during vertebrate development (Chung et al., 2012; Lemeille et al., 2020) or in non-brain-related processes like spermatogenesis (Wu et al., 2016). Following stable *RFX2* knockout, we detected defects of cilia function, a significant downregulation of several ciliary genes involved in relevant ciliary signaling pathways, and cilia structure. *RFX2* -/- cilia resulted significantly longer than the WT ones. Although previous works observed shorter/truncated cilia in *RFX2* -/- cells, these mutations were carried out in different organisms (Zebrafish, Xenopus), with a transient and not stable gene expression downregulation (Bisgrove et al., 2012; Chung et al., 2012). Therefore, RFX2 in LUHMES neurons may actually act as inhibitor of ciliogenesis. Also, ciliation is slightly accelerated in *RFX2* -/- neurons and, based on our transcriptomics data, it is the only RFX TF highly expressed in the early days of differentiation (d0-d2, when general ciliation is low) before being downregulated during the subsequent maturation days (in contrast with ciliation increase).

Taking advantage of the non-synchronous differentiation of LUHMES cells, we examined two important aspects of neuron differentiation (axon outgrowth, axon branching) in WT and *RFX2* -/- ciliated and non-ciliated neuronal populations, specifically in the early time window of differentiation. While in the WT background having a cilium correlates with a higher percentage of neurons able to break the bipolar symmetry and outgrow the axon (stage 3), *RFX2* -/- neurons showed no axon outgrowth differences between ciliated and non-ciliated cells, delaying the overall differentiation process. Similarly, axons of ciliated WT cells were more branched than the non-ciliated ones, and, once again, no difference was observed

in *RFX2* -/- neurons. Interestingly, axons of non-ciliated *RFX2* -/- neurons appeared as branched as the ciliated ones, potentially implicating *RFX2* as a negative regulator of axon branching, facilitating the axon pruning process neurons normally undergo (Creighton et al., 2021). To confirm the cilium as being responsible for regulating these differentiation aspects, we transiently downregulated the expression of two well-known components of the IFT-B complex (*IFT88*, *IFT172*) to perturb ciliogenesis (Wang et al., 2021). We then performed the same cellular observations, detecting fewer ciliated *IFT88* and *IFT172* KD neurons with significantly shorter cilia in comparison to the control WT. As expected, ciliated *IFT88* and *IFT172* KD neurons also resulted in being less competent to break the bipolar symmetry and reach stage 3 (axon outgrowth), and, in contrast to *RFX2* -/- neurons, to develop axon branches. This suggests how different aspects of neuron differentiation may be regulated by independent mechanisms and strengthens the hypothesis of *RFX2* involvement in trimming the excess of branches of developing axons.

After an extensive cellular study, we performed the molecular transcriptomics analysis of LUHMES neurons. Applying the STRT RNA-seq method we compared the LUHMES WT gene expression profile with available data from *in vivo* human fetal midbrain during the differentiation of precursor cells into neurons (La Manno et al., 2016), finding highly similar patterns indicating the experimental value of LUHMES neurons as *in vitro* cell model. By STRT RNA-seq we also compared the differentiation time course between WT and *RFX2* -/- neurons. This confirmed the overall delay by which *RFX2* -/- neurons undergo the maturation process already in the early time window of differentiation, resulting from a significant downregulation of genes enriched with GO terms related to neuron differentiation. Therefore, alterations, even mild ones, of cilia function and/or structure seem able to disrupt relevant aspects of the process, where also proper timing represents an essential factor in the organization of a such precise sequence of cellular events. To further uncover the molecular mechanisms that govern human neuron differentiation, we applied two more RNA-seq methods to study the main genomic *cis*-regulatory elements. We integrated CAGE and NET-CAGE analyses (Hirabayashi et al., 2019) to identify promoters and the lesser-explored enhancer sequences at the beginning, the middle, and at the end of neuron differentiation, almost tripling the amount of LUHMES neuronal enhancers currently known. Different from promoters, enhancers modulate gene expression in a more cell and tissue type-specific fashion, therefore studying their sequences becomes crucial

to elucidate how complex systems are molecularly regulated. Detection of enhancer-associated sequence motifs gave us important clues to which TFs are dynamically expressed in different phases of neuron differentiation and allowed to reveal enhancer SNPs-associated variants potentially related to the pathogenesis of common neuropsychiatric conditions that still lack effective treatments, in particular Parkinson's disease, schizophrenia, and bipolar disorder (Carullo and Day, 2019). Thus, among downregulated genes of *RFX2* -/- cells (STRT RNA-seq data) and upregulated promoters and enhancers (CAGE and NET-CAGE data), motif analysis showed a consistent enrichment of TF binding motifs of members of the AP-1 transcriptional complex activated by ciliary non-canonical WNT signaling.

The WNT signaling pathway participates in multiple processes to control nervous system development (Inestrosa and Varela-Nallar, 2015; Arredondo et al., 2020). Its canonical and non-canonical activation is finely tuned during the different phases of neurodevelopment and a predominant non-canonical activation was found essential to mediate the early differentiation of human neurons (Bengoa-Vergniory et al., 2014). Due to its complex signaling cascade, the WNT pathway can be modulated at different levels with small molecules (Tran and Zheng, 2017). As LUHMES cells are an *in vitro* model only exposed to well-defined culture media, to study WNT signaling effects on neuron differentiation we chose to use Wnt-C59 compound to inhibit the expression and activation of all WNT ligands endogenously produced by the cell (Motono et al., 2016). Treatment of WT neurons led to a decrease of stage 3 neurons in the population in comparison to the control (vehicle), mimicking the defect observed in *RFX2* -/- neurons already at the basal level. To investigate the effect of Wnt-C59 inhibition on cilia function, we analyzed ciliated and non-ciliated neurons in both genetic backgrounds. While functional (WT) cilia still promoted axon outgrowth process in untreated conditions, treatment with the Wnt-C59 antagonist resulted in a reduction of axon outgrowth regardless of the presence or absence of cilia, as well as *RFX2* -/- neurons (with altered cilia) always displayed axon outgrowth defects under any circumstance. The same pattern was observed when axon branching was analyzed, where WNT pathway inhibition led to an increased branching of axons of non-ciliated neurons, like occurred in non-ciliated *RFX2* -/- neurons. Altogether, these results confirm that WNT signaling is one of the pathways involved in the independent regulation of axon outgrowth and branching with WNT ligands functioning upstream the ciliary signaling machinery and suggest that the WNT

pathway, like the *RFX2* gene, may be implicated in promoting the axon pruning process.

Canonical and non-canonical WNT pathways are always active simultaneously where the transcriptional co-activator β-catenin represents the main mediator to stimulate the canonical activity (Qin et al., 2023). Cilia have been found important to control the balance between the two activation levels mediating the cytoplasmic degradation of β-catenin and consequent reduction of the canonical activity (May-Simera and Kelley, 2012). While the total β-catenin expression levels were comparable in WT and *RFX2* -/- neurons, we found increased levels of the active (non-phosphorylated) form of β-catenin in *RFX2* -/- neurons, indicating a variation of the pathway regulation in comparison to WT neurons. To explore the ciliary connection to this different regulation, we analyzed the cellular distribution of the active β-catenin in ciliated and non-ciliated neurons. When the cilium was extended in WT neurons, the ratio of active β-catenin intensity was lower than in non-ciliated cells, suggesting a ciliary role in diminishing its nuclear translocation potentially promoting its cytoplasmic degradation. By contrast, altered cilia of *RFX2* -/- neurons showed high ratio levels like the non-ciliated counterpart, with an increased nuclear expression of active β-catenin. These differences may explain the neuron differentiation delay previously detected in the *RFX2* -/- background, considering the required switch from canonical to non-canonical WNT activation during the process. Accordingly, canonical activation was found important to promote the centrosome-related first steps of neuron differentiation (neuron polarization, axonogenesis, initial axonal growth) (Stanganello et al., 2019), when ciliation is relatively low, then neurons need to switch to non-canonical WNT activation in order to promote cytoskeleton rearrangement, massively required during the early time window of differentiation (axon outgrowth/branching, ciliation increases and peaks at d3) (Bengoa-Vergniory et al., 2014). Finally, a last canonical WNT activation was found to be prosynaptogenic while non-canonical WNT stimuli functioned as antisynaptogenic (Davis et al., 2008) (cilia start being disassembled after d3 to reduce β-Catenin degradation and increase canonical WNT pathway activation). We speculate that ciliation of LUHMES neurons may be also influenced by the cellular demand of activate either the canonical or the non-canonical WNT signaling, although ciliogenesis seems not to be affected by the pathway itself (Bernatik et al., 2021).

One of the main functions of the non-canonical WNT/PCP signaling is to influence cytoskeletal rearrangements during neuron differentiation (May-Simera and

Kelley, 2012). To test this hypothesis, during treatment of LUHMES neurons with Wnt-C59 antagonist we also assessed the effects of WNT pathway inhibition on cytoskeleton remodeling. In WT neurons, this resulted in a remarkable downregulation of key remodeler and non-canonical WNT target genes at a similar basal expression level of *RFX2* -/- neurons. Conversely, the expression of canonical WNT target genes did not decrease with the treatment. This further suggested a predominant non-canonical activation over a canonical one in the middle of the neuron differentiation process (d3). Moreover, the affected genes were also candidate genes for various neurodevelopmental disorders (Yamakawa, 2016; Chia et al., 2018; Yang et al., 2019; Delprato et al., 2022), potentially linking cilia defects (*RFX2* -/-) to aspects of their pathogenesis.

Our work in LUHMES neurons on the ciliary contribution to promote neuron (axon) differentiation and on the study of the molecular machinery that regulates the process, fits and integrates well with current studies on similar topics. Neuronal primary cilia were mostly studied in human iPSC-derived neurons, with lengthy and complex culturing processes of above a month (Miki et al., 2019; Bieder et al., 2020; Schmidt et al., 2022), and non-human models like mouse, zebrafish, *C. elegans*, and *Drosophila* (Arellano et al., 2012; Lepanto et al., 2016; Bae and Barr, 2008; Jana et al., 2016). Cilia and ciliary signaling were found critical for axonal development and connectivity, determining proper branching and growth cone formation through the PI3K/AKT pathway (Guo et al., 2019). Cilia were also shown to regulate the ciliary cAMP/PKA pathway to promote migration of neurons (Stoufflet et al., 2020) approaching their final brain destination, where they were then required for dendritic refinement and synapse formation (Kumamoto et al., 2012; Guadiana et al., 2013). Ciliary defects were proven to perturb several signaling pathways like WNT, with a concomitant enhanced canonical activation which may cause cytoskeleton remodeling and developmental defects at the origin of certain brain malformations (May-Simera and Kelley, 2012; Park et al., 2019). Interestingly, and even though ciliary signaling was associated with modifications of chromatin accessibility (Sheu et al., 2022), little is known about the molecular regulation of neurodevelopment and an exhaustive identification of enhancer-promoter interactions is still missing. Thus, neuronal enhancers were mostly studied in relation to DNA breakage repair systems, only partially revealing their importance in relation to brain conditions (Carullo and Day, 2019; Wu et al., 2021).

Our research complements previous studies as we offer the possibility to precisely investigate cilia of human neurons at molecular and cellular levels covering the entire differentiation process, including the proliferating and the fully differentiated phases. We strengthened the connection between cilia and WNT signaling in assisting the neuron differentiation process through cytoskeleton rearrangement mediated by the non-canonical activation of the pathway. We discovered new regulatory elements and how the molecular landscape dynamically changes during the process, providing insight into the pathogenesis of neuropsychiatric disorders. Although LUHMES is a simple *in vitro* model of a single neuronal population without the physiological interaction with different cell types and neurotransmitters, it shows similar gene expression profile to the corresponding *in vivo* neuronal population, and it facilitates the study of every single neuron differentiation step during time course analyses. As *in vitro* model, it lacks the essential migration step, but *in vitro* assays exist to faithfully replicate and also assess this process (Azzarelli et al., 2017).

Neurons are traditionally difficult to transfect, including LUHMES cells. However, efficient electroporation and lipid-based transfection protocols have been developed to produce either stable mutants (by CRISPR/Cas9) (Shah et al., 2016; Calamini et al., 2021) or transient siRNA KDs (Paper II). Several ciliary signaling pathways are transduced and cross-talks in the developing brain, including WNT (Park et al., 2019). To accurately understand the role of WNT signaling during neuron differentiation, further investigation is required but it is possible to interfere with the pathway at many different levels using available small molecules (Tran and Zheng, 2017).

In conclusion, with different and complementary methods we showed how and when cilia and ciliary signaling are important to promote aspects of neuron differentiation. We propose a model where the differentiation of neuron precursor is initiated by the centrosome (polarization, axonogenesis) followed by an increased ciliation and ciliary signaling during the early time window of differentiation (migration, axon outgrowth, axon branching) to mediate and sustain the anatomical changes before dendrite generation and maturation and the establishment of functional synapses with a concomitant gradual loss of cilia. After a first connection between cilia and NDD candidate genes (Massinen et al., 2011; Chandrasekar et al., 2013), not surprisingly, several ciliary defects have been found to be implicated in the onset of brain disorders (Valente et al., 2014; Reiter and Leroux, 2017; Lauter et al., 2018). For an enhanced comprehension of their

pathogenesis, as a future perspective, it will be of great help to research cilia structure and function in more complete and complex systems like 3D brain organoids and patient-derived samples, and following the whole cilia biogenesis and dynamics through live cell imaging, with the possibility to selectively collect ciliated and non-ciliated cells to further elucidate the etiological mechanisms underlying cilia/centrosome-related disorders.

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