Title: Roles of a novel serine/threonine protein kinase Ulk4 in mouse brain development and functions

Author(s): Liu, Min

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Roles of a Novel Serine/Threonine Protein Kinase Ulk4 in Mouse Brain Development and Functions

A thesis submitted to the National University of Ireland (NUI) Galway
in fulfillment of the requirement for the degree of

Doctor of Philosophy

By

Min Liu

Regenerative Medicine Institute (REMDI),
National University of Ireland (NUI) Galway
Thesis supervisor: Prof. Sanbing Shen
Co-supervisor: Dr Una FitzGerald
Submitted: October 2015
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DECLARATION

I declare that the work described in this thesis is solely that of the author. None of this work has been submitted for any other qualification at this or any other university. ________________

Min Liu

October 2015
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I would like to dedicate this thesis to my family.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>anterior commissure</td>
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<tr>
<td>Actr3 (=Arp3)</td>
<td>Arp3 Actin-Related Protein 3 Homolog (Yeast)</td>
</tr>
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<td>Adra2a</td>
<td>Adrenoceptor Alpha 2A</td>
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<td>Ak7</td>
<td>Adenylate Kinase 7</td>
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<tr>
<td>Ak8</td>
<td>Adenylate Kinase 8</td>
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<td>a-Parvin (Parva)</td>
<td>Parvin, Alpha</td>
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<td>Aqp4</td>
<td>Aquaporin-4</td>
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<td>Asap1</td>
<td>ArfGAP With SH3 Domain,</td>
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<td>Avil</td>
<td>Advillin</td>
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<td>BB</td>
<td>Basal bodies</td>
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<td>BBGRE</td>
<td>Brain and Body Genetic Resource Exchange program</td>
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<tr>
<td>BLA</td>
<td>Basolateral nucleus of the amygdala</td>
</tr>
<tr>
<td>Blbp</td>
<td>Brain lipid binding protein</td>
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<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
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<tr>
<td>CC</td>
<td>Corpus callosum</td>
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<td>Ccdc123/Cep123</td>
<td>Centrosomal Protein 89kDa</td>
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<td>Ccp110</td>
<td>Centriolar Coiled Coil Protein 110kDa</td>
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<td>Chrna4</td>
<td>Cholinergic Receptor, Nicotinic, Alpha 4 (Neuronal)</td>
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<td>Chibby</td>
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<td>Centromere Protein J</td>
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<td>Cerebrospinal fluid</td>
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<td>Cut-Like Homeobox 1</td>
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<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
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<td>DBP</td>
<td>Diastolic blood pressure</td>
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<td>Ccde67</td>
<td>Coiled-Coil Domain Containing 67</td>
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<td>Dpcd</td>
<td>Deleted In Primary Ciliary Dyskinesia Homolog</td>
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<td>Drd3</td>
<td>Dopamine Receptor D3</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases,</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>Forkhead Box J1</td>
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<td>GABA_A</td>
<td>Gamma-Aminobutyric Acid (GABA) A Receptor</td>
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<td>Gad2</td>
<td>Glutamate Decarboxylase 2</td>
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<td>Gad67</td>
<td>Glutamate decarboxylase 1 (brain, 67kDa)</td>
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<td>Gfap</td>
<td>Glial Fibrillary Acidic Protein</td>
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<td>Gria1</td>
<td>Glutamate Receptor, Ionotropic, AMPA 1</td>
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<td>GT335 (=C21orf33)</td>
<td>Chromosome 21 Open Reading Frame 33</td>
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<td>GWASs</td>
<td>Genome-wide association studies</td>
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<td>HDAC</td>
<td>Histidine Decarboxylase</td>
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<tr>
<td>HTR1A</td>
<td>5-Hydroxytryptamine (Serotonin) Receptor 1A, G Protein-Coupled</td>
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<tr>
<td>Hydin</td>
<td>HYDIN, Axonemal Central Pair Apparatus Protein</td>
</tr>
<tr>
<td>hyh</td>
<td>Hydrocephalus with hop</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Iba1</td>
<td>Allograft Inflammatory Factor 1</td>
</tr>
<tr>
<td>ID</td>
<td>Intellectual disability</td>
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<td>IFT</td>
<td>Intraflagellar transport</td>
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<td>JNK</td>
<td>C-Jun N-terminal kinases</td>
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<td>Kif3B</td>
<td>Kinesin Family Member 3B</td>
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<td>KO</td>
<td>Knockout</td>
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<td>LICAM</td>
<td>L1 Cell Adhesion Molecule</td>
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<td>Mboat</td>
<td>Membrane Bound O-Acyltransferase Domain Containing 7</td>
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<td>MBP</td>
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<td>TNF Receptor-Associated Factor 3 Interacting Protein 1</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>MV</td>
<td>Microvilli;</td>
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<td>MZ</td>
<td>Marginal zone</td>
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<td>Nme7</td>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>NSCs</td>
<td>Neural stem cells</td>
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<td>Nurr1</td>
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<td>Odf2</td>
<td>Outer Dense Fiber Of Sperm Tails 2</td>
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<td>Ofd1</td>
<td>Oral–facial–digital syndrome gene OFD1</td>
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<td>Olig2</td>
<td>Oligodendrocyte Lineage Transcription Factor 2</td>
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<td>OPCs</td>
<td>Oligodendrocyte progenitor cells</td>
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<td>p38</td>
<td>P38 mitogen-activated protein kinases</td>
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</table>
PAC1 gene (ADCYAP1R1)  Pituitary Adenylate Cyclase Activating Polypeptide 1 Receptor Type I

PCR  
Polymerase chain reaction

Pdgfrα  
Platelet-Derived Growth Factor Receptor, Alpha Polypeptide

PFA  
Paraformaldehyde

Ph3  
Phospho-Histone H3

PKA  
Protein kinase A

PKC  
Protein kinase C

Pkd1  
Polycystin-1 (PC-1)

Plk4  
Polo-Like Kinase 4

Poc5  
POC5 Centriolar Protein

PTPN23  
Protein Tyrosine Phosphatase, Non-Receptor Type 23

Rab8  
Member RAS Oncogene Family

RF  
Reissner’s fibre

RFX  
Regulatory factor X

Rfx2  
Regulatory Factor X, 2

Rfx3  
Regulatory Factor X, 3

Sas-6 (Sass6)  
Centriolar Assembly Protein

Satb2  
Satb Homeobox 2

SBP  
Systolic blood pressure

SCO  
Subcommissural organ

SEM  
Scanning electron microscopy

sem  
standar error of mean

Shh  
Sonic hedgehog

SLC17A8  
Solute Carrier Family 17 (Vesicular Glutamate Transporter), Member 8

SLC6A12  
Solute Carrier Family 6 (Neurotransmitter Transporter), Member 12

SNP  
Single nucleotide polymorphisms

Sox3  
Transcription factor Sox-3

SP  
Subplate

Spag6  
Sperm-associated antigen 6
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Sstr2</td>
<td>Somatostatin Receptor 2</td>
</tr>
<tr>
<td>Stil</td>
<td>SCL/TAL1 Interrupting Locus</td>
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<tr>
<td>STK</td>
<td>Potential serine/threonine kinase</td>
</tr>
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<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
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<td>Tbr1</td>
<td>T-Box, Brain, 1</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<td>ULK4</td>
<td><em>UNC-51-like kinase 4</em></td>
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<td>VZ</td>
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<td>Wave Associated Rac-GAP</td>
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<td>Soluble N-ethylmaleimide-sensitive factor attachment protein alpha</td>
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**SUMMARY**

*UNC-51-like kinase 4 (ULK4)* is a novel gene encoding a potential serine/threonine kinase (STK), and little is known about its functions. Limited studies have been carried out so far, and most of the current 12 publications are genetic studies, which suggest that ULK4 may be involved in hypertension, myeloma and neurodevelopmental disorders. We were the first to show that ULK4 gene can be a rare risk factor for neurodevelopmental/ neuropsychiatric disorders including schizophrenia, autism, bipolar disorder and depression (Lang, Pu et al. 2014).

Increased copy number variations (CNVs) were detected in patients of schizophrenia and autism, and single nucleotide polymorphisms (SNP) of the ULK4 were also found to be associated with the major depression (Lang, Pu et al. 2014). Functional analyses of the ULK4 gene was carried out *in vitro* by a knockdown approach in neuroblastoma cells, which showed that depleted ULK4 expression disrupted the composition of microtubules, and compromised neuritogenesis and cell motility. ULK4 also modulated multiple signaling pathways including ERK, p38, PKC, and JNK, which were implicated in neurodevelopmental disorders and involved in stress response. Ulk4 expression was regulated by developmental cues, and there was a developmental switch in Ulk4 isoform expression during mouse brain development and neuronal maturation. I contributed to the publication (Lang, Pu et al. 2014) by providing evidence that targeted deletion was shown to compromise the integrity of the corpus callosum in newborn mice, a phenotype which is commonly associated with ciliopathies and neurodevelopmental disorders.

We have additional unpublished data that ULK4 is deleted in a small proportion of patients with heterogeneous clinical features including developmental delay, language delay and severe intellectual disability. In a recent comparative study, we characterized the Ulk4 gene expression during *Xenopus* development, and found co-expression of the Ulk4 mRNA with Sox3 (a neural stem/progenitor cell marker) and Blbp (a radial glial marker) in the ventricular regions of the forebrain (Dominguez,
Schlosser et al. 2015). However, in both mouse and human adult brains, ULK4 was co-expressed with GAD67+ GABAergic neurons in cerebral cortex and hippocampus (Lang, Pu et al. 2014), highlighting its potential function of ULK4 gene in excitation/inhibition balance.

The aim of this PhD project is to investigate the roles of Ulk4 in brain development and function. I have carried out a systematic study in the Ulk4−/− mice and littermate controls, and the research outcomes of this PhD project are summarized in four Results Chapters, which demonstrate that (1) ULK4 gene is crucial for cilia development and flow of cerebrospinal fluid (CSF), (2) ULK4 is essential for white matter integrity and myelination, (3) ULK4 regulates cell cycle and neurogenesis, and (4) ULK4 regulates excitation and inhibition balance and anxiety-related behavior.

Firstly in Chapter 3, I described that (1) Ulk4 is predominantly expressed in mouse ventricular system, (2) Ulk4−/− ependymal cells display reduced and disorganized cilia with abnormal microtubular ultrastructure, (3) CSF flow is functionally impaired in the Ulk4−/− mice, (4) Ulk4 regulates Foxj1, a master regulator of ciliogenesis and a range of other cilia components and ciliogenesis genes. This provides the subcellular and molecular mechanisms and networks how disruption of Ulk4 can lead to non-communicating hydrocephalus phenotype. I also proposed that Ulk4 may act as a scaffold protein, in this aspect, regulating different processes of ciliogenesis and coordinating cilia formation and ciliary beating, as Ulk4 lacks evolutionally conserved critical amino acid in the N-terminus (see Chapter3) as a kinase.

In Chapter 4, I followed up the early observation that the integrity of the corpus callosum was compromised in Ulk4−/− newborn mice (Lang, Pu et al. 2014), and presented the evidence that (1) Ulk4 is involved in white matter integrity in postnatal mice, (2) oligodendrocyte production and myelination are significantly reduced in Ulk4−/− mice, and (3) the Ulk4−/− mice exhibit an increased neuro-inflammation which includes activation of astrocytes and microglia (see Chapter 4).
In Chapter 5, I started with an initial observation that in the *Ulk4*^{-/-} newborn mice the subventricular zone (SVZ), where adult neural stem cells reside, was substantially smaller. I followed up this initial observation and presented evidence that, (1) *Ulk4* is expressed in a cell cycle–dependent manner *in vivo* and *in vitro*, with a peak of expression in the G2 and M phases of the cell cycle; (2) *Ulk4* deficiency reduces middle neurogenesis and compromises the generation of layer II-IV pyramidal neurons, which are commonly implicated in neurodevelopmental disorders including schizophrenia; (3) In the absence of the *Ulk4*, the neural stem cell pool is dramatically reduced in the SVZ of newborn mice, which is the starting point of adult neurogenesis and neuronal renewal, and this will have a wide implication in both neurodevelopmental and neurodegenerative disorders. In addition, I identified a cluster of genes, which are involved in cell cycle regulation and neural stem cell proliferation, but dysregulated in the *Ulk4*^{-/-} mice. This suggests that *Ulk4* regulates neurogenesis through the Wnt signaling (see Chapter 4).

In order to determine how *Ulk4* deletion may impact on behavior, we performed a series of behavioral tests on the *Ulk4*^{+/-} mice, in collaboration with Dr Michelle Roche in the NUI Galway and Dr Steve Clapcote in the University of Leeds. In Chapter 6, I described that *Ulk4*^{+/-} mice display an anxiety-related phenotype. I also provided the evidence that a reduction in GABAergic neurons in amygdala and hippocampus may partially account for the mechanisms of the anxiety-related behavior in the *Ulk4*^{+/-} mice. In addition, I presented the negative data that the immobility of depressive tests was not altered in forced swimming and tail suspension tests.

In summary, I demonstrated, through this PhD project, that *Ulk4* is critical for proper brain development and function. Deletion, mutation and/or polymorphism of the *Ulk4* gene may have an implication in a wide range of neurodevelopmental and neuropsychiatric disorders.
CHAPTER 1

GENERAL INTRODUCTION
ULK4 is a relatively novel gene and little is known about its function. In this PhD project, I systematically investigated the roles of Ulk4 in several biological processes, such as CSF flow and ciliogenesis, white matter development and myelination, cell cycle and neurogenesis, GABAergic neuronal production and anxiety and depression-related behaviour.

1.1 Unc-51-like protein kinase family

Unc-51-like serine/threonine kinase (STK) family is comprised of Ulk1, Ulk2, Ulk3, Ulk4 and Stk36. Ulk1/Ulk2 are known as the major regulators of autophagy which is a basic catabolic mechanism and an adaptive stress response to degrade un-essential or dysfunctional cellular components through the actions of lysosome (Egan, Shackelford et al. 2011; Lee and Tournier 2011). Ulk3 is involved in the regulation of the sonic hedgehog signaling pathway which is critical for brain development (Fuccillo, Joyner et al. 2006; Maloverjan, Piirsoo et al. 2010), and Ulk2 is reported to be involved in the habenula development in the brain (Taylor, Qi et al. 2011).

1.2 Biochemistry and Biology of Ulk4

The human ULK4 gene is located on chromosome 3p22.1 and there are four predicted isoforms in the UCSC database. The longest isoform encodes 1275 amino acids via 36 exons across 722kb of genomic DNA. Two additional isoforms at the 5’ end of the ULK4 gene are predicted, with 580 aa and 732aa coded by exons 1-16 and exons 1-20, respectively. At the 3’ end of the ULK4 gene, a non-coding splice variant is predicted to contain exons 35-36 of the full-length gene (see image below).

![Image of UCSC Genome Browser](image.png)

**Figure 1-1.** Human ULK4 splice variants predicted by UCSC database.
In mouse, the *Ulk4* gene is located on chromosome 9 (chr9:120,964,000-121,279,000) and comprises of >315kb of genomic DNA. There are five predicted isoforms in the UCSC database (Figure 1-2). The longest isoform also encodes 1275 amino acid via 36 exons; the 1118 aa isoform is encoded by exons 1-33; the 1145 aa isoform is encoded by exons 2-33 with an alternative exon 1; the 910 aa isoform is encoded by exons 1-24; and 405 aa isoform is encoded by exons 9-24. The knockout construct (see Chapter 3, Figure 3-1) was designed to delete exon 7 and replace it with a knock-in cassette harbouring *SA-IRES-LacZ-Neo*. Therefore, theoretically, four of the five isoforms will be disrupted, whereas the 405 aa isoform encoded by exons 9-24 may still remain intact. It is worth noting the alternative non-coding transcript at the 3’ of the human ULK4 gene was not predicted in mouse.

![UCSC Genome Browser on Mouse Dec. 2011 (GRCm38/mm10) Assembly](image)

Figure 1-2. Mouse Ulk4 splice variants predicted by UCSC database. aa for amino acids. E for exon.

For ULK4 protein domain function, the bioinformatic database was searched, and it was found that the 1275aa full-length ULK4 isoform is predicted to contain four structural domains from N- to C-terminus: a Kinase-like domain from at aa 2-282, and three Armadillo-type folds at aa 507-762, 793-854 and 889-1249, respectively (see image below).
Table 1-1. The critical Lysine (K) residue is conserved among ULK1, ULK2, ULK3, STK36, but not ULK4 during evolution.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human_ULK1</td>
<td>MEPGRGTTKVKEFSRKLHIGGFAVVFGRHRKDKDLEVAYKCIANKNLAKSQTLL</td>
<td>60</td>
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<tr>
<td>Human_ULK2</td>
<td>MEKVGDGEYKRDVLYGAFAVVFGRHRKQFDKDEVAYKCIANKNLAKSQTLL</td>
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</tr>
<tr>
<td>Human_ULK3</td>
<td>MAGPWPPPLDGLPILRGLGTYATYAYAKEDPFEVAYKCIANKNLAKSQTLL</td>
<td>56</td>
</tr>
<tr>
<td>Human_STK36</td>
<td>MEKYLVMEGESFGRYKGRK YSDAQVKLPPLPKRGRSKEELK</td>
<td>47</td>
</tr>
<tr>
<td>Drosophila_UCN-51</td>
<td>MN1VGEYEYSKMLHGAFAVVFGRHRKMPVAKITKKQIKNLAKSQTLL</td>
<td>52</td>
</tr>
<tr>
<td>C.elegans UNC-51</td>
<td>MEQFDGEYSRDKLLHGAFAVVFGRHRKAIKKNISKSNL</td>
<td>53</td>
</tr>
<tr>
<td>Human_ULK4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RPEI</td>
<td>43</td>
</tr>
<tr>
<td>Mouse_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RPEI</td>
<td>43</td>
</tr>
<tr>
<td>Rat_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RPEI</td>
<td>43</td>
</tr>
<tr>
<td>Chimpanzee_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RPEI</td>
<td>43</td>
</tr>
<tr>
<td>Cattle_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCR----RPEI</td>
<td>43</td>
</tr>
<tr>
<td>Chicken_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RAEI</td>
<td>43</td>
</tr>
<tr>
<td>Zebrafish_Ulk4</td>
<td>---------------MENFILYEEGRGSKTVYKGRRKYGINFVAILSTDKCK----RFEL</td>
<td>43</td>
</tr>
</tbody>
</table>

The family members ULK1 and ULK2 have already been investigated in detail previously. The amino acid K46 of ULK1 and the corresponding amino acid K39 in ULK2 are critical for the kinase activity. Mutation of the Lysine (K) to Arginine (R) caused ULK1 and 2 to become kinase dead (Chan, Longatti et al. 2009). I carried out a bioinformatic analysis with CLUSTAL 2.0.10 multiple sequence alignment and showed that this Lysine residue is conserved among human ULK1-3, STK36, Drosophila and C. elegans Unc51 (highlighted in the sequence alignment below). Surprisingly, this Lysine residue was altered to Leucine (L) in ULK4 genes from human, mouse, rat, chimpanzee, cattle, chicken and to Isoleucine (I) in zebrafish (Table 1-1.). This suggests that ULK4 may be a pseudokinase.
However, the evolutionary conservation of the amino acid L34 among all species (except Zebrafish) indicates that ULK4 may have a unique role which is fundamentally different from other family members. We speculate that ULK4 may exercise its function through interactions with other proteins via its three ARM domains, and subsequently activate and/or inhibit phosphorylation of the binding partners, leading to activation and/or inhibition of ULK4 complex members for various cellular functions.

In the Genecard database, Ulk4 is shown to be widely expressed in different systems, including nervous system, immune system and secretion system. We also have unpublished Western Blotting data showing that different sizes of Ulk4 proteins are detected in the CNS and all peripheral organs examined. While less is known about the localization of ULK4 in subcellular compartments, weak levels of ULK4 protein were detected in the nucleus, cytosol and mitochondrion, which implies that ULK4 may be involved in a range of cellular functions. However, this has rarely been investigated so far.

Recently, genome-wide association studies suggest that ULK4 may be involved in the function of peripheral organs and tumorigenesis, as single-nucleotide polymorphism (SNP) markers rs1717027 and rs9815354 located in the ULK4 gene are associated with hypertension (Levy, Ehret et al. 2009; Franceschini, Fox et al. 2013; Lu, Wang et al. 2015). The haplotype containing SNP rs2700464 on ULK4 was reported to be strongly associated with hypertension (Lang, Pu et al. 2014; Shen, Espin-Garcia et al. 2014). Genome-wide association studies (GWASs) in the Chinese population have identified rs9815354 on ULK4 was associated with diastolic blood pressure (Lu, Wang et al. 2015). Konigorski showed that the whole genome sequence variants in the gene ULK4 on chromosome 3 suggest that inference obtained from a copula model can be more informative than findings from systolic blood pressure (SBP)-specific and diastolic blood pressure (DBP)-specific univariate models alone (Konigorski, Yilmaz et al. 2014).

It also was reported that rs1052501 on ULK4 is associated with multiple myeloma (Broderick, Chubb et al. 2012; Greenberg, Lee et al. 2013). Recently, ULK4 was
identified as one gene which is under positive selection for somatic mutations in endometrial carcinoma and clear cell renal carcinoma respectively (Lebovitz, Robertson et al. 2015).

1.3 Logic of the experimental approach

While these genetic studies suggest that ULK4 plays important roles in blood pressure and tumorigenesis, my interest has been limited to the neuroscience aspect in this PhD project. Previous research from our group demonstrated that the ULK4 gene could be a rare risk factor for neurodevelopmental and neuropsychiatric conditions including schizophrenia, autism, bipolar disorder and depression in humans (Lang, Pu et al. 2014). Functional analyses of the ULK4 gene by a knockdown approach in neuroblastoma cells showed that depleted ULK4 expression disrupted the composition of microtubules, compromised neuritogenesis and cell motility, and modulated multiple signalling pathways which were known to be implicated in schizophrenia. Additionally, Ulk4 expression was shown to be regulated by developmental cues, and there was a developmental switch in Ulk4 isoform expression during mouse brain development and neuronal maturation. I have participated in this research and showed that targeted deletion also compromised the integrity of the corpus callosum in mice (Lang, Pu et al. 2014).

Meanwhile our team member Dominguez et al also examined Ulk4 gene expression during Xenopus development, and found that Ulk4 was co-expressed with Sox3 (a neural stem/progenitor cell marker) and Blbp (a radial glial marker) in the forebrain ventricular regions throughout development and in the adulthood, suggesting that Ulk4 can be involved in neurogenesis (Dominguez, Schlosser et al. 2015). These data suggest that Ulk4 could be critical for the development and function of the nervous system. Interestingly, in a recent genetic screening study, two Ulk family members, Ulk4 and Stk36 were identified among the 12 genes (Ak7, Ak8, Celsr2, Dpce, FZD3, Kif27, Mboai7, Nme5, Nme7, RIKEN 4930444A02, Stk36, Ulk4) associated with congenital hydrocephalus in genetically modified mice (Vogel, Read et al. 2012).
Our further genetic data (Chapter 5, Figure 5-1) showed that ULK4 is deleted in 1.2/1000 patients with pleiotropic developmental symptoms affecting both the central and peripheral systems, leading to developmental delay, language delay and intellectual disability. Therefore, this PhD thesis was set to investigate the following aspects: (1) I characterize the pathology of hydrocephalus-related phenotype (Chapter 3) and how it relates to the pathology seen following ULK4 deletion and in particular, the relevance of ULK4 to human brain; (2) I examined embryonic neurogenesis (Chapter 5) and investigated how ULK4 deletion affects grey matter and brain formation; (3) Appropriate neuronal communications require white matter, I therefore followed up the early discovery of Ulk4 effects on integrity of the corpus callosum (Lang, Pu et al. 2014), and further studied the white matter and oligodendogenesis, how ULK4 deletion affects the white matter and oligodendrogenesis (Chapter 4); (4) Finally animal behaviour studies were carried out in collaborations (Chapter 6), as this would enhance our understanding how ULK4 lesion may affect behaviour in patients.

1.4 Hydrocephalus

Since Ulk4−/− mice developed hydrocephalus, so in this part I introduced the definition, causes and treatment of hydrocephalus. I also reviewed the genetics of congenital hydrocephalus, dysfunctional motile cilia and hydrocephalus. For a better understanding of the complex progresses of cilia formation, three main steps of ciliogenesis were described in detail.

1.4.1 Overview of hydrocephalus

The term hydrocephalus comes from two Greek words "hydro" and "cephalus", so the hydrocephalus was once known as “water on the brain”. Actually “water” is cerebrospinal fluid (CSF), which is a colorless bodily and serves as nutrients supplier, cushion and disposer of waste in the brain and spinal cord. CSF is manly produced from arterial blood by the choroid plexuses of the lateral and fourth ventricles. The CSF circulation within the ventricular systems is driven by the pulsation of the choroid plexus and coordinated beating of motile cilia on the
ependymal cells. The pathway of CSF is from lateral ventricle to the third ventricle via foramen of monro and then to the fourth ventricle through the aqueduct of Sylvius. Then CSF flows from fourth ventricle to subarachnoid space through one foramen of magendie and two foramen of luschka. CSF is believed to return to the vascular system in the subarachnoid space by the arachnoid villi or it is drained in to lymphatic channels around the cranial cavity and spinal canal.

Although the high prevalence of hydrocephalus, the treatments options are limited. Currently, there are two common treatments for hydrocephalus. One is shunt placement, which is considered the best treatment for hydrocephalus and postoperative mortality rate for shunt placement is less than 5%. However it has several drawbacks: like shunt failure occurs at a rate of approximately 20% and surgical infection occurs at a rate of 5% to 15% (Hoppe-Hirsch, Laroussinie et al. 1998). Another approach is endoscopic third ventriculostomy which is to create an

Figure 1-4 Cerebrospinal fluid pathway (cited from Virtualmedstudent.com)

Hydrocephalus is characterized by the excessive accumulation of CSF within brain ventricles, which results in ventricular dilatation and damage to the surrounding brain parenchyma. The initial causes of hydrocephalus can be divided roughly into two cases. One is the imbalance of production and absorption, which can be induced by excessive CSF production or reduced CSF absorption. Another case is impaired flow of CSF, which CSF can’t pass smoothly from production areas to the final absorption area. Base on the different pathological conditions, hydrocephalus is commonly classified into communicating or non communicating (also known as
obstructive) hydrocephalus. Hydrocephalus may present at any time during the life, so it is subdivide into congenital and acquired hydrocephalus according to the time when the disorder occurs. It is considered to be congenital if the hydrocephalus is present at birth or embryonic stages. The incidence of congenital hydrocephalus presently is 1 to 3 of 1000 children at birth (Lang, Song et al. 2006). Acquired hydrocephalus may develop at any time after birth and is usually associated with traumatic injury, disease, tumor obstruction, intracranial hemorrhage, and infection. The complex nature of hydrocephalus make it is difficult to determine the overall incidence of hydrocephalus.

opening in the floor of the third ventricle to enable the passage of CSF. These treatments of hydrocephalus often require long-term care and lifelong follow up, especially in children and neonates in whom there is a congenital cause. So a better understanding of the underlying molecular mechanisms of the disease is an urgency which may help identify some promising interventions and offer alternative treatment strategies.

1.4.2 Genetics in congenital hydrocephalus

Congenital hydrocephalus is a complex and multifactorial disorder, which presents a challenge to uncover its pathophysiology. The consequences of congenital hydrocephalus are various and significant due to ventricular dilation and increased CSF pressure. Before understanding the pathophysiology of congenital hydrocephalus, it is important to clarify two mechanisms: the primary genetic defects which may contribute to congenital hydrocephalus and secondary effects which may result from dilation of ventricles.

Genetic studies in human and animal models suggest that many genetic loci are associated with hydrocephalus. Hydrocephalus animal models may be the effective tools to understand the unclear pathogenesis of hydrocephalus. Although genetic research about the human hydrocephalus is limited, similar abnormalities in both animal models and humans may guide us to know better about the human hydrocephalus. And also identification of genes and signaling pathways in animal
models can be candidates of concern in humans. Review of hydrocephalus disease from genetic aspect may provide new drug targets for this neurological disorder.

Compared to animal studies, genetic factors contributing to human congenital hydrocephalus have not been defined. It is estimated that about 40% of hydrocephalus cases have a possible genetic etiology (Haverkamp, Wolfle et al. 1999). At present, only one human gene (X-linked) was reported to be involved in the human congenital hydrocephalus. *L1CAM* (L1 Cell Adhesion Molecule) is a Protein Coding gene located at Xq29. It encodes an axonal glycoprotein which is a member of the immunoglobulin superfamily. This cell adhesion molecule has an important role in the different processes of nervous system development, such as neuronal migration and differentiation. It was the only gene that has been identified in human to associate with hydrocephalus, and it accounts for 5% to 15% of likely hereditary cases. This X-linked hydrocephalus characterized at the molecular level has been estimated to constitute 2–5% of all cases of isolated congenital hydrocephalus. The causes were unknown or poorly recognizable in 50% of congenital hydrocephalus cases (Vogel, Read et al. 2012). A newly report found that 57 clinical cases with deleterious *L1CAM* mutations present 100% hydrocephalus. It was well-known that LICAM (L1 Protein) involved in several development processes including neuronal adhesion, migration, neurite outgrowth and development of ventricular system. Nevertheless, how hydrocephaly develops in the *L1CAM* mutant patients remains unclear (Adle-Biassette, Saugier-Veber et al. 2013).

In addition, 3.54Mb deletion of 17q22-q23.1 in one female was reported to develop human hydrocephalus. This patient displayed enlarged lateral ventricle and reduced cortex at the first of life and severe hydrocephalus at the second week of her life. This is a direct evidence emphasized the role of genetic factors in the development of human hydrocephalus. Genes located in this region may be the candidates factors involved in the hydrocephalus formation (Khattab, Xu et al. 2011).

In contract, numerous genes have been determined by establishing genetically engineered hydrocephalus animal models. Products of these genes can be divided into important cytokines, growth factors or related molecules, which play important
roles in cellular signal pathways during the brain development (Batiz, Paez et al. 2006). The establishment of animal models provides us effective tools to explore the cause and pathogenesis of the hydrocephalus. Understanding of the underlying molecular signals and mechanisms in animal models may offer us a way to know the uncertain and complex pathogenesis in human hydrocephalus. Characterization of the various phenotypes in different models and identification of the underlying signals occurred in the development of hydrocephalus may be the first step to enhance the understanding of human hydrocephalus.

At present, there were several animal models of hydrocephalus involving spontaneous gene mutations, transgenic mutations, gene overexpression or immunoneutralization of brain protein (Luis Federico Ba´tiz and Jose´ Manuel Pe´rez-Fi´gares 2006). A hereditary hydrocephalus animal model was established by over-expressing of human PAC1 receptor in mouse. PAC1 gene (ADCYAP1RI) is located at Chr7p15 and clinical evidence suggests that duplication of chromosomal region 7p15 in a patient developed the communicating hydrocephalus. The transgenic mice were characterized by dilated ventricular systems and abnormal cerebral cortex, corpus callosum, subcommissural organ (SCO) and ependymal cilia. The mechanism lying in this hydrocephalus model may be the abnormalities in the neuronal proliferation or apoptosis during the embryonic development. PAC1 is a G-protein coupled receptor. Binding of neuropeptide PACAP activates multiple signaling pathways including PKA, PKC and Ca++ signaling. The component CREB, a substrate of PKA, was detected has an abnormal expression in the ependymal cilia at the molecular level (Lang, Song et al. 2006). This work shows that elevated signaling of G-protein coupled receptors may cause hydrocephalus.

Another report demonstrated that hydrocephalus with hop gait (hyh) mice developed hydrocephalus in two to four days after birth (Wagner, Batiz et al. 2003). Later, the inherited hydrocephalus was also found in the hyh mouse carrying a point mutation (M1051) in protein α-SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein alpha). This protein way encoded by Napa gene located on chr7. The neuropathological studies in hyh mice found that maternal- and sex-related factors,
other than the a-SNAP mutation, were also associated with the development of hydrocephalus (Batiz, Paez et al. 2006).

As an important gene associated with obstructive hydrocephalus, Aqp4 mutants developed hydrocephalus in both rat and mouse (Mao, Enno et al. 2006; Shen, Miyajima et al. 2006). Characterizations of Aqp4 null mice were well described. It was reported that the Aqp4 null mice displayed three-fold increase in ventricular size compared with wild-type mice and obstruction of the cerebral aqueduct. Aqp4 encodes protein Aquaporin-4 (Aqp4) which highly expressed in glia and ependymal cells. Aqp4 was also associated with various processes of brain development including brain water balance, glial cell migration and neuronal excitation (Feng, Papadopoulos et al. 2009).

Recently, the gene Wave Associated Rac-GAP (Wrp) was also reported to associate with obstructive hydrocephalus. It was involved in the process of cytoskeletal organization and also was identified as a factor of syndromic and psychiatric disorders in human. Besides the common hydrocephalic phenotypes, massive cystic cavities and debris also were predominantly observed in the corpus callosum. They proposed that formation of cystic cavities and debris was induced by the disruption of the corpus callosum where the neural progenitor was abnormally located. Then debris produced in corpus callosum was released into the ventricular space and result in the blockage of the aqueduct. So the abnormalities in the migration of neural progenitor may be the initial cause of obstructive hydrocephalus (Kim, Carlson et al. 2012). Another study has independently strengthened the role of neural progenitors in the pathogenesis of hydrocephalus. Increased apoptosis and reduced proliferation of Ng2+/Pdgfrα+ neural progenitors induced the development of hydrocephalus (Carter, Vogel et al. 2012). This may provide us therapeutic targets to treat the common disorder.

Polycystin-1 (PC-1), encoded by Gene Pkd1 was identified as a genetic factor of hydrocephalus. It is expressed strongly and restrictedly in the ependymal and choroid plexus of brain. PC-1 mice by inactivation of gene Pkd1 exhibited hydrocephalus phenotypes both in embryos and newborn stages. The morphology of
choroid plexus and ependymal cilia were normal in model, suggesting that the PC-1 involved in the ciliary function rather than in genesis of cilia (Wodarczyk, Rowe et al. 2009).

Recently, 12 lines of genetically engineered mice were established and all of them displayed with autosomal recessive congenital hydrocephalus. 12 specific genes \((Ulk4, \text{Nme5, Nme7, Kif27, Stk36, Dpcd, Ak7, Ak8, RIKEN4930444A02, Celsr2, Mboat7, and FZD3})\) were indentified in knockout or overexpression mice. They all played major roles in the development of congenital hydrocephalus. Among them, 7 genes encode kinases and the rest of genes encode microtubule motor, acyltransferase, polymerase or Gpcr (Vogel, Read et al. 2012). Some genes are novel with unclear function, while functions of some genes were well described in the previous studies. \textit{Celsr2} was well studied and it played a major role in regulating neurons migration and growth of axons and dendrite in the brain development. As a member of NME/NM23 family, some studies reported that \textit{Nme5} was involved in numerous regulatory processes including proliferation, development and differentiation. All mutants of 12 lines exhibited various degree congenital hydrocephalus form mild to severe. The common phenotypes were dome head and enlarged ventricles system. Histological studies on these mice, dysfunctional motile cilia may be the cause of the hydrocephalus. In \textit{Ulk4} mutants, the cilia on respiratory epithelium and ependymal cells were reported to be shorter than those in wild type littermates, while cilia of \textit{Nme5} mice have the normal appearance (Vogel, Read et al. 2012).

Previous studies showed that the maturation of ependymal motile cilia occur form P5-P10 (Zhang, Williams et al. 2006). According to histological studies, hydrocephalus was present from P0 in some knockout mice, in which dilation of lateral ventricle and third ventricle were observed. The timing of initial hydrocephalus observed was prior to the cilia maturation. This mismatching time of hydrocephalus development and cilia maturation may suggest that dysfunctional motile cilia are not the only mechanism of pathophysiology of hydrocephalus or
onset of hydrocephalus in these mutants occurs independently of ependymal motile cilia function.

1.5 Dysfuntional motile cilia and hydrocephalus

The pathology of hydrocephalus is complex and one major cause is the dysfunction of motile cilia. Motile cilia are the complex and highly ordered organelle which is lining on the ependymal cells in the ventricular system. They have a basic structure called axoneme which is consisted of nine peripheral microtubule doubles and a central pair of microtubules, termed (“9+2”). Motile cilia are crucial for the CSF flow, because their synchronized beating is the driving force for the CSF circulation within brains and spinal cord. Abnormal or disturbed motility of ependymal cilia are unable to drive the flow of CSF within ventricular system, which leads to excessive CSF accumulation in the ventricle systems, and development of hydrocephalus. Up to now, numerous hydrocephalus animal models were showed to associate with disturbed ciliary motility, although the precise mechanisms of how these genes regulated cilia formation or functional maturation were not well understood.

Growing studies showed that the defective ciliogenesis resulting in altered CSF dynamics is a prevalent cause for hydrocephalus. For example, disruption of the outer dynein arm protein Mdnah5 results in impaired cilia motility on ependymal cells, which leads to hydrocephalus (Ibanez-Tallon, Pagenstecher et al. 2004). In Sperm-associated antigen 6 (Spag6) null mice, a significant number of mice died with hydrocephalus and the abnormalities in cilia included a reduction of cilia density, disorganized cilia arrays, random axoneme orientation, reduced number of basal feet (Sapiro, Kostetskii et al. 2002). Also Spag6 deficiency exhibited defects in ciliogenesis and cilia function: polarity, density, and beating (Teves, Sears et al. 2014). Hydin mutants have defective central pair resulting in impaired ciliary motility and CSF flow in the brain, which leads to hydrocephalus (Lechtreck, Delmotte et al. 2008).

The cellular processes affected in the ciliary defects include loss and abnormal length of cilia. Recently several regulators have been identified. It is worth to note
that the studies uncovered the mechanism of ciliogenesis may focus on the cells like human RPE1 cells or non-mammalian vertebrate animals like zebrafish, paramecium tetraurelia and Xenopus. The number of studies in mammalian models is limited due to complexities of cilia types and functions and complicated pathway signaling controls by other systems.

Recently, two gelsolin (GSN) family proteins GSN and AVIL were reported to involve the regulating of cilia number. Depletion of actin filament severing proteins GSN and AVIL by two independent siRNAs significantly reduced ciliated cell numbers (Kim, Lee et al. 2010). Katanin p80, coding by Katnb1, was recently shown to have a role in regulating overall centriole, mother centriole and cilia number. Loss of Katanin p80 resulted in over-duplication of centrioles and excessive maternal centrioles, which subsequently leading to supernumerary cilia (Hu, Pomp et al. 2014). PTPN23, a non-transmembrane tyrosine phosphatase, was expressed at the basal bodies. It was required for the ciliary vesicle targeting and silencing of PTPN23 markedly reduced the number of ciliated cells (Doyotte, Mironov et al. 2008). Knockdown of ASAP1, a gene required for pericentrosomal enrichment of recycling endosomes, was showed to decrease the number of ciliated cells, which linked the recycling endosomes and ciliogenesis (Inoue, Ha et al. 2008). Depletion of MKS3 (also known as Tmem67) exhibited global shortening and loss of cilia (Picariello, Valentine et al. 2014).

Actin-related protein ACTR3 (also known as ARP3) was showed to be a negative regulator of cilium length. It combines another actin-related protein ARP2 to form the ARP2/3 complex, which is essential for nucleating actin polymerization at filament branches. It was reported that loss of ACTR3 leads to an increase in ciliary length, suggesting an inhibitory role of branched actin network formation in the controlling of cilia length during ciliogenesis (Cooper and Schafer 2000). Focal adhesion complex was known to regulate actin cytoskeletal dynamics and depletion of a component of this complex, a-PARVIN (PARVA), led to lengthened cilia. 3-OST-5, a family member of 3-OST family, was showed to work together with FGF
signaling pathway to control cilia length via the transcription factors FoxJ1a and Rfx2 (Neugebauer, Cadwallader et al. 2013).

Foxj1 (also known as Hyh4), encodes a member of the forkhead family of transcription factors. Growing evidences shown that this gene was an important regulator for ciliogenesis, specifically it has a role in the biogenesis of motile cilia. The link between Foxj1 and motile ciliogenesis has been confirmed in different species. Foxj1 mutant mice exhibited loss of cilia on the ependymal cells, which subsequently resulted in hydrocephalus (Chen, Knowles et al. 1998). Also Foxj1 knockout mice showed a complete loss of the axonemes of motile multicilia of airways cells, and disrupted the 9+2 motile cilia, whereas the 9+0 immotile primary cilia were not altered. The defects in ciliogenesis were due to impaired process of basal body docking to the apical cell membrane. Thus, in multiciliated cells, Foxj1 is essential for basal body docking and for all of the subsequent steps involved in ciliary differentiation. Knockdown of Foxj1 in both Xenopus and zebrafish caused a loss of all motile cilia, and this further supported a role for Foxj1 in the cilia formation (Stubbs, Oishi et al. 2008; Caron, Xu et al. 2012). Taken together, it appears that Foxj1 plays an important regulatory role in the biogenesis of the motile cilia. RNA sequencing data showed that RNA expression of Foxj1 was increased in Ulk4−/− mice compared with littermate controls, suggesting everexpression of the Foxj1 may also be pathogenic to ciliogenesis.

Stk36 (serine/threonine kinase 36), a family member of Ulk4, was showed to be required for the central pair apparatus and cilia orientation of motile cilia in mouse. Stk36 deficient mice developed hydrocephalus and approximately 60% of Stk36 cilia exhibited abnormal ciliary ultrastructure. Loss of the central pair apparatus in motile cilia axonemes or disturbed cilia orientation resulted in defects in cilia motion abilities. The Stk36−/− cilia exhibited stiff movement and significant reduced stroke amplitude, even some cilia were immotile. Also in contrast to the coordinated beating in the wild types, cilia of Stk36 deficient mice beat disoriented with respect to their neighbours (Nozawa, Yao et al. 2013). Similar hydrocephalic phenotypes and ciliary defects were present in Ulk4−/− mice and this promoted us to seek the links
between *Ulk4* and *Stk36*. The RNA expression level of *Stk36* was not changed in *Ulk4*−/− compared to littermate control mice, suggesting there was no compensational changes in these two genes, and both are essential for ciliogenesis.

Sperm-associated antigen 6 (*Spag6*), was an axonemal protein which was essential for stability of the axoneme central apparatus. Both studies of *Spag6*-deficient mice showed that a significant number of mice died with hydrocephalus. They exhibited defects in brain including reduced cilia density, disorganized cilia arrays, random axoneme orientation and decreased number of basal feet. *Spag6* was not only important for the motility of ependymal cilia, but also essential for the sperm flagellar motility. *Spag6* deficient mice exhibited abnormal sperm without sperm head. The structures of flagella have been disturbed, including loss of the central pair of axonemes and disorganization of the outer dense fibers and fibrous sheath (Sapiro, Kostetskii et al. 2002; Teves, Sears et al. 2014). In *Ulk4*−/− mice, the RNA expression of *Spag6* was increased compared with controls.

*Celsr2* and *Celsr3*, encode the planar cell polarity cadherins, was showed to govern the formation and function of ependymal cilia. Mice lacking *Celsr2* have abnormal ependymal ciliogenesis resulting in defective CSF circulation and hydrocephalus. In double mutant of *Celsr2* and *Celsr3*, the ependymal cilia formation was significantly disturbed, leading to lethal hydrocephalus (Tissir, Qu et al. 2010). The membrane distribution of another two key planar cell polarity proteins, *Vangl2* and *Fzd3*, were affected in the absent of *Celsr2* separately or both of *Celsr2* and *Celsr3*. These emphasized the role of planar cell polarity signaling in the controlling of ependymal cilia development and in the pathophysiology of hydrocephalus (Tissir, Qu et al. 2010). In our study, there were no changes of RNA expression of *Celsr2*, *Celsr3*, *Vangl2* and *Fzd3* in *Ulk4*−/− mice and litter controls, but significant reduction of their family members including *Vangl1*, *Fzd2*, *Fzd6*, *Fzd10*, indicating there was potential regulation relationship between Ulk4 and these regulators of ciliogenesis, and they may participate in different signaling pathways including Wnt for governing the cilia formation.
CEP83 is a key gene encoding one component of distal appendages, which was essential for the docking and anchoring of the mother centriole to the cellular membrane during ciliogenesis. CEP83 is localized at the base of both primary and motile cilia. Inactivation of Cep83 led to ciliary dysfunction phenotype associated with primary cilia defects like nephronophthisis and motile cilia defect like hydrocephalus. A recent study of nephronophthisis-related ciliopathy gene screening showed that all eight of CEP83 biallelic affected patients had infantile nephronophthisis and four individuals presented hydrocephalus and other neurological alterations including learning and language disability. During ciliogenesis, the function of CEP83 is recruiting its partner CEP164/NPHP15 to the mother centriole, which is required for the docking of the mother centriole to the primary ciliary vesicle (Failler, Gee et al. 2014). In Ulk4−/− mutant, the RNA expression of Cep83 was significantly increased and RNA level of Cep164 was not altered compared with controls. This is not the only case, although the mechanism remains to be characterized. Cell culture of primary skin fibroblasts from two heterozygous Cep83 mutations indicated that Cep83 level was reduced whereas Cep164 level was unchanged, suggesting that Cep83 variations affect recruitment of Cep164 at the centrosome without any change in its total protein level. Also overexpression of Cep83 might somehow prevent the coordinated assembly of Cep/Daps onto the mother centroles (Failler, Gee et al. 2014).

Cep120, which encodes a core centriolar protein, was shown to be associated with hydrocephalus and skeletal ciliopathy like skeletal dysplasia. Knockdown Cep120 in zebrafish led to hydrocephalus (Shaheen, Schmidts et al. 2015). Also inactivation of Cep120 in mouse brain resulted in hydrocephalus, due to the absence of cilia on ependymal cells (Wu, Yang et al. 2014). These indicated the role of Cep120 in the cilia formation and function. In Ulk4−/− mice, RNA level of Cep83 and Cep120 was significantly increased compared with controls, suggesting there was direct or indirect relation between them, and the signaling pathway and mechanism remain to be investigated.
KIF19A, belonging to the kinesin superfamily, is a ciliary tip protein. It has ATP-dependent microtubule-depolymerizing activity. Recent genetic and biochemical evidence showed that KIF19A is an essential for the controlling the optimal length of motile cilia in mice. *Kif19a* mutant presented ciliary phenotypes including hydrocephalus and female infertility. The pathology of hydrocephalus observed in *Kif19a* mice might be due to the elongated cilia in the lateral ventricle, which were not able to produce proper fluid flow. The mechanism of controlling the ciliary length was that *Kif19a* regulated the length of microtubules polymerized from axonemes (Niwa, Nakajima et al. 2012). In *Ulk4*−/− mice, the RNA expression level is significantly decreased than that in wildtype mice.

1.5.1 Overview of Ciliogenesis

Cilia are the complex organelles which are found in eukaryotic cells and have various biological functions. They may serve as sensory organelles, clear the pathogen from airways, mediate signal transduction in vision and olfaction, drive CSF movement within the brain and affect fertility. Base on the different functions that cilia are involved, they show diverse changes in their shape, size, ultrastructural details, numbers per cell, motility patterns and sensory capabilities, despite the basic morphology is conserved. Cilia are generally divided into two types: motile cilia and primary cilia. The axoneme of the motile cilia is composed by nine radially arranged microtubule doublets with a central pair of singlet microtubules (termed “9+2”), which is important for the movement of cilia. The axoneme of primary cilia lacks the central pair apparatus, and is therefore termed “9+0”.

Ciliogenesis is a complicated and precisely controlled process, in which hundreds or even thousands of components are participated. Generally, ciliogenesis can be defined as three main stages: (1) generation of centrioles, (2) migration of amplified centrioles, and (3) Intraflagellar transport and cilia elongation. During each step, numerous component proteins and transcriptional regulators are involved and they exactly orchestrated with others.
1.5.2 Generation and duplication of centrioles:

Generally, in mouse ependymal multiciliated cells, there are two independent pathways to generate hundreds of centrioles (Al Jord, Lemaitre et al. 2014). A common centriolar pathway occurs during the S and G2 phases of dividing cells. Another is acentriolar/deuterosome pathway which usually occurs at the G0–G1 phase in terminally differentiated multiciliated cells (Tang 2013; Al Jord, Lemaitre et al. 2014). The significance of hiring two independent pathways to generate the same organelle is unknown.

In a recent study, Adel et al (Al Jord, Lemaitre et al. 2014) have suggested that the so-called ‘centriolar’ and ‘deuterosome’ pathways are the outcomes of a single pathway orchestrated by the centrosome, and that only one daughter centrosomal centriole is involved in the formation of deuterosome which produced over 90% of the final centriole population (Al Jord, Lemaitre et al. 2014). Also they created a new model of centriole amplification in multiciliated cells to further clarify the complex progress.

They divided the process of centriole biogenesis in multiciliated cells into two stages, the procentriole initiation (halo stage/deuterosome formation) stage and the late phases of centriole assembly (flower stage). The nascent halos budded from the wall of one centrosomal centriole, detached and accumulated in the nearby cytoplasm. By examination of the structural protein Sas-6 (Sass6) and the key regulators of centriole biogenesis Cep152 and Plk4, they found there was a switch between symmetric centriole duplication in cycling progenitors to asymmetric centriole duplication in postmitotic ependymal progenitors. In the proliferating cells, progenitors duplicate their centrosome, and these proteins were stained symmetrically on both mother and daughter centrosomal centrioles. However, in the postmitotic differentiating cells, these proteins activities were mainly detected in the daughter centrioles, not the mother centrioles.
Figure 1-5 Model of centriole amplification in multiciliated cells, Adapted from (Al Jord, Lemaitre et al. 2014).

The centrosomal daughter centrioles are mainly contributed to the production of procentrioles which form halos and are released into the cytoplasm. As the cells entered the halo stage, high level expression of Deup1 (encoded by Ccdc67 gene) in daughter was shown to trigger deuterosome formation. During the halostage/deuterosome formation, procentrioles displayed a tube-like structure organized around a cartwheel containing microtubule singlets (Al Jord, Lemaitre et al. 2014). Immediately after the formation of the last halo/ deuterosome at the daughter centriole, all the halo cells simultaneously transformed into flower-like structures. During the flower stage, the increasing rate of the precentrioles was significant than the procentrioles in halo-stage, because all procentriole sizes were similar and they grew synchronously. At the molecular level, the early markers of centriole biogenesis (Cep152, Plk4, Sass6, Stil, Ccap (=Sass4), Cep120 and Ccp110 were expressed in the halo stage procentrioles. While the markers for the late stages of centriole assembly like Poc5 and GT335 (=C21orf33) were detected in the flower procentrioles (Al Jord, Lemaitre et al. 2014).
1.5.3 Basal body formation, migration and docking

During ciliogenesis, the centriole becomes a basal body once the adornment with appendages projecting from the centriolar barrel. The appendages include distal appendages which are far from the nucleus and sub-distal appendages which are closest to the nucleus. Distal appendages are essential for the mother centriole
anchors to a membrane of the ciliary vesicle or plasma membrane and then become the pin wheel shaped transition fibres or alar sheets. In mammalian cells, distal appendage structures contain Cep164, Ccdc123/Cep123 and Ofd1 (oral–facial–digital syndrome gene OFD1).

Ofd1 is a domain distal centriole component and localizes to the central region of distal centriole. Ofd1 is required for controlling the process of recruiting Cep164 to the centriole and loss of Ofd1 results in failure of distal appendage formation and affects the subsequent processes like centriole docking to membranes and cilium formation (Singla, Romaguera-Ros et al. 2010). The Ofd1 is regulated by another distal centriole component Poc5.

Ninein and Odf2 protein belong to sub-distal appendages. Odf2 is essential for basal foot formation, which is critical for the consistent beating of motile cilia (Kunimoto, Yamazaki et al. 2012). Another sub-distal appendage component Ninein is recruited by the Odf2 protein, and Ninein promotes centriolar microtubule nucleation and anchoring (Singla, Romaguera-Ros et al. 2010).

After basal bodies maturation, small vesicles generated from Golgi apparatus are recruited and attach to the distal appendages of basal body. Subsequently, they fuse with each other to form a large membranous cap, the so-called ciliary vesicle, at the distal end of basal body. The ciliary vesicles migrate to the cell surface and the vesicles undergo fusion with apical membranes, allowing the basal bodies to anchor to the cell surface. Recently a new hypothesis is merged that the basal bodies migrate directly to the cell surface without attachment to vesicle, although details are not clear (Reiter, Blacque et al. 2012).

The processes of basal bodies migration and docking to the cell surface are complicated and numerous regulators were identified to control the processes. Distal appendage protein Cep164 is indispensable for the docking of vesicles at the distal appendages (Schmidt, Kuhns et al. 2012). Recent report showed that basal body component Chibby (Cby) is essential for efficient docking of basal bodies to the apical cell surface in airway ciliated cells (Burke, Li et al. 2014). Moreover, Helen R
et al demonstrated that Cby interacts with CEP164 and Rabin8 (encoded by *Rab3ip*) to promote the timely recruitment of Rab8 vesicles to the basal bodies before docking to the apical membrane (Burke, Li et al. 2014). Actin-myosin system was shown to be involved in basal bodies migration (Dawe, Farr et al. 2007).

The forkhead box protein J1 (FOXJ1), a transcription factor expressed in ciliated cells, was revealed to be central in centriole migration and apical membrane docking during ciliogenesis (Vladar and Stearns 2007). Centriole docking itself requires many other centrosomal proteins including Talpid3, Chibby (Cby1), Ofd1, Odf2 (Avasthi and Marshall 2012). The original positioning and orientation of the basal bodies is important for the alignment of the forming cilia. Once the alignment is determined, axonemal microtubules extend from the basal body and go beneath the developing ciliary membrane, forming the cilia.

![Image](image.png)

**Figure 1-7 Substructures of the centriolar barrel**, with the reported localization of several components shown, adopted from (Reiter, Blacque et al. 2012).

### 1.5.4 Intraflagellar transport and cilia elongation

The final step of ciliogenesis is the extension of axonemal microtubules from the basal bodies. During this process, proteins building for the axoneme were transported to the tip of the cilium by intraflagellar transport (IFT). These proteins cannot be synthesized within cilia and must be synthesized in the cytoplasm of the cell. IFT is a bidirectional movement along axonemal microtubules. There are two
different microtubule-based motors are responsible for the movement of IFT particles along the microtubules. The anterograde movement is driven by kinesin-2 (KIF3A, KIF3B, and KAP in mammals) and the retrograde is powered by cytoplasmic dynein 2 (=DYNC2H1) in vertebrates. When the cilium is completely formed, it continues to incorporate new tubulin at the tip of the cilia. This won’t affect the cilia length, because older tubulin is simultaneously degraded. The regulation role of IFT in two-way movements of tubulin and proteins suggests that the ciliary length may be determined by the balancing assembly and disassembly of tubulin (Avasthi and Marshall 2012). The ciliary length is not determined by total amount IFT protein in flagella, but is controlled by the size and number of IFT trains in flagella.

During the process of cilia elongation, some regulators have been identified. A member of the kinesin-2 family, Kinesin-II, was required for the assembly of central pair of axoneme of motile cilia on sea urchin embryos. Morris and Scholey microinjected the anti–kinesin-II monoclonal antibody into fertilized sea urchin eggs, and found the antibody injected embryo exhibited abnormal ciliary axoneme that lacked the central pair (Morris and Scholey 1997). The stability and motility of cilia were dependent on the post-translational modification state of tubulin. The acetylation, detyrosination, polygutamylation and polyglycylation of microtubules were involved in controlling the assembly and motility of cilia (Choksi, Lauter et al. 2014).
Figure 1-8 Direct and indirect targets of ciliary transcription factors (Choksi, Lauter et al. 2014).

Transcription factors regulatory factor X (RFX) family is essential for both motile and primary cilia generation by activating core components necessary for both types of cilia. One family member, $Rfx3$, is expressed in ciliated cell types in brain. In early stages of mouse brain development, $Rfx3$ is detected in ciliated ependymal cells lining the ventricular. During later stages, $Rfx3$ expression becomes progressively restricted to the cortex and to midline structures, such as the choroid plexus (CP), subcommissural organ (SCO) and the cortical septal boundary (Baas, Meiniel et al. 2006; Benadiba, Magnani et al. 2012). In line with these expression patterns, mice deficient in $Rfx3$ exhibit the disruption of the differentiation of ciliated cells of the CP and SCO, which leads to the disorganization of these structures and the development of severe hydrocephalus (Baas, Meiniel et al. 2006). Loss of $Rfx3$ is also associated with the malformation of the corpus callosum.
1.6 White matter, myelin, myelination and myelination disorders

In the Chapter 4 we have demonstrate that Ulk4 is involved in white matter integrity, oligodendrocyte production and myelination. For a better understanding of the white matter and related development processes, here I described some of the background information and reviewed white matter related disorders.

1.6.1 White matter and its functions in brain

A major difference of the brain from other organs is the overwhelming connectivity. Early neuroanatomy divided brain into gray matter (cell bodies) and white matter (cell projections). White matter is a component of the central nervous system and it comprises over half the human brain, a far greater proportion than in other animals (Fields 2008).

Within the white matter, there are three different types of tracts or bundles of axons. First type is commissural tracts which connects the two sides of the cerebrum to communicate with each other. As we known, corpus callosum, anterior and posterior commissures were belonged to this kind of commissural tracts. Another type is the association tracts which link the different area within the same side of the brain. The third type of tract is used for carrying the information between the cerebrum or the higher brain and spinal cord centers.

White matter is composed of bundles of myelinated axons that connect various grey matter areas of the brain to each other, and carry nerve impulses between neurons. For carrying the information between areas or hemispheres, the very important structure is the myelin of the tracts. Myelin is a fatty white substance that surrounds the nerve fiber and its insulating character makes it to increase the speed of electrical communication between neurons. Myelination is the production process of the myelin sheath. The myelin sheath is a layer wrapping around the axon of a neuron and is formed by the cell membranes of oligodendrocytes in CNS or Schwann's cells in peripheral system. Myelination is an important process in brain development and it greatly increases the speed and power of nervous system function (Bullock, 1984).
Also myelin formation is critical for the proper communication and survival of axons (Popko 2010).

In humans, myelination begins in the 14th week of fetal development and the process extends at least through the first 20 years of life. The prolonged period of myelination in humans coincides with the same period in which the human cerebral cortex undergoes massive remodeling of synaptic connections, which are understood to modify the brain according to experience. During infancy, myelination occurs quickly, leading to a child's fast development, including crawling and walking in the first year. In some animals, myelination is nearly completed by birth, such as horses, which are precocial and can walk and feed independently soon after birth.

Myelinogenesis occurs predominantly postnatally, within the first 3 weeks in rodents and first 2 years in humans which coincides with many neurodevelopmental disorders (From, Eilam et al. 2014). During the stages of oligodendrocyte development, oligodendrocyte progenitor cells (OPCs) undergo proliferation and differentiation into mature myelinating oligodendrocytes through distinct stages, characterized by sequential expression of developmental markers (Baumann and Pham-Dinh 2001).

Corpus callosum is the major white matter fiber tract in the brain that connects the left and right hemispheres. In mouse corpus callosum, myelin sheaths were first seen at 11 postnatal days, and the most rapid phase of myelination occurred between 14 and 45 days when 13.5% of axons were myelinated, but myelination continued at a reduced rate up to 240 days when 28% of axons were myelinated (Sturrock 1980). The corpus callosum changes structurally throughout life, but most dramatically during childhood and adolescence and finally it reaches to the adult morphology. The number of callosal fibers is already fixed around birth, but the thickness of the corpus callosum changes during postnatal development due to the degree of fiber myelination, redirection and pruning (Luders, Thompson et al. 2010).
1.6.2 Defects in white matter and disorders

White matter abnormalities have been involved in a wide range of psychiatric illness including schizophrenia, chronic depression, bipolar disorder, obsessive-compulsive disorder and posttraumatic stress disorder. Changes in white matter also are associated with neurodevelopmental, cognitive and emotional disorders including autism, dyslexia and attention-deficit hyperactivity disorder (Fields 2008). For example, schizophrenia patients typically present white matter reduction, including altered morphology, decreased dendritic arborization and agenesis of corpus callosum (Douaud, Smith et al. 2007; Bose, Mackinnon et al. 2009; Fornito, Yucel et al. 2009; Francis, Bhojraj et al. 2011). Corpus callosum is the major white matter fiber tract in the brain, which coordinates the activity of the left and right hemispheres. Growing evidence suggests that disruption of the corpus callosum development is associated with cognitive and behavioral deficits. Although a complete agenesis of the corpus callosum exists in schizophrenia but rare (Motomura, Satani et al. 2002; Chinnasamy, Rudd et al. 2006; Paul, Brown et al. 2007), mild alterations in inter-hemispheric callosal connections is not only common in schizophrenia (Wolf, Hose et al. 2008), but also in autism (Innocenti, Ansermet et al. 2003; Wolff, Gerig et al. 2015), bipolar (Caetano, Silveira et al. 2008), and attention-deficit disorder (Hynd, Lorys et al. 1991). Furthermore, agenesis of the corpus callosum is also a common pathology among >80 human congenital syndromes including ciliopathies and developmental disorders (Magnani, Morle et al. 2015).

Numerous studies have also provided a link between disrupted callosal development and genetic models of neurological disorders including hydrocephalus. For example, expression of the truncated Disrupted-in-schizophrenia 1 (Disc1) led to partial agenesis of the corpus callosum in Disc1tr transgenic mice (Shen, Lang et al. 2008). Overexpression of a G-protein coupled receptor PAC1 was associated to corpus callosum agenesis and hydrocephalus (Lang, Song et al. 2006). Mutations in LICAM also resulted in hydrocephalus, mental retardation and agenesis of the corpus callosum (Chidsey, Baldwin et al. 2014).
1.6.3 Relationship between whiter matter development and neuroinflammation

The development of white matter was not only affected by the genetic lesion, but also was influenced by the early life events like inflammatory. For example, to determine whether the inflammatory disrupted the development of white matter, Lieblein-Boff, McKim created an acute brain inflammation animal model by injection the *Escherichia coli* to the mice during the perinatal stages. They found a significantly hypomyelination and a decreased number of oligodendrocytes in subcortical region and motor cortex, which suggested that the white matter development was impaired by the infection at early life (Lieblein-Boff, McKim et al. 2013). Also another study evidenced that the systemic inflammation could alter the myelin formation by examining the mice with repeat IL-1β injections. These mice exhibited defects in oligodendrocyte maturation and further myelination (Favrais, van de Looij et al. 2011). The mechanism of early life inflammation disrupted the white matter development are poorly understood. Recently it was believed that the altered iron homeostasis in the brain may link early life inflammatory events to subsequent myelin-related deficits in brain and behavior (Lieblein-Boff, McKim et al. 2013). The white matter is not only co-related with myelination, but also the length/abundance of projections as well as number of projection neurons.

1.7 Cortex development and neurogenesis

In the Chapter 5 we showed that *Ulk4* is involved in cortex formation and neurogenesis including cell cycle, proliferation rate and stem cell pool. So in this part, we described the processes of and current understanding of cortiogenesis during embryonic and postnatal stages.

1.7.1 Overall of cerebral cortex

The cerebral cortex is the most key structure in the mammalian brain, involved in various cognitive processes such as leaning, memory, language, consciousness, awareness, reasoning and thought. The performance of highly complicated neural processes is based on the hundreds of distinct types of neurons. There are two main kinds of neurons in the cortex, the excitatory pyramidal neurons and inhibitory
interneurons. Different parts of cerebral cortex are associated with different specific function like sensory, motor, language, numeric processing etc, and association areas contain specific groups of neurons that are specialized in particular tasks.

The cerebral cortex is laminated structure which consists of distinct layers. The newest part of the cerebral cortex, the neocortex, is comprised by six horizontal layers, while the more ancient parts of the cerebral cortex, the hippocampus and olfactory cortex are differentiated into three cellular layers.

1.7.2 Corticogenesis

Corticogenesis is the process by which the cerebral cortex is formed by the generated cortical neurons. In mouse brain, it starts from embryonic days 10 to 11.5 (E10 to E11.5) and is completed largely at birth. All pyramidal neurons and a fraction of the inhibitory neurons arise from two germinal compartments, the ventricular zone (VZ) and subventricular zone (SVZ). Before the neurogenesis beginning, the neuroepithelial progenitors in the VZ undergo symmetric divisions to continually replenish the progenitor pool. Around embryonic day E10.5, radial glial cells are generated from the neuroepithelial progenitors and became the progenitors for the projection neurons. Radial glial cells undergo directly symmetric neurogenic divisions and indirectly give rise to inter precursor cells. The inter precursor cells will migrate away from the VZ to create SVZ, an additional proliferative zone, to under further neurogenic divisions after only once or twice symmetric divisions.

The preplate is formed by the first-born or pioneer neurons and it is located between the pia and the ventricular zone. There also are predecessors in the preplate, which later will become to subplate. At E11.5, the final cortical plate is created by the first projection neurons generated from VZ. When the cortical plate formed, the preplate are divided into marginal zone (MZ) and subplate (SP), which are important for the migration of later cortical plate neurons. From E11.5 to E16.5, various subtypes of cortical neurons are generated in an inner-out manner. The later born cortical neurons will across the first born neurons and settle within the superficial location of the cortical plate to form the upper layers. The first born cortical neurons will form
the relative deeper layers. It is believe that the lower layer neurons are generated by the VZ, and the upper layer neurons are produced by the SVZ (Dehay and Kennedy 2007).

Each cortical layer contains different neuronal shapes, sizes and density as well as different organizations of nerve fibers. Layer I, also called molecular layer, is firstly produced during early neurogenesis, E10.5-E12.5 in mouse. It contains two kinds of cells, Cajal-Retzius neurons and pyramidal cortical neurons. Reelin and transcription factor T-box brain 1 is expressed in these cells. Layer II-III is known as external granular layer and the external pyramidal layer. Cortical neurons in these two layers are created at E13.5-E16 during mouse embryonic development. Various types of cells including pyramidal neurons, astrocytes, stellates and radial glial cells are located in these layers. SATB2 and CUX1 are expressed in the pyramidal and stellate neurons. Layer IV is known as the internal granular layer. The function of this layer is receiving thalamocortical connections, especially from the specific thalamic nuclei. This is most prominent in the primary sensory cortices. Layer V is called the internal pyramidal layer. The pyramidal cells are the main cell type within layers V. These cells can be extremely large in layer V of the motor cortex, giving rise to most corticobulbar and corticospinal fibers. Layer V gives rise to all of the principal cortical efferent projections to the basal ganglia, brain stem and spinal cord. Layer VI is the multiform or fusiform layer, projects primarily to the thalamus.
1.7.3 Wnt signaling pathway and neurogenesis

Increasing evidence shows that Wnt signaling is essential for both embryonic and adult neurogenesis. During early development Wnt signaling is involved in regulation of various progresses of nervous system, including cell fate specification, cell polarity and cell migration (Bielen and Houart 2014). For example, Wnts are secreted glycoproteins which are necessary for the differentiation of precursor cells. Also other Wnt signaling components like morphogens and gradients of Wnt will have a key role in differentiation and migration of neural progenitor cells. In addition, Wnt signaling is involved in a series of fundamental biological processes like neuronal formation, dendrite and axon development, dendritic spine formation and synaptogenesis (Inestrosa and Varela-Nallar 2015). In the adult brain, Wnts have key regulation roles in hippocampal plasticity, synaptic transmission, and neurogenesis (Inestrosa and Varela-Nallar 2015). Dys-regulation of Wnt signaling pathway has been implicated in neurodegenerative diseases including Alzheimer's...
disease (AD) and Parkinson's disease (Berwick and Harvey 2012; Inestrosa and Varela-Nallar 2014). Abnormalities in Wnt signaling may have an aberrant regulation of new neuron development (Hussaini, Choi et al. 2014).

1.8 Anxiety

1.8.1 Overview of anxiety

Anxiety disorders are a category of mental disorders including generalized anxiety disorder, panic disorder and phobias, which are characterized by feelings of anxiety and fear. They are the most common mental health illness with an estimated prevalence of 20%, and approximately 69 million people are affected in the EU alone (Wittchen, Jacobi et al. 2011). The causes of anxiety disorders can be genetic abnormalities or/and drug use.

Recently Viggiano et al (Viggiano, Cacciola et al. 2015) reported that 67 genes were associated with anxious behavior. Among them, 33 genes whose deletions are accompanied with increased anxious behavior and 34 other genes whose deletions are related to decreased anxious behavior. Interestingly, they also show that presynaptic genes are involved in the emergence of anxiety, and postsynaptic genes in the reduction of anxiety after gene deletion by using gene enrichment analysis (Viggiano, Cacciola et al. 2015). For example, deletion of different neurotransmitter related genes for glutamatergic (GRM8, GRIN3B, SLC17A8), GABAergic (GAD2, SLC6A12), serotonergic (HTR1A, HDC), noradrenergic (ADRA2A), cholinergic (CHRNA4), somatostatinergic (SSTR2) systems induces higher anxiety; whereas the absence of some other genes for glutamatergic (GRIN2D, GRIA1) and dopaminergic (DRD3, NURR1) signaling reduces anxiety.

For the pharmacological treatment of anxiety disorders, anxiolytic drugs with different advantages and disadvantages are available. For example, benzodiazepines is well known anxiolytic drug which was enhancing the affinity of the natural agonist to the GABA_A receptor by acting at the benzodiazepine binding site on the
GABA<sub>A</sub> receptor (Sigel and Buhr 1997). Now diazepam is probably the best known anxiolytic drugs with relative safety. Lines of evidence showed that some neuropeptides such as substance P, corticotrophin-releasing factor and neuropeptide Y were associated with the anxiety and may potentially serve as the therapeutic targets for the treatment of anxiety disorders (Madaan and Wilson 2009).

**1.8.2 The Amygdala and anxiety**

The amygdala, belonging to the limbic system, was reported to play a key role in emotions and emotional behavior. Alterations in neuronal excitability in the amygdala are associated with psychiatric disorders including anxiety disorders and depressive disorders. Basolateral nucleus of the amygdala (BLA), a key component of amygdala, was particularly involved in anxiety. For example, growing studies showed that facilitation of serotonin-2C receptor (5-HT2CR)-mediated neurotransmission in the basolateral nucleus of the amygdala (BLA) is associated with anxiety generation (Vicente and Zangrossi 2012). Neuropeptide Y (NPY), a 36 aa peptide, is highly expressed in the amygdala. NPY binding with the presynaptic receptor Y2 has a potential anxiolytic effects.
1.9 Objectives

*Unc-51-like kinase 4 (ULK4)* was a relatively novel gene encoding a potential serine/threonine kinase (STK), and little was known about its functions. The objective of this PhD project was to investigate the roles of *Ulk4* in brain development and function using *Ulk4*−/− mice. Systematic analyses were carried out in this project, which uncovered the roles of *ULK4* in ciliogenesis and CSF flow (Chapter 3), in cell cycle and neurogenesis (Chapter 5), in white matter integrity and myelination (Chapter 4), and in anxiety-related behavior (Chapter 6).
CHAPTER 2

MATERIAL AND METHODS
2.1 Material

2.1.1 Instruments list

Upright Fluorescent Microscope (Olympus BX51)
Stereomicroscope (ZEISS Germany, stemi SVII)
Cryostat (Leica CM1850)
Microtome (Leica RM2235)
Tissue cold plate (Leica EG1130)
Heated paraffin dispensing module (Leica EG1150 H)
Tissue processor (Leica ASP300)
Nanodrop Spectrophotometer (Thermo Scientific Nanodrop ND-1000)
Mini-Protean Tetra Cell Electrophoresis Tank System
Alpha Innotech Gel Doc/Chemiluminescence System
PCR machine (G-STORM)
24 Place Microlitre centrifuge (Heraeus Biofuge Pico75003235)
Electric homogenizer
UV Transilluminator (VWR)
Perfusion pump

2.1.2 Ulk4 gene targeting construct

Ulk4 targeted, conditional ready, lacZ-tagged vector (PRPGS00083_A_C05) was constructed using knockout-first strategy. Methods used to create the CSD targeted alleles have been published (Skarnes, Rosen et al. 2011). The 5’ homologous recombination arm was comprised of 5682 bp Ulk4 genomic DNA which harbored 2398 bp of intron 3, exon 4-6 and 1401 bp of intron 6. The 3’ homologous recombination arm (4084 bp) contained 951 bp of intron 7, exon 8-12 and 610 bp of intron 12.

After targeted integration, the L1L2_Bact_P cassette was inserted at position Chr9:121,260,507(Build GRCm38), upstream of the critical exon(s). The cassette was composed of an FRT site followed by lac sequence and a loxP site. This first loxP site was followed by neomycin under the control of the human beta-actin
promoter, SV40 polyA, a second FRT site and a second loxP site. A third loxP site was inserted downstream of the targeted exon(s) at position Chr9:121,259,772. Further information on targeting strategies can be found at http://www.knockoutmouse.org/aboutkompstrategies. The exon 7 with 76 bp Ulk4 coding sequence was deleted by targeted integration, and replaced by a cassette containing SA-IRES-lacZ sequence, so that lacZ reporter gene can be expressed under the endogenous mouse Ulk4 regulatory machinery.

2.1.3 Ulk4 knockout mice

The ES cell clone EPD0182_4_E12 was generated by the Wellcome Trust Sanger Institute using the knockout construct. This was used to create Ulk4 knockout mouse strain by the KOMP Repository (WWW.KOMP.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. We acquired Ulk4+/− breeding pairs on C57BL/6N strain background from KOMP Repository (knockout mouse project, USA) at UC Davis. All experimental procedures were conducted under a license approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland (NUI) Galway, Ireland.

Ulk4−/−, Ulk4+/− and WT littermates were obtained from Ulk4+/− x Ulk4+/− mating. Mice were genotyped by PCR, using genomic DNA with two pairs of DNA primers: Ulk4EndE7For (5’-TAACTTGCTGGACGGATTGCTG-3’) and Ulk4EndIn7Rev (5’-TGATCTGTAATCGCAGTGCAGG-3’) amplifying a 271bp DNA fragment from the WT allele, and Ulk4KOMPKOFor (5’-GAGATGGCGCAACGCAATTAATG-3’) and Ulk4KOMPKORev (5’-CTGAGGAGACAATGTGACCAG-3’) to synthesize a 621bp DNA fragment from the knockout allele, respectively.
2.1.4 A list of primary and secondary antibodies, justification of choice and other details

Rabbit polyclonal Anti-ULK4 (NBP1-20229, Novus Biologicals, 1:1000) against aa 750-800 of full length ULK4 protein; Other ULK4 antibodies included N-15 against aa 1-50, F-18 against aa 1000-1050 and Y-13 against aa 1150-1200 (Santa Cruz);

Mouse monoclonal Anti-Acetylated Tubulin (Sigma-Aldrich, T7451-200UL, 1:1000), ciliary marker;

Mouse monoclonal Anti-Olig2 (Millipore, MABN50, 1:1000), oligodendrocyte marker;

Rabbit polyclonal Anti-MBP (82-87), RTX-1ML (Millipore, MAB386, 1:1000), myelin marker;

Rabbit polyclonal Anti-GFAP (Z0334, Dako, 1:1000), astrocyte marker;

Rabbit polyclonal Anti-Iba1 (Wako, 019-19741, 1:1000), microglia marker;

Mouse monoclonal Anti-Phospho-Histone H3 (Ser10) (6G3) (Cell Signaling Technology #9706, 1:1000), M phase marker;

Rabbit polyclonal Anti-Ki67 (Abcam, ab16667, 1:500), proliferating cell marker;

Mouse monoclonal Anti-Human Ki-67, Clone B56 (RUO) (BD, 550609, 1:1000), proliferating cell marker;

Rabbit polyclonal Anti-Cux1 (Santa Cruz biotechnology, sc-13024, 1:1000), cortex layer II-IV marker;

Rabbit polyclonal Anti-Ctip2 (Abcam, ab18465, 1:500), cortex layer V-VI marker;

Rabbit polyclonal Anti-Tbr1 (Abcam, ab31940, 1:500), cortex layer VI marker;

BrdU In-Situ Detection Kit (BD Bioscience, 551321, 1:10);

Mouse monoclonal Anti-GAD67 (Millipore MAB5406 1:1000) for Gabaergic neurons;

Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor®555 Conjugate) (Cell Signaling Technology #4409, 1:1000);

Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor®488 Conjugate) (Cell Signaling Technology #4412, 1:1000);

Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor®555 Conjugate) (Cell Signaling Technology #4413, 1:1000).
2.2 Methods

2.2.1 Preparation of genomic DNA for genotyping

A Tail Lysis Buffer (100 mM Tris.Cl pH8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) was used to extract mouse genomic DNA. Adult mice were locally anesthetized before tail biopsy, and embryos were decapitated before tail sampling. A piece of tail (about 5 mm long) was obtained and transferred into an Eppendorf tube. A 0.5 ml Tail Lysis Buffer and 5-10 µl of 20 mg/ml Proteinase K were then added into each tube. The tubes were incubated and shaken at 50-55°C overnight. Efficient digestion would be critical. The next day, the tubes were vortexed and microcentrifugated at the top speed for 10 min to pellet undigested hair, and the supernatant was transferred to a new Eppendorf tube (Omit this step for embryonic tails). A 0.5 ml isopropanol was added and tubes inverted a few times. Genomic DNA was pelleted by microcentrifugation at the top speed for 10 min at 4°C, and the supernatant was poured off, DNA pellets were washed with 70% ethanol, dried by Speed Vac (5 min, setting at Medium) or room temperature, and resuspended in 100 µl of dH2O. DNA was dissolved by incubation at 50°C with regular vortex. A 1-2 µl of DNA (100 ng) was used for each PCR reaction in a total PCR volume of 25 µl for genotyping.

2.2.2 Polymerase chain reaction (PCR)

For amplification of DNA fragments from genomic DNA, PCR was performed. About 100 ng of DNA template was used with the 2x PCR Mastermix in 25 µl total reaction volume. The PCR reactions (25 ul in total) were prepared as the following: 1 µl of DNA (100ng), 1.5 µl of MgCl2 at 25mM; 0.125 µl of each of primers at 100 pmol., Master Mix 2X (12.5 µl), Top up with H2O to 25 µl per reaction.

The following cycles were passed:

\[
\begin{align*}
95°C & \quad 2\text{min} \quad 1 \text{ cycle} \\
95°C & \quad 20 \text{ seconds} \\
64°C & \quad 30 \text{ seconds} \\
72°C & \quad 30 \text{ seconds}
\end{align*}
\]

5 cycles
94°C 30 seconds
62 °C 30 seconds
72 °C 30 seconds
72°C 5min 1 cycle

25 cycles

PCR products were stored at 4°C before agarose electrophoresis.

2.2.3 Electrophoresis

A 1.5% agarose gel was used to resolve amplified DNA products. Weigh out 1.5 g agarose into an Erlenmeyer flask. Add running buffer (1x TAE) to the flask and swirl to mix. Melt the agarose/buffer mixture by heating in a microwave. Add SYBR safe 1:10,000 dilution into the flask. Place an appropriate comb into the gel mold to create wells. Pour the molten agarose (cooled to ~60°C) into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box with 1x TAE running buffer to cover the gel. Add loading dye to the PCR products. Program the power supply to 120-150V. Carefully load DNA samples into wells of the gel with a lane of 1kb DNA ladder for each row. Run the gel until the dye migrated to an appropriate distance. Capture an image of the gel on the UV transilluminator. Examine the DNA bands for anticipated sizes to genotype mice.

2.2.4 RNA isolation from mouse brain tissue and quality assessment (Followed the protocol of Dr.Enda O’Connell RNA Isolation & Quality Assessment)

Use filter tips and Nuclease-free tubes. Spray bench and pipettes with RNase Zap. Add 1ml TRI Reagent into the frozen mouse tissue. Homogenize fully and transfer the lysed tissue to new 1.5 ml tube, then incubate on ice for 5 min. Add 200 µl chloroform (per 1 ml Tri reagent) and shake vigorously for 15 sec to allow layers to separate for 15 min on ice. Centrifugate the tubes at 12,000 x g at 4°C for 15 min. Remove clear upper aqueous phase (≈ 650 µl) and transfer to new 1.5 ml tube. Slowly add 1 volume of 70% Ethanol (in 3 approximately equal aliquots) and mix by inversion. The remaining steps were performed according to manufacturer’s instructions of RNeasy Mini Kit (Qiagen, Netherlands). Nanodrop was used to
quantify RNA at O.D. 260 and calculate O.D. 260/280 and 260/230 ratios. Total RNA in 1000 ng aliquots was stored at -80°C.

### 2.2.5 Transcardial Perfusion of Mice

The mice were weighed and humanely terminated individually with over-dose of sodium pentobarbitone. The skin was removed and the heart of mouse was exposed. A syringe needle was inserted into the apex of the left ventricle and the needle was connected with the perfusion pump by long tube. Then a cut was made at the right atrium using a pair of scissors at right angle, and the pump was turned on to drain the 5ml PBS into the heart. The blood was flowing out from the left ventricle to the right atrium. After draining of PBS, 10ml 4% paraformaldehyde (PFA) at 4°C was slowly pumped into the heart. The brain was dissected and post-fixed in 4% PFA for 24 hours.

### 2.2.6 Cryosection

After perfusion brains were post-fixed overnight at 4°C in 4% PFA and equilibrated in 25% sucrose solution o/n at 4°C. Brains with freezing medium OCT were frozen on dry ice or at -20°C and fixed on an object holder. On a cryostat, brain slices of 30 µm were cut and mounted onto the slides, then the slides were kept in -20°C freezer before use.

### 2.2.7 Paraffin section

1) Paraffin embedding

After perfusion brains were post-fixed for 24 hours in 4% PFA at RT, dehydrated in an ascending ethanol scale, and equilibrated and embedded in paraffin. The automated embedding machine was used in this step.

2) Cutting paraffin embedded tissue with the microtome

Paraffin embedded brain tissue was first mounted on a tissue cassette with paraffin and fixed on the microtome. Sections at 20 µm thickness were cut and put into a water bath (37-42°C) for flattening. Sections were mounted on slides and dried on a
heating plate and/or in an incubator at 37°C. Slides with brain sections were stored at 4°C or used directly.

2.2.8 Hematoxylin and Eosin (H&E)

Paraffin section slides were placed in a slide holder. The sections were deparaffinized and rehydrated for 2 x 8 minutes in Xylene, 2 x 5 minutes in 100% ethanol, then sequentially dipped in following solutions: 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, then into dH2O. They were stained with Hematoxalin for 10 minutes, washed in flowing tap water for 15 minutes, and then rinsed in dH2O and in 90% ethanol. After that, slides were stained with Eosin for 30 seconds, washed for 3 x 2 min in 95% ethanol, 3 x 5 min in 100% ethanol, and cleared for 3 x 5min in Xylene. The slides were covered by coverslip with mounting medium and dried overnight in the hood.

2.2.9 Immunohistochemistry

Firstly preheat brain sections at 60°C for 20 min, then deparaffinize sections in 2 changes of xylene, 10 minutes each and hydrate sections in 2 changes of 100% ethanol for 5 minutes each, 95% ethanol for 1 minute, then rinse in distilled water. Pre-heat water bath with staining dish containing antigen retrival solutions and load the section slides into the dish until temperature reaches 60°C. Incubate the sections in the solutions for 20 minutes at 95-100°C. After that, turn off water bath and allow the slides to cool for at least 20 minutes. Rinse sections in PBS pH7.0 for 3x10 min, and treat with 3% H2O2 for 10 min. Block sections with 10% normal goat serum for 60 minutes, then incubate sections with primary antibody at appropriate dilution in primary antibody dilution buffer overnight at 4 °C in a humidity box. Next day leave the humidity box at the RT for 20 min firstly, and rinse sections with PBS for 3x10 min. Block sections with peroxidase blocking solution for 30 minutes. Rinse with PBS for 3x10 min before applying the ready-to-use Streptavidin-HRP to each slide, and incubate for 30 minutes at room temperature. Rinse slides 3x in PBS, 2 minutes each time. Prepare DAB substrate solution by adding 1 drop of DAB chromogen to 1 ml of DAB Buffer. Apply DAB substrate solution to cover the tissue sections and
incubate for 5 minutes or less until the desired color intensity is developed. Rinse the slides 3× in water, 2 minutes each time. Stain in hematoxylin for 30 – 60 seconds and rinse thoroughly in water. Dehydrate through 4 changes of alcohol (95%, 95%, 100%, 100%) for 5 minutes each. Clear in 3 changes of xylene (or xylene substitute) and mount with a coverslip.

2.2.10 Immunofluorescence

The first few steps were exactly same as immunohistochemistry. After the incubation in antigen retrieval solution, skip the H2O2 treatment step and continue with the blocking with 10% normal goat serum for 60 minutes. After incubation in primary antibody, secondary antibody Anti-rabbit IgG (H+L), F(ab’)2 Fragment (Alexa Fluor®488 Conjugate) and/or Anti-rabbit IgG (H+L), F(ab’)2 Fragment (Alexa Fluor®555 Conjugate) were used. Images were captured by upright fluorescent microscope (Olympus BX51).

2.2.11 Data acquisition of the immunohistochemistry and Immunofluorescence

A series of attempts were made to minimise the potential for bias in the statistical analysis of immunohistochemical and immunofluorescence assays; (1) Littermate mice were used to avoid age interference; (2) a minimum of 3 mice (n>3) from each genotype group were used and the exact number for each experiment was documented in each Chapter; (3) identical thicknesses of serial brain sections were produced from each mouse; (4) sections were immunohistochemically processed with the same durations for respective steps to maximise comparability of staining intensity between different repeats; (5) images were acquired from only anatomically comparable brain sections; (6) for each mouse in each experiment, 3-6 comparable brain sections were imaged, quantified, and only the average was taken as the representative data for the individuals; and (7) the images were blindly and independently validated by volunteers, summer students and honours project students.
2.2.12 Proliferation assay
The day plug found was recorded as E0.5. Time-mated pregnant females were injected with a pulse of BrdU (i.p., 50 mg/kg body weight) at E15.5. 2 hours later, embryos were fixed into 4% PFA overnight. BrdU incorporation assay (BD Biosciences) was executed according to the manufacturer’s instruction.

2.2.13 X-gal staining for brain sections
At P14, mice were briefly perfused. Brains were dissected, post-fixed in 4% PFA for 10 min, then were washed in PBS twice for 5 min each. Brains were cryoprotected in 30% sucrose in PBS overnight at 4°C until brains were saturated and sunk to the bottom of the tubes. Brains were embedded in OCT and immersed in isopentane bath pre-cooled with liquid nitrogen. The samples were sectioned on a cryotome at 30 μm and mounted onto VWR® Superfrost® Plus Micro slides. The sections were post-fixed in 4% PFA for 10 minutes on ice, rinsed twice in 1X PBS for 10 minutes each on ice, placed in detergent rinse (2mM MgCl2, 0.02% Nonidet P-40, 0.01% Sodium deoxycholate in PBS) for 10 minutes on ice, and stained in X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) staining solution (1 mg/ml X-Gal, 5mM Potassium ferricyanide, 5mM Potassium ferrocyanide, 2mM MgCl2, 0.02% Nonidet P-40, 0.01% Sodium deoxycholate in PBS) overnight at 37°C in the dark. Next day slides were washed twice in 1X PBS for 5 minutes each at room temperature. Then X-gal stained sections were rinsed in distilled H2O, counter-stained for 30 seconds in the Eosin, washed three times in distilled H2O for 5 minutes each, and dehydrated through ethanol (5 minutes each in 50%, 70%, and 100% ethanol). Sections were cleared twice for 5 minutes each in xylene before mounting for microscopic examination.

2.2.14 Scanning Electron Microscope
Lateral wall of the lateral ventricles dissected from mice was fixed in primary fixative solution (2.5% glutaraldehyde/ 2% paraformaldehyde/ 0.1M sodium cacodylate/HCl buffer, pH7.2) overnight at 4 °C. After dehydration through graded alcohols, tissue samples are transferred to Hexamethyl disilizane (HMDS) for 2x15
min changes. Then samples are allowed to dry for at least 2 hours in a fume hood. Dried samples are fixed to metal stubs using either Carbon adhesive tabs or silver dag paint, gold sputter coated and viewed on a Hitachi S2600N Variable Pressure Scanning Electron Microscope.

2.2.15 Transmission Electron Microscope

Lateral wall of the lateral ventricles dissected from mice was fixed in primary fixative solution (2.5% glutaraldehyde/ 2% paraformaldehyde/ 0.1M sodium cacodylate/HCl buffer, pH7.2) overnight at 4 °C. After primary fixative, tissues were fixed in secondary fixative solution (1% Osmium Tetroxide in 0.1 M Sodium Cacodylate/HCl buffer pH 7.2) for 4 hours. Then tissues were dehydrated through a series of graded alcohols 50%, 70%, 90%, 95% and 100%. Then tissues were placed in Propylene Oxide to replace the pure ethanol. After that, tissues were transferred to a series of mixture of resin and Propylene Oxide, 50:50, 75:25, pure resin. Finally, tissues were transferred to flat embedding moulds, clearly labeled and placed in 65 degree oven for 48 hours and allowed to polymerise. After polymerization, blocks are sectioned at 100nm, lifted onto 3mm copper grids, stained for 30min in 1.5% aqueous Uranyl Acetate for 10 min in Lead Citrate. Sections are allowed to dry and then viewed on the Hitachi H7000 Transmission Electron Microscope.

2.2.16 CSF Circulation

P12 days old mice of Ulk4^−/− hydrocephalic and litter controls were anesthetized. Then a 0.5-mm burr hole was drilled 3 mm posterior and 3 mm lateral to the Bregma as described previously study (Feng, Papadopoulos et al. 2009). Evans blue dye solution (4 µl, 4% in phosphate-buffered saline, Sigma-Aldrich) was injected into the right lateral ventricle through the hole by a 30-gauge needle attached to a 10-IL Hamilton syringe. 20 min later, mice were humanely killed with an overdose of pentobarbital sodium, and whole brains with a portion of the spinal cord were surgically removed and fixed in 4% formalin solution for 24 hr. The fixed brains were coronally sliced at 1-mm intervals from the anterior horn of the lateral ventricles to the medulla oblongata with a sections chopper. The blockage site of
CSF flow pathway in hydrocephalic mice was determined from the Evans blue dye distribution (Feng, Papadopoulos et al. 2009).

2.2.17 Statistical analysis

Most statistical analyses were carried out by one-way ANOVA with two-tail to compare the WT and homozygously disrupted mice and SPSS statistical package was used to analyze all data in Chapter 6 between WT and heterozyhous mice as specified. The n number was larger than 3 but varied as specified in each experiment in the figure legends. Most of the experiments were successfully repeated at least three times. Anatomically comparable and multiple sections were imaged and quantified, and their average numbers were taken as the representative number for each mouse. At least three coronal brain serial sections per mouse were analyzed. Image J software package was used to quantify the brain areas from comparable sections. The data were presented as mean ± SEM. One-way ANOVA or SPSS statistical package was carried out with a two-tail comparison. A p-value of less than 0.05 (*) indicated statistical significance, and a p-value of less than 0.01 (**) indicated statistically very significant.

2.2.18 Whole genome RNA sequencing

To reveal the molecular network of Ulk4, we carried out whole genome RNA sequencing with cortical RNA. Three pairs of Ulk4−/− mice and WT littermate controls at P12 were used in this study for whole genome RNA sequencing due the costly nature of the experiments. Mice were euthanized by CO2 exposure, and then humanely terminated by cervical dislocation. Mouse cortexes were quickly dissected and snap frozen in liquid nitrogen. An RNeasy Kit (QIAGEN) was used for RNA extraction and sample preparation procedures were performed according to the manufacturer’s instructions. The RNA concentration was measured using a Nanodrop Spectrophotometer. Equal amounts of total RNA (6µg/sample) were sent to BGI for commercial sequencing service. Libraries were constructed to convert RNA to cDNA, and quantitative RNA sequencing was carried out by using the Illumina HiSeq2000 next generation sequencer.
A total of 19,651 reads of RNA sequences were acquired, screened with gene IDs validated from DNA database (See Figure 2-1A for scatter plot below). One-way ANOVA was carried out to statistically compare the RNA transcriptional levels of individual genes with one–tail due to small sample size (n=3 each). A total of 2829 genes with significant change had been identified. Among them, 1005 genes were significantly downregulated and 1824 genes were significantly upregulated. The low expression genes were filtered out with an expression value of 1 in either WT or Ulk4−/− mice. Subsequently, the gene list was further reduced to 618, with >20% reduction or >150% increase in the Ulk4−/− mice (See Figure 2-1B).

Figure 2-1. Scatter plot of gene expression from whole genome RNA sequencing. (A) Scatter plot of quantitative expression of 19651 genes in the WT (Y axis) and Ulk4 knockout (X axis) cortex. (B). Scatter plot of quantitative RNA expression of 618 dys-regulated genes in the WT (Y axis) and Ulk4 knockout (X axis) cortex. The diagonal lines in red indicate the position if they have identical levels of gene expression. The majority of genes are not grossly deviated from the diagonal line.

Subsequently, the dys-regulated genes were analyzed for pathways using the free-online software STRING program (http://string-db.org/). The 618 Ulk4 targets from the whole genome RNA sequencing were analysed for pathways in the STRING program, which provided the following of information for each pathway (Table 2-1.), i.e.
1. “GO” number (i.e. GO:0048709);
2. Pathway name, i.e. oligodendrocyte differentiation;
3. Significance of the pathway with three p values of i.e. 2.62E-06, 3.52E-02, 1.35E-03;
4. Number of genes involved, i.e. 8,
5. The gene names, i.e. Plp1, Cnp, Gm98, Fa2h, Olig2, Tspan2, Sox10, Dusp10.

Table 2-1. An example of output of GO pathway analyses from STRING program.

<table>
<thead>
<tr>
<th>GO number</th>
<th>Pathway</th>
<th>No of genes</th>
<th>p</th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0048709</td>
<td>oligodendrocyte differentiation</td>
<td>8</td>
<td>2.62E-06</td>
<td>3.52E-02</td>
<td>1.35E-03</td>
</tr>
</tbody>
</table>

Plp1
Cnp
Gm98
Fa2h
Olig2
Tspan2
Sox10
Dusp10

Then, the expression data of individual genes were pulled out from the original RNA sequencing dataset (Table 2-2). The statistics was applied to calculate the mean of WT (M-WT) and KO (M-KO) mice; the standard error of mean (SEM); the expression ratio (KO/WT), p value. For example,

Table 2-2. An example of whole genome RNA sequencing and data analyses.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Transcript ID</th>
<th>KO1</th>
<th>KO2</th>
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The final list of 618 Ulk4 dys-regulated genes related to this thesis was not presented in an appendix, however, it is acknowledged here that: (1) these data require further experimental validation; (2) neither qRT-PCR nor Western blotting is sufficiently sensitive to match the sensitivity of quantitative RNA sequencing; and (3) these data are confidential before formal publications.
CHAPTER 3

ULK4 IS ESSENTIAL FOR CILIOPHORESIS AND CSF FLOW.
3.1 Abstract

Ciliopathies are an emerging class of devastating disorders with pleiotropic symptoms affecting both the central and peripheral systems, which are commonly associated with hydrocephalus. Despite ciliary components and three master transcriptional regulators identified, little is known about the signaling molecules involved. We previously identified a novel *Unc51-like-kinase 4 (ULK4)* as a risk factor of neurodevelopmental disorders. Here we took multidisciplinary approaches and uncovered essential roles of *Ulk4* in ciliogenesis. We show that *Ulk4* is predominantly expressed in the ventricular system, and *Ulk4*−/− ependymal cells display reduced/disorganized cilia with abnormal axoneme. *Ulk4*−/− mice exhibit dysfunctional subcommissural organ, obstructive aqueduct and impaired cerebrospinal fluid flow. Mechanistically, we performed whole genome RNA sequencing and discovered that *Ulk4* regulates the *Foxj1* pathway specifically and an array of other ciliogenesis molecules. This is the first evidence demonstrating that *Ulk4* plays a vital role in ciliogenesis and deficiency of *Ulk4* can cause hydrocephalus and ciliopathy-related disorders.
3.2 Introduction

Ciliopathies are associated with neurodevelopmental disorders of pleiotropic clinical symptoms, including Joubert syndrome, Bardet-Biedl syndrome, Meckel-Gruber syndrome, Oral–facial–digital syndrome type 1 and Nephronophthisis, all of which are commonly associated with hydrocephalus (Lee and Gleeson 2011). Handedness, linked to high rates of dyslexia and schizophrenia, is also shown to involve ciliopathies (Brandler and Paracchini 2014). MIPT3, an interactive protein of Disrupted-In-Schizophrenia 1 (DISC1), which is truncated in a large Scottish schizophrenic family (Millar, Wilson-Annan et al. 2000), functions synergistically with the Bardet-Biedl syndrome protein Bbs4 and plays a critical role in assembling intraflagellar transport (IFT) particle complexes (Li, Inglis et al. 2008). Remarkably, when 41 candidate genes associated with schizophrenia, bipolar affective disorder, autism spectrum disorder and intellectual disability were investigated in cultured cells, 23 were found to regulate cilium length (Marley and von Zastrow 2012). Several lines of evidence point to a link of the Unc51-like-kinase 4 (ULK4) gene to human diseases which involve neurological abnormalities. We recently demonstrate that the ULK4 gene is a rare risk factor for schizophrenia, autism and bipolar disorder (Lang, Pu et al. 2014). We have unpublished data that ULK4 is deleted in some patients with heterogeneous clinical features including developmental delay, language delay and severe intellectual disability. Depleted ULK4 expression in neuroblastoma cells disrupts microtubular composition, compromises neuritogenesis and cell motility, and modulates multiple signaling pathways which are associated with schizophrenia. Ulk4 is developmentally regulated by morphogens, and there is a switch in Ulk4 isoforms during mouse brain formation and neuronal maturation. Targeted Ulk4 deletion compromises the integrity of the corpus callosum in mice (Lang, Pu et al. 2014), and agenesis of the corpus callosum is a frequent brain disorder found in >80 human congenital syndromes including ciliopathies and neurodevelopmental disorders (Laclef, Anselme et al. 2015).

To investigate the role of Ulk4 during brain formation, we recently characterized Ulk4 gene expression during Xenopus development, and found co-expression of Ulk4 mRNA with Sox3 (a neural progenitor cell marker) and Blbp (a radial glial
marker) in the ventricular zone of the forebrain (Dominguez, Schlosser et al. 2015). To understand the consequence of genetic lesions, here we systematically investigated $Ulk4^{+/ -}$ mice. We demonstrate that $Ulk4$ is predominantly expressed in the cells lining the ventricles and essential for ciliogenesis. $Ulk4^{+/ -}$ mice exhibit dysfunctional subcommissural organ (SCO), obstructive aqueduct and non-communicating hydrocephalus. The CSF flow is impaired in $Ulk4^{+/ -}$ mice, and interestingly, $Ulk4^{+/ -}$ ependymal cells display reduced/disorganized/dysfunctional cilia with abnormal axoneme, which is required for directional beating of ependymal cilia. Furthermore, we present molecular mechanism that $ULk4$ regulates expression of $Foxj1$, a master switch of ciliogenesis, and numerous other ciliogenesis molecules. Therefore, our findings indicate that $ULk4$ may act as a scaffold protein regulating different processes of ciliogenesis and coordinated beating, and $ULk4$ may be associated with human diseases of heterogeneous clinical symptoms in relation to cilia dysfunction.

### 3.3 Materials and Methods

#### 3.3.1 $Ulk4$ knockout mice:

The $Ulk4$ mutant strain used for this research project was created from ES cell clone EPD0182_4_E12 generated by the Wellcome Trust Sanger Institute and made into mice by the KOMP Repository (WWW.KOMP.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. Methods used to create the CSD targeted alleles was published elsewhere (Skarnes et al, 2011). Breeding pairs of $Ulk4^{+/ -}$ mice on C57BL/6N strain background were purchased from KOMP Repository. All experimental procedures were conducted under a license approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland (NUI) Galway. $Ulk4^{+/ -}$, $Ulk4^{+/ +}$ and WT littermates were obtained from $Ulk4^{+/ -} \times Ulk4^{+/ +}$ mating. Each mouse was genotyped by genomic
PCR with two pairs of PCR primers: Ulk4EndE7For (5’-TAACTTGCTGGACGGATTGCTG-3’) and Ulk4EndIn7Rev (5’-TGATCTGTAATCGCAGTGCAGG-3’) to uniquely amplify a 271bp DNA fragment from exon 7 in the WT allele, and Ulk4KOMPKOFor (5’-GAGATGGCGCAACGCAATTAATG-3’) and Ulk4KOMPKORev (5’-CTGAGGAGACAATGTAACCAGC-3’) to produce a 621bp DNA fragment solely from the knockout allele.

3.3.2 Histology:

*Ulk4*−/− and 4 litter controls were deeply anesthetized and intra-cardiaca1ly perfused with 20 ml of 4% paraformaldehyde in PBS. The brains were dissected and post-fixed in the fixative solution for 24 hours. Then brains were embedded into paraffin blocks. Serial coronal sections at 10um were cut by a microtome (SM2000R, Leica Instruments). The sections were stained with hematoxylin-eosin and imaged under a bright filed microscope (IX41, Olympus) equipped with a camera.

3.3.3 CSF Circulation assay:

Mice at P12 (3 *Ulk4*−/− and 4 litter controls) were anesthetized by intraperitoneal injection of ketamine ketamine (150 mg/kg)/xylazine (15 mg/kg). The 30-gauge needle attached to a 10-μL glass syringe was positioned at 0.1 mm posterior and 1.0 mm lateral to the Bregma on the head. 4% Evans blue dye in PBS was slowly injected into the lateral ventricle of mice, and 20min later mice were sacrificed and whole brains with a portion of the spinal cord were dissected and fixed into 4% paraformaldehyde in PBS. 48 hours later, coronal sections at 1mm thick were made by hand or tissue chopper. Images at 1x magnification were captured by a stereomicroscope with a digital camera.

3.3.4 X-gal staining for brain sections:

P14 days mice were perfused and brains were dissected out. Brains were post-fixed in 4% PFA for 10 min, then washed in PBS twice for 5 min. Brains were cryoprotected in 30% sucrose in PBS overnight at 4°C until brains sink to the bottom.
Brains were embedded in OCT and immersed in pre-chilled isopentane bath cooled with liquid nitrogen. Section the sample on a cryotome at 30μm and mount onto coated slides. Postfix the sections in fixative solution (4% PFA) for 10 minutes on ice. Rinse the slides twice in 1X PBS twice for 10 minutes each on ice. Place the slides in detergent rinse (2mM MgCl2, 0.02% Nonidet P-40, 0.01% Sodium deoxycholate in PBS) for 10 minutes on ice. Stain the sections in X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) staining solution (1 mg/ml X-Gal, 5mM Potassium ferricyanide, 5mM Potassium ferrocyanide, 2mM MgCl2, 0.02% Nonidet P-40, 0.01% Sodium deoxycholate in PBS) overnight at 37°C in the dark. Next day slides were washed twice in 1X PBS for 5 minutes each at room temperature. Then Rinse the slides in distilled H2O. Counter stain the sections for 30 seconds in the Eosin. Wash three times in distilled H2O for 5 minutes each. Dehydrate the sections through methanol (5 minutes each in 50%, 70%, and 100% methanol). Sections were cleared twice for 5 minutes each in xylene.

3.3.5 Immunohistochemistry:

Juvenile mice were humanely terminated with over-dose of sodium pentobarbitone and newborn mice were humanely terminated by decapitation. Brains were post-fixed in PBS containing 4% paraformaldehyde (PFA) at 4°C overnight. They were then embedded in paraffin and sectioned at 10μm for immunohistochemistry or 30um for X-gal staining. Sections were immunostaining using the following primary antibodies mouse monoclonal anti-Acetylated-α- tubulin (1:1000, sigma), Fluorescent secondary antibodies for immunofluorescence staining were Alexa Fluor 488 goat anti-rabbit or 555 goat anti-mouse (1:500, Cell signalling). Sections were mounted with mounting medium containing DAPI (Sigma).

3.3.6 Scanning Electron Microscope:

Lateral walls of the lateral ventricles dissected from P18 mice were fixed in primary fixative solution (2.5% glutaraldehyde/ 2% paraformaldehyde/ 0.1M sodium cacodylate/HCl buffer, pH7.2) overnight at 4 °C. After dehydration through graded alcohols, tissue samples are transferred to Hexamethyl disilizane (HMDS) for 2x15
min changes. Then samples are allowed to dry for at least 2 hours in a fume hood. Dried samples are fixed to metal stubs using either Carbon adhesive tabs or silver dag paint, gold sputter coated and viewed on a Hitachi S2600N Variable Pressure Scanning Electron Microscope.

3.3.7 Transmission Electron Microscope:

Lateral walls of the lateral ventricles dissected from P18 mice were fixed in primary fixative solution (2.5% glutaraldehyde/ 2% paraformaldehyde/ 0.1M sodium cacodylate/HCl buffer, pH7.2) overnight at 4 °C. After primary fixative, tissues were fixed in secondary fixative solution (1% Osmium Tetroxide in 0.1 M Sodium Cacodylate/HCl buffer pH 7.2) for 4 hours. Then tissues were dehydrated through a series of graded alcohols 50%, 70%, 90%, 95% and 100%. Then tissues were placed in Propylene Oxide to replace the pure ethanol. After that, tissues were transferred to a series of mixture of resin and Propylene Oxide, 50:50, 75:25, pure resin. Finally, tissues were transferred to flat embedding moulds, clearly labeled and placed in 65 degree oven for 48 hours and allowed to polymerise. After polymerization, blocks are sectioned at 100nm, lifted onto 3mm copper grids, stained for 30min in 1.5% aqueous Uranyl Acetate for 10 min in Lead Citrate. Sections are allowed to dry and then viewed on the Hitachi H7000 Transmission Electron Microscope.

3.3.8 RNA sequence

Three pairs of Ulk4−/− mice and wildtype controls at P12 were used in this study. All mice were euthanized by a two minutes exposure to CO2. Then cervical dislocation was carried out to humanely terminate the mice. Cortexes of mice were quickly dissected out and snap frozen in liquid nitrogen. RNeasy Kit (QIAGEN) was used for RNA extraction and sample preparation procedures were performed according to the manufacturer’s instructions. The concentration of RNA was measured by the Nanodrop Spectrophotometer. Equal amounts of RNA (~6µg per sample) were sent for sequencing (BGI). Libraries were constructed to convert to cDNA, and RNA sequencing was carried out by using the Illumina HiSeq2000 next generation sequencer.
Using bioinformatics tools and databases, tens of thousands genes (19,652 genes) have been screened. The overall transcriptome analysis by comparing \( \text{Ulk4}^{+/+} \) mice and wildtype controls, 2,829 genes with significant change had been identified. Among them, 1,005 genes were significantly downregulated and 1,824 genes were significantly upregulated. In this study, we screened a small proportion of genes which are important for ciliogenesis and they were categorized by their functions during different stages of cilia formation. A \( p \)-value of less than 0.05 indicates statistically significant. Statistical analyses were carried out by one-way ANOVA with one-tail due to the small sample size.

3.4 Results

3.4.1 Lack of Ulk4 leads to significant growth retardation and pre-weaning loss

To investigate \( \text{Ulk4} \) gene function, we acquired \( \text{Ulk4}^{+/+} \) breeding pairs on the C57BL/6N background from KOMP Repository and the Mouse Biology Program at the UC Davis. The gene targeting strategy was designed to replace the exon 7 of the \( \text{Ulk4} \) with an IRES-\text{lacZ} reporter cassette (Fig.3-1 a-b). The breeding of \( \text{Ulk4}^{+/+} \) male with wild type (WT, \( \text{Ulk4}^{+/+} \)) female resulted in offspring of expected Mendelian ratio. For example, 117 pups were born from 19 litters. The average litter size (6.2±0.6, mean±sem) was standard for the C57BL/6N strain. Among the 111 mice (95%) survived to the weaning stage, 52 were genotyped as WT and 59 as \( \text{Ulk4}^{+/+} \). Therefore, one copy of the \( \text{Ulk4} \) deletion did not affect mouse survival.

However, a significant pre-weaning loss was observed from \( \text{Ulk4}^{+/+} \times \text{Ulk4}^{+/+} \) mating. Among the 82 pups born from 12 litters (6.8±0.5/litter), 53 (64.6%) survived to the weaning stage, with a ratio of \( \text{Ulk4}^{+/+}:\text{Ulk4}^{+/+}:\text{Ulk4}^{+/-} = 16:37:10 \). To determine if \( \text{Ulk4}^{+/-} \) mice were embryonic lethal, we genotyped additional 4 liters of 27 pups from \( \text{Ulk4}^{+/-} \times \text{Ulk4}^{+/-} \) mating (6.7 pups/litter) at birth (P0), and detected a ratio of \( \text{Ulk4}^{+/-}:\text{Ulk4}^{+/-}:\text{Ulk4}^{-/-} = 9:11:8 \), showing that \( \text{Ulk4}^{-/-} \) mice were not underrepresented at birth. Subsequent genotyping further confirmed that \( \text{Ulk4}^{-/-} \) mice were born in Mendelian ratio but rarely survived to P28.
Ulka<sup>−/−</sup> mice also displayed marked postnatal growth retardation. At P12, the body weight of the Ulka<sup>−/−</sup> mice was 5.10±0.65g (n=5, p<0.01), ~30% lighter than that of Ulka<sup>+/+</sup> (7.10±0.15g, n=12, Fig.3-1 e-g) or Ulka<sup>+/−</sup> littermates (7.20±0.21g, n=12). However at P0, the body weight of the Ulka<sup>−/−</sup> mice (1.52±0.02g, n=5) did not differ from WT (1.53±0.04g, n=8; Fig.3-1 h) or Ulka<sup>+/−</sup>. Therefore, Ulka gene is essential for postnatal growth and survival.

**Figure 3-1. Ulka is essential for postnatal growth and survival.** The mouse Ulka genomic structure was presented in panel c. The 5’ targeting arm was a 5682bp fragment containing exons 4-6, and the 3’ arm was a 4084bp fragment containing exons 8-12 (b). The exon 7 was replaced by a knock-in cassette (a) harbouring SA-IRES-LacZ-Neo. Genotyping was carried out by PCR with two pairs of primers to amplify a 271bp DNA from the exon 7 of the WT allele and a 621bp band from the knockout (KO) allele (d). The body weight of P12 (g, Ulka<sup>+/+</sup> n=12, Ulka<sup>+/−</sup> n=12, Ulka<sup>−/−</sup> n=5, p<0.01) and P0 (h, Ulka<sup>+/+</sup> n=8, Ulka<sup>+/−</sup> n=9, Ulka<sup>−/−</sup> n=5) mice were quantified statistically. At P12 the Ulka<sup>−/−</sup> mice (f) were significantly smaller than WT littermates (e). +/+ for Ulka<sup>+/+</sup>, +/− for Ulka<sup>+/−</sup> and −/− for Ulka<sup>−/−</sup>. ** for p<0.01. The arrow in f highlighted a domed-shaped head. Data were analyzed by t-test and presented as mean ± SEM. p<0.01 was considered to be statistically significant.
3.4.2 Ulk4−/− mice exhibit hydrocephalus-like phenotype

The majority of Ulk4−/− mice exhibited a typical hydrocephalus appearance with domed-shaped heads after 2 weeks of birth (arrow, Fig.3-1 f). To investigate the neuroanatomical abnormalities, four pairs of P12 Ulk4−/− and Ulk4+/+ littermates were histologically examined. Ventriculomegaly was evident in the Ulk4−/− mice, which included dilation of the lateral ventricle (LV, Fig.3-2 a-f) and third ventricle (3V, Fig.3-2 g-h), although there was no significant difference of brain size (Fig.3-2 q, WT 46.0±4.1mm²; Ulk4−/− 45.2.0±4.5mm², p=0.79). The Ulk4−/− LV size (7.00±1.48 mm²) at the anterior commissural level was 17.5-fold larger (p<0.01) than that of WT littermates (0.40±0.11 mm², Fig.3-2 r), and the Ulk4−/− 3V (0.122±0.070 mm², Fig.3-2 h,s) was also significantly dilated (Fig., p=0.03) in comparison to the controls (0.026±0.003 mm², Fig.3-2 g).

The subcommissural organ (SCO) is important for the CSF flow within the ventricles, and the relationship between the abnormal SCO development and hydrocephalus has been well documented in animal studies (Lang, Song et al. 2006; Lee, Tan et al. 2012). We quantified the cell number in the SCO, and found no significant difference between the Ulk4−/− and WT mice (Fig.3-2 i,j,t, p=0.67), despite the ventricle at the posterior commissural (pc) level was 5-fold dilated in the Ulk4−/− mice (Fig.3-2 i,j,u, p<0.05).

The SCO generates and secrets the Reissner’s fibre (RF) into the CSF, which grows and extends caudally through the cerebral aqueduct to the fourth ventricle and the spinal cord. Impaired RF production and secretion can lead to aqueduct stenosis causing obstructive hydrocephalus (McAllister 2012). Whereas the RF was apparent in the WT littermates (arrow, Fig.3-2 i), we failed to detect RF extension in the Ulk4−/− mice (Fig.3-2 j), suggesting a dysfunction of the Ulk4−/− SCO.

Aqueduct of Sylvius is a narrow channel connecting the 3V and fourth ventricles (4V). Remarkably, the Ulk4−/− aqueduct was not overtly dilated (Fig.3-2 l,k). Further scanning electron microscopy (SEM) showed the blockage of the aqueduct in the mutant mice (Fig.3-2 n,p), which was not present in the WT aqueduct (Fig.3-2 m,o).
These data demonstrated that *Ulk4*−/− hydrocephalus was associated with SCO dysfunction and aqueduct stenosis.

**Figure 3-2.** *Ulk4*−/− mice display hydrocephalus phenotype at P12. Brain sections of P12 *Ulk4*−/− (d,e,f,h,j,l) and WT (a,b,c,g,i,k) littermate brains were analysed statistically, showing significantly LV (r), 3V (g-h,s), and SCO ventricle (i-j,u), but not the aqueduct (k-n) of the *Ulk4*−/− mice. SEM images showed the blockage in the mutant aqueduct (n,p), but not in the WT aqueduct (m,o). Scale bar=1 mm in a-f; 50 µm in g,h,i,j; 100 µm in k, l; 500 µm in m-n, 200 µm o.p. * for *p*<0.05; ** for *p*<0.01. 3V, third ventricle; LV, lateral ventricles; pc, posterior commissure; RF, Reissner’s fibre; SCO, subcommissural organ.
3.4.3 The Ulk4<sup>−/−</sup> hydrocephalus is congenital

Congenital hydrocephalus accounts for approximately 55% of all hydrocephalus cases (Carter, Vogel et al. 2012). To determine whether the hydrocephalus in the Ulk4<sup>−/−</sup> mice was congenital or acquired, we histologically analyzed Ulk4<sup>−/−</sup> brains and littermate controls at P0, when they did not show gross abnormality. A significant dilation of the LV and 3V was detected in P0 Ulk4<sup>−/−</sup> mice (Fig.3-3). However the LV enlargement in P0 mice (Fig.3-3 d, 5-fold) was not as dramatic as in P12 (Fig.3-2 r, 17.5-fold). These results showed that Ulk4<sup>−/−</sup> hydrocephalus was congenital, but the phenotype was progressively worsened during the early postnatal period, which coincided with the development and maturation of ependymal cilia, and suggested that the CSF flow might be compromised in the Ulk4<sup>−/−</sup> mice.

3.4.4 CSF flow is obstructed in the Ulk4<sup>−/−</sup> aqueduct

The majority of CSF is produced by the choroid plexus in the LV. The CSF flows sequentially to the 3V, aqueduct and 4V. It then enters the subarachnoid space which covers the brain and spinal cord, and is drained into the blood primarily through arachnoid granulations. Aqueduct obstruction is the common cause of hydrocephalus in animal models. Evans Blue is an azo dye with a high affinity binding serum albumin. The dye can flow with the CSF after injecting into the LV, and is therefore used to trace the CSF flow in animals (Kim, Carlson et al. 2012)

To investigate the CSF flow in the Ulk4<sup>−/−</sup> mice, we injected 5µl of Evans Blue dye (4% in PBS) into the LV of P12 mice, and 20min later examined the dye distribution (Fig.3-4). The dye in WT littermates (n=4) was detected throughout all ventricles, including the LV (Fig.3-4 a), dorsal 3V (Fig.3-4 b), ventral 3V (Fig.3-4 b), SCO (Fig.3-4 c), aqueduct (Aq, Fig.3-4 g), 4V (Fig. 3-4 h) and spinal canal (SC, Fig. 3-4 i). However, there was little evidence of CSF flow in the Ulk4<sup>−/−</sup> brains (n=3). Although the dye was strongly detected in the LV (Fig. 3-4 d) and 3V (Fig. 3-4 e), very little dye was reached to the SCO ventricle (Fig. 3-4 f) in the Ulk4<sup>−/−</sup> mice, even 20 min after dye injection. No dye was accumulated in the Ulk4<sup>−/−</sup> aqueduct (Fig. 3-4 j), 4V (Fig. 3-4 k), or SC (Fig. 3-4 l). These functional data demonstrated that the
lack of Ulk4 was a pathological factor of non-communicating hydrocephalus and that Ulk4 deficiency impaired CSF circulation.

Figure 3-3. The Ulk4<sup>−/−</sup> hydrocephalus is congenital. Brain sections from P0 WT (a,e) and Ulk4<sup>−/−</sup> (b,f) mice were stained with H/E. The relative brain section size (c), the LV (d) and 3V at the SCO position (g) were quantified, * for p<0.05, ** for p<0.01. Scale bar=1mm in a-b, 100µm in e-f. pc, posterior commissure.
Figure 3-4. Impaired CSF circulation in the *Ulk4^−/−* mouse brain. Mice were intraventricularly injected with 5 µl of Evans Blue dye (4% in PBS), and 20min later, mouse brains were fixed and sectioned. The distribution of the dye in the anatomical positions of the LV, 3V, SCO, aqueduct (Aq), 4V and spinal canal (SC) of WT littermate brains (a, b, c, g, h, i) and *Ulk4^−/−* mouse brains (d, e, f, j, k, l) was arrowed. Note the dye was completely absent in the Aq/4V/SC of the *Ulk4^−/−* brain (j, k, l), demonstrating an impaired CSF flow in the *Ulk4^−/−* mice. Scale bar =1mm.
3.4.5 Ulk4 is predominantly expressed in the ventricular system

The impaired CSF flow suggested a failure of ependymal function. To explore the pathological mechanisms of hydrocephalus in the Ulk4−/− mice, we firstly investigated Ulk4 expression pattern using X-gal staining (Fig.3-5), since that a SA-IRES-lacZ reporter gene was knocked into the exon 7 of the Ulk4 locus, therefore, an Ulk4-IRES-lacZ fusion mRNA could be transcribed under the endogenous Ulk4 promoter. We stained the P14 brain sections with X-gal and observed weak β-gal activity in the choroid plexus where the CSF was produced (CP, Fig.3-5 a), and in the subfornical organ (SFO, Fig.3-5 c) which was previously reported to be involved in hydrocephalus.

Ependymal cells are a single layer lining on the ventricles of the brain. They are covered by motile cilia which are complex and highly ordered organelle, which are essential for CSF flow. The most intense lacZ staining was detected in the ventricular system, including the ependymal cells in the LV (Fig.3-5 b), 3V (Fig.3-5 c,d), SCO (Fig.3-5 e) and aqueduct (Aq, Fig.3-5 f). Notably Ulk4 was highly expressed in the lateral wall of the aqueduct with motile cilia (arrowheads, Fig.3-5 f), whereas almost no expression was present on the roof of the aqueduct which was occupied by non-ciliated cells (Collins 1983) (arrows, Fig.3-5 f). The predominant Ulk4 expression in the mature ependymal cells also suggests that Ulk4 play a vital role in the function of the CSF circulation.
Figure 3-5. Ulk4 is expressed in the ventricular system, and Ulk4−/− ependymal cilia are disorganised. (a-f) The X-gal staining (in blue) was carried out to reveal Ulk4 expression pattern. A moderate level of lacZ expression was observed in the choroid plexus (CP, a) and subfornical organ (SFO, c), and a high level of β-gal activity was detected in the ependymal cells along the LV (b), dorsal 3V (D3V, c), 3V (d), SCO (e) and aqueduct (Aq, f). Black arrows in f showing no expression in the roof of aqueduct which was occupied by non-ciliated ependymal cells (Collins, 1983), and black arrowheads showing strong expression at the lateral wall of the aqueduct with motile cilia. (g-h) Immunohistochemical staining with an anti-acetylated α-tubulin showing densely clustered cilia in the WT (white arrowhead, g), but fewer and disorganised cilia (white arrowhead, h) and absence of cilia (white arrow, h) in the Ulk4−/− mice. (i-j) Scanning electronic microscopy (SEM) confirmed highly organised cilia in Ulk4+/− (i) mice, but disorganised/dysfunctional cilia, with the absence of cilia in large areas of the Ulk4−/− (j) mice. Bar =100µm in a-h, i-j.
3.4.6 *Ulk4*⁻/⁻ mice display impaired motile cilia

CSF circulation is largely considered uni-directional, and directional flow of the CSF is driven by coordinated beating of motile cilia of the ependymal cells. To investigate the development of the ependymal cilia in the *Ulk4*⁻/⁻ mice, we firstly carried out immunohistochemical staining with an antibody against a ciliary marker, acetylated α-tubulin in P14 mice when motile cilia were fully developed and matured. Highly organized cilium bundles were shown to line on the *Ulk4*⁺/⁻ ependymal wall (arrowhead, Fig. 3-5 g). In contrast, *Ulk4*⁻/⁻ cells exhibited disrupted, less dense and disorganized cilia (arrowhead, Fig. 5-5 h), or absence of cilia (arrow, Fig. 3-5 h). Shorter cilia were previously reported in hydrocephalus models (Vogel, Read et al. 2012), however, the cilia length appeared comparable between the *Ulk4*⁻/⁻ and control mice (Fig. 3-5 g,j).

We subsequently performed SEM to examine the LV surface (Fig. 3-5 k-l) and compared the morphological difference of cilia between *Ulk4*⁺/⁻ and *Ulk4*⁻/⁻ mice. The ependymal cilia were largely normal on the LV wall of the *Ulk4*⁺/⁻ mice, and most cilia bundles were highly organized and orientated towards the same direction (Fig. 3-5 i), indicative of coordinated and directional beating. In the respective area of the *Ulk4*⁻/⁻ mice, the number of cilia bundles was dramatically reduced (Fig. 3-5 j), and a large proportion of cells did not have cilia arrays, suggestive of either underdevelopment and/or a massive loss of cilia. Although the length of *Ulk4*⁻/⁻ cilia was not shortened, they were highly disorganized and randomly scattered on the surface of the ependymal wall, a sign of dysfunctional cilia with no directional beating (Fig. 3-5 j).
Figure 3-6. *Ulk4* mutation impairs the axoneme structure and ciliary function. TEM images of radial sections of ependymal cells (a-f). Cluster of multi-cilia with same orientation appeared on P18 WT ependymal cells (a,d, arrowed), but reduced (b,c,e) or absent (f) in the *Ulk4*<sup>−/−</sup> (b,c,e,f). Black box in (b,f) showed an immature cilium, namely BB- vesicle fusion complex, beneath the *Ulk4*<sup>−/−</sup> cell membrane,
which appeared normally in the first postnatal week. Cilium length were not shortened in the *Ulk4* mice (a,c,d,e), but the BB orientation was dispersed in the *Ulk4* (a,d). Arrows showed normally embedded BBs in WT (d), but differently orientated in the *Ulk4* (e). Black box in (e) showed cross-section view of a BB foot in a vertical orientation. Black box in (f) showed a BB - vesicle fusion complex orientated in parallel to ependymal membrane, with 90° difference to normal situation. WT littermates presented normal axoneme of “9 + 2” (g). Some *Ulk4* motile cilia exhibited “9 + 2” (h), but others either lost the central doublets without (“9 + 0”, i) or with disorganization of a peripheral doublet (also “9 + 0”, j), or missed a peripheral doublet but with supernumerary central doublet (“8 + 4”, k), or lost a peripheral and central doublets (“8 + 0”, l). Scale bars represent 1000nm in a,b,c, 500 nm in d,e,f, and 100 nm in g-l. Insets in b,e,f were magnified 3 times from the respective images. BB, basal bodies; KO, knockout; MV, microvilli; N, nucleus; WT, wild type.

3.4.7 *Ulk4* mice display major defects in cilium development, axonemal organization and basal body orientation

Motile cilia have a basic structure called axoneme which consists of nine peripheral doublets and a central pair of microtubules, termed (“9+2”). Synchronized beating of the motile cilia is the driving force of CSF circulation. To study the effects of Ulk4 inactivation on cilium development, we carried out transmission electron microscopy (TEM) to examine multiciliated ependymal cells lining the LV at P18. In WT ependymal cells, multi-cilia were fully developed, matured and organized into clusters, and oriented in a consistent direction (Fig.3-6 a,d). The length of the *Ulk4* cilia, if developed (Fig.3-6 a,c), were not markedly shortened. However, fewer cilia or even the absence of cilia were observed on the *Ulk4* ependymal cells. Instead, abundant microvilli of smaller and shorter projections were evident on the surface of the *Ulk4* ependymal cells (Fig.3-6 b,c,e,f). The presence of immature cilia, i.e., basal body (BB) - vesicle complex, was found beneath the ependymal cell membrane in P18 *Ulk4* mice (Fig.3-6 b,f, black box).

To further investigate the underlying mechanism of disorganized cilia observed in the *Ulk4* mice, we examined the position of BB, which determined the cilium
orientation during ciliogenesis. In the WT mice, all the BBs were lined in parallel and leaned towards the same direction (BB, arrows, Fig.3-6 a,d). However, dispersed and randomly orientated BBs were present in the Ulk4−/− mice (Fig.3-6 b,c,e,f). Some BBs were orientated towards completely different directions, and this was evident from the detection of cross-section view of a BB foot (see black box in Fig.3-6 e) and BB - vesicle fusion complex orientated in parallel to ependymal cell membrane (Fig.3-6 f). These situations could only be observed when the cilium (Fig.3-6 e) and fusion complex (Fig.3-6 f) were organized 90° to the normal orientation.

Next we examined the ultrastructure of ciliary microtubule by TEM. In the Ulk4+/+ mice, all the axonemes exhibited a typical “9+2” microtubular ultrastructure on motile cilia across sections (Fig.3-6 g). In the Ulk4−/− mice, some motile cilia exhibited normal structure (Fig.3-6 h), whereas others showed various defects in axonemes, i.e., missing the central doublets (“9 + 0”, Fig.3-6 i), with a disorganized peripheral doublet and lacking the central pair (also termed as “9 + 0”, Fig.3-6 j), losing a peripheral doublet but gaining supernumerary central pair (“8 + 4”, Fig.3-6 k), and missing the central and a peripheral microtubule doublets (termed as “8 + 0”, Fig.3-6 l). Together, these data showed that the Ulk4−/− ependymal cilia were underdeveloped, disorganized, structurally errored and functionally impaired.

3.4.8 Alterations of molecular pathways associated with Ulk4−/− hydrocephalus

Ulk4 is a novel Ser/Thr kinase, and virtually, nothing is known about the function of the Ulk4. To understand the molecular mechanisms of cilium dysfunction in the Ulk4−/− hydrocephalus, we carried out whole genome RNA sequencing and analyzed RNA expression profiles in P12 cortex. DNA sequence databases and bioinformatic tools were used to identify and validate 19652 genes after filtering. We then statistically compared gene expression levels among the three Ulk4−/− and three WT controls, and identified 1824 genes significantly up-regulated and 1005 genes significantly (p<0.05) down-regulated in the P12 Ulk4−/− cortex.
Figure 3-7. Changes in mRNA expression of genes associated with ciliary formation and function in the *Ulk4*⁻/⁻ mice. Whole genome RNA sequencing was carried out with P12 RNA from 3 WT and 3 KO cortex. The abundance of RNA expression was statistically analysed by one way ANOVA. (a) The *Ulk4* family members showed no compensation changes. (b,c) *Ulk4* regulated *Foxj1* but not other master ciliogenesis genes. Key genes involved in (d,e) centriole amplification, (f) axoneme ultrastructure, (g) basal body orientation, (h) IFT and (i) ciliary beating were statistically analyzed. Data were presented with folds of changes in the *Ulk4*⁻/⁻ mice (KO/WT, mean±sem). * represented $p<0.05$ and ** represented $p<0.01$. 

*a ULK4 family members
*b Cilia master genes
*c Rfx family
*d Centriole duplication
*e Centriole duplication
*f Axoneme structure
*g Basal body orientation
*h IFT
*i Ciliary beating
We first analyzed the expression of the master regulators Foxj1, Rfx3 and Mcidas. Neither the 8 Rfx family members (Rfx1-8, p>0.05) nor the Mcidas (30% reduced, p= 0.098) was significantly altered in the Ulk4−/− mice (Fig.3-7 a,b). However, the expression of Foxj1 and Foxj3 was increased by 1.59-fold (p= 0.027, Fig.3-7 a) and 1.33-folds (p= 0.043), respectively. Despite that no literature supports the involvement of the Foxj3 in ciliogenesis, the Foxj1 has been overwhelmingly shown as a master regulator, which activates a series of ciliogenic genes (Yu, Ng et al. 2008; Skarnes, Rosen et al. 2011; Choksi, Lauter et al. 2014). Therefore Ulk4 gene deletion specifically altered Foxj1 pathway.

Ciliogenesis is a complicated and precisely controlled process, and amplification of tens or hundreds of centrioles is an initiation step. Among the key genes of Avil, Tmem67, Gsn, Katnb1, Asap1, Pttn23, Poc5, Cep120, Cep152, Deup1, Cede78, Poc1 and Plk4, which were involved in the centriole amplification (Keller, Geimer et al. 2009; Al Jord, Lemaitre et al. 2014), five of them Avil (2.09-fold, p=0.003), Tmem67 (1.28-fold, p=0.013), Poc5 (1.27-fold, p=0.037), Cep120 (1.19-fold, p=0.038) and Cep152 (1.26-fold, p=0.049) were significantly up-regulated in P12 Ulk4−/− mice. On the other hand, Gsn was 40% down-regulated (p=0.033), and Poc1b was reduced 20% with a marginal significance (p=0.066). By P12, the ependymal cilia should completely be formed and matured. Increased expression of procentriole components (Cep152, Cep120 and Poc5) suggested that ciliogenesis could be delayed in the Ulk4−/− mice.

It was shown that Odf2, Nphp4, Tmem67, Vangl1, Pcm1, Nedd1 Celsr2 and Celsr3 regulate orientation of the BBs (Ansley, Badano et al. 2003; Arts, Doherty et al. 2007; Tissir, Qu et al. 2010; Kunimoto, Yamaoka et al. 2012; Vladr, Bayly et al. 2012; Leightner, Hommerding et al. 2013) While the expression of the Odf2 (1.11-fold, p=0.049), Nphp4 (1.43-fold, p=0.024) and Tmem67 (1.28-fold, p=0.013) was significantly increased, the Vangl1 was 33% (p=0.017) down-regulated in Ulk4−/− mice. Previous report showed that in the absence of Vangl1 and Vangl2, cilia are positioned randomly(Song, Hu et al. 2010).
A centriolar satellite component, PCM1, is involved in ciliogenesis. PCM1 was shown to interact and co-localize with BBS8 to centrosomes and basal bodies, a gene was mutated in a family with randomization of left-right body axis symmetry and known ciliary defects (Ansley, Badano et al. 2003). Pcm1 was 19% increased in the knockout mice (p=0.014). On the other hand, the neural precursor cell expressed, developmentally down-regulated gene 1 (Nedd1) is a key component of the basal body localized at the root of ciliated microtubules (Manning, Colussi et al. 2008), and a 30% reduction of the Nedd1 in the Ulk4−/− mice (p=0.031).

Two gelsolin (Gsn) family proteins, Gsn and Avil, were shown to regulate cytoskeletal actin organization by severing actin filaments, and regulate the cilia number (Kim, Carlson et al. 2012). While Avil expression was increased (p<0.01), the Gsn expression was decreased by 40% in the Ulk4−/− mice (p=0.033). It is worth to note that Avil was expressed at an extremely low level in P12 cortex, while Gsn expression was hundreds-fold more abundantly.

The TEM data showed that Ulk4 was required for the axonemel assembly both in central pair formation and peripheral microtubule doublet organization. The genes Spag6, Rsph4a, Rsph9 and Rsph1 encode the central pair components, which are also the target genes of Foxj1 (Choksi, Lauter et al. 2014). RNA sequencing data showed that Foxj1 and its three target genes, Spag6 (1.46-fold, p<0.001), Rsph4a (1.75-fold, p=0.002) and Rsph9 (1.31-fold, p=0.006), were significantly up-regulated in the Ulk4−/− mice. Dnah9, an axonemal dyneins (outer) coding genes, was also up-regulated (2.1-fold, p=0.006).

C14ORF179, Wdr35 and Ttc30b genes encode IFT43, Ift121 and Ttc30b, which are subunits of the IFT complex A (IFT-A) machinery involved in retrograde ciliary transport along axonemal microtubules (Arts et al., 2011; Mill et al., 2011; Howard et al., 2013). In the Ulk4−/− brain, the Ift43 was 34% reduced (p=0.03), whereas expression of Wdr35 and Ttc30b was 1.34-fold (p=0.016) and 1.35-fold (p=0.015) increased in the Ulk4−/− mice. Cluap1 is localized to the base and tip of cilia and is required for ciliogenesis in the mouse embryo. The Cluap1−/− mice showed impairment of ciliogenesis with pronounced down-regulation of the Gdf1 gene.
(Botilde, Yoshiba et al. 2013). Gdf1 expression was also 19% reduced in the Ulk4\textsuperscript{−/−} mice (p=0.047). Therefore, Ulk4 deletion may be associated with disturbed intraflagellar transportation.

In addition, Odf2, Spag6, Lgals3, Tekt2, Dnah10, Dnal1, Ttll6 and Drc1 genes were known to involve in ciliary beating (Mitchell, Jacobs et al. 2007; Suryavanshi, Edde et al. 2010; Mazor, Alkrinawi et al. 2011; Kunimoto, Yamazaki et al. 2012; Wirschell, Olbrich et al. 2013; Clare, Magescas et al. 2014), and four of them, Spag6 (1.46-fold, p=0.001), Tekt2 (1.38-fold, p=0.034), Odf2 (1.11-fold, p=0.049), Lgals3 (7.48-fold, p=0.007) and Lgals3bp (1.59-fold, p=0.007) were significantly up-regulated in the Ulk4\textsuperscript{−/−} mice. On the other hand, the expression of the Ttll6 gene encoding a tubulin tyrosine ligase was reduced by 49% (p=0.06). Dnal1, encoding a component of outer dynein arms, is the highest expressing gene of the axonemal dyneins, and in the Ulk4\textsuperscript{−/−} brain, the Dnal1 expression was reduced by 10% (p=0.035).

Together, these data showed that Ulk4 deficiency disturbed expression of the Foxj1 and a series of ciliogenenic genes, which consequently compromised the ciliary development, axoneme structure, intraflagellar transport and coordinated ciliary beating (Fig.3-8).
Figure 3-8. A working model for the molecular pathways leading to the hydrocephalus in the Ulk4−/− mice. Key genes, that were altered in the Ulk4−/− mice and involved in either the centriole amplification, basal body orientation, cilia number, axoneme structure, IFT or cilia beating, were listed above. Arrows in the boxes showed the up-regulation (↑) or down-regulation (↓) of mRNA expression in the Ulk4−/− mice. Genes colored in red are known targets of the Foxj1. Genes in blue were not listed as direct Foxj1 targets, but altered in the Ulk4−/− mice. Ulk4 is likely to act as a scaffold protein, modulates expression of the Foxj1, a master regulator, and as well as an array of other ciliogenesis factors, and therefore regulate ciliogenesis and cilia function. Deficiency of the Ulk4 led to malformation and dysfunction of cilia and thereby caused hydrocephalus.
3.5 Discussion

As introduced in the General Introduction Chapter, the mouse *Ulk4* gene is located on chromosome 9 and predicted to encode five splicing isoforms by the UCSC database (Figure 1-2). The longest isoform of 1275 amino acids is encoded by exons 1-36; the 1118 aa isoform by exons 1-33; the 1145 aa isoform by exons 2-33 with an alternative exon 1; the 910 aa isoform by exons 1-24; and 405 aa isoform by exons 9-24.

During the design of the Wellcome Knockout construct, not much was known about the *Ulk4* gene, but exon 7 was considered as a critical exon: (1) it contains 76 bp, therefore, the deletion will introduce a reading frame shift, if an alternative splicing exits to skip the exon 7 and/or the system leaks; (2) an intervention of *SA-IRES-lacZ* cassette with transcriptional termination signal (PolyA signal) to ensure that transcription is stopped after the insertion cassette.

Now an alternative splicing isoform of 405 aa is predicted to be coded by exons 9-24 with an alternative promoter. The actual existence of this predicted isoform requires confirmation by cDNA cloning. If it does exist, this isoform may still be present intact in the knockout mice. In addition, another alternative promoter is predicted in the 3’ of the human *ULK4* gene which transcribes a non-coding transcript comprising of Exons 35-36 of full length ULK4. It requires further bioinformatic and experimental analyses to validate if this splicing variant also exists in the mouse *Ulk4* gene.

The theoretical presence of additional Ulk4 isform(s) may partially explain why Ulk4 antibodies detected bands in the Ulk4 KO tissues (data not shown), although it was questionable if the antibodies are suitable for immunoblotting.

The knockout allele will encode a fusion mRNA transcript *Ulk4-En2-SA-IRES-lacZ-P A* (*Table 3-1*), comprising *Ulk4* exons 1-6 (underlined, 727 nt), 184 nt of *En2* exon (highlighted in Red), 594 nt of IRES (colored in Purple), and 3060 nt of *lacZ* coding sequence (colored in Blue). *Table 3-1*. Predicted fusion mRNA transcribed from the *Ulk4* knockout allele.
from this fusion mRNA, two proteins can be translated: the β-Galactosidase encoded by the lacZ gene (in blue), and a 343 amino acid fusion protein (39kD) from the 5’ end of chimeric mRNA transcript. The Ulk4 exon 1-6 contains only 727 coding nucleotides for the first 242 amino acid of the Ulk4, whereas the three predicted Ulk4 protein interaction domains are situated in regions of aa 507-762, 793-854, 889-1249, and the kinase-like domin (aa 2-282) may have no kinase activity. Therefore it is unlikely that the Ulk4+/− mice have any gain of function effects, due to lack of kinase activity or protein interaction domains. However, we have no data to exclude this possibility either. Nevertheless, the knockout construct would disrupt 4 of the 5 predicted mouse Ulk4 isoforms.

Ciliopathies are pathogenic features of numerous human diseases including hydrocephalus. Here we have characterized Ulk4−/− mice, and showed that targeted disruption of the Ulk4 caused congenital hydrocephalus, which became severer postnatally. Histological, SEM, TEM and functional analyses revealed the nature of obstructive aqueduct, dysfunctional SCO, impaired CSF flow and non-communicating hydrocephalus. The subcellular changes include reduced/disorganized/nonfunctional ependymal cilia with defects in axoneme organization. RNA sequencing analyses uncovered molecular mechanism of dysregulation of Foxj1 and other ciliogenesis genes in Ulk4−/− mice. Therefore, Ulk4 is likely to act as an essential scaffold protein regulating ciliogenesis (Fig.3-8).

The Ulk4 family consists of five members. Ulk1/2 are major regulators of autophagy, a catabolic mechanism and an adaptive stress response to degrade unessential/dysfunctional cellular components (Egan, Shackelford et al. 2011; Lee and Tournier 2011). Ulk3 is involved in sonic hedgehog signaling (Fuccillo, Joyner et al. 2006).
We reported that ULK4 was required for neuritogenesis *in vitro* and *in vivo* (Lang, Pu et al. 2014). *Ulk4* deficiency compromised neurite outgrowth in neuroblastoma cells and the integrity of the corpus callosum in mice (Lang, Pu et al. 2014). Our unpublished data showed that *ULK4* can be a rare risk factor (1/1000) for patients with developmental delay, language delay and intellectual disability. Lack of *Stk36* was reported to cause hydrocephalus in mice (Merchant, Evangelista et al. 2005), however, neither *Stk36* nor other *Ulk4* family members was compensationally changed in *Ulk4*−− mice (Fig.7g). Therefore both *Ulk4* and *Stk36* are indispensible for proper CSF circulation.

Congenital hydrocephalus occurs in approximately 1~3/1000 child births. We analyzed the neuroanatomy of newborn mice and identified significantly enlarged ventricles before the maturation of motile cilia. We, therefore, consider the *Ulk4*−− hydrocephalus congenital. However, the phenotype was worsened during the postnatal period. The aqueduct is a narrow join connecting the 3V and 4V, and commonly blocked in non-communicating hydrocephalus. Our SEM study also demonstrated aqueduct stenosis in *Ulk4*−− mice. Functional analysis of CSF circulation using Evans Blue further confirmed the impairment of CSF flow, as little dye reached the SCO in *Ulk4*−− mice, after 20 min of injection.

The SCO cells secrete high molecular weight glycoproteins to form RF, which is required to maintain the aqueduct patency and CSF flow. Impaired production/secretion of the RF can lead to obstructive hydrocephalus (McAllister 2012). Overexpression of *Sox3* was shown to result in SCO dysplasia, abnormal RF formation and obstructive hydrocephalus (Lee, Tan et al. 2012). We showed co-localization of *Sox3* protein with *Ulk4* mRNA in *Xenopus* brain (Dominguez, Schlosser et al. 2015), but currently have no evidence to support *Sox3* dysregulation in *Ulk4*−− mice currently. The SCO morphology and cell number were not overtly altered. However, the RF extension was not detected in *Ulk4*−− SCO. Therefore both the aqueduct stenosis and SCO dysfunction may contribute to *Ulk4*−− hydrocephalus.

The characteristic feature of hydrocephalus is an expanded ventricular system with excessive CSF accumulation. The experiment with Evans blue showed no
directional CSF flow, but passive diffusion of the dye in *Ulk4*<sup>−/−</sup> brain. Directional CSF flow requires motile cilia on ependymal cells, which are developed and matured within the first postnatal week, and this coincided with progressive severity of *Ulk4*<sup>−/−</sup> phenotype. Indeed, Ulk4 is highly expressed in ependymal cells of ventricles, SCO and aqueduct consisting of multiciliated cells. Interestingly, Ulk4 is not expressed on the roof of the aqueduct, which is populated with non-ciliated cells, suggesting the function of Ulk4 may be specifically related to motile cilia.

Multicilia from a single ependymal cell beat coordinately in one direction, and the sum of the beats from multiple cells produces stereotypical flow of the CSF (Breunig, Arellano et al. 2010). Clinically, abnormal cilia are associated with hydrocephalus in patients (Fliegauf, Benzing et al. 2007; Lee and Gleeson 2011). In mice, mutations in *Foxj1, Ro1, Polaris/Ift188, Mdnah5, Hydin* and *Spag6* also result in dysfunctional ependymal cilia and hydrocephalus (Huh, Todd et al. 2009). Our immunohistochemical study showed abnormal and disorganized cilia in *Ulk4*<sup>−/−</sup> mice, which was consistently supported by SEM and TEM evidence. Many *Ulk4*<sup>−/−</sup> ependymal cells lacked motile cilia. *Ulk4*<sup>−/−</sup> cilia were highly disorganized and randomly scattered on the ependymal wall. They showed no consistent orientation, which was strikingly different from the WT cilia, which were tightly clustered and uniformly orientated for coordinated beating. The TEM also revealed fewer cilia with dys-alignment of BBs, which provided the subcellular mechanism for disorganization of the *Ulk4*<sup>−/−</sup> cilia bundles. The “9+2” microtubular ultrastructure is the distinguishing feature of motile cilia, and the central pair is crucial for beating. *Ulk4*<sup>−/−</sup> mice displayed an array of abnormal microtubular organization including “9+0”, “8+4” and “8+0”. Together, these data showed Ulk4 is fundamental to cilia development and function.

ULK4 is a hypothetical kinase and nothing is known about its substrates or interactive partners. Other Ulk4 family members have kinase activities with a critical Lysine residue conserved at *ULK1<sup>K46</sup>, ULK2<sup>K39</sup>, ULK3<sup>K26</sup> and STK36<sup>K33</sup>. Mutations of *ULK1<sup>K46N</sup> or ULK2<sup>K39T</sup> inactivate kinase activity and exert a dominant-negative effect (Chan, Longatti et al. 2009). However, the respective residue *Ulk4<sup>33</sup> is a
Leucine, not Lysine. It would be interesting to test whether Ulk4 has kinase activity by further biochemical assays. We performed whole genome sequencing to identify genes which were dysregulated in Ulk4−/− cortex. Foxj1, Rfx3 and Mcidas are master ciliogenesis genes regulating overlapping sets of target genes (Yu, Ng et al. 2008; El Zein, Ait-Lounis et al. 2009; Thomas, Morle et al. 2010; Choksi, Lauter et al. 2014). We showed that Foxj1 was specifically altered in Ulk4−/− brain. This is the first evidence suggesting that elevated Foxj1 signaling may also be pathogenic to the ciliogenesis and function.

Consistent with Foxj1 upregulation, a whole array of ciliogenesis genes were modulated in Ulk4−/− mice. For example, among genes involved in centriole amplification (Keller, Geimer et al. 2009; Al Jord, Lemaitre et al. 2014), Tmem67, Poc5, Cep120 and Cep152 were upregulated, meanwhile, Gsn was 40% down-regulated, consistent with a previous study that knockdown of Gsn significantly reduced ciliated cell numbers (Kim, Lee et al. 2010).

The BBs were dys-orientated in Ulk4−/− mice. Odf2, Nphp4, Pcm1 and Tmem67 were shown to involve in BB formation and orientation (Ansley, Badano et al. 2003; Arts, Doherty et al. 2007; Kunimoto, Yamazaki et al. 2012; Leightner, Hommerding et al. 2013), and their expression was significantly increased. In contrast, Nedd1 was 30% down-regulated in Ulk4−/− mice, in line with the study that Nedd1 is a critical BB component localized at the root of ciliated microtubules (Manning, Colussi et al. 2008). Vangl1 was also 33% down-regulated, and interestingly, mutations of VANGL1 were identified in sporadic and familial neural tube defects (Kibar, Torban et al. 2007), and loss of Vangl2 caused neural tube defects in Lp/Lp mice (Kibar, Vogan et al. 2001). Therefore decreased expression of Nedd1 and Vangl1 may contribute to dys-alignment of BBs in Ulk4 mutants.

Ulk4−/− mice showed defects in axoneme ultrastructure which is vital for motor function. Tubb4a, the predominant subtype of β-tubulins in the brain (Leandro-Garcia, Leskela et al. 2010) was significantly reduced in Ulk4 mutants. A kinesin protein Kif5a functioning as a microtubule motor also was 19% decreased. In addition, the expression of Dnall and Till6 were downregulated by 10% and 49%,
respectively. This is consistent with a role of Tll6 in ciliary motility through posttranslational modification of inner dynein arm (Suryavanshi, Edde et al. 2010), and role of DNAL1 as a component of outer dynein arms (Mazor, Alkrinawi et al. 2011). DNAL1 mutations were identified in patients with CILD16, a disorder with abnormalities of motile cilia (Mazor, Alkrinawi et al. 2011). Together, these data showed that Ulk4 disruption resulted in disturbed balance of an array of ciliogenesis genes, which consequently impaired cilia development, axoneme structure, and coordinated beating.

We also found that Ulk4−/− mice died early postnatally. Hydrocephalus phenotype may partially contribute to this, but peripheral defects are likely. ULK4 is associated with hypertension (Levy, Ehret et al. 2009) and multiple myeloma (Broderick, Chubb et al. 2012). We had no evidence of tumor formation, or difference in gross morphology of heart, liver, lung, kidney and spleen. Ulk4−/− stomach was smaller but colored white, suggestive of reduced food intake. Functional defects in lung and kidney are likely, given the major phenotype in motile cilia. The current study revealed defects in the structure, abundance, organization and function of ependymal motile cilia in Ulk4−/− mice, together with aqueduct obstruction, SCO dysfunction and impaired CSF flow. This is the first comprehensive report demonstrating that Ulk4 is crucial for survival, postnatal growth and ciliogenesis, which support a wide implication of the ULK4 gene in human conditions.
CHAPTER 4

ULK4 IS ESSENTIAL FOR OLIGODENDROGENESIS AND WHITE MATTER INTEGRITY
4.1 Abstract

White matter integrity is implicated in neurodevelopmental and neuropsychiatric illness, in which Unc-51-like kinase 4 (ULK4) gene is proposed as a rare risk factor. Defects in myelination of white matter are increasingly recognized as a common pathology underlying brain injuries, neurodegenerative, neuropsychiatric and neurodevelopmental disorders. Key transcription factors of oligodendrogenesis are identified, but less is known about how they are regulated. Here we discover a novel role of Ulk4 as a key regulator oligodendrogenesis and white matter integrity. Ulk4 deficiency compromised the corpus callosum integrity and reduced myelination by half. RNA sequencing analyses demonstrated that both the genesis of axons and dendrites was impaired in the Ulk4−/− mice. Ulk4 was required for proper expression of key myelination regulators including Olig2, Olig1, Myrf, Sox10 and Sirt2. Oligodendrocyte progenitors, differentiation, maturation and myelination were severely affected in the Ulk4−/− mice. ULK4 may therefore become a novel drug target for hypomyelination-associated diseases.
4.2 Introduction

The most distinct feature of the brain is cell connectivity, achieved through the white matter that occupies almost one half of brain volume. It is not surprising that changes in white matter are widely associated with cognitive, emotional, neurodevelopmental and neuropsychiatric disorders including dyslexia, attention-deficit hyperactivity disorder, autism, schizophrenia, chronic depression, bipolar, obsessive-compulsive and posttraumatic stress disorder (Fields 2008). For example, schizophrenic patients typically present with white matter reduction, altered morphology, decreased dendritic arborization and agenesis of the corpus callosum (CC) (Douaud, Smith et al. 2007; Bose, Mackinnon et al. 2009; Fornito, Yucel et al. 2009; Francis, Bhojraj et al. 2011). A complete CC agenesis is rare but exists in schizophrenia (Matomura, Satani et al. 2002; Chinnasamy, Rudd et al. 2006; Paul, Brown et al. 2007), while mild alterations in inter-hemispheric callosal connections are not only common in schizophrenia (Swayze, Johnson et al. 1997; Wolf, Hose et al. 2008), bipolar disorder (Caetano, Silveira et al. 2008), but also in attention-deficit disorder (Hynd, Lorys et al. 1991) and autism (Innocenti, Ansermet et al. 2003; Wolff, Gerig et al. 2015). The agenesis of the CC is also a common pathology among >80 human congenital syndromes including ciliopathies and other developmental disorders (Benadiba, Magnani et al. 2012; Laclef, Anselme et al. 2015).

The CC is a nerve fiber tract in the brain that coordinates the left and right brain activity. Disruption of CC development is associated with animal models of neurological disorders. For example, L1CAM mutations resulted in hydrocephalus, mental retardation and agenesis of the CC in humans, with failure of callosal axons to cross the midline in mice (Demyanenko, Tsai et al. 1999). Overexpression of a G-protein coupled receptor PAC1 was linked to CC agenesis and hydrocephalus (Lang, Song et al. 2006). An idiopathic model of autism, BTBR mice, exhibited selective impairment in the long-range intra-hemispheric connectivity in the fronto-cortical area (Sforazzini et al., 2014). Expression of the truncated Disrupted-in-schizophrenia 1 (Disc1) gene led to partial agenesis of the CC in Disc1tr transgenic mice (Shen, Lang et al. 2008). A moderate reduction (40%) of Zic2 expression in
Zic2 (kd/+), mice displayed the schizophrenic neuropathology including thinning of the cerebral cortex and CC (Hatayama, Ishiguro et al. 2011). Abnormal myelination can therefore be an underlying mechanism of impaired white matter integrity in animal models.

Myelinogenesis occurs predominantly within the first 3 weeks of postnatal period in rodents and the first 2 years in humans, a period that coincides with many neurodevelopmental disorders (From, Eilam et al. 2014). During oligodendrogenesis, oligodendrocyte progenitor cells (OPCs) undergo proliferation and sequential differentiation into pro-oligodendrocytes, immature oligodendrocytes, non-myelinating mature oligodendrocytes, and myelinating mature oligodendrocytes (Baumann and Pham-Dinh 2001). Key transcription factors have been identified and Olig2 is proposed as a master regulator of oligodendrogenesis, because the oligodendrocyte lineage is absent in Olig2−/− mice (Zhou, Choi et al. 2001; Lu, Sun et al. 2002). Olig2 regulates and coordinates other transcription factors, including Olig1, Sox10, Myrf, Ascl1, Nkx2-2, Yy1, Tcf4, Id2, Id4, Hes5, Sox6 and Sox5 (Emery, Agalliu et al. 2009).

In a recent genetic screening for association of serine/threonine kinase genes with neurodevelopmental disorders, we discovered that Unc-51-like kinase 4 (ULK4) is hemizygously deleted in approximately 1/1000 patients with autism, schizophrenia or bipolar disorder (Lang, Pu et al. 2014). A SNP of ULK4 is also associated with bipolar disorder and depression (Lang, Pu et al. 2014). In addition, our unpublished genetic data showed that ULK4 is hemizygously deleted in ~1.19/1000 patients with heterogeneous clinical features including developmental delay, language delay and intellectual disability (Liu et al., in revision of Stem Cells). Interestingly, depleted ULK4 expression in neuroblastoma cells was shown to disrupt neuritogenesis. Targeted Ulk4 deletion also compromises the integrity of the CC in newborn mice (Lang, Pu et al. 2014), suggesting an important role of ULK4 in white matter development.
To understand the relationship between Ulk4 lesions and white matter development, in this study we investigated the CC in the Ulk4−/− mice, and showed compromised integrity of the CC at postnatal stages. The molecular network of the genesis of axons and dendrites were systematically downregulated, whereas axonogenesis inhibitory molecules encoded by Cdh1, Vim and Gfap genes were induced in Ulk4−/− mice. Ulk4−/− mice displayed hypomyelination with a reduction of ~50% in myelin, and a systematic reduction in expression of myelin components. Oligodendrogenesis factors including Olig2, Olig1, Nkx2-2, Sox10 and Myrf were significantly downregulated, suggesting that Ulk4 is critical for myelination. Taken together, this is the first report demonstrating that Ulk4 acts as a key regulator of oligodendrogenesis and white matter integrity, which may have implications in a wide range of neurodevelopmental and neuropsychiatric disorders.

4.3 Materials and Methods

4.3.1 Ulk4 knockout mice:

The Ulk4 mutant strain used for this research project was created from ES cell clone EPD0182_4_E12 generated by the Wellcome Trust Sanger Institute and made into mice by the KOMP Repository (WWW.KOMP.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. Methods used to create the CSD targeted alleles was published elsewhere (Skarnes et al, 2011). Breeding pairs of Ulk4+/− mice on C57BL/6N strain background were purchased from KOMP Repository. All experimental procedures were conducted under a license approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland (NUI) Galway. Ulk4−/−, Ulk4+/− and WT littermates were obtained from Ulk4+/− x Ulk4+/− mating. Each mouse was genotyped by genomic PCR with two pairs of PCR primers: Ulk4EndE7F or (5′- TAACTTGGCTGGACGGATTGCTG-3′) and Ulk4EndIn7Rev (5′- TGATCTGTAATCGCAGTGCAGG-3′) to uniquely amplify a 271bp DNA
fragment from exon 7 in the WT allele, and Ulk4KOMPKOFor (5’-GAGATGGCGCAACGCAATTAATG-3’) and Ulk4KOMPKORRev (5’-CTGAGGAGACAATGTAACCAGC-3’) to produce a 621bp DNA fragment solely from the knockout allele.

4.3.2 Histology:

Four pairs of *Ulk4*−/− and litter controls at P12 were deeply anesthetized and intracardiacally perfused with 20 ml of 4% paraformaldehyde in PBS. Brains were dissected and post-fixed in the fixative solution for 24 hours. Then brains were embedded into paraffin blocks. Serial coronal sections at 10μm were cut by with a microtome (SM2000R, Leica Instruments). The sections were stained with hematoxylin-eosin and imaged under a bright field microscope (IX41, Olympus) equipped with a camera.

4.3.3 Immunohistochemistry:

P12 mice were humanely terminated with over-dose of sodium pentobarbitone and newborn mice were humanely terminated by decapitation. Brains were post-fixed in PBS containing 4% paraformaldehyde (PFA) at 4°C overnight. They were then embedded in paraffin and sectioned at 10μm for immunohistochemistry. Sections were immunostaining using the following primary antibodies: rabbit polyclonal anti-Anti-rabbit-Cux1 (Santa Cruz biotechnology, sc-13024, 1:1000), Anti-rabbit-Ctip2 (Abcam, ab18465, 1:500), Anti-rabbit-Tbr1 (Abcam, ab31940, 1:500), rabbit polyclonal anti-GFAP (1:1000, Dako), rabbit polyclonal anti-Iba1 (1:000, Wako), rabbit polyclonal anti-MBP (1:500, Millipore), mouse monoclonal anti-Olig2 (1:1000, Millipore). Fluorescent secondary antibodies for immunofluorescence staining were Alexa Fluor 488 goat anti-rabbit or 555 goat anti-mouse (1:500, Cell signallng). Sections were mounted with mounting medium containing DAPI (Sigma).
4.3.4 Transmission Electron Microscopy:

Lateral ventricle walls were dissected from P18 mice and fixed in 2.5% glutaraldehyde/2% paraformaldehyde/0.1 M sodium cacodylate/HCl buffer (pH7.2) overnight at 4 °C. Tissues were subsequently immersed in secondary fixative solution containing 1% Osmium Tetroxide in 0.1 M Sodium Cacodylate/HCl buffer (pH 7.2) for 4 h. They were then dehydrated through a series of graded alcohols (50%, 70%, 90%, 95% and 100%), placed in Propylene Oxide, and transferred to a series of mixture of resin and Propylene Oxide, (50:50, 75:25, pure resin). Finally, tissues were transferred to flat embedding moulds, clearly labeled and placed in a 65°C oven for 48 h to polymerize. After polymerization, blocks were sectioned at 100 nm, lifted onto 3 mm copper grids and stained for 30 min in 1.5% aqueous Uranyl Acetate for 10 min in Lead Citrate. Sections were dried and viewed on the Hitachi H7000 Transmission Electron Microscopy.

4.3.5 RNA sequencing

Three pairs of *Ulk4<sup>-/-</sup>* mice and wildtype controls at P12 were used in this study. All mice were euthanized by a two minutes exposure to CO<sub>2</sub>. Then cervical dislocation was carried out to humanely terminate the mice. Cortexes of mice were quickly dissected out and snap frozen in liquid nitrogen. RNeasy Kit (QIAGEN) was used for RNA extraction and sample preparation procedures were performed according to the manufacturer’s instructions. The concentration of RNA was measured by the Nanodrop Spectrophotometer. Equal amounts of RNA (~6µg per sample) were sent for sequencing (BGI). Libraries were constructed to convert to cDNA, and RNA sequencing was carried out by using the Illumina HiSeq2000 next generation sequencer.

Using bioinformatics tools and databases, tens of thousands genes (19652 genes) have been screened. The overall transcriptome analysis by comparing *Ulk4<sup>-/-</sup>* mice and wildtype controls, 2829 genes with significant change had been identified. Among them 1005 genes were significantly downregulated and 1824 genes were significantly upregulated. In this study, we screened a small proportion of genes
which are important for ciliogenesis and they were categorized by their functions during different stages of cilia formation. A $p$-value of less than 0.05 indicates statistically significant. Statistical analyses were carried out by one-way ANOVA with one-tail due to the small sample size.

4.3.6 Statistical analysis.

At least three different mice per genotype from two or three litters were used in per experiment. For histology experiment, at least three coronal brain serial sections per mice were analyzed. Image J software package were used to quantify the brain areas from comparable sections. Statistical significance was evaluated by two-tailed Student’s t-test, with $p < 0.05$ as indicative of statistical significance. The number of animals used for quantification is indicated in the figure legends. Most of the experiments were successfully repeated at least three times.

4.4 Results

4.4.1 Defects of the corpus callosum (CC) in P0 $Ulk4^{-/-}$ mice

In a previous report, we showed that knockdown of ULK4 in human neuroblastoma cells reduced neurite outgrowth and branching, and that in newborn (P0) mice, $Ulk4^{-/-}$ deficiency led to compromised integrity of the CC, with numerous pockets of tissue un-occupied by nerve fibers (Lang et al., 2014). To follow up this observation, we investigated white matter development in $Ulk4^{-/-}$ mice.

In the anterior mouse brain, two distinct bundles of nerve fibers connect the two cerebral hemispheres across the midline: the dorsal CC, projecting from the majority of intracortical neurons, and the ventral anterior commissure (AC), projecting from the most lateral cortical areas. We compared coronal brain sections isolated from 5 P0 WT, with 5 from $Ulk4^{-/-}$ littermates. The CC in WT mice was densely occupied by cortical projections (Fig.4-1A,C). However in 5 $Ulk4^{-/-}$ littermates, the CC was filled with numerous spaces lacking nerve fibers (arrow, Fig. 4-1D). The posterior commissure (PC) above the subcommissural organ also displayed pockets of un-
occupied space in the Ulk4−/− mice (arrow, Fig. 4-1F). However, the integrity of the AC appeared less affected (Fig. 4-1A-D).

The CC changes structurally throughout life, but most dramatically during childhood and adolescence before CC finally attains adult morphology (Luders, Thompson et al. 2010). To investigate the effects of ULK4 deletion on postnatal white matter development, we examined the CC in P12 brain sections and found deterioration of CC integrity (Fig.4-1M-P). In contrast to a thick layer of dense nerve fibers in the CC of WT mice (Fig. 4-1M,O), the integrity of the CC across the midline in Ulk4−/− mice, was severely damaged (Fig. 4-1N). The nerve fibers were substantially reduced in the Ulk4−/− CC and were structurally disrupted and non-directional when crossing the midline (Fig. 4-1N) or at the dorsal-lateral position (Fig. 4-1P). In contrast, highly organized and directionally orientated nerve fibers were densely occupying the CC in the controls (Fig. 4-1M,O). These data demonstrate that the Ulk4 plays an important role in the postnatal development of the white matter.

The agenesis of the CC can result from reduced numbers of intracortical neurons. We therefore stained the P0 section with anti-NeuN, which demonstrated a subtle reduction of mature neurons in the mutants (Fig. 4-1G-H). This was confirmed with layer-specific markers (Fig. 4-1I-L). Although the deep layer neurons in P0 mice showed comparable number of Ctip2+ cells (Fig. 4-1K-L), the superficial layers exhibited a mild but reduction of CDP+ neurons in the Ulk4−/− mice (Fig. 4-1I-J). In agreement with deteriorated white matter in P12 Ulk4−/− mice (Fig. 4-1N,P), the cortical thickness was more dramatically reduced in P12 mutants (not shown). These data demonstrate that the reduction of intracortical neurons can partially account for reduced nerve fibers in the Ulk4 mutants.
Figure 4-1. Compromised integrity of the white matter in the Ulk4−/− mice. P0 (A-L) and P12 (M-P) brain sections were stained with Nissl (A-D), H/E (E-F, M-P), or antibodies against NeuN (G-H), CDP/Cux1 (I-J), or Ctip2 (K-L). (A-B) P0 whole brain images. (C-D) Enlarged images showing damaged integrity in the CC (arrow in D), not much in the AC, of KO mice. (E-F) The integrity of the PC was also affected in the KO mice (arrow in F). (G-H) Anti-NeuN staining, showing a mild reduction of mature neurons in P0 mutants. (I-J) A mild but significant decrease of layer II-IV neurons in P0 KO mice. (K-L) Anti-Ctip2 staining, showing no significant effect on deep layer neurons. At P12, the CC at the midline (M-N) or dorsal-lateral position (O-P) were more severely damaged (N, P) than at P0 Ulk4−/− mice. While CC in Ulk4−/− mice had abnormal structure with loose, fractured, dys-orientated and collapsed fibers, the CC in WT mice had a normal structure, with dense orientated fibers. AC, anterior commissure; CC, corpus callosum; KO, knockout mice; PC, posterior commissure. Bar=1000 µm in A-B, =100 µm in C-D, =50 µm in E-P.
4.4.2 Significant down-regulation of the neuronal projections in the Ulk4<sup>−/−</sup> mice

The apparent agenesis of Ulk4<sup>−/−</sup> CC may result from shorter cortical projections and/or reduced branching. To objectively analyze the molecular bases of CC agenesis, we carried out pathway analyses for the 618 Ulk4 targets from the whole genome RNA sequencing, which were significantly altered by a reduction of more than 20% or by an induction of 1.5-fold or more (p<0.05; RNA expression level >1) in P12 Ulk4<sup>−/−</sup> brain. The pathway analyses from the STRING program (http://string-db.org/) demonstrated a systematic down-regulation of genes associated with white matter, including both dendrites and axons in the Ulk4<sup>−/−</sup> mice (Fig. 4-2). These included 21 genes in the “somatodendritic compartment” (Fig. 4-2A, p=0.022), 6 genes in the “Dendrite morphogenesis” (Fig. 4-2B, p=1.40E-04), 6 genes in “Regulation of dendrite development” (Fig. 4-2C, p=0.020); 9 genes in “Axonogenesis” (Fig. 4-2D, p=0.045), 4 genes in “Regulation of axonogenesis” (Fig. 4-2E, p=0.192), 6 genes in “Axon Guidance” (Fig. 4-2F, p=0.064) and 13 genes in “Axon ensheathment” (Fig. 4-2G, p=2.30E-09).

In contrast, Cdhl, Vim and Gfap genes were induced by 2.82-fold (p=0.011), 1.91-fold (p=0.014) and 2.86-fold (p=0.036), respectively, in the Ulk4<sup>−/−</sup> mice. These were consistent with their negative roles in axonogenesis. For example, Vimentin and Gfap are major intermediate filaments of reactive astrocytes, and both have inhibitory effects on axonal growth. Knockout of the Gfap and/or Vim increases plastic sprouting of axons (Menet, Prieto et al. 2003; Ribotta, Menet et al. 2004). Cdhl knockdown is also shown to dramatically increase axonal length, both accelerating the rate of growth and significantly augmenting the final axonal length (Konishi, Stegmuller et al. 2004). Therefore, the quantitative RNA sequencing data provides convincing evidence that the development of the white matter in P12 Ulk4<sup>−/−</sup> mice is grossly reduced. Meanwhile down-regulation of the 13 genes in “Axon ensheathment” pathway suggests an involvement of oligodendrocytes (Fig. 4-2G).
Figure 4-2. Systematic under-development of the white matter in the *Ulk4<sup>−/−</sup>* mice. (A) 21 genes in the “Somatodendritic compartment” (Adcy4, Apc, Atp1a2, Bcan, Casr, Ddc, Ernn, Grin2c, Grm3, Homer2, Itga1, Lpar1, Lrp4, Mbp, Myo1d, Ntsr2, Pura, Pvalb, Shank1, Sirt2 and Slc1a2, p=0.022); (B) 6 genes relating to “Dendrite morphogenesis” (Ephb1, Fmn1, Lrp4, Prex2, Shank1, Shank3, p=1.40E-
04); (C) 6 genes in “Regulation of dendrite development” (Caprin2, Lpar1, Pdlim5, Sema4d, Shank1, p=0.020); (D) 9 genes in “Axonogenesis” (Apc, Cnp, Cxcl12, Ephb1, Etv4, Fgfr2, Nfasc, Sema6a, Unc5b, p=0.045); (E) 4 genes in “Regulation of axonogenesis” (Cxcl12, Dnm2, Lrp4, Sema4d, p=0.192); (F) 6 genes in “Axon Guidance” (Cxcl12, Ephb1, Etv4, Nfasc, Sema6a, Unc5b, p=0.064) and (G) 13 genes in “Axon ensheathment” (Arhgef10, Cldn11, Fa2h, Gal3st1, Gjc3, Myrf, Mbp, Nfasc, Olig2, Plp1, Sirt2, Tspan2, Ugt8a, p=2.30E-09). Data were presented with mean fold of changes in relation to WT as 1, with SEM (n=3 each). All these genes were down-regulated significantly in Ulk4−/− mice. * for p<0.05; ** for p<0.01.

### 4.4.3 Reduced expression of myelin basic protein (Mbp) P12 Ulk4−/− mice

The white matter is myelinated by oligodendrocytes and this occurs predominantly within the first 3 postnatal weeks in rodents (From, Eilam et al. 2014). To investigate the potential involvement of myelination in reduced CC in the Ulk4 mice, comparable brain sections were immunostained with anti-Mbp, a marker of myelin produced by mature oligodendrocytes. In WT mice, Mbp was abundantly expressed in myelinated fiber tracks of the CC (Fig. 4-3A). However, the Mbp was barrenly expressed in the Ulk4−/− brain (Fig. 4-3D). In the Ulk4−/− CC fibers, Mbp was restricted to the dorsal-most line of the projections (CC, Fig. 4-3D). In the dorsal lateral ependymal region, the projections from the cortex were dramatically reduced (CX, Fig. 4-3E). In the lateral cortex close to the SVZ region, the dense axonal bundles projecting to the thalamus (CPU, Fig. 4-3C) were lacking, and projections from the deep cortex were markedly reduced (CX, Fig. 4-3F). Mbp is produced by mature oligodendrocytes. Therefore, these data demonstrated a marked impairment of mature oligodendrocyte function in the Ulk4−/− mice.
Figure 4-3. Ulk4−/− mice exhibited decreased myelination in the CC and cortex at P12. Immunohistochemical staining of the WT (A-C) and Ulk4−/− (D-F) brain sections with anti-Mbp, showed dense and directional nerve fibers in the CC of WT mice (A-C), while CC in the Ulk4−/− brain (D-F) showed a dramatic reduction in anti-MBP staining. Bar=100 µm in A-F. CC, corpus callosum; CPU, caudate putamen; CX, cortex; LV, lateral ventricle.

The RNA sequencing data provided strong support for myelination deficiency in Ulk4−/− mice. Consistent with the reduced anti-Mbp staining, Mbp mRNA expression in the Ulk4−/− cortex (1318.23±0.14) was decreased by 53%, compared with that in the WT controls (2775.67± 0.11, p=0.02). In contrast, there was no difference on the Nefh mRNA expression between the WT (19.72±0.08) and Ulk4−/− cortex (19.98± 0.14, p=0.469). The Nefh encodes the heavy neurofilament protein, which plays an important function in mature axons, and is commonly used as a biomarker of neuronal damage and susceptibility to amyotrophic lateral sclerosis. The type IV intermediate filament heteropolymers are composed of light, medium, and heavy chains. They comprise the axoskeleton and are functionally involved in the maintenance of neuronal caliber and in the mutual sustenance of axons and myelin (Larsen, DaSilva et al. 2006). There was no significant change in Nefl (1.01-fold, p=0.47) or Nefm expression (0.95-fold, p=0.36). These data suggested that neurofilaments as axonal skeleton in the cortex were not affected by Ulk4 deletion.
The overall under-development of the CC may therefore be significantly attributed to reduced myelination.

**4.4.4 Reduced Olig2$^+$ cells in the CC of the *Ulk4$^{-}$* mice**

In the CNS, mature oligodendrocytes synthesize myelin, extending processes to contact multiple neighboring axons and enwrapping short axonal segments (Baumann and Pham-Dinh 2001). To become functional, oligodendrocyte progenitor cells undergo differentiation, maturation and finally become myelinating oligodendrocytes (Baumann and Pham-Dinh 2001). To access the effect of *Ulk4* deficiency on the development of the oligodendrocyte population, we first investigated the expression Olig2, which is restricted to the oligodendrocyte lineage and is expressed in progenitors and myelinating oligodendrocytes.

In the whole regions of the CC including the middle (Fig. 4-4B) and lateral (Fig. 4-4D) parts, the number of Olig2$^+$ cells in *Ulk4$^{-}$* mice was markedly reduced compared with that in the WT littermates (Fig. 4-4A,C). Quantification of Olig2$^+$ cells in comparable regions of the CC showed a modest reduction (~17%) of Olig2$^+$ cells in the *Ulk4$^{-}$* (Fig. 4-4F,G) compared with that in the control mice (Fig. 4-4E). In addition, based on Hoechst staining, the total cell numbers in the CC were comparable (Fig. 4-4E-G). The loss of 17% Olig2$^+$ progenitors in the CC partially contributed to 53% decrease of *Mbp* mRNA expression. This suggested that in the absence of *Ulk4*, the generation of oligodendrocyte population was compromised, while the maturation of oligodendrocytes was more severely affected.
Figure 4-4 Anti-Olig2 staining showed a significant reduction of Olig2+ cells in the CC of the Ulk4+/− mice. P12 brain sections from WT littermate controls (A, C, E) and Ulk4−/− mice (B, D, F) were stained with anti-Olig2. The number of Olig2+ cells was quantified in defined areas (500 μm x 100 μm) in E and F, and showed a loss of 17% Olig2 positive cells in the Ulk4−/− mice (G, n=4, * for p<0.05). Bar=100 μm in A-F.

4.4.5 Ulk4 deficiency leads to fewer myelinated axons and decreased axon caliber at P18

To determine whether the decreased myelination in the Ulk4+/− mice was transient or sustained, we investigated the myelination of the CC at a later stage, P18. Transmission electron microscopy showed less myelin in the Ulk4−/− mice (Fig. 4-5D).
compared to the WT littermates (Fig. 4-5A). In addition to a reduced number of myelinated axons, a decreased diameter of axons was also seen in the \textit{Ulk4}^{-/-} CC (Fig. 4-5B,E). We failed to detect differences in the structure of the myelin sheath between the WT and \textit{Ulk4}^{-/-} mice, as most axons in both genotypes displayed loose wrapping (Fig. 4-5C,F). Most \textit{Ulk4}^{-/-} mice had died by P18 and we could not perform similar experiments on older mice. The electron microscopic images of P18 CC provided further evidence of reduced number of axons and a decreased size of axons sizes together with reduced myelination in the \textit{Ulk4}^{-/-} mice, suggesting that \textit{Ulk4} deficiency may delay maturation of myelinating oligodendrocytes.

\begin{figure}[h]
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\caption{Electron microscopic images of the white matter tracts in cross-sections of the CC of P18 mice. Fewer myelinated axons were present in the CC of \textit{Ulk4}^{-/-} mice (* in D showing un-myelinated areas) compared with the WT littermates (A), which was fully occupied by myelinated axons. The diameter of myelinated fiber was substantially smaller in the \textit{Ulk4}^{-/-} mice (E) compared to WT controls (B). It appeared that there was more space between the different layers of the myelin sheath in \textit{Ulk4}^{-/-} mice (F), and therefore \textit{Ulk4}^{-/-} sheaths seemed less compacted than WT controls (C), consistent with reduced Mbp which has role in myelin compaction. Bar=2 µm in A and D, 100 nm in B, C, E and F.}
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4.4.6 Elevated reactive astrogliosis in the Ulk4−/− mice

Oligodendrocytes and astrocytes are differentiated from glial progenitors, which depart from the neuronal progenitor lineage during CNS development. Also, astrogliosis is a common phenotype in hydrocephalic animals (Takei, Shapiro et al. 1987). In a parallel study, we showed that Ulk4−/− mice developed congenital hydrocephalus (manuscript under review), which became severe at an early postnatal stage due to impaired CSF flow and abnormal ciliary ultrastructure and function. To determine the gliosis status in the Ulk4−/− mice, we carried out immunohistochemical staining on P12 brain sections with anti-GFAP, a marker for astrocytes.

Elevated GFAP expression was detected in the CC and deep layer of the Ulk4−/− cortex at the level of the lateral ventricles (Fig. 4-6B) when compared to littermate controls (Fig. 4-6A). Under higher magnification, resting astrocytes with lightly stained cell body and thin processes predominantly occupied the corpus callosum and deep cortical gray layer in the WT mice (Fig. 4-6C). In contrast, massive reactivated astrocytes with enlarged cell bodies and thicker processes appeared in equivalent areas of the Ulk4−/− mice (Fig. 4-6D). The same phenomenon was observed in the posterior part of the CC at the level of the hippocampus (Fig. 4-6F). The altered cell morphology indicated that astrocytes in the Ulk4−/− mice were in the status of activation, which was different from the resting astrocytes in the WT littermates (Fig. 4-6E).

During glial differentiation, Olig2 is expressed in progenitors of both oligodendrocyte and astrocyte lineages. A recent study using short-term lineage tracing showed that Olig2+ cells in the astroglial lineage were the essential source of reactive astrocytes (Chen, Miles et al. 2008). To determine if Olig2+ cell reduction in the Ulk4−/− CC was accompanied with changes in astrocyte lineage, we performed double staining with anti-Olig2 and anti-Gfap (Fig. 4-6A-B, A’-B’). There was no co-localization of Olig2 and GFAP detected. Therefore lack of Olig2+/GFAP+ cells showed that reduced Olig2+ oligodendrocytes were not a consequence of increased astrocyte production.
4.4.7 Reactivation of microglia in periventricular white matter and deep cortex.

The presence of predominant reactivated astrocytes suggested a higher degree of neuroinflammation in $\textit{Ulk4}^{-/}$ mice. To further validate the neuroinflammation status, we examined alterations in microglial cells, which are considered as the most sensitive sensors of brain pathology. Microglia undergo a complex and multistage activation process that converts them into “activated microglial cells” upon detection of lesion signs or nervous system dysfunction (Kettenmann, Hanisch et al. 2011).

We performed immunostaining with anti-Iba1, an antibody against the ionized calcium binding adaptor molecule 1, a marker of microglia. Iba1$^+$ cells were detected throughout the cortex in both $\textit{Ulk4}^{-/}$ and WT mice (Fig. 4-6E-H’). In the superficial cortex, there was no obvious change in the number or morphology of the Iba1$^+$ cells between the $\textit{Ulk4}^{-/}$ mice (Fig. 4-6F) and the controls (Fig. 4-6E). These cells displayed a typical ramified morphology as resting microglia. However, there was a dramatic increase of the Iba1$^+$ cells in the deep cortex and periventricular white matter of the $\textit{Ulk4}^{-/}$ mice (Fig. 4-6H,H’) compared with the respective regions in the WT mice (Fig. 4-6G,G’). Examination of the Iba1$^+$ cells under higher magnification revealed dramatic alterations of microglial morphology in the $\textit{Ulk4}^{-/}$ mice, with characteristics of activation: they became amoeboid with round cell shape, and had reduced or an absence of processes (Fig. 4-6H’). This did not couple with increased microglial differentiation in the whole cortex and the expression of a key molecule $\textit{Tspan2}$ for microglial differentiation was actually 40% reduced ($p=0.024$). However, the “microglial cell activation” molecule $\textit{Tlr7}$ gene for Toll-like receptor 7 was induced 2.22-fold in the $\textit{Ulk4}^{-/}$ mice ($p=0.047$). Together these data showed that $\textit{Ulk4}^{-/}$ mice are undergoing striking neuroinflammation.
Figure 4-6. Elevated neuroinflammation in P12 \textit{Ulk4}\(^{-}\) mice. WT (A,A’,C,E,G,G’) and \textit{Ulk4}\(^{-}\) (B,B’,D,F,H,H’) coronal brain sections were stained with anti-GFAP for astrocytes (green, A-D,A’B’), anti-Olig2 for oligodendrocytes (red, A-B’), anti-Iba1 for microglia (E-H’) and count-stained with DAPI for nuclei (blue). Images of A and B were sampled from the CC adjacent to the lateral ventricle, and A’ and B’ were the higher magnification images of A and B respectively. Images of C and D were sampled from the hippocampal level showing reacted astrocytes in the mutant (D). Images in E and F showed the superficial layers and in G and H from the deep cortical layers. Images in G’ and H’ were magnified view of the boxed areas in C and D respectively. +/+and -/- indicate the \textit{Ulk4} genotype status. Note that increased numbers of reactivated GFAP\(^{+}\) cells were present in both anterior (B) and posterior parts (D) of the CC of \textit{Ulk4}\(^{-}\) mice compared to the WT littermate controls (A,C). There was no co-labeling of anti-Olig2 and anti-Gfap in WT or \textit{Ulk4}\(^{-}\) mice. Also, microglia in deep cortical layers were activated (H,H’). Scale bar =100 \(\mu\)m in A,B,C,D,E,F,G,H; 50 \(\mu\)m in A’B’; 20 \(\mu\)m in G’H’.
Oligodendrocyte differentiation, maturation and myelination were disrupted in the \textit{Ulk4}^{−/−} brain

Oligodendrogenesis in the vertebrate CNS is regulated by both intracellular transcriptional factors and extracellular signals (Emery 2010). To build a developmental profile and gain a better understanding of how \textit{Ulk4} deficiency affected oligodendrocyte lineage progression, we carried out pathway analyses on the whole genome RNA sequencing data, which identified a series of genes which were not only involved in oligodendrocyte lineage differentiation but also myelination. Remarkably, all of them were significantly down-regulated in the \textit{Ulk4}^{−/−} brain.

Among the 618 \textit{Ulk4} targets for which mRNA expression was significantly reduced by >20% or was induced by >1.5-fold, 8 genes (Cnp, Dusp10, Fa2h, Myrf, Olig2, Plp1, Sox10, Tspan2) were identified that related to “Oligodendrocyte differentiation” \(p=2.62\times10^{-06}\), Fig. 4-7D) and these were significantly reduced by 23-50%. Three genes (Dusp10, Olig2, Sirt2) associated with the “Regulation of oligodendrocyte differentiation” were decreased by 33-36% (Fig. 4-7F, \(p<0.05\)). Twelve genes (Arhgef10, Fa2h, Gal3st1, Gjc3, Myrf, Mbp, Nfasc, Olig2, Plp1, Sirt2, Tspan2, Ugt8a) related to “Myelination” \(p=1.59\times10^{-08}\) were down-regulated by between 30% and 53% (Fig. 4-7E, \(p<0.05\)), and 18 genes (Atp1a2, Car2, Cldn11, Cnp, Ermn, Gjc2, Gjc3, Mag, Mbp, Mobp, Mog, Myo1d, Nfasc, Pllp, Plp1, Rap1a, Sirt2, Tspan2) involved in the “Myelin sheath” \(p=1.12\times10^{-08}\), Fig. 4-7G) were reduced by 23-56%. These data comprehensively support the immunohistochemical observation that oligodendrocyte development and maturation were compromised in the \textit{Ulk4}^{−/−} mice.

Gliogenesis includes production of astrocytes and oligodendrocytes. We next investigated if the reduced oligodendrogenesis is accompanied with an increase in astrocyte production. Fourteen \textit{Ulk4} targets (Arhgef10, Cnp, Dusp10, Erbb3, Fa2h, Gcm1, Myrf, Lamac3, Lef1, Olig2, Plp1, Sirt2, Sox10, Tspan2) were identified as “Gliogenesis” genes \(p=0.014\), and they were reduced by 22-47% (Fig. 4-7A). Two genes (Gcm1 and Olig2) in “Glial cell fate commitment” \(p=0.024\) were reduced by
33-36%, 3 genes (Mag, Olig2 and Sox10) in “Positive regulation of gliogenesis” (p=6.21E-02) and 2 genes (Dusp10 and Sirt2) in the “Negative regulation of gliogenesis” (p=1.48E-01) were also down-regulated by 23-49% (Fig. 4-7C). Four genes (Gcm1, Lamc3, Plp1, Tspan2) in the "astrocyte differentiation" pathway (p=0.002), the Mag as "positive regulation of astrocyte differentiation", and 3 genes (Grm3, Nfasc, Sirt2) involved in "Astrocyte projection" were all down-regulated (Fig. 4-7A). These data together showed that Ulk4 deficiency systematically reduced the whole gliogenesis in the mutants.

In contrast, mRNA expression of Ccl3, Gfap, Vim and Tlr7 genes was induced by 6.16, 2.86, 1.91 and 2.22-fold, respectively (Fig. 4-7F), which were more likely to co-relate with the inflammatory response in the Ulk4−/− mice. The Tlr7 is known to be involved in microglia reactivation. The Ccl3 is chemokine (C-C motif) ligand 3 that binds CCR1, CCR4 and CCR5, and has inflammatory and chemokinetic properties, which play a role in responding to brain injuries. Vimentin is a class-III intermediate filament and a marker for relatively later steps of gliogenesis. Gfap is one of the major intermediate filament proteins of mature astrocytes and a marker to distinguish mature astrocytes from other glial cells during development. Its upregulation is consistent with increased reactivated astrocytes detected by IHC. Therefore, upregulated expression of these markers is likely to reflect astrogliogenesis and reactivation of astrocytes and microglia in the Ulk4−/− mice.

Oligodendrogenesis can be divided into 4-5 stages, which can be identified by morphological changes and overlapping markers: bipolar progenitors (A2B5, GD3, NG2, Pdgfra, DM20, Cnp), pro-oligodendrocytes (A2B5, GD3, NG2, Pdgfra, DM20, Cnp, O4), immature oligodendrocytes (DM20, Cnp, O4, Rip, GalC), non-myelinating mature oligodendrocytes (DM20, Cnp, O4, Rip, GalC, Mbp, Plp1, Mag) and myelinating mature oligodendrocytes (DM20, Cnp, O4, Rip, GalC, Mbp, Plp1, Mag, Mog) (Mattan, Ghiani et al. 2010). (Fig. 4-8).
A. Gliogenesis

B. Regulation of gliogenesis

C. Oligodendrocyte differentiation

D. Key factors regulating oligodendrocyte differentiation and maturation

E. Myelination

F. Inflammation

G. Myelin sheath
Figure 4-7. Quantitative genome RNA sequencing revealed a systematically reduced gliogenesis in the Ulk4−/− mice. The STRING program identified (A) 14 Ulk4 target genes in “Gliogenesis” (p=0.014); (B) 2 genes (Gcm1 and Olig2) in “Glial cell fate commitment” (p=0.024), 3 genes (Mag, Olig2 and Sox10) in “Positive regulation of gliogenesis” (p=6.21E-02) and 2 genes (Dusp10 and Sirt2) in the “Negative regulation of gliogenesis” (p=1.48E-01); (C) 8 genes in the “Oligodendrocyte differentiation” (p=2.62E-06); (E) 12 genes in “Myelination” (p=1.59E-08), and (G) 18 genes in the “Myelin sheath” (p=1.12E-08). All of these genes were down-regulated in the Ulk4−/− mice. (D) 17 key genes relating oligodendrocyte differentiation and maturation. (F) Four genes (Ccl3, Gfap, Vim and Tlr7) related to neuroinflammation were induced in the Ulk4−/− mice by 6.16, 2.86, 1.91 and 2.22-fold. * for p<0.05, and ** for p<0.01.

The transcription factors Id2, Id4, Hes5 and Sox6 are required to maintain OPCs in their undifferentiated state and to repress myelin gene expression. Ulk4−/− brain expressed 119% of Id2 (p=0.134), 99% of Id4 (p=0.477), 77% of Hes5 (p=0.088) and 82% of Sox6 (p=0.107) of that in the WT littermates (Fig. 4-7D), suggesting that the Ulk4 null mutation had no significant effect on factors repressing myelin gene expression.

There was no significant change in mRNA expression for Pdgfra (1.04-fold, p=0.311) or Galc involved in the lysosomal catabolism of galactosylceramide that is a major lipid in myelin. St8sia1 encoding an enzyme that produces ganglioside GD3 was upregulated by 1.22-fold (p=0.004). Ng2 (=Cspg4) mRNA was reduced by 17% (p=0.085) and CNPase by 47% (p=0.027), showing a modest effect of Ulk4 on progenitors, pro-oligodendrocytes or immature oligodendrocytes. The Myrf is induced concurrent with terminal differentiation and expressed only by postmitotic oligodendrocytes (Cahoy et al., 2008). A 38% reduction in Myrf mRNA expression (p=0.034) suggested a significant loss of the birth of postmitotic oligodendrocytes (Fig. 4-7C).

Olig2 is a master regulator of the oligodendrogenesis, and the initial specification of the oligodendrocyte lineage is reliant on the transcription factor Olig2. Oligodendrocyte lineage is absent in Olig2−/− mice (Zhou, Choi et al. 2001; Lu, Sun
et al. 2002). A 33% reduction in the master regulator Olig2 had a subsequent knock on effect of 50% reduction of myelination in the Ulk4−/− mice. Sox10 is another transcription factors expressed in all stages, and it was 33% down-regulated as well. The downstream of the Olig2 include Olig1 (30%↓, p=0.053), Ascl1 (18%↓, p=0.147), Nkx2-2 (30%↓, p=0.091), Sox10 (34%↓, p=0.026), Yy1 (104%, p=0.218) and Tcf4 (8%↓, p=0.197), which are required for the generation of postmitotic oligodendrocytes (Wegner 2008). All, except Yy1, were down-regulated in the Ulk4−/− mutants (Fig. 4-7D, Fig. 8).

The Apc was down-regulated by 25% (p=0.018), Mbp by 53% (p=0.020), Plp1 by 45% (p=0.023) and Mag by 49% (p=0.023), demonstrating a much severer effect of Ulk4 on the maturation of oligodendrocytes. Meanwhile, a 47% down-regulation (p=0.015) in Mog expression, a unique marker for myelinating mature oligodendrocytes, and a 48% decrease in CGT, an enzyme encoded by Ugt8 and played a role in myelin compaction and lipid synthesis, illustrating a detrimental effect of Ulk4 deficiency on the maturation and myelination of oligodendrocytes, which eventually resulted in a half of myelination in P12 Ulk4−/− mice (Fig. 4-8).

![Figure 4-8. Changes in mRNA expression of genes associated with myelination in the Ulk4−/− mice.](image_url)

Whole genome RNA sequencing was carried out with P12 RNA from 3 WT and 3 KO cortex. The abundance of RNA expression was statistically analyzed by one-way ANOVA. Data were presented with percentage (%) of reduction in the Ulk4−/− mice.
4.5 Discussion

White matter reduction is an underlying pathway in neurodevelopmental and neuropsychiatric disorders, and myelination plays an important role in the integrity of white matter. We recently showed that the \textit{ULK4} gene is a rare risk of neurodevelopmental disorders and essential for neuritogenesis (Lang, Pu et al. 2014). In this study, we for the first time present evidence that Ulk4 is critical for progenitor maintenance, oligodendrocyte differentiation, maturation and myelination. Deficiency of the Ulk4 reduced myelination by \~50\% and compromised the integrity of the CC in the \textit{Ulk4}\textsuperscript{−/−} mice.

White matter reduction and decreased dendritic arborization have been reported in schizophrenia patients (Douaud, Smith et al. 2007; Bose, Mackinnon et al. 2009; Fornito, Yucel et al. 2009; Francis, Bhojraj et al. 2011) and autistic brains (Paul, Brown et al. 2007; Sajan, Fernandez et al. 2013). White matter tract integrity and callosal thickness were reported to affect cognitive performance and associate with intelligence, processing speed and problem solving abilities in childhood (van Eimeren, Niogi et al. 2008; Hutchinson, Mathias et al. 2009; Penke, Maniega et al. 2010). Our unpublished genetic evidence shows that ULK4 is deleted in 1.12/1000 patients with heterogeneous clinical features including developmental delay, language delay and severe intellectual disability, and these are developed during early childhood and can be related to their white matter development, synapses, and brain connectivity.

We investigated this hypothesis in \textit{Ulk4}\textsuperscript{−/−} mice. White matter is largely developed within the first 3 postnatal weeks in mice. Histological examination of P12 mice showed severely disrupted integrity of the \textit{Ulk4}\textsuperscript{−/−} CC, suggesting a crucial role for Ulk4 in the postnatal white matter development. RNA sequencing data uncovered a global reduction of both axons and dendrites in the \textit{Ulk4}\textsuperscript{−/−} brain. This is consistent with previous reports that \textit{Unc}-51 is essential for axonal elongation in \textit{C. elegans} (Ogura, Wicky et al. 1994), that knockdown of \textit{ULK4} impairs neuritogenesis in human neuroblastoma cells, and that disruption of \textit{Ulk4} compromises the CC at birth (Lang, Pu et al. 2014). From neuroimaging studies, agenesis of the CC is widely
implicated in autism, epilepsy, schizophrenia, and intellectual disability (Motomura, Satani et al. 2002; Grewal, Almullahassani et al. 2007; Lau, Hinkley et al. 2013). Therefore a correct dose of the *ULK4* gene is likely critical for the development of white matter in both mice and men.

In healthy individuals, callosal fibers are present at birth, but the functional connectivity via the CC increases as the fibers myelinate from about 4 postnatal months into young adulthood (Giedd, Blumenthal et al. 1999). Myelin formation is critical for proper electrical impulse conduction. In mice, myelin is constituted by oligodendrocytes, starting from ~P5 and ending at ~P20 in the CNS (Fern, Davis et al. 1998). During this period, oligodendrocyte progenitor cells, generated from subventricular cells, divide, migrate and then terminally differentiate into premyelinating oligodendrocytes. These immature oligodendrocytes will then mature and myelinate nearby receptive axons. These processes may be affected by environmental cues but also through regulation of the master transcription factor Olig2 and other intrinsic factors including Olig1, Ascl1, Nkx2-2, Sox10, Yy1, Tcf4, Id2, Id4, Hes5, and Sox6, Hadc1 and Hdac2, Sirt2, Dusp10 (Emery 2010). We show here that Ulk4 regulates a core set of these factors, and Ulk4 deficiency significantly down-regulates the key factors including Olig2, Olig1, Nkx2-2, Sox10, Sirt2 and Dusp10.

Myelin controls the speed of impulse conduction through axons, and the synchrony of impulse traffic between distant cortical regions is critical for optimal mental performance and learning (Fields 2008). White matter myelination was suggested as an indicator of functional brain maturation, as children with developmental delay showed a significant reduction in the relative content of myelinated white matter (accounting for 19.8% of brain volume in patients and 21.4% in control subjects, $p=0.005$) (Pujol, Lopez-Sala et al. 2004). However, this 1.6% difference is substantially less than ~50% reduction of myelination that we observed in the *Ulk4* mice. This may help explain why half of the *ULK4* deletion patients manifest severe learning difficulty and language delay.
The consequences of *Ulk4* deficiency on myelinogenesis can thus be comprehended by its effect on the oligodendrocyte population. Consistently, a series of genes involved in the whole process of oligodendrocyte development were significantly dysregulated in the *Ulk4*<sup>-/-</sup> mice. Our present work provides insight into how *Ulk4* may involve may be involved in oligodendrocyte maturation and myelination.

This was demonstrated in this study first by a 17% reduction of Olig2<sup>+</sup> cell number in the *Ulk4*<sup>-/-</sup> CC and a 34% decrease in overall Olig2 mRNA in P12 *Ulk4*<sup>-/-</sup> brain. Olig2 is a master regulator of oligodendrocytes that is continuously expressed throughout all stages of oligodendrogenesis, from progenitors to myelinating oligodendrocytes, and Olig2<sup>-/-</sup> mice show an absence of the oligodendrocyte lineage (Zhou, Choi et al. 2001; Lu, Sun et al. 2002). A 34% decrease in total cortical mRNA for Olig2 and a 17% decrease in Ng2/Cspg4 mRNA, a marker for progenitors, pro-oligodendrocytes or immature oligodendrocytes, indicated a significant role for Ulk4 in the maintenance of the bipolar progenitors.

*Myrf* is a marker of postmitotic oligodendrocytes, vital for the generation of CNS myelination during development and maintenance in the adult (Bujalka, Koenning et al. 2013). Conditional inactivation of *Myrf* in the oligodendrocyte lineage causes differentiation to stall at an early premyelinating stage, and the cells are unable to express myelin genes. Conversely, forced expression of *Myrf* in oligodendrocyte progenitors causes a precocious expression of myelin proteins (Emery, Agalliu et al. 2009). *Myrf* mRNA expression is 38% reduced in (*p*=0.034), which suggests a strong effect of Ulk4 on the birth of postmitotic oligodendrocytes.

Expression of *Cnp*, *Plp1*, *Mag*, *Mbp* and *Mog* are all attenuated in the *Ulk4* deficient mice by ~50%. Cnp is a critical component of the molecular machinery that mediates early events in myelinogenesis, in particular the elaboration of oligodendrocyte processes. Mag is involved in mediating glial-axon interactions during myelination (Marcus, Dupree et al. 2002), Mbp is a major component of myelin, and Mog is specific to the CNS and is located on the surface of oligodendrocytes and myelin sheaths (Brunner, Lassmann et al. 1989). *Mobp* plays a
role in myelin compaction or stabilization (Montague, McCallion et al. 2006) and was 56% reduced \((p=0.015)\) in Ulk4\(^{-/-}\) mice. Plp1 mRNA was 45% reduced. Plp1 is a transmembrane proteolipid protein, a predominant component of myelin. Ugt8, which encodes the enzyme CGT, involved in myelin compaction and lipid synthesis, was 48% reduced. Oligodendrocyte myelin paranodal and inner loop protein (Opalin) is enriched in myelin of the CNS and is specifically localized to the membranes of paranodal and inner loops (Yoshikawa, Sato et al. 2008). Opalin mRNA was 53% downregulated in the Ulk4\(^{-/-}\) mice \((p=0.013)\). These data together convincingly demonstrate critical roles of Ulk4 in the maintenance of oligodendrocyte progenitors, oligodendrocyte differentiation, maturation and myelination, through regulation of key transcription factors, which subsequently contribute to white matter integrity.

In contrast, molecules which inhibit axon growth are induced in the Ulk4\(^{-/-}\) mice, which include 2.82-fold increase of Cdh1, 1.91-fold elevation of Vim and 2.86-fold induction of Gfap genes. Cdh1 knockdown has been shown to dramatically increase axonal length (Kunimoto, Yamazaki et al. 2012). Gfap and/or Vim knockout increases plastic sprouting of axons (Menet, Prieto et al. 2003; Ribotta, Menet et al. 2004). Gfap is an astrocyte marker and astrocytes are reactivated in the Ulk4\(^{-/-}\) mice, together with microglia reaction, suggesting a high level of neuroinflammation present in the Ulk4\(^{-/-}\) mice, which may also contribute to the compromised integrity of the white matter.

In this Chapter, I largely focused on the integrity of white matter and oligodendrocyte production. The shortcoming of the study in particular is the possibility that the phenotype may be related to axon loss or a failure of axon growth. It was previously suggested that axons provide molecular cues necessary for oligodendrocyte myelination and appropriate sheath lengths. This view was based upon the observation that axon diameters correlate with myelin sheath length (Hildebrand, Remahl et al., 1993; Murray & Blakemore1980; Ibrahim, Butt et al., 1995). A recent study showed that oligodendrocytes have regional identity and generate different sheath lengths that mirror internodes, for example, oligodendrocytes respond to fiber diameters, and spinal cord oligodendrocytes
generate longer sheaths than cortical oligodendrocytes [Bechler, Byrne et al., 2015]. It must be noted that oligodendrocytes require axons for survival and normal proliferation, so a reduction in axon caliber at P18 in Ulk4 mutants could explain the phenotype observed on oligodendrocytes. This will require future experiments to validate if this is the case.

In summary, we discovered an essential role for Ulk4 in myelination. Ulk4 regulates key transcription factors of oligodendrogenesis including Olig2, Olig1, Sox10 and Myrf. Ulk4 deficiency leads to 50% hypomyelination, which contributes to damaged white matter integrity with increased neuroinflammation in the Ulk4−/− mice. These phenotypes were observed in mice with both Ulk4 alleles disrupted. Some of these phenotypes may extend mildly to pathology in patients with a single copy of ULK4 deletion, as often same dose of alterations have a more severe phenotype in humans than in mice. Therefore, ULK4 may become a novel biomarker and drug target for diseases associated with white matter integrity and hypomyelination.
CHAPTER 5

ULK4 IS ESSENTIAL FOR MAINTAINING THE NEURAL STEM CELL POOL
5.1 Abstract

Neural stem cell pool at birth determines the starting point of adult neurogenesis. Abnormal neurogenesis was associated with major mental illness, in which ULK4 was previously identified as a rare risk factor. Little is known about the function and signaling mechanism of the ULK4. Here we provide further evidence that ULK4 is deleted in approximately 1/1000 patients with developmental disorders of pleiotropic symptoms including severe language/speech delay and learning difficulties. Targeted disruption of the Ulk4 significantly reduces cerebral cortex in postnatal mice. Ulk4 is expressed in a cell cycle-dependent manner and peaked in the G2/M phases. Ulk4 deficiency compromises mid-neurogenesis and dramatically reduces the neural stem cell pool in the subventricular zone at birth. Pathway analyses of the dysregulated targets support Ulk4 as a key regulator of neural progenitor proliferation and cell cycle, partially through regulation of Wnt signaling. ULK4 may therefore significantly contribute to human developmental disorders.
5.2 Introduction

We recently investigated the involvement of serine/threonine-protein kinase genes in neuropsychiatric and neurodevelopmental disorders, and identified *Unc-51-like kinase 4* gene (*ULK4*) as a rare susceptibility gene for schizophrenia, autism and major depression (Lang, Pu et al. 2014). Both embryonic and adult neurogenesis are essential for brain formation and proper function, and abnormal neural proliferation is an underlying neuropathology associated with the major mental illness. Cortical neurogenesis in the mouse starts from E10.5 and is largely thought to be completed by E17.5. Cells in the proliferating ventricular zone (VZ) of the dorsolateral telencephalon undergo a maximum of 11 cell divisions, and neurons at different layers are generated in a cell cycle number-dependent manner (Dehay and Kennedy 2007). Therefore embryonic cell cycle regulation plays a crucial role in cortical lamination and proper brain function.

Both increased and decreased embryonic neurogenesis are associated with neurodevelopmental diseases. For example reciprocal deletions and duplications (copy number variants) of ~600kb region at 16p11.2 are strongly associated with behavioral disorders, cognitive deficits and brain volume. Deletions are associated with neurodevelopmental disorders, especially speech, language and motor deficits, intellectual impairment, restrictive and repetitive behaviors and macrocephaly (Shinawi, Liu et al. 2010; Hanson, Bernier et al. 2015), whereas duplication are associated with ADHD, schizophrenia and microcephaly (McCarthy, Makarov et al. 2009; Shinawi, Liu et al. 2010); A study by Golzio et al (Golzio, Willer et al. 2012) in zebrafish and mouse embryos found that, of the 29 known genes in the region, KCTD13 is a key driver of neuronal proliferation and growth abnormalities in the developing brain, with macrocephaly caused by increased proliferation, and microcephaly caused by increased apoptosis and decreased proliferation of neuronal progenitors.

Decreased cortical and hippocampal volumes are associated with schizophrenia (Harrison 1999), in which the first ULK4 CNVs were described (Lang, Pu et al. 2014). *Disrupted-in-Schizophrenia 1* (*DISC1*) is the first gene identified to cause
psychiatric illness and DISC1 truncation co-segregates with the illness in a large Scottish schizophrenia family (Stclair, Blackwood et al. 1990; Millar, Wilson-Annan et al. 2000). Disc1 mutants are reported to have a reduced brain volume (Clapcote, Lipina et al. 2007). Expression of the truncated Disc1 shows specific corticogenesis defects in mice, with reduced layers II/III (Shen, Lang et al. 2008), which correspond to the peak of Disc1 expression during middle neurogenesis in embryos (Schurov, Handford et al. 2004), and are the layers which are altered in schizophrenia (Harrison 1999). DISC1 appears to play rather different roles during adult neurogenesis. Downregulation of DISC1 leads to accelerated neuronal integration, mispositioning of new dentate granule cells, accelerated dendritic development and synapse formation and enhanced excitability (Duan, Chang et al. 2007). The neural progenitor proliferation and behavioral defects caused by DISC1 deficiency can be normalized by inhibitors of Gsk3, a component of canonical Wnt signaling pathway (Mao, Enno et al. 2006).

Wnt signaling is an essential regulator of adult neurogenesis. It is inhibited by the tumor suppressor adenomatous polyposis coli (APC), which is a multifunctional protein promoting β-catenin degradation by forming destructive complex with β-catenin, axin and Gsk3β. Binding of the ligands, Wnts, to their receptor (fzd1-10) complex and prevents β-catenin destruction, and induces nuclear translocation and transcriptional activation of target genes in association with the Tcf/Lef family. APC itself is also shown to regulate newly generated cells, as well as maintaining neural stem cells (NSCs) in the adult neurogenic niche. Loss of APC results in a marked reduction of GFAP-expressing NSC-derived new neurons in the subventricular zone (SVZ) and hippocampal dentate gyrus, leading to the decreased volume of olfactory granule cell layer (Imura, Wang et al. 2010).

Brain overgrowth including megalencephaly is also associated with risk of epilepsy, intellectual disability (ID), autism, and hydrocephalus (Mirzaa and Poduri 2014). This is probably due to a change in the proliferation of neural progenitors in the brain (Chenn and Walsh 2002; Bernier, Golzio et al. 2014). Mutations in repressor genes of the mammalian target of rapamycin (mTOR) pathway, such as PTEN
increase protein translation in neurons and at the synapse (Auerbach, Osterweil et al. 2011). Patients carrying single-copy of functional \textit{PTEN} suffer from autism, macrocephaly, mental retardation, and epilepsy (Butler, Dasouki et al. 2005; Herman, Butter et al. 2007; Orrico, Galli et al. 2009; Varga, Pastore et al. 2009; McBride, Varga et al. 2010; O’Roak, Vives et al. 2012). Macrocephaly was a clinical indicator of genetic subtypes in autism (Hobert, Embacher et al. 2014; Vanderver, Tonduti et al. 2014). PTEN is a dual protein/lipid phosphatase that dephosphorylates PIP3 and inhibits the mTOR/AKT pathway. This inhibition decreases protein translation, cell proliferation and cell growth. Inhibition of PTEN leads to increased stem cell proliferation, somatic/dendritic/axonal growth, accelerated spine maturation, diminished synaptic plasticity, and altered intrinsic excitability.

The mTOR is an evolutionarily conserved serine/threonine kinase that is a member of the PI3K-related kinase (PIKK) family. The mTORC1 has emerged as a central regulator of cell proliferation, cellular differentiation, cellular metabolism, autophagy and immune response regulation; whereas mTORC2 participates in cell survival and the regulation of actin and cytokeratin organization. Deregulation of the mTOR pathway is associated with increased cell proliferation, loss of autophagy, and diseases including cancer and autism in humans (Ma and Blenis 2009). These mutations can also lead to hyperactivation of mTOR, and consequently impairs autophagy. It may result in an increase in dendritic spine density in layer V of the temporal lobes in post-mortem brains from patients with autism (Tang, Gudsnuk et al. 2014). These reductions in autophagy and developmental spine pruning were suggested to be the origin of the macrocephaly that is present in the majority of the individuals with such mutations (Williams, Dagli et al. 2008). ULK1 is a Ser/Thr kinase that plays a major role in inducing autophagy. ULK1 is phosphorylated and negatively regulated by the mTOR complex 1 (mTORC1). Evidence is merging that ULK1 may in return negatively regulate the kinase activity of mTORC1 and cell proliferation. Deficiency or knockdown of ULK1 or ULK2 is also shown to enhance cell proliferation rates and mTORC1 signaling (Jung, Seo et al. 2011).
Evidence is emerging that some ULK family members may be involved in cell cycle progression and tumorigenesis. The ULK family members include ULK1, ULK2, ULK3, ULK4 and STK36. Knockdown of STK36 appeared to be associated with increased sensitivity to PARP inhibitor-induced cell death, and this was also thought to be cell cycle-related (Turner, Lord et al. 2008). Interestingly, a STK36 Ala687Thr mutation was identified in a patient as one of the genes associated with differential responsiveness to temozolomide chemotherapy in glioblastoma multiforme. Although the biological mechanism(s) of the mutation was unknown, the patient cells showed higher level of STK36 mRNA, altered cell-cycle G2/M-checkpoint-regulation and elevated sonic hedgehog (Shh) pathways (Biswa, Chandra et al. 2015). Ulk3 also plays a role in regulating Shh signalling, by interacting with the Suppressor of Fused (Sufu), a protein required for negative regulation of Gli proteins (Maloverjan, Piirsoo et al. 2010). Overexpression of ULK3 is shown to induce autophagy, delay cell doubling and promote premature senescence in human diploid fibroblasts (Young, Narita et al. 2009).

Both ULK1 and ULK2 was hypermethylated and down-regulated in glioblastoma, the most aggressive type of brain tumor. The ULK2 kinase activity was required for inhibiting tumor growth, and kinase mutant of ULK2 failed to induce autophagy or inhibit the growth (Shukla, Patric et al. 2014). A single-nucleotide polymorphism rs1052501 in ULK4 gene, that changes codon 542 from Lysine to Threonine, was associated with multiple myeloma (Broderick et al., 2011; Greenberg et al., 2013). Although functional consequence of this protein polymorphism is currently unclear, the studies suggest that ULK4 may be involved in cell cycle control.

In a previous study, we investigated evolutionary conservation and developmental expression of the Ulk4 gene in Xenopus. To our surprise, Xenopus Ulk4 gene was found highly expressed along the VZ and SVZ of forebrain regions throughout development and in the adult. Xenopus Ulk4 mRNA expression was highly co-localized with Sox3 - a neural stem/progenitor cell marker in Xenopus, and a radial glial marker Blbp, highlighting a potential role of Ulk4 in embryonic neurogenesis (Domínguez et al., 2015).
In the present study, we have provided further genetic evidence that ULK4 is deleted in ~1/1000 of patients with heterogeneous clinical features including developmental delay, language delay and intellectual disability. We have investigated the role of the Ulk4 in neurogenesis in Ulk4−/− mice, and discovered for the first time that Ulk4 is expressed in a cell cycle–dependent manner in vivo and in vitro. Targeted disruption of the Ulk4 impairs embryonic neurogenesis and dramatically reduces the neural stem cell pool in early postnatal mice. In addition, we identified a range of dysregulated genes in the Ulk4 deficiency mice, which are involved in controlling the cell cycle and neural stem cell proliferation, including Wnt signaling pathway. These data provide plausible biological basis for investigating patients of neurodevelopmental and neuropsychiatric disorders associated with human ULK4 lesions.

5.3 Materials and Methods

5.3.1 Diagnostic referral cases.

The tested cohort consisted of patients referred to Guy’s and St Thomas NHS Foundation Trust from regional paediatricians and other health specialists, as well as from genetics centres both in and outside the region (SE Thames). Array CGH analysis was initiated to determine the causes of developmental delay, neurocognitive disability, learning difficulties, behavioral abnormalities or birth defects or to confirm a clinical diagnosis of a suspected syndrome. All patient tests were carried out as part of standard clinical care, either as clinical referrals for array CGH testing following a normal karyotyping, or those having array CGH as a first-line test in place of karyotyping. All data were anonymized.

5.3.2 Array CGH analysis

Testing was carried out at a regional cytogenetics CPA accredited laboratory, according to published protocols (Ahn et al., 2010), using an Agilent oligonucleotide 60 K array platform (designs 028469 and 017457) with a total imbalance detection
rate of 24%. Genomic data and referral phenotype information was recorded in a clinical database. Copy number variants in this population are available in the Brain and Body Genetics Resource Exchange (BB-GRE; https://bbgre.brc.iop.kcl.ac.uk).

5.3.3 Ulk4 gene targeting construct

Ulk4 targeted, conditional ready, lacZ-tagged vector (PRPGS00083_A_C05) was constructed using knockout-first strategy. Methods used to create the CSD targeted alleles have been published (Skarnes et al., 2011). The 5’ homologous recombination arm was comprised of 5682 bp Ulk4 genomic DNA which harbored 2398 bp of intron 3, exon 4-6 and 1401 bp of intron 6. The 3’ homologous recombination arm (4084 bp) contained 951 bp of intron 7, exon 8-12 and 610 bp of intron 12.

After targeted integration, the L1L2_Bact_P cassette was inserted at position Chr9:121,260,507(Build GRCm38), upstream of the critical exon(s). The cassette was composed of an FRT site followed by lac sequence and a loxP site. This first loxP site was followed by neomycin under the control of the human beta-actin promoter, SV40 polyA, a second FRT site and a second loxP site. A third loxP site was inserted downstream of the targeted exon(s) at position Chr9:121,259,772. Further information on targeting strategies can be found at http://www.knockoutmouse.org/aboutkompstrategies. The exon 7 with 76 bp Ulk4 coding sequence was deleted by targeted integration, and replaced by a cassette containing SA-IRES-lacZ sequence, so that lacZ reporter gene can be expressed under the endogenous mouse Ulk4 regulatory machinery.

5.3.4 Ulk4−/− mice

The ES cell clone EPD0182_4_E12 was generated by the Wellcome Trust Sanger Institute using the knockout construct. This was used to create Ulk4−/− mouse strain by the KOMP Repository (WWW.KOMP.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. We acquired Ulk4−/− breeding pairs on C57BL/6N strain background from KOMP Repository (knockout mouse project, USA) at UC Davis. All experimental procedures were conducted
under a license approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland (NUI) Galway, Ireland. Ulk4−/−, Ulk4+/− and WT littermates were obtained from Ulk4−/− x Ulk4+/− mating. Mice were genotyped by PCR, using genomic DNA with two pairs of DNA primers: Ulk4EndE7For (5’-TAACCTTGCTGGACGGATTGCTG-3’) and Ulk4EndIn7Rev (5’-TGATCTGTAATCGCAGTGCAGG-3’) amplifying a 271bp DNA fragment from the WT allele, and Ulk4KOMPKOFor (5’-GAGATGGCGCAACGCAATTAATG-3’) and Ulk4KOMPKORev (5’-CTGAGGAGACATAATGTAACCAGC-3’) to synthesize a 621bp DNA fragment from the knockout allele, respectively.

5.3.5 Histology and Immunohistochemistry

Young adult mice at 2 month or newborn mice were humanely terminated with overdose of sodium pentobarbitone and brains were immersed overnight in PBS containing 4% paraformaldehyde (PFA) at 4°C. They were then embedded in paraffin and sectioned at 10μm. Brain sections were prepared for hematoxylin and eosin (H&E) or immunofluorescence staining. Primary antibodies used in the following experiment were: rabbit anti-CDP/Cux1 (1:500, sc-13024, Santa Cruz), rabbit anti-Ctip2 (1:1000, ab18465, Abcam), rabbit-anti-Tbr1 (Abcam, ab31940, 1:500), mouse Anti-mouse-Phospho-Histone H3 (Ser10) (6G3) (Cell Signaling Technology #9706, 1:1000), rabbit Anti-rabbit-Ki67 (Abcam, ab16667, 1:500), rabbit anti-Ulk4 (1:500, NBP1-20229, Novus Biologicals), BrdU In-Situ Detection Kit (BD Bioscience, 551321, 1:10). Fluorescent secondary antibodies for immunofluorescence staining were Alexa Fluor 488 or 594 conjugated. Images were captured by Olympus BX51 Upright /Fluorescent Microscope.

5.3.6 BrdU labeling index

Bromodeoxyuridine (BrdU) is synthetic nucleoside that is an analog of thymidine. BrdU can be incorporated into the newly synthesized DNA of proliferating cells
during the S phase of the cell cycle, so it is commonly used to detect the proliferating cells. The fraction of progenitor cells in S phase, a labeling index, was determined by scoring the percentage of BrdU-labeled cells over total neural progenitor cells (Ki67-positive).

The day plug found is determined as E0.5. Time-mated pregnant females were injected with a pulse of BrdU (i.p., 50 mg/kg body weight) at E15.5. 2 hours later, embryos were fixed into 4% PFA overnight. BrdU incorporation assay (BD Biosciences) was executed according to the manufacturer’s instructions. The labelling index was determined by counting BrdU + cells over total cells within 100 um bins extending from ventricular surface to VZ/SVZ boundary (Takahashi et al., 1993).

5.4 Results

5.4.1. Rare ULK4 deletion in patients with developmental disorders

Genomic imbalances can be associated with both complex and Mendelian genetic disorders. Copy number variation (CNV) studies from large samples can uncover the common genetic factors involved. The Brain and Body Genetic Resource Exchange (BBGRE) program has collected and genotyped CNVs of approximately 5891 patients (3747 male and 2144 female) with developmental disorders (Ahn et al., 2010 and 2013), and this provides a rich resource for studying the genetic association of the human diseases (https://bbgre.brc.iop.kcl.ac.uk).

A search for the ULK4 gene in the BBGRE database (BBGRE V3) led to the identification of 7 patients with CNVs including this gene (Fig.5-1, and Table 5-1), therefore, the ULK4 CNVs occur in a rate of 1.19/1000 patients in BBGRE with developmental disorders. Previously, we identified 4 ULK4 deletions from 3,391 ISC schizophrenic patients, 2 in 708 Icelandic schizophrenia cases, 2 in 1,136 Icelandic bipolar patients, and 1 in 507 Icelandic autism cases (Lang et al., 2014). However, ULK4 CNVs was absent in the 3,181 ISC controls, whereas small
deletions affecting exons 33-34 were detected in 37 of 98,022 Icelandic controls (Lang et al., 2014). Taken together, ULK4 CNVs occur in 16/10,925 in the disease populations, at a rate of 1.46/1000, which is 4.01-folds enriched in comparison to a total of 37 carriers in 101203 controls (3.65E-4). In addition, there are 14 patients with CNVs on ULK1, 19 on ULK2, 5 on ULK3, and 2 on STK36, totaling 47 Cases in 5891 patients. Although most of these CNVs involve multiple genes, together, it does highlight the importance of the ULK family in contributing to developmental disorders.

Six patients carry a ULK4 intragenic deletion (Fig.5-1 in red, Case 1-6) but no other CNV, with an exception of Case 6, who has deletion also on a non-coding gene LINC01098 at chr4:78860573-179094829. Interestingly, all 6 patients (4 male and 2 female) have the identical 143,487 bp deletion at Chr3:41611009-41754496 (hg19), which comprises of exons 25-30 of the ULK4 gene. This is nested within the exon 21-34 region deleted in Scottish schizophrenic patients (Lang et al., 2014). The Case 7 (BBGRE ID: 108064) harbors a large chromosomal duplication at Chr3:249726-43129680 (hg19), and his clinical features are therefore likely to result from the combinational effects of 294 genes including ULK4 (Fig.5-1 in blue).

Five couples out of the 6 ULK4 deletion families were also genotyped. Interestingly, these were not de novo mutations, three cases were inherited from paternal and 2 from maternal alleles. Therefore, the deletion is un-bias in parental transmission, and single copy of the intragenic ULK4 deletion is unlikely to affect sexual development or function. At the current stage, it is unknown if these 6 ULK4 patients originate from a common ancestor.

The clinical symptoms of the 6 ULK4 deletion patients are heterogeneous (Table 5-1). ULK4 deletion may affect peripheral function, as general developmental delay, short stature, enamel hypoplasia and hypotonia are observed in several cases. Two patients (Case 5 and 6) are diagnosed with typical cognitive development but behavioral problems. Four other patients (Cases1-3 and 5) are associated with severe learning difficulties, severe language delay, speech delay, and hyper mobility, which highlight that ULK4 deletion affect brain development and/or function.
Figure 5-1. Seven *ULK4* CNVs are present in the BBGRE cohort of patients. Seven patients were identified to carry CNVs on the *ULK4* gene including one duplication (in blue, 3 copies) and 6 deletions (in red, 1 copy).

Table 5-1. Clinical and genetic information of the 7 patients with ULK4 CNVs

<table>
<thead>
<tr>
<th>BBGRE ID</th>
<th>Sex</th>
<th>Age in 2015</th>
<th>Chromosome</th>
<th>Size</th>
<th>Genes involved</th>
<th>Copy</th>
<th>Inheritance</th>
<th>Clinical phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>132678</td>
<td>F</td>
<td>4</td>
<td>Chr3:41611008-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>N/K</td>
<td>Speech delay, bum shuffling, FTND</td>
</tr>
<tr>
<td>121612</td>
<td>F</td>
<td>10</td>
<td>Chr3:41611009-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>Maternal</td>
<td>Severe learning difficulties, speech delay, eczema, enamel hypoplasia, short stature</td>
</tr>
<tr>
<td>115861</td>
<td>M</td>
<td>11</td>
<td>Chr3:41611009-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>Paternal</td>
<td>Severe language delay, mild general delay</td>
</tr>
<tr>
<td>116054</td>
<td>M</td>
<td>6</td>
<td>Chr3:41611009-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>Paternal</td>
<td>Developmental delay, hypotonia</td>
</tr>
<tr>
<td>111565</td>
<td>M</td>
<td>10</td>
<td>Chr3:41611008-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>Maternal</td>
<td>Typical cognitive development, behavioral problems, hyper mobility, neck webbing, hearing, high arched palate,</td>
</tr>
<tr>
<td>113015</td>
<td>M</td>
<td>43</td>
<td>Chr3:41611009-41754496</td>
<td>143,487</td>
<td>ULK4 (E25-30)</td>
<td>1</td>
<td>Paternal</td>
<td>Typical cognitive development, minor tremors, slightly short sighted,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>chr4:78860573-179094829</td>
<td>234,256</td>
<td>LINC01098 on-coding</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>108064</td>
<td>M</td>
<td>3</td>
<td>Chr3:249726-43129680</td>
<td>42,879,954</td>
<td>ULK4 + 293 others</td>
<td>3</td>
<td>N/K</td>
<td>Dysmorphic features, Micrognathia, preterm (32+1 weeks), prominent features, scaphocephaly</td>
</tr>
</tbody>
</table>
5.4.2 Ulk4 deficiency leads to thinner cortex in mice

The CNS-related clinical features in the ULK4 deletion patients prompted us to investigate brain development in mice. Whereas Ulk+/− mice did not show phenotype in survival or gross neuroanatomy, a large proportion of Ulk4−/− mice died before weaning. Therefore, most of the studies here were carried out at ~2 weeks after birth. We first examined the neuroanatomy of the cerebral cortex, and morphometric analyses showed that the cerebral cortex was significantly thinned in the Ulk4−/− mice (Fig. 5-2A-D). At postnatal day 12 (P12), the in thickness of cerebral cortex in the WT mice was 1.51±0.01 mm (n=4) at the anterior commissure level, and this was reduced by 24.5% in the Ulk4−/− mice (1.14±0.06mm, n=4, p<0.01, Fig. 5-2E).

The mammalian cortex is organized into six neuronal layers, and cells in different cortical layers display distinct morphology, for example, layer IV cells are much smaller, and layer V neurons are substantially larger. We divided cortical layers based on histological sections (Fig. 5-2C-D), and quantified the thickness of individual layers based on H&E staining (Fig. 5-2F-G). Six sections at frontal cortex were measured per mouse at the anterior commissure level. The quantitative analyses showed a reduction of 20% in layer I (0.11 ±0.002mm in WT vs 0.088 ±0.004mm in Ulk4−/− n=4), 20% in layers II-IV (0.54 ±0.021mm in WT vs 0.43 ±0.017mm in Ulk4−/−, n=4), 25.8% in layer V (0.31 ±0.014 mm in WT vs 0.23 ±0.011 mm in Ulk4−/−, n=4) and 29.6% in layer VI (0.55 ±0.005 mm in WT vs 0.387±0.035 mm in Ulk4−/−, n=4), respectively. The morphometric analyses of the bright-field images suggest a generalized thinning in all layers of P12 Ulk4−/− cortex.
Figure 5-2. Disruption of Ulk4 led to reduced cerebral cortex in P12 mice. H&E-stained coronal sections of the cerebral cortex at the anterior commissure level were quantified from the WT (A) and Ulk4<sup>+/−</sup> (B) mice. The cortex was markedly thinned with enlarged lateral ventricles in the Ulk4<sup>+/−</sup> mice (B) compared with the WT littermate controls (A). Quantification of the cerebral cortex showed a significant reduction of the thickness in the Ulk4<sup>+/−</sup> mice (C) in comparison to controls (D). Measurement of cortical layers suggested that layers I-VI of the Ulk4<sup>+/−</sup> mice were all significantly thinner than that in the controls, which suggested a general reduction rather than layer specific (F), and the reduction of each layer was in the range of 20%-29.6% (G). Bars in A-B =1000um, in C-D =100um.
5.4.3. Ulk4 is required for neuronal production and cortical cyto-architecture

Cells in different cortical layers express distinct molecular markers. We next performed immunochemistry using layer-specific markers, and quantified the relative number of cortical projection neurons at P12. We used Cdp/Cux1 as a marker for layers II-IV cortical neurons (Fig. 5-3A-B) and Ctip2 for layers V and VI (Fig. 5-3C-D) (Lizarraga, Margossian et al. 2010). At P12, Cux1\(^+\) cells were abundantly detected in the superficial area, and Ctip2\(^+\) cells in the deeper layers of cortex in both WT and mutant mice (Fig. 5-3A-B), suggesting there was no gross migration error in the Ulk4\(^{-/-}\) mice. The Cux1\(^+\) cells exhibited a significant reduction (20\%) in the Ulk4\(^{-/-}\) mice (827 ± 24 in WT vs 691± 39 in KO, n=4, p=0.02, Fig. 5-3F), with a reduced thickness (Fig. 5-3E) and unaltered cell density (2028± 60 in WT vs 2062± 54 in KO, n=4, p=0.69, Fig. 5-3G).

Ctip2\(^+\) cells in layer V of the Ulk4\(^{-/-}\) mice (97.9 ±9.8) appeared to be similar to the WT controls (89.5 ±8.3, p=0.57), however, the layer V cell density was higher in the Ulk4\(^{-/-}\) mice (583.7± 52.7) than that in controls (393.2±24.3, p=0.029), which was largely due to reduced thickness in the Ulk4\(^{-/-}\) (230.2 ±10.1) compared to WT (307.8 ±14.3, p=0.021).

The Ctip2-labeled cells in layer VI (Fig. 5-3C-D) were quantified from the Ulk4\(^{-/-}\) mice (342.5±20) and from WT (427.3±17.7, p=0.033), and detected 19.8\% loss of the Ctip2 cells in the Ulk4\(^{-/-}\) cortex (Fig. 5-3H). The layer VI thickness was also reduced in the Ulk4\(^{-/-}\) cortex (386.8±34.7, Fig. 3I), compared with WT controls (548.7± 4.8, p=0.017), therefore, the Ctip2 cell density was not significantly different between the Ulk4\(^{-/-}\) (1180.9±90.1, Fig. 3J) and WT controls 1042.3 ±45.4, p=0.19). Taken altogether, these results demonstrate a profound defect in neuronal generation, despite the cortical lamination was relatively preserved in the Ulk4\(^{-/-}\) mice.
5.4.4. A subtle reduction in the superficial layers of P0 *Ulk4*<sup>−/−</sup> cortex

The six-layered cortical structure is largely formed in the embryonic day 12.5-17.5 in mouse embryos, in an inside-out fashion. Whereby the later born cortical neurons
will cross the deepest layers which are formed earlier, to generate the superficial layers (Dehay and Kennedy 2007). To evaluate relative contribution of embryonic neurogenesis to the reduced cortex observed at P12, we examined histological sections of newborn (P0) mice (Fig. 5-4A-B). At this stage, the hydrocephalus phenotype was subtle (Liu et al., manuscript under review), and the intracranial pressure was relatively lighter. Quantitative analysis of the P0 brain sections showed that the overall cortical thickness (716.6±19.9 in WT; 609.8±30.0 in Ulk4−/−, n=5 each) was slightly but significantly (p=0.018) reduced in mutant mice compared with controls (Fig. 5-4D). Thickness of cortex without corpus callosum (399.8±14.2 in WT, 370.3±12.1 in Ulk4−/−, n=5 each; p=0.038) was also thinner than that of littermate controls at this stage (Fig. 5-4C). This suggested that embryonic neurogenesis was subtly affected in the Ulk4−/− mice.

The mammalian cortex is organized into six neuronal layers. These cortical layers are largely formed in the E12.5-17.5 in mouse embryos. Neurons are generated in an inside-out fashion, whereby the later born cortical neurons will cross the deepest layers which are formed earlier, to generate the superficial layers (Dehay and Kennedy 2007). To further evaluate the defective neurogenesis in the Ulk4−/− mice, P0 cerebral cortex was examined using specific layer markers (Fig. 5-4). The superficial layers identified by anti-Cux1/CDP showed reduced Cux1+ cells in P0 Ulk4−/− mice (291.5±27.2, p=0.022) compared with controls (382.9±17.6, n=5). Also, the thickness of the Cux1/CDP+ cell layer was reduced from 105.5±2.7 µm in WT to 92.3±4.9 µm in P0 Ulk4−/− mice (p=0.047).

However, there was no significant difference in the number of Ctip2+ cells, or in the thickness of layer V-VI between P0 Ulk4−/− mice and controls (Fig. 5-4H-J). This was supported by further examination of E15.5 embryo, at which time the layer VI was formed. Immunohistochemical staining with anti-Tbr1, another marker of layer VI, showed no significant alteration of Tbr1+ cells between E15.5 Ulk4−/− and control embryos (Fig. 5-5A-B). Thus, we conclude that Ulk4 plays a role in later-born superficial neurons affecting mid-neurogenesis onwards.
Figure 5-4. Decreased cortical thickness and superficial layers in Ulk4−/− newborn mice. (A-B) Histological sections from P0 mice and thickness of P0 cortex with (C) or without (D) corpus callosum (cc) was quantified. (C-D) Statistical analyses showed a reduction in Ulk4−/− cortex with (C) or without corpus callosum, separately. Bar = 100 μm in A-B. (E-G) P0 sections from WT (E) and Ulk4−/− mice were stained with anti-Cux1 and Cux1+ cells were quantified, which showed a significant reduction in Cux1+ cell number (G) and in Cux1+ cell layer thickness. (H-I) P0 sections were stained with anti-Ctip2 to identify the neurons in layer V-VI (J). There was no alteration of early-born neurons of layer V or VI identified by Ctip2 between WT and Ulk4−/− cortex. Bars=100 μm. Four pairs of WT and KO mice were used in this experiment. Cell numbers of 6 brain sections from each mouse were quantified. Data were statistically analyzed by one-way ANOVA and presented as mean ± SEM. p <0.05 was considered to be statistically significant.
5.4.5 Reduction of BrdU incorporation in E15.5 Ulk4⁻/⁻ mice

It is known that later birth neurons (layer II-IV) are mostly generated from SVZ progenitors during mid- stages (E15.5) of cortical neurogenesis (Dehay & Kennedy, 2007). To assess the total progenitor population at E15.5, we performed the anti-Ki67 staining to label cells at all cycling phases (Fig. 5C-D). Quantification of Ki67⁺ VZ/SVZ showed no significant difference in thickness of Ki67⁺ cells between E15.5 Ulk4⁻/⁻ and control embryos (Fig.5E). Next we examined neural stem cells in the S phase. E15.5 pregnant females were injected with BrdU, a nucleotide analog, and 2-hour later, embryos dissected and anti-BrdU staining performed (Fig. 5E-F'). BrdU⁺ cells were quantified from 6 sections of each embryo at comparable anatomic level. The percentage of BrdU⁺ cells (0.26%± 0.01, n=3) over the total cell number of the selected areas was significantly less in E15.5 Ulk4⁻/⁻ embryos than that in WT controls (0.35%± 0.03, p=0.039, n=3 Fig.5F). These data suggested a significant reduction of neural progenitors entering the DNA synthesis in E15.5 Ulk4⁻/⁻ embryos, which led to significant reduction of layer II-IV in P0 and P12 mice.
Figure 5-5. A reduced S phase neural progenitors in E15.5 $Ulk4^{-/}$ embryos. E15.5 embryonic sections were processed with anti-Tbr1 (A-B), anti-Ki67 (C-D), or anti-BrdU (E-F), which was pre-injected with BrdU 2-hour before embryo dissection. The Ki67$^+$ (E) and BrdU$^+$ (F) cells were quantified and presented with M+SEM. (G) Quantification of anti-Tbr1 staining showed no significant difference between E15.5 WT (A) and $Ulk4^{-/-}$ (B) sections for layer VI formation. n=3, * for $p<0.05$. Bars=50 in A-D, Bar=100 µm in E-F. Three pairs of WT and KO mice were used in this experiment. Cell numbers of 6 brain sections from each mouse were quantified. Data were statistically analyzed by one-way ANOVA and presented as mean ± SEM. $p<0.05$ was considered to be statistically significant.
5.4.6 Ulk4 is expressed highly in the G2 and M phases of neural proliferative cells

Previously we showed a wide expression of Ulk4 throughout the brain with a high level in the cortex and hippocampus of adult mouse and human brain (Lang et al., 2014). In Xenopus embryo, Ulk4 was shown to express in the VZ and SVZ of the developing forebrain, and co-localized with SOX3 and Blbp (Domínguez, Schlosser et al. 2015). To explore the biological base of function of the Ulk4 gene during corticogenesis, we characterized the Ulk4 expression in mouse developing cortex.

At E12.5 of early neurogenesis, the anti-Ulk4 immunohistochemical staining showed the presence of Ulk4 immuno-reactivity throughout the developing cortex, including the pre-plate and VZ (Fig. 5-6A-C). Co-staining with Ki67 demonstrated a good co-localization of Ulk4 immuno-reactivity with Ki67 in the proliferating neural progenitors (white arrowheads, Fig. 5-6A-C). The strongest anti-Ulk4 staining was localized in the VZ of the dorsal telencephalon, and co-labelled with anti-phosphohistone H3 (PH3), a marker for the M phase of mitotic cells. All PH3+ cells lined the ventricular wall were highly expressing Ulk4 (white arrowheads, Fig. 5-6D-F). Some cells, however, were strongly expressing Ulk4, but negative for PH3 (red arrows Fig. 5-6F, I). This suggested that Ulk4 was also highly expressed in another phase of cell cycle. A similar pattern was observed at E15.5 of mid-neurogenesis, and Ulk4 was expressed highly in all PH3+ cells in the VZ (white arrowheads, Fig. 5-6G-I), but Ulk4+/PH3- cells were also present (red arrows, Fig. 5-6G-I). Together, the in vivo expression data demonstrated high Ulk4 expression in the neural proliferating zones during early and middle phases of corticogenesis, which supported a role of Ulk4 in the generation of pyramidal neurons.
Figure 5-6 High expression of Ulk4 is co-related with cell cycle in vivo and in vitro. Sections from E12.5 (A-F') and E15.5 (G-I') embryos were stained with anti-Ulk4, anti-Ki67 and anti-PH3. Ulk4 (green) is expressed in Ki67+ cells (Red) (white arrows, A, B, C) and strongly co-localized with PH3 (white arrows, D-F'). At E15.5, a similar pattern of Ulk4 is detected with strong Ulk4 expression in the PH3+ cells (G, H, I). G’-I’ were low magnification images to give a view of larger areas from which images G-I were taken, respectively. (J-M) High Ulk4 expression in the G2 and M phases of human neuroblastoma cells. SH-SY5Y cells were stained with anti-ULK4 (in green) and count-stained with DAPI (in blue). Low expression of Ulk4 was detected in all SH-SY5Y cells (J-K) but strong immune-reactivity of Ulk4 was identified in the G2 and M-phases (M). Bar =100 µm in J-K and =10 µm in M.

The presence of high ULK4+/PH3− cells in vivo prompted us to investigate cell cycle-related expression in cultured cells of human origin. We carried out anti-ULK4 staining on SH-SY5Y cells. Whereas low level of ULK4 expression was detected in every neuroblastoma cells, strong anti-ULK4 staining appeared in limited cells, which displayed either large nuclei and/or with two adjacent nuclei (Fig. 5-6J-K). In the absence of unique cycle markers for individual phases and/or sub-phases, we took advantage of the distinct nuclear morphology at different phases and four subphases (prophase, metaphase, anaphase and telophase) of cell cycle and investigated Ulk4 expression under high microscopic magnification.

We examined all 108 brightly stained cells based on nuclear morphology (Fig. 5-6M), and quantify the ratio of Ulk4+ cells in different phases of cell cycle (Fig. 5-6L). Approximately 18.1% of strongly Ulk4+ cells was in the G2 (Fig. 5-6M), with a smooth and round edge but substantially larger nuclei than others, 36.2% of Ulk4-expressing cells were identified as prophase, which had big round nuclei but punctuated morphology on the edge, as chromosomes started to condense; 18.1% cells in the metaphase with 2 sets of chromosomes lining in the middle, 10.5% cells in the anaphase with 2 sets of chromosomes just about being separated; and 17.1% cells in the telophase with formation of two smaller adjacent nuclei (Fig. 5-6M). The Ulk4 expression was sharply declined at the G1 when two daughter cells were completely formed and separated (white arrowheads, Fig. 5-6M) or in S the phase
with no co-labelling anti-BrdU (Lang, Pu et al. 2014). Therefore, both in vivo and in vitro expression studies demonstrate is cell cycle–dependent of the Ulk4 gene, with high expression in the G2 and M phases of neural proliferating cells.

5.4.7 Lack of Ulk4 dramatically reduces the neural stem cell pool in P0 mice

To investigate the effects of the Ulk4 deficiency on neurogenesis, we examined P0 brain histological sections and discovered a marked reduction of the SVZ region of all Ulk4−/− newborn brains (Fig. 5-7H-K) in comparison to WT littermates (Fig. 5-7D-G). We further examined the stem cell pool in the SVZ by anti-Ki67 staining, which showed a significant reduction of Ki67+ cells in P0 Ulk4−/− brains (Fig. 5-7D-K). The Ki67+ cells in the Ulk4−/− mice (530±46, n=4, Fig.5-7L) were decreased by 23.9% in comparison to the equivalent regions in the controls (696±23, n=4, Fig.5-7M). We also quantified the relative populations of neural progenitors in defined areas. The thickness of both dorsal (dSVZ, 55.7±2.5 in Ulk4−/− vs 91.3±3.9 in WT) and ventral SVZ (vSVZ, 77.9 ±14.2 in Ulk4−/− vs 123.5 ±4.1 in WT) occupied by Ki67+ cells was significantly reduced in the Ulk4−/− mice. We carried out co-staining with anti-Ulk4 and Ki67, and identified strong co-expression in the SVZ region of P0 mice (Fig. 5-7A-C), whereas Ulk4 expression in areas neighbouring SVZ was substantially weaker (Fig. 5-7A). Together these data showed that Ulk4 is highly expressed in the SVZ at birth, and Ulk4 is required for the maintenance of the neural stem cell pool. Ulk4 depletion reduces neural proliferation from mid-neurogenesis and results in a decreased neural stem cell pool at birth. Therefore, ULK4 regulates both embryonic and adult neurogenesis.
Figure 5-7. Intense Ulk4 expression in P0 SVZ and reduced neural stem cells in P0 Ulk4<sup>−/−</sup> mice. (A) Anti-Ulk4 staining was co-localized with Ki67 (B) in the SVZ region (C) of the P0 brain. Four pairs of P0 WT (D-G) and Ulk4<sup>−/−</sup> (H-K) brain sections were processed for anti-Ki67 staining. The Ki67 positive cells in the areas of the SVZ of WT (L,N,P) and Ulk4<sup>−/−</sup> (M,O,Q) newborn brain were quantified, which showed a significant reduction in neural stem cell pool (R) and in thickness of SVZ subregions (S,T) in the Ulk4<sup>−/−</sup> mice. Bars=100 µm. Four pairs of WT and KO mice were used in this experiment. Cell numbers of 6 brain sections from each mouse were quantified. Data were statistically analyzed by one-way ANOVA and presented as mean ± SEM. p <0.05 was considered to be statistically significant.
5.4.8. Ulk4 regulates cell cycle and neural proliferation, partially through WNT pathway

To reveal the molecular network of the Ulk4, we carried out whole genome RNA sequencing with cortical RNA, and obtained 19,652 reads of sequences. The RNA expression data were statistically analyzed by one-way ANOVA, with \( p<0.05 \) as a cut-off point. The extremely low expressing genes were filtered out with an expression value of 1 in either WT or Ulk4\(^{-/-}\) mice. Subsequently, the gene list was further reduced with >20% reduction or >150% increase in the Ulk4\(^{-/-}\) mice. This narrowed down the list to 618 genes regulated by Ulk4, with 469 genes down-regulated and 149 upregulated.

These dys-regulated genes were analyzed on the STRING program. The GO pathway analyses pointed to significant association with “neural precursor cell proliferation” and “cell cycle” pathways. For example, sixteen genes (Cenpw, Clgn, Eml1, Itgb3bp, Mecom, Nedd1, Nek3, Paf15 (=KIAA0101), Sept4, Setd8, Setd8, Ska3, Sox17, Stra13, Sycp3 and Trim36) were involved in “cell cycle process” \( (p=0.0133) \), all of which were significantly down-regulated. Eight genes (Fgfr2, Lef1, Pdgfrb, Sgo2, Sirt2, Tbx3, Xrcc3 and Ctgf) were “positive regulators” of cell cycle, and 7 of them were reduced in mRNA expression, except Ctgf gene which was often up-regulated in response to trauma, scar formation and excitotoxicity in the CNS. Among the 6 “negative regulators” identified, four (Apc, AY074887, Fgfr2, Pura) were decreased, whereas other two (Ier3 and Ptgs2) were significantly increased. Remarkably, 6 cell cycle phase transition genes (Apc and AY074887 for cell cycle arrest, Taf10 for G1/S transition, Pura as S phase checkpoint, Abcb1a for G2 to M, Arhgef10 in mitotic spindle formation for G1/S to M phase) were reduced in expression, whereas two negative regulators (Parp3 with negative influence on G1/S progression, Ier3 as G2 DNA damage checkpoint) of phase transition were significantly up-regulated.
Figure 5-8. Genes dys-regulated by Ulk4 deficiency. The whole genome sequencing identified 19652 transcripts from 3 WT and 3 Ulk4<sup>−/−</sup> mouse cortex. The expression data were statistically analyzed for Ulk4 targets with three criteria: (1) \( p < 0.05 \), (2) expression value >1, (3) reduced by >20%, or increased by >1.5 fold. The 618 resulting targets were analyzed by STRING. The genes altered in NPC (A,B) and cell cycle pathways (C-F) were presented by folds of changes in the Ulk4<sup>−/−</sup> mice in relation to the WT value (as “1”). * for \( p < 0.05 \), and ** for \( p < 0.01 \).

Six genes were identified to involve in “neural precursor cell proliferation” \((p<0.01)\), Apc, Fzd6, Fgfr2, Lef1, Ephb1, Eml1, and all of which were down-regulated from 23% to 39% in the Ulk4<sup>−/−</sup> brain. Five genes, Ell3, Hdac5, Sox10, Lims2, Sirt2, were involved in “regulation of neural precursor cell proliferation”, and they were all down-regulated by 22-41%. Remarkably, several are directly involved in (Apc, Fzd6,
Lef1, Ephb1) or connected with (Fgfr2) Wnt signaling pathway, which plays a critical role in neurogenesis.

These results suggest that Ulk4 serves as a scaffold protein during cell division. Ulk4 deficiency may systematically dump down cell proliferation, partially through dys-regulation of the Wnt signaling, which dramatically reduces the neural stem cell pool in the SVZ of the newborn mice, compromising both embryonic and adult neurogenesis.

5.5 Discussion

Embryonic and adult neurogenesis are crucial for brain formation and normal function. Disturbed neural proliferation is associated with neurodevelopmental and neuropsychiatric illness, including schizophrenia, autism and depression, which are linked to ULK4 (Lang, Pu et al. 2014). In this report we showed that ULK4 is deleted in 1.19/1000 patients with heterogeneous clinical symptoms including severe language delay, speech delay and learning difficulties. Together with previous datasets, 16 ULK4 CNVs are present in 10,925 cases of schizophrenia, autism, bipolar patients, and developmental disorders, while 37 ULK4 CNVs are identified among the 101203 controls (Lang, Pu et al. 2014), showing a 4-fold enrichment of ULK4 CNV in the disease populations.

We showed here that Ulk4 is essential for neurogenesis, and targeted disruption of the Ulk4 gene reduces middle neurogenesis and dramatically reduces the neural stem cell pool in newborn mice. Mechanistically, we demonstrate that Ulk4 expression is cell cycle-dependent, and most abundantly in the G2 and M phases, in the neural progenitors lining the ventricular surface of developing cortex. This observation is compatible with the notion that xUlk4 mRNA is most abundantly expressed in the VZ and SVZ regions, co-localizing with a neural stem cell marker of Sox3 in Xenopus embryo (Dominguez, Schlosser et al. 2015). Ulk4 deficiency systematically damps down pathways in cell cycle and neural progenitor proliferation. On the other
hand, polymorphisms of ULK4, most likely leading to gain-of-function, may cause tumorigenesis in human (Broderick, Chubb et al. 2012; Greenberg, Lee et al. 2013). We demonstrate that Ulk4 regulates WNT signalling pathways by quantitative whole genome RNA sequencing. Many key molecules in the Wnt pathway, including Apc, Fzd10, Fzd2, Fzd6, Lef1, Porcn, Sox17, were significantly reduced in the Ulk4−/− mice. Wnt signaling is fundamental to brain development and function via both canonical and non-canonical pathways. The canonical pathway regulates cell fate determination, and its activation is required for the nuclear accumulation of β-catenin, which interacts with Tcf1/Lef at Wnt response elements and maintains stemness. The non-canonical pathways regulate planar cell polarity, cell adhesion, and motility, through actions of Tcf3, which antagonizes the canonical pathway by competing for target genes (O’Roak, Vives et al. 2012).

The APC negatively regulates canonical Wnt signaling by forming destruction complex with β-catenin, axin and Gsk3β. APC is highly expressed in the brain. Consistent with reduced neurogenesis and low abundance of APC in the Ulk4−/− mice, conditional deletion of APC from Gfap-expressing cells causes a marked reduction of neural stem cell-derived new neurons (Imura, Wang et al. 2010).

Binding of Wnts to their receptors (Fzd1-10) prevents the destruction of β-catenin and induces gene transcription. Out of 10 Wnt receptors, three (Fzd2, Fzd6 and Fzd10) are reduced to 73%, 61% and 70% respectively in the Ulk4−/− brain. In relation to this, high FZD6 expression is linked to poor survival in neuroblastoma patients, and FZD6+ neuroblastoma cells form neurospheres with high efficiency and grow more aggressively in immunodeficient mice (Cantilena, Pastorino et al. 2011). In contrast, mutations on human FZD6 are 5.1-fold enriched in cases of neural tube defects, and double mutations of Fzd3+/Fzd6− were associated with high penetrant occurrence of neural tube defects in mice (De Marco, Merello et al. 2012). Together, the data suggest that downregulation of the 3 Wnt receptors play a key role in mediating Ulk4 effects on neural stem cells.
$\text{CHD}8$ and $\text{CTNNB}1$ genes in the Wnt pathways are major risk factors of autism (Zahir, Firth et al. 2007; Neale, Kou et al. 2012; O'Roak, Vives et al. 2012; O'Roak, Vives et al. 2012; Bernier, Golzio et al. 2014). CHD8 acts as a negative regulator of the Wnt signalling by repressing CTNNB1 activity (Bourgeron 2015) and $\text{CHD}8$ mutation is accounted for 0.2% of patients (most with ID). While $\text{Ctnnb}1$ is unaltered, the Chd8 expression is upregulated by 1.15 fold with a marginal $p$ value of 0.052 in the $\text{Ulk}4^{-/}$ mice. This is consistent with the reports that $\text{CHD}8$ mutations cause larger heads, whereas mutations in $\text{CTNNB}1$ lead to microcephaly (O'Roak, Vives et al. 2012; Bernier, Golzio et al. 2014). Also transgenic mice overexpressing $\text{CTNNB}1$ in neural precursors have increased cerebral cortical surface area and folding (Chenn and Walsh 2002), while reduced $\text{CHD}8$ expression in zebrafish resulted in a larger brain (Bernier, Golzio et al. 2014).

WNT signaling cascades are known to network with Notch, FGF, BMP and Hedgehog signaling cascades to regulate the balance of stem cells and progenitor cells. $\text{Fgfr}2$ is shown to robust express in Gfap populations in the SVZ and SGZ (Chadashvili and Peterson 2006). Its ligand, FGF2, is also involved in maintaining the stem cell pool in the SVZ, and the absence of Fgf2 gene reduces the dividing progenitor population of the anterior SVZ by 50% (Zheng, Nowakowski et al. 2004). In line with this, $\text{Fgfr}2$ is reduced by 23% in the $\text{Ulk}4^{-/}$ mice.

EphB1 also networks with the Wnt signaling pathway, EphB1 is a receptor tyrosine kinase, and a member of EPH family receptors for Ephrin ligand family. EphB1-3 and their transmembrane ligands are expressed by the SVZ cells. Infusion of the ectodomain of either EphB2 or ephrin-B2 into the lateral ventricle increased cell proliferation and disrupted neuroblast migration (Conover, Doetsch et al. 2000). Mice lacking EphB1, and more profoundly EphB1 and EphB2, have significantly fewer neural progenitors in the hippocampus (Chumley, Catchpole et al. 2007) In the $\text{Ulk}4^{-/}$ mice, EphB1 is reduced by 28%, with 24% reduction of neural stem cells.

Wnt target genes are also dysregulated in the $\text{Ulk}4^{-/}$ mice. For example, Nr5a2 is a stemness maintenance gene in ES cells and is reduced to 37% ($p=0.042$), in contrast Cyr61 as a differentiation gene is 1.86-fold increased ($p=0.042$) in the $\text{Ulk}4^{-/}$ mice.
Wnt signaling is shown to regulate intermediate precursor production in the postnatal dentate gyrus via Cxcl12 signaling (Choe and Pleasure 2012), and Cxcl12 expression is 23% reduced ($p=0.028$) in the $Ulk4^{-/-}$ mice. Expression of Vim, a target gene of non-canonical Wnt signaling cascades, is 1.91 fold increased in the $Ulk4^{-/-}$ brain ($p=0.013$), and another astrocyte marker GFAP expression is also 2.86-fold induced ($p=0.036$). Increased expression of astrocyte molecules in $Ulk4^{-/-}$ mice is consistent with the literature that astrocytes negatively regulate neurogenesis. For example both basal and injury-induced hippocampal neurogenesis are increased in mice lacking Gfap and Vimentin (Wilhelmsson, Faiz et al. 2012).

In this neurogenesis study, there are shortcomings which will require further experiments to address. For example, people may argue that there can be two proliferative populations: stem cells and amplifying precursors. A stem cell has a tendency to differentiate into a specific type of cell, whereas a progenitor cell is already more specific and is pushed to differentiate into its target cell. The most important difference between stem cells and progenitors is that stem cells can replicate indefinitely, whereas progenitors can divide only a limited number of times. Recently, neural stem cells and neural progenitors are often referred to same cells. In this study, we investigated proliferating cells during embryonic development and in newborn mice, and used the terminology of neural stem cells. If there are two kinds of proliferating cells presented at these stages that I investigated, we have not addressed this in the study.

Does the physical disruption of the hydrocephalus explain the neurogenesis effect? So far we have evidence that reduced embryonic proliferation leads to alteration of layer II-IV in P0 and in P14 mice. At P0, when the mild hydrocephalus is present but not so severe, the neural stem cell pool is already grossly reduced. Therefore, the physical effect will be less obvious before birth. At 2-3 weeks of postnatal brain, the cortex reduction is more dramatic in mutant mice in proportion to CSF accumulation. This is likely to result from combinational effects of both physical disruption (CSF pressure), and effects on the neural stem cells during postnatal development, and it would be a challenging task to separate them.
The genomic sequencing analysis of human subjects with developmental delay cohort also has its limitations. We have checked with clinicians that six $ULK4$ deletion carriers are not related. Five couples of the 6 families were genotyped, and showed inheritance of 3 cases from father and 2 from mother. These parents do not present major clinical phenotype. These data showed that $ULK4$ deletion is unbiased in parental transmission to the next generation, and a single copy of functional $ULK4$ does not grossly affect sexual development or function. The clinical symptoms of the 6 patients are also heterogeneous. The deletion may affect peripheral organs, as developmental delay, short stature, enamel hypoplasia and hypotonia are observed. There are three possible explanations for the lack of clinical phenotype in these parents:

1. they might have some clinical sympotoms at their childhood, but these were not alarming enough for clinical examination or they were missed out due to lack of health facility and knowledge at the time;
2. some symptoms could be less prominent in adulthood, when subjects passed the education phase and physical developmental stage
3. there could be genetic background effect, which modulates the penetration of the genetic lesion.

In summary, we showed that ULK4 copy number variation is a risk factor associated with disturbed brain function. Ulk4 is a key regulator of cell cycle and neural progenitor proliferation. ULK4 deficiency leads to dramatic reduction of neural stem cell pool at birth, which will have knock on effect in adult neurogenesis. Therefore, ULK4 dysfunction may be widely associated with tumorigenesis and brain malfunction.
CHAPTER 6

BEHAVIORAL CHARACTERIZATION OF

$ULK4^{+/-}$ MICE
6.1 Introduction

Anxiety disorders are a category of mental disorders including generalized anxiety disorder, panic disorder and phobias characterized by feelings of anxiety and fear. They are the most common mental health illness with an estimated prevalence of 20%, and approximately 69 million people affected in the EU alone (Wittchen, Jacobi et al. 2011). The causes of anxiety disorders can be genetic abnormalities or drug use. Recently Viggiano et al reported that 67 genes were associated with anxious behavior. Among them, 33 genes whose deletions are accompanied with increased anxious behavior and 34 other genes whose deletions are related to decreased anxious behavior (Viggiano, Cacciola et al. 2015). Interestingly, they also show that presynaptic genes are involved in the emergence of anxiety, and postsynaptic genes in the reduction of anxiety after gene deletion by using gene enrichment analysis (Viggiano, Cacciola et al. 2015). For example, deletion of different neurotransmitter related genes for glutamatergic (GRM8, GRIN3B, SLC17A8), GABAergic (GAD2, SLC6A12), serotonergic (HTR1A, HDC), noradrenergic (ADRA2A), cholinergic (CHRNA4), somatostatinergic (SSTR2) systems induces higher anxiety; whereas the absence of some other genes for glutamatergic (GRIN2D, GRIA1) and dopaminergic (DRD3, NURR1) signaling reduces anxiety.

Clinical evidence suggests that alterations in normal gaminobutyric acid (GABA) transmission contribute to the pathophysiology of anxiety disorders in humans. The inhibitory neurotransmitter GABA is synthesized from glutamate by two distinct enzymes, GAD67 encoded by the Glutamic Acid Decarboxylase 1 (GAD1) and GAD65 encoded by the GAD2, with the cofactor pyridoxal phosphate (Soghomonian and Martin 1998; Fenalti, Law et al. 2007). GAD67 plays a particularly important role in the alteration of inhibitory synaptic transmission, although the mechanisms underlying changes in GABAergic neurotransmission are poorly understood. Mice lacking Gad67 exhibit a 93% reduction in cerebral GABA levels, while the effects of Gad65 knockout are less dramatic in mice. Gad2−/− mice live to adulthood, and exhibit susceptibility to seizures (Kash, Johnson et al. 1997; Stork, Ji et al. 2000);
with unaltered spontaneous inhibitory postsynaptic currents (sIPSC) amplitudes (Tian, Petersen et al. 1999).

GABAergic neurons are commonly defective in schizophrenia. In a previous study it was shown that ULK4 was co-expressed with GAD67 in cortex and hippocampus of both mouse and human adult brains (Lang, Pu et al. 2014). ULK4 deletions and polymorphisms were also associated with neuropsychiatric disorders such as schizophrenia, autism, bipolar disorder and major depression(Lang, Pu et al. 2014), We have unpublished data that ULK4 is deleted in some patients with a diversity of clinical features including developmental delay, language delay and severe intellectual disability. In addition, ULK4 modulates multiple signaling pathways including ERK, p38, PKC, and JNK, which are implicated in neurodevelopmental disorders and involved in stress response. Whether Ulk4 deficiency has direct effects on the GABAergic system, anxiety- and/or depression-like behavior are unknown.

To investigate potential behavioral changes associated with Ulk4 gene deletion, in this study, we firstly performed a battery of behavioral tests for locomotion and exploratory, anxiety-like and depressive-like behavior on the Ulk4 heterozygous mice, since most Ulk4 homozygous mutants died within the first 3 weeks after birth. We found that the Ulk4+/− mice exhibited anxiety-related phenotypes. To further uncover the cellular mechanism of the behavioral alternation in the Ulk4 heterozygous mice, we examined the anatomical changes of Ulk4+/− mice brains and compared the Gad67 expression in the hippocampus and amygdala, which were important neuroanatomic sites for the anxiety disorders. Our results demonstrated a significant reduction of Gad67-positive cells in both hippocampus and amygdala, while the gross neuroanatomy was not altered in the Ulk4+/− mice. This reduction in GABAergic functions within the hippocampus and amygdala of the Ulk4+/− mice may partially underlie the mechanisms of the anxiety-related behavior in the Ulk4+/− mice. This was the first report that Ulk4 lesions can be a rare genetic risk factor implicated in anxiety-related disorders.
6.2 Animals and materials:

6.2.1 *Ulk4* het mice

*Ulk4*<sup>+/−</sup> mice on C57BL/6N strain background were purchased from KOMP Repository (knockout mouse project, USA). All experimental procedures were conducted under a license approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland Galway, Galway, Ireland. *Ulk4*<sup>+/−</sup> and WT littermates were obtained from *Ulk*<sup>+/+</sup> x *Ulk4*<sup>+/−</sup> mating. Mice were genotyped by PCR of genomic DNA with two pairs of PCR primers: *Ulk4*EndE7For (5' - TAACCTTGCTGGACGGATGCTG-3') and with *Ulk4*EndIn7Rev (5' - TGATCTGTAATCGCAGTGAGG-3') amplifying a 271bp DNA fragment from the WT allele, and *Ulk4*KOMPKOFor (5' - GAGATGGCGCAACGCAATTAATG-3') and *Ulk4*KOMPKORev (5' - CTGAGGAGACCAATGTAACCAGC-3') to produce 621bp DNA fragment from the knockout allele, respectively.

6.2.2 Histology

*Ulk4*<sup>−/−</sup> and 4 litter controls were deeply anesthetized and intra-cardiacally perfused with 20 ml of 4% paraformaldehyde in PBS. The brains were dissected and post-fixed in the fixative solution for 24 hours. Then brains were embedded into paraffin blocks. Serial coronal sections at 10um were cut by with a microtome (SM2000R, Leica Instruments). The sections were stained with hematoxylin-eosin and imaged under a bright filed microscope (IX41, Olympus) equipped with a camera.

6.2.3 Immunohistochemistry

Young adult mice (2 months) were anesthetized with lethal dose of sodium pentobarbitone and transcardiacaclly perfused with 4% cold paraformaldehyde in phosphate buffer (pH 7.4). Brains were dissected from skull and post-fixed in the same solution overnight. Then brains were then embedded into paraffin. Serial coronal sections were cut at 20um on a microtome. Antigen retrieval was performed at 95°C for 20 min in 10mM citrate buffer (pH 6.0). The primary antibodies were
mouse anti-GAD67 (1:1000; MAB5406, Millpore). The secondary antibodies included biotinylated goat anti-mouse (2.0μg/ml, Catalog No. 71-00-18, KPL) and Gad67-positive neurons were stained with brown colour by diaminobenzidine (DAB).

6.2.4 Cell number counting in amygdala and hippocampus
Brain structures were delineated in sections according to the mouse brain atlas (Franklin and Paxinos 2001). The amygdala is a complex structure consisting of multiple nuclei. Here, we performed cell counting of the portion of the basolateral amygdaloid complex that is surrounded by the amygdalar capsule (amc) (Bregma-1.34 mm to Bregma-1.70mm). Images were taken at 4x objective lens as specified, under an Axiovert 40CFL microscope (Zeiss, Germany). For each mouse, the number of cells was determined in 6-10 coronal serial sections and the mean values were used for statistics. Data were presented as mean and SEM, and analyzed by one-way ANOVA for statistical significance. Sample sizes were specified in the Results section. *p*<0.05 was considered to be significant.

Figure 6-1 Mouse brain atlas showing the amygdala and hippocampal regions which were quantified for Gad67-positive cells in the WT and *Ulk4*+/− mice.
6.2.5 Animals for behavioral tests

Male and female, 8-week old, C57BL/6N wildtype (WT) and \( Ulk4^{+/−} \) mice (weight 16-25g) were obtained from the National Centre for Biomedical Engineering Science (NCBES), National University of Ireland (NUI) Galway. Animals were housed in groups of 2 to 4, according to gender and genotype, in plastic-bottomed cages containing wood shavings as bedding. Animals were maintained in a constant temperature (21±2°C) under standard lighting conditions (12:12 hr light-dark, lights on from 07.00 to 19.00 hr). All experiments were carried out during the light phase, between 10.00 and 17.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Welfare Committee, National University of Ireland, Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

For the behavioral tests, the PPI was performed in collaboration with SJC; FST and TST of 7 months old mice were done by myself, and the other behavioral tests were carried out by Marie Fitzgibbon and Michelle Roche.

Experimental Design

This study investigated and compared behavioral phenotypes in C57Bl/6 WT and \( Ulk4^{+/−} \) mice. Characteristics assessed include social behavior, communication deficits, and stereotyped or repetitive behavior. Anxiety- and depressive-like behavior and thermal nociceptive response was also analyzed. Behavioral testing began following 5 days of acclimatization to the animal unit. The sequence of behavioral testing over the course of the study is depicted in Fig 6-1.

Behavioral Tests

Behavioral testing was performed between the hours of 10.00 and 17.00 each day. While some of the tests were carried out in the home room (open field, sociability, marble burying and hot plate tests), a separate room was assigned for 3-chamber social ability, olfactory habituation dishabituation (OHD), elevated plus maze (EPM)
and the social interaction test. In most cases, animals were tested immediately without time for habituation to the new environment, except in the case of OHD whereby animals were given 30 minutes to habituate upon introduction to the new cage and odourless cotton bud. After each session, animals were returned to the cages in their own room. The only exception to this occurred after the hot plate test when the animals were singly housed for the social interaction test which took place the following day. All tests were recorded by video camera and analyzed at a later date, using Ethovision video tracking software (Noldus).

**Figure 6-2 Timeline for study investigating and comparing behavioral responding in C57Bl/6 WT and *Ulk4*<sup>+/−</sup> mice.** (OFT, open field test; MBT, marble burying test; OHD, olfactory habituation dishabituation; EPM, elevated plus maze; SI, social interaction).

**3-Chamber Sociability Test**: The sociability test was conducted on day 1 of the study following two days of handling by the tester. The test involved a novel 3-chamber apparatus which allows for the measurement of social approach and social preference (Crawley 2004; Nadler, Moy et al. 2004). The arena itself was composed of three communicating chambers separated by Perspex walls with central openings allowing access to all chambers. Upon testing, each animal was initially placed into the centre of the arena and allowed access to all chambers for 10 minutes. Distance moved and time spent in the various compartments was assessed during this time to evaluate general locomotor activity and ensure that animals did not have a preference for a particular side of the arena. Following this acclimatization period, animals were briefly confined to the central chamber while an unfamiliar stimulus mouse confined in a small wire cage was placed in one of the outer chambers. An identical empty wire cage was placed in the other chamber. The unfamiliar mouse
was randomly assigned to either the right or left chamber of the arena. The test animal was then allowed to freely explore the entire arena for a further 10 minutes. Distance moved in the arena, time spent in each chamber as well as the duration and frequency of engaging in investigatory behavior with the novel mouse and novel object (empty cage) was evaluated. Following this, the test animal was again, briefly confined to the central chamber while a second unfamiliar mouse was placed in the empty wire cage. The test animal was then allowed to freely explore the entire arena for a final 10 minutes. Distance moved in the arena, time spent in each chamber as well as duration and frequency of engaging in investigatory behavior with the novel and familiar mouse was assessed. All such behaviors were evaluated with the aid of EthoVision XT software (Noldus Netherlands) in order to examine social approach and preference.

**Social Interaction (SI) test:** The social interaction test has routinely been used to evaluate social behavior in rodents. The test was carried out employing a modified version of a previously described and validated method (Bolivar, Walters et al. 2007). Mice were singly housed 24 hours before the test. On the day of testing, the test mouse was placed under a dim light, lux 25, in a separate procedure room, and allowed to explore freely for 5 minutes. At the end of the 5-minute session, a second novel conspecific mouse of the same sex was added to the cage, and subsequent behavior was recorded onto a DVD for 5 minutes. Thus, one mouse, the test animal, was the resident of the cage and the other an intruder. At the end of the session, both mice were removed and returned to their original home cages. The amount of time during which the test mouse engaged, or attempted to engage in social interactions (e.g., sniffing, following, allogrooming, biting, chasing, mounting, wrestling) with the unfamiliar stimulus animal was recorded and analyzed with the aid of EthoVision XT software (Noldus Netherlands).

**Open Field and Grooming Test:** Locomotor activity and grooming behavior (excessive grooming indicative of repetitive behaviour) were assessed in the open field arena. The apparatus consisted of a wooden arena, divided into four equal quadrants forming four individual open fields (30 x 30 x 30 cm). Mice from each
cage were tested simultaneously for a period of twenty minutes and behavior was recorded onto a DVD. Distance moved and time spent grooming (total and per 5-minute time bins) was assessed using Ethovision XT software (Noldus Netherlands). The floors and walls of the apparatus were cleaned at the beginning of the day and between each trial.

**Olfactory Habituation/ Dishabituation (OHD):** The OHD test was carried out similar to that previously described (Yang and Crawley 2009; Yang, Abrams et al. 2012). Each subject mouse was tested in a clean mouse cage containing a thin layer of fresh bedding. Cotton tipped swabs were used to deliver odor stimuli. In order to reduce novelty-induced exploratory activities, each subject was first habituated to the testing cage equipped with a clean dry, cotton swab for 45 min before testing. Testing itself consisted of a sequence of fifteen 2-minute odor exposures: three presentations of tap water, followed by three presentations of lemon odor (prepared from lemon extract, 1:100 dilution in tap water), followed by three presentations of almond odor (prepared from almond extract, 1:100 dilution), followed by three presentations of odor from a cage of unfamiliar conspecific animals of the same sex, then finally followed by three presentations from a cage containing unfamiliar animals of the opposite sex. Water, lemon and almond odors were prepared by dipping the cotton tip in the solution. Social odors were prepared by wiping a swab in a zigzag pattern across a soiled cage of the unfamiliar mice. Time spent sniffing the swab was observed and recorded onto DVD.

**Marble Burying Test (MBT):** The MBT is a well-validated screen for stereotyped/repetitive behavior and/or anxiety-related/neophobic behaviour. This test was carried out as previously described (Njung’e and Handley 1991; Deacon 2006) with minor modifications. A novel home cage contained 15 cm deep wood shavings bedding, with 15 black glass marbles arranged in an equidistant 5 × 3 grid on top of the bedding. Each test animals was placed in cage and allowed access to the marbles for 20 minutes. After this time, the number of buried marbles (2/3 of the marble covered by the bedding) was recorded.
**Elevated Plus Maze (EPM):** The EPM arena consisted of a wooden apparatus, elevated to a height of 55 cm above the floor. The maze itself consisted of two open (50 x 10 cm, lux 65) and two closed arms (50 x 10 x 30 cm, lux 30) extending from a central platform (10 x 10 cm). Mice were placed individually on the central platform facing an open arm and behavior recorded for a period of five minutes. The maze was cleaned at the beginning of the day and between each trial. Anxiety was analyzed by assessing the time spent in open and closed arms, as well as the number of entries by the subjects into the open arms. Locomotor activity was assessed as distance moved over the duration of the trial.

**Forced Swim Test:** The FST is a reliable, commonly used method of identifying anti-depressant-like and depressive-like behavior in mice and rats (Porsolt, Le Pichon et al. 1977). In brief, mice were placed in a vertical glass cylinder filled (dimensions) with 25 cm deep water at 25 ± 2 °C for six minutes and behavior was recorded. Mice were deemed immobile when they floated in an upright position or when only small movements were made to keep the head above water. Immobility was assessed over the six minute trial period, of which the last four minutes were used in analysis as previously described (Porsolt, Bertin et al. 1977).

**Hot Plate Test:** This test of thermal hyperalgesia involved placing mice individually onto a hot ceramic plate (temperature 55 ± 1 °C) and the latency (seconds) to lick the front paws only front paws was recorded with a cut off time of 40 sec to avoid potential tissue damage. The hot plate apparatus itself was cleaned with acetic acid between each subject trial.

**Tail Suspension Test:** To suspend the mouse, electrical tape was attached to the tail, near the tip, which was affixed to a structure that allowed the mouse to be suspended 20 cm from the ground. The trial lasted for 6 minutes, during which duration of immobility was measured. Immobility was defined as the lack of all movement except that required for respiration. At the completion of the trial, the mouse was removed and returned to the home cage.
Prepulse Inhibition: In a separate group of animals not previously undergoing behavioural testing, mice were tested for acoustic startle reactivity (ASR) and prepulse inhibition (PPI) using the SR-LAB startle response system (San Diego Instruments), which consisted of a sound attenuating box housing an animal enclosure platform, a fan and a speaker, all of which were lit from above by LEDs. Acoustic stimuli were delivered through a speaker at 70 dB, 80 dB, 85 dB, 90 dB and 120 dB, which was calibrated using a decibel counter (A weighting; Radio Shack). The animal enclosure platform that contained an accelerometer was calibrated before testing commenced using the San Diego Instruments Standardization Unit. Initial pilot data (not shown) suggested that a standardized value of 1000 mV from the platform was necessary to have sufficient measurable startle responses at low dB to measure ASR.

ASR and PPI were measured within the same trial, as outlined in the EMPReSS standard operating procedure (http://empress.har.mrc.ac.uk/viewempress/pdf/ESLIM_011_001.pdf). ASR was measured at ‘no sound’ (65 dB; BN; background noise), 70 dB, 80 dB, 85 dB, 90 dB and 120 dB using a single 40 ms pulse (‘no sound’ was a trial with only the background noise audible). PPI was measured by the delivery of a tone at either 70 dB, 80 dB, 85 dB, 90 dB (prepulse) for 10 ms followed by a 100 ms gap at background noise and then a 120 dB ‘startle’ tone for 40 ms. During the trials, background noise at 65 dB presented throughout. Intra-trial intervals were averaged at 25 seconds.

A trial consisted of a 5 minute habituation period, during which the 65 dB noise was presented. After this, five 120 dB startle pulses were delivered, although these were subsequently excluded from the analysis. PPI and ASR were then tested in 10 blocks, of which each block contained 10 trials (ASR: ‘no sound’, 70 dB, 80 dB, 85 dB, 90 dB and 120 dB; PPI: 70 dB, 80 dB, 85 dB, 90 dB). The delivery of the tones was pseudorandom and the pattern was never repeated across blocks. Startle responses were detected by an accelerometer on the platform, digitized and saved to computer
in waveform format, with a post-stimulus time window of 300 ms and resolution of 1 kHz.

ASR was analyzed by measuring the $V_{\text{max}}$ of the mV waveform from 0 to 100 ms. ASR was then averaged for all 10 trials at each dB intensity. PPI trials were analyzed using the same method, except that the waveform was measured in response to the second, ‘startle’ pulse from 120 to 220 ms. mV values were again averaged for each dB intensity. PPI was calculated as $(\text{mean 120 startle alone} - \text{mean PPI dB startle}) / \text{mean 120 startle alone} \times 100$, where mean PPI dB startle represents a separate calculation at each of the 70 dB, 80 dB, 85 dB, 90 dB PPI trials.

6.2.6 Statistical analysis:

SPSS statistical package was used to analyze all data in this Chapter. Behavioral data were analyzed using unpaired, two tailed, t-tests with comparisons between Het and WT mice. IHC data were analyzed using paired, two tailed, t-tests analysis. Six brain sections per mouse and five mice per genotype were used in this experiment. All results were presented as means ± SEM. Data were considered significant when $p < 0.05$.

6.3 Results

6.3.1 Ulk4 heterozygous ($\text{Ulk4}^{+/-}$) mice are born at a Mendelian rate and survive well

$\text{Ulk4}^{+/-}$ mice were bred with WT littermates, and 192 mice were born from the first 27 litters and 180 (93.8%) were survived to the weaning time. The average litter size was $7.11 \pm 0.64$, which was comparable to average litter size (6~8) of C57BL/6N strain from Jax laboratory, indicating that there was no significant prenatal lethality. The 180 mice were genotyped after weaning, which identified 86 as WT and 94 as $\text{Ulk4}^{+/-}$ mice, and showed no major deviation from the expected 1:1 Mendelian ratio (Table 6-1, $p=0.71$). $\text{Ulk4}^{+/-}$ mice did not display any noticeable difference in body size, appearance, or activity. Therefore, we conclude that deletion of one copy of the
Ulk4 gene neither affects embryonic development to term, nor compromise postnatal growth or survival of the heterozygous mice.

Table 6-1. Ulk4+/− mice are born at a Mendelian ratio and survived normally

<table>
<thead>
<tr>
<th>Total born</th>
<th>survival</th>
<th>Ulk4+/+</th>
<th>Ulk4+/−</th>
<th>expected ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT x Het (12 litters)</td>
<td>192</td>
<td>180 (93.8%)</td>
<td>86 (57F+39M)</td>
<td>94 (50F+44M)</td>
</tr>
<tr>
<td>Litter size (M±SEM)</td>
<td>7.1±0.6</td>
<td>2.4±0.4</td>
<td>3.5±0.5</td>
<td>p=0.71</td>
</tr>
</tbody>
</table>

6.3.2 Young Ulk4+/− mice do not exhibit major neuroanatomical changes

Previously, we identified copy number variation of the ULK4 gene in human as a rare risk factor of neurodevelopmental disorders including autism and schizophrenia (Lang et al., 2014), which are associated with subtle neuroanatomical alterations. In Chapter 3, we described that deletion of both copies of the Ulk4 gene in mice led to hydrocephalus with impaired ciliogenesis and ventriculomegaly. Here we histologically analyzed 8 Ulk4+/− mice and 7 WT littermate controls at 2-month young adult stage (Table 6-2 and Fig. 6-3), comparable to sexually matured adolescence stage (18~25), in which period the first wave of psychosis is peaked in human.

We quantified the brain section size at the anterior commissural (AC, Fig. 6-3. A-C) level, and found no significant differences in brain size between the WT (44.5 ± 4.9 mm²) and the Ulk4+/− (47.3±5.2 mm², p=0.337, Fig. 6-3 C). We measured lateral ventricle (LV) size from the WT (1.4 ±0.9 mm², Fig. 6-3 A,D) and the Ulk4 heterozygous mice (1.1±0.6 mm², Fig. 6-3 B,D, p=0.272) and did not detect genotype-associated dilation. We quantified the cerebral cortex thickness in WT (1316 ±119 μm) and Ulk4+/− (1336±130 μm, p=0.770, Fig. 6-3 E) mice, and did not find significant alterations.
Figure 6-3. No significant neuroanatomical differences between 2-month WT (A, F, I) and *Ulk4*+/− (A, F, I) mouse brains. Morphometric analyses were carried out to quantify the brain section sizes (A-C), the LV size (D), the cortex thickness (E), the thickness of corpus callosum (F-H), and SCO cell number (I-K). No statistical difference was found (*p*>0.05 for all).
Table 6-2. Morphometric analyses of 2-month mouse brains

<table>
<thead>
<tr>
<th></th>
<th>WT (M±SEM)</th>
<th>HET (M±SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Brain section areas at the AC level (mm²)</td>
<td>44.5 ± 4.9</td>
<td>47.3 ± 5.2</td>
<td>0.337</td>
</tr>
<tr>
<td>LV size (mm²)</td>
<td>1.4 ± 0.9</td>
<td>1.1 ± 0.6</td>
<td>0.272</td>
</tr>
<tr>
<td>Cortex thickness (um)</td>
<td>1316 ± 119</td>
<td>1336 ± 130</td>
<td>0.770</td>
</tr>
<tr>
<td>Corpus callosum thickness (um)</td>
<td>178 ± 10.3</td>
<td>210 ± 17</td>
<td>0.128</td>
</tr>
<tr>
<td>SCO cell number</td>
<td>194 ± 15.2</td>
<td>189 ± 25.3</td>
<td>0.272</td>
</tr>
</tbody>
</table>

We also measured the thickness of the corpus callosum at the AC level, because the agenesis of the corpus callosum is commonly associated with neurodevelopmental disorders. The corpus callosum in the $Ulk4^{+/\text{-}}$ mice (210 ± 17 um) was not reduced in comparison to that in the WT mice (178 ± 10.3 um, p=0.128, Fig. 6-3 F-H). We counted cell numbers in the subcommisural organ (SCO), as $Ulk4^{+/\text{-}}$ mice developed hydrocephalus and SCO was critical for the flow of cerebrospinal fluid (CSF). The cell number in the SCO of the $Ulk4^{+/\text{-}}$ (189 ± 25.3, p=0.272) mice was not markedly decreased in comparison to that in the WT mice (194 ± 15.2) at the comparable postcommisural level (pc, Fig. 6-3 I-K). Therefore, these morphometric measures revealed no significant neuroanatomical difference between the $Ulk4^{+/\text{-}}$ and WT mice (Table 6-2).

6.3.3 $Ulk4^{+/\text{-}}$ mice exhibit impaired in sociability and social novelty preference.

We next carried out a battery of behavioral tests on two batches mice. The first batch included 18 WT female, 16 WT male, 7 $Ulk4^{+/\text{-}}$ female, and 14 $Ulk4^{+/\text{-}}$ male; and second batch included 17 WT female, 16 WT male, 7 $Ulk4^{+/\text{-}}$ female, and 14 $Ulk4^{+/\text{-}}$ male mice. They were littermates of 14 breeding pairs set up at the same time. The tests were carried out sequentially from 2~2.5 month old mice, as this age in mice coincided with the early peak of psychosis at adolescence stage in human patients.
Defects in social interaction and communication are common in neurodevelopmental and neuropsychiatric disorders including schizophrenia, autism and depression which were associated with rare copy number variation of the ULK4 gene (Lang, Pu et al. 2014), and we therefore examined the effect of heterozygous deletion of the Ulk4 gene on the sociability and social interaction in mice.

**Habitation in Three-Chamber apparatus**

Three-Chamber apparatus was used to investigate the Sociability and Social Novelty recognition of four groups: WT male (n=16), WT female (n=20), Ulk4<sup>+/−</sup> male (n=19) and Ulk4<sup>+/−</sup> female (n=14). Each was individually placed in the centre of the Three-Chamber apparatus for 10 min for habitation, mouse activity was video-recorded, and the duration of time that mice spent in each chamber was recorded quantified (Fig. 6-4A). During the habitation period, WT and Ulk4<sup>+/−</sup> male mice displayed no side preference with respect to time spent in each zone. WT female animals appeared to spend less time in the centre compared with both the left and right chambers. Ulk4<sup>+/−</sup> female mice spent more time in the right chamber. However, statistically, there was no zone preference prior to sociability testing, neither associated with gender nor with genotype (Fig. 6-4A). **Ulk4<sup>+/−</sup> female mice spend less time interacting with novel animal when compared to male counterparts.**

Next, the test animals was briefly confined to the centre chamber and a novel object (wire cage) was placed in one side chamber and a novel mouse (same sex) under a wire cage in another side chamber test mice were then allowed to explore the chambers for a further 10 min (Fig. 6-4B). All animals spent substantially less time in the centre zone and significantly more time in the chamber containing the novel or novel animal with a significant preference for the chamber containing the novel animal (Fig. 6-4B).

We then quantified the duration time spent directly interacting with the novel object or novel animal. All animals also spend more time interacting with the novel animal in comparison with the novel object (Fig. 6-4C). Male Ulk4<sup>+/−</sup> mice interact with the novel animal for a longer period than female Ulk4<sup>+/−</sup> counterparts, an effect not
evident in WT animals. We also counted the frequency of the test animal approaching the novel object or novel animal. The male (WT and Ulk4\(^{+/−}\)) mice made less approaches towards the novel object than female counterparts. There was no statistical significance among the four groups in frequency of interacting with the novel animal (Fig. 6-4D). These data demonstrated that Ulk4\(^{+/−}\) male mice display increased social interactions with the novel animal when compared to female counterparts. Thus the data showed possible sex-related difference may exist in relation to social ability in Ulk4\(^{+/−}\) mice.

**Ulk4\(^{+/−}\) male mice spend less time interacting with the novel animal than WT mice.**

We subsequently replaced the object with a novel animal (same sex) in the Three-Chamber apparatus, and tested mice for novel animal recognition (Fig. 6-4 E-G). All mice in four groups spent significantly increased time in the chamber housed the novel animal (**p < 0.001 for all 4 groups), when compared to time in the centre or in side chamber containing familiar animal (Fig. 6-4 E). WT male spent more time interacting with the novel animal than WT female animals, an effect not observed in Ulk4\(^{+/−}\) male mice (Fig 6-4F). WT male mice have less number of interactions (frequency) with the familiar mouse when compared to female WT counterparts and Ulk4\(^{+/−}\) male mice also exhibited less frequent approaches to the novel animals than Ulk4\(^{+/−}\) female mice, an effect not observed in the WT mice (Fig. 6-4 G). These data suggest that Ulk4\(^{+/−}\) male mice may have an impairment in social responding to a novel mouse, when compared to WT counterparts.
Figure 6-4 Evaluating sociability and social novelty preference in male and female Ulk4+/- mice. (A.) Mice showed no chamber preference during the habitation period. (B.) The time spent in each zones after introduction of novel object and novel animal. ***p<0.001 for time spent in the arena of the novel stimulus animal vs. time in centre and time in arena of novel object. (C) The duration of interaction with novel cage or novel animal. #p<0.05 for difference interacting with the novel animal between Ulk4+/- male and female mice. (D) The frequency of the test mice approached the novel object or novel mouse. *p<0.05 for male Ulk4+/- vs. female Ulk4+/- animals; **p<0.01 for difference in contacting novel object between WT male and WT female. (E) The time spent in each zones following introduction of a novel animal and familiar animals into the chambers, showing all groups of mice spent significantly more time in the chamber with the novel animal (***p<0.001 for all 4 groups), than time spent in the arena with a familiar animal or in central arena. (F) The duration of interaction with familiar or novel animal. #p<0.05 showing WT male spent more time interacting with the novel animal than WT female animals. *p<0.05 showing Ulk4+/- male spent significantly less time with the novel animal in comparison to the WT male mice. (G) The frequency of the test mice approached the familiar animal or the novel animal. #p<0.05 indicating that WT male mice visited the familiar animal less frequently than WT female mice. ##p<0.01 showing that Ulk4+/- male mice visited the novel animal significantly less frequently than the Ulk4+/- female mice.
6.3.4 Social Interaction Test: Males interact less with novel co-specific when compared to female counterparts

We then carried out direct social interaction test, by placing a stimulus animal (matched for sex, age, weight) into the home cage of the test animal for 5 min, and recorded the duration and frequency of the contacts. The WT male mice spent less time interacting with the stimulus animal than WT females, an effect also observed, but not significant, in Ulk4+/- animals (Fig. 6-5 a). WT male mice made fewer contacts with the co-specific, although not statistically significant (Fig. 6-5 b).

![Direct Social Interaction](image)

Figure 6-5 .a, Duration of interaction and b, frequency of interaction of male and female C57BL/6N WT and Ulk4+/- mice. Data are expressed as mean ± SEM; n= 13-20 per group; *p<0.05, male WT vs. female WT animals.

6.3.5 Ulk4+/- female mice show diminished interest in opposite sex odor

This experiment measures olfaction, memory, and social interest. Test mice were first presented with three non-social odor cues of water, lemon and almond, then three trials of the urine of same sex followed by three trials of the urine of opposite sex. A lack of habituation to a new smell or the inability to distinguish a new smell from an old one during the non-social phase may indicate deficits in general olfaction. Poor habituation and dishabituation to the urine may suggest a social deficit.
All animals were indistinguishable in habituation to non-social odor cues (Fig. 6-6). They also exhibited a heightened response to the presentation of a social odor, from the same sex, in comparison with non-social odors, suggesting that the basic olfactory function was intact in the $Ulk4^{+/+}$ mice. Female (WT and $Ulk4^{+/+}$) mice habituated to the social cue of the same sex odor at a faster rate than male counterparts. An increased response (time sniffing) was also observed among animals upon presentation of a novel social odor from the opposite sex. $Ulk4^{+/+}$ female mice habituated to this novel odor faster than WT counterparts and male animals (Fig. 6-6). This diminished response and lack of interest may be indicative of social communicative deficits or impairments in the $Ulk4^{+/+}$ females.

**Olfactory Habituation Dishabituation**

![Olfactory Habituation Dishabituation graph](image.png)

**Figure 6-6. Olfactory Habituation Dishabituation test was carried out in male and female $Ulk4^{+/+}$ mice and WT littermate controls.** Each of the non-social (water, lemon and almond) and social (urine of same or opposite sex) were given three consecutive trials (1 min gap between the trails). The time that the test mouse spent on sniffing the odor in 2-minute test was quantified. The Data are expressed as mean ± SEM; n= 13-20 per group; a, ANOVA revealed the effect of sex; $p<0.05$ between $Ulk4^{+/+}$ male and $Ulk4^{+/+}$ female; $# p<0.01$ between WT male and $Ulk4^{+/+}$ females; $p=0.06$ between WT female and $Ulk4^{+/+}$ female mice.
6.3.6 *Ulk4*+/– female mice show more repetitive behavior in Marble Burying Test

Marble burying test is commonly used to assess repetitive, anxiety-like, obsessive and compulsive behavior. *Ulk4*+/– female mice buried significantly more marbles when compared with WT counterparts, an effect not observed in male subjects. This may indicate that *Ulk4*+/– female display more repetitive and/or anxiety-like behavior in this test when compared to WT animals. Similarly, increased marble burying may also indicate neophobia behavior among *Ulk4*+/– female mice (Fig. 6-7).

![Marble Burying Test](image)

**Figure 6-7.** *Ulk4*+/– mice buried significantly more marbles in the marble burying test. The test was carried out in 20 min with in a home cage. The number of marbles buried was quantified among the groups, n= 13-20 per group. Data are expressed as mean ± SEM; *p*<0.05 shows that *Ulk4*+/– female mice is significantly different from WT female.

6.3.7 *Ulk4*+/– male mice spend more time grooming and exhibit less locomotor activity

Excessive grooming is a type of repetitive activity and mice were assessed for grooming activity over 5-min time bins following exposure to the open field arena for 20 min (Fig. 6-8). Quantification of the activity revealed that in the first 5 and latter 10 minutes, male *Ulk4*+/– mice spend significantly more time engaged in grooming behavior when compared to female counterparts, an effect not observed in WT animals (Fig. 6-8 b). The initial excessive grooming is indicative of enhanced
repetitive behavior and combined with reduced locomotor activity may represent an anxiety-like phenotype. Analysis during the latter 10 minutes of the trial revealed that $Ulk4^{+/−}$ female mice failed to increase grooming behavior (as would be expected) towards the end of the trial period. This failure to acclimatize to the arena indicates possible anxiety-related behavior in the $Ulk4^{+/−}$ female mice.

**Figure 6-8. Locomotor activity (a) and grooming behavior (b) of mice.** Mice were individually placed into an open field arena of (30 x 30) cm² for 20 minutes. The locomotor activity (a) and grooming behavior were collected over 5-min bins. Test mice were divided into 4 groups of WT male, WT female, $Ulk4^{+/−}$ male and $Ulk4^{+/−}$ female, with n= 14-20 per group. Data are expressed as mean ± SEM. ### $p<0.001$ in (A) shows WT female had significantly higher locomotor activity than WT male. ++ $p<0.01$ in (A) indicates that male $Ulk4^{+/−}$ male mice have lower locomotor activity than $Ulk4^{+/−}$ female mice. + $p<0.05$ in (b) shows that $Ulk4^{+/−}$ male mice have increased grooming activity than $Ulk4^{+/−}$ female mice.

**6.3.8 $Ulk4^{+/−}$ mice spend more time in the closed arms of the Elevated Plus Maze.**

The Elevated Plus Maze (EPM) test is commonly used to assess anxiety-related behavior in animals (Walf and Frye 2007). The EPM apparatus consists of a “+”-shaped maze elevated above the floor with two oppositely positioned closed arms, two oppositely positioned open arms, and a center area. The preference for being in closed arms over open arms is a measure of increased anxiety-like behavior. Following the initial observation of the anxiety-like behavior in the $Ulk4^{+/−}$ mice, we carried out the EPM tests. Each animal was placed on the central area of EPM, and
the mouse was allowed to move freely for a period of 5-min and the activity was recorded and analyzed.

Both WT and $Ulk4^{+/–}$ female mice exhibited slightly greater locomotor activity in the EPM when compared to male counterparts (Fig. 6-9 a). However, this was not significant when statistically analyzed across the 4 groups. Both $Ulk4^{+/–}$ male and female mice spent significantly less time in the open arms of the EPM (Fig. 6-9 c) and slightly increased time in the closed arms when compared to WT counterparts (Fig. 6-9 b), indicating that both $Ulk4^{+/–}$ male and female mice display enhanced anxiety-related behavior over the EPM apparatus.

**Figure 6-9.** a, Distance moved; b, time spent in the closed arms; c, time spent in open arms and d, percentage of time spent in the open arms of male and female C57BL/6N WT and $Ulk4^{+/–}$ mice subjected to the EPM. Data are expressed as mean ± SEM; n= 12-19 per group; a, ANOVA revealed an overall effect of sex in female WT and $Ulk4^{+/–}$ mice vs. male counterparts; b, ANOVA revealed an overall effect of gene in combined male and female $Ulk4^{+/–}$ mice versus WT; *p<0.05, female $Ulk4^{+/–}$ vs. WT; †p<0.05, male $Ulk4^{+/–}$ vs. WT.
6.3.9. No effect of sex or gene on duration of immobility in the FST

The Porsolt or forced swimming test (FST) is known as the behavioral despair test, which is the most commonly used test for assessment of depression in animal models. At 2-3 month of age, 4 groups of mice did not show sex or genotype-specific effect on the duration of immobility in the FST (Fig. 6-10). This indicates the absence of increased despair- or depressive-like behavior in the Ulk4+/− mice.

To examine if there were age-specific effect on depressive behavior, we tested FST mice at the age of 7-10 months, which correlated with the second wave of psychosis in human subjects. WT male (n=16), WT female (n=10), Ulk4+/− male (n=13) and Ulk4+/− female (n=9) mice were tested for immobility in 6-min test, and accumulated immobility time were shown in Table 6-3 and Fig. 6-11. No genotype or sex-specific difference was seen.

**Forced Swim Test**

![Forced Swim Test](image)

Figure 6-10. a, Duration of immobility over time and b, total immobility (T2-6) in male and female C57BL/6 WT and Ulk4+/− mice. Data are expressed as mean ± SEM; n= 7-14 per group.

**Table 6-3. Immobility time (sec) of WT and Ulk4+/− mice in FST.**

<table>
<thead>
<tr>
<th></th>
<th>WT(M)</th>
<th>HET(M)</th>
<th>WT(F)</th>
<th>HET(F)</th>
<th>WT(T)</th>
<th>HET(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>13</td>
<td>10</td>
<td>9</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>M±SEM</td>
<td>169.4±15.5</td>
<td>134.6±19.0</td>
<td>148.7±19.3</td>
<td>184.8±10.2</td>
<td>161.4±12.0</td>
<td>155.2±12.9</td>
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<tr>
<td>p (genotype)</td>
<td>0.170</td>
<td>0.120</td>
<td>0.725</td>
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</table>
6.3.10. *Ulk4*+/− mice show no difference in the duration of immobility in Tail suspension Test (TST).

To further evaluate if *Ulk4*+/− female mice were associated with depressive behavior, we performed TST on 16 WT male, 9 WT female, 13 *Ulk4*+/− male and 13 *Ulk4*+/− female mice at age of 7-10 months, and analyzed the accumulated immobility time from 6-min test (Table 6-4 and Fig. 6-12). There was no significant difference in accumulated immobility time in TST. Therefore, a single copy of Ulk4 gene deletion is not associated with depressive behavior in mice.
Table 6-4. Immobility time (sec) in tail suspension test (TST).

<table>
<thead>
<tr>
<th></th>
<th>WT(M)</th>
<th>HET(M)</th>
<th>WT(F)</th>
<th>HET(F)</th>
<th>WT(T)</th>
<th>HET(T)</th>
</tr>
</thead>
<tbody>
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<td>13</td>
<td>9</td>
<td>13</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>M±SEM</td>
<td>195.7±14.0</td>
<td>210.1±8.5</td>
<td>155.6±17.3</td>
<td>159.2±17.8</td>
<td>177.4±12.9</td>
<td>194.1±9.9</td>
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<td>p (genotype)</td>
<td>0.921</td>
<td>0.935</td>
<td>0.856</td>
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</tbody>
</table>

Figure 6-12. *Ulk4*<sup>+</sup>/<sup>−</sup> mice show no difference from WT mice at 7-10 months in TST. TST was carried out on 16 WT male, 9 WT female, 13 *Ulk4*<sup>+</sup>/<sup>−</sup> male and 13 *Ulk4*<sup>+</sup>/<sup>−</sup> female mice and accumulated immobility time in 6-min test were analyzed, which showed no genotype- or sex-specific effect.

6.3.11. *Ulk4*<sup>+</sup>/<sup>−</sup> mice show no difference in Prepulse Inhibition (PPI) test

For abnormalities in cognitive dysfunction and affective symptoms, some proxy measures have been developed to test in animals. For example, PST and TST can indicate a depression-related phenotype, whereas prepulse inhibition (PPI) and latent inhibition (LI) are commonly used to detect attention-related deficits. Schizophrenic patients often have defects in prepulse inhibition (Braff, Geyer et al. 2001) and latent inhibition (Rascle, Mazas et al. 2001). Some animal models of psychosis also have profound deficits in latent inhibition and prepulse inhibition (Clapcote, Lipina et al. 2007; Shen, Lang et al. 2008), whereas some other transgenic mice did not show
robust changes in prepulse inhibition (Hikida, Jaaro-Peled et al. 2007; Pletnikov, Ayhan et al. 2008).

We next evaluated $Ulk4^{+/−}$ mice in PPI test. This was carried out in collaboration with Dr Steve Clapcote in the University of Leeds. PPI was performed on 10 WT male (average body weight 34.28±0.44g), 10 WT female (27.27±1.33g), 8 $Ulk4^{+/−}$ male (33.45±0.28g) and 10 $Ulk4^{+/−}$ female (26.59±0.89g) at the age of ~10 month old. Mice were tested for acoustic startle reactivity (ASR) and PPI using the SR-LAB startle response system (San Diego Instruments). Acoustic stimuli were delivered through speaker at 70 dB, 80 dB, 85 dB, 90 dB and 120 dB. Initial pilot data suggested that a standardized value of 1000 mV from the platform was necessary to have sufficient measurable startle responses at low dB to measure ASR.

ASR and PPI were measured within the same trial, as outlined in the EMPReSS standard operating procedure (http://empress.har.mrc.ac.uk/viewempress/pdf/ESLIM_011_001.pdf). ASR was measured at ‘no sound’ (65 dB; BN; background noise), 70 dB, 80 dB, 85 dB, 90 dB and 120 dB using a single 40 ms pulse. PPI was measured by the delivery of a tone at either 70 dB, 80 dB, 85 dB, 90 dB (prepulse) for 10 ms followed by a 100 ms gap at background noise and then a 120 dB ‘startle’ tone for 40 ms. Intra-trial intervals were averaged at 25 seconds.

A trial consisted of a 5 minute habituation period with background noise. Then, five 120 dB startle pulses were delivered, although these were subsequently excluded from the analysis. PPI and ASR were then tested in 10 blocks, of which each block contained 10 trials (ASR: ‘no sound’, 70 dB, 80 dB, 85 dB, 90 dB and 120 dB; PPI: 70 dB, 80 dB, 85 dB, 90 dB). Startle responses were detected by an accelerometer on the platform, digitized and saved to computer in waveform format, with a post-stimulus time window of 300 ms and resolution of 1 kHz.
Table 6-5. Acoustic Startle reactivity (ASR) in tested mice.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Gender</th>
<th>BN</th>
<th>70dB</th>
<th>80dB</th>
<th>85dB</th>
<th>90dB</th>
<th>120dB</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, n=20</td>
<td>M+F</td>
<td></td>
<td>14.8±1.0</td>
<td>18.8±5.1</td>
<td>75.3±12.8</td>
<td>79.0±12.6</td>
<td>100.3±15.1</td>
</tr>
<tr>
<td>Ulk4+/-, n=18</td>
<td>M+F</td>
<td></td>
<td>13.3±1.1</td>
<td>17.0±1.9</td>
<td>57.5±11.1</td>
<td>80.5±20.6</td>
<td>99.2±20.1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>p (total)</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>WT, n=10</td>
<td>M</td>
<td>14.5±1.9</td>
<td>23.4±10.2</td>
<td>95.9±23.7</td>
<td>106.1±20.9</td>
<td>134.3±25.7</td>
<td>315.1±40.3</td>
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<tr>
<td>Ulk4+/-, n=8</td>
<td>M</td>
<td>11.5±1.0</td>
<td>13.1±0.9</td>
<td>79.8±22.1</td>
<td>111.6±44.6</td>
<td>134.9±41.8</td>
<td>405.4±78.2</td>
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<table>
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<th></th>
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<tbody>
<tr>
<td>WT, n=10</td>
<td>F</td>
<td>15.0±0.9</td>
<td>14.2±1.7</td>
<td>54.7±5.7</td>
<td>51.8±8.5</td>
<td>66.4±6.5</td>
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<tr>
<td>Ulk4+/-, n=10</td>
<td>F</td>
<td>14.8±1.8</td>
<td>20.2±3.0</td>
<td>39.7±5.7</td>
<td>55.5±6.8</td>
<td>70.5±8.9</td>
<td>219.1±33.9</td>
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<table>
<thead>
<tr>
<th></th>
<th>p (female)</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>WT, n=10</td>
<td>F</td>
<td>41.7±5.1</td>
<td>83.4±2.0</td>
<td>80.3±1.7</td>
<td>83.7±2.4</td>
<td>81.2±1.7</td>
<td>82.0±1.7</td>
</tr>
<tr>
<td>Ulk4+/-, n=10</td>
<td>F</td>
<td>33.1±6.1</td>
<td>79.5±2.3</td>
<td>83.4±2.0</td>
<td>83.7±2.4</td>
<td>81.2±1.7</td>
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</tr>
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</table>

Table 6-6. Prepulse inhibition (PPI) in tested mice

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Gender</th>
<th>70dB</th>
<th>80dB</th>
<th>85dB</th>
<th>90dB</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, n=20</td>
<td>M+F</td>
<td>37.4±4.9</td>
<td>80.3±2.7</td>
<td>84.7±1.7</td>
<td>85.4±1.9</td>
</tr>
<tr>
<td>Ulk4+/-, n=18</td>
<td>M+F</td>
<td>39.9±5.6</td>
<td>82.1±1.9</td>
<td>85.3±1.6</td>
<td>86.0±1.5</td>
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<table>
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<th>P (total)</th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, n=10</td>
<td>M</td>
<td>33.1±8.5</td>
<td>77.2±4.9</td>
<td>85.8±2.6</td>
<td>86.0±3.2</td>
</tr>
<tr>
<td>Ulk4+/-, n=8</td>
<td>M</td>
<td>48.4±9.6</td>
<td>85.4±2.8</td>
<td>90.4±1.4</td>
<td>91.0±1.3</td>
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<table>
<thead>
<tr>
<th></th>
<th>P (male)</th>
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<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>WT, n=10</td>
<td>F</td>
<td>41.7±5.1</td>
<td>83.4±2.0</td>
<td>83.7±2.2</td>
<td>84.9±2.2</td>
</tr>
<tr>
<td>Ulk4+/-, n=10</td>
<td>F</td>
<td>33.1±6.1</td>
<td>79.5±2.3</td>
<td>81.2±1.7</td>
<td>82.0±1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>p (female)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, n=10</td>
<td>F</td>
<td>0.292</td>
<td>0.222</td>
<td>0.375</td>
<td>0.326</td>
</tr>
<tr>
<td>Ulk4+/-, n=10</td>
<td>F</td>
<td>0.292</td>
<td>0.222</td>
<td>0.375</td>
<td>0.326</td>
</tr>
</tbody>
</table>
ASR was analyzed by measuring the Vmax of the mV waveform from 0 to 100 ms. ASR was then averaged for all 10 trials at each dB intensity. PPI trials were analyzed using the same method, except that the waveform was measured in response to the second, ‘startle’ pulse from 120 to 220 ms. mV values were again averaged for each dB intensity. PPI was calculated as (mean 120 startle alone – mean PPI db startle) / mean 120 startle alone x 100, where mean PPI db startle represents a separate calculation at each of the 70 dB, 80, 85 dB, 90 dB PPI trials.

The ASR (Table 6-5) and PPI (Table 6-6) data are statistically analyzed, no significant difference was found among the 4 groups. Therefore there was no genotype- or sex-associated alteration in ASR or PPI. One copy of the Ulk4 gene deletion does not affect related behavior.

Figure 6-13. *Ulk4*/*- mice show no alteration in difference in Acoustic Startle reactivity (ASR) or Prepulse inhibition (PPI). The PPI test was carried out in 10 WT male, 10 WT female, 8 Ulk4/*- male and 10 Ulk4/*- female at the age of ~10 month old. Both the ASR (A-C) and PPI (D-F) did not show significant difference in gender or genotype.
6.6.12 *Ulk4*+/– mice show no difference in thermal nociceptive responding from WT mice.

Thermal Nociceptive Behavior test was performed to examine the pain response in animals. To examine if Ulk4 affects pain response, we carried out the hot plate test. Male mice (*Ulk4*+/– and WT) exhibit lower latency to respond in the hot plate. Both WT and *Ulk4*+/– male mice exhibited lower latency in response to a noxious thermal stimulus (55 ±/1°C) when compared to female counterparts, however there was no genotype-specific differences. This is a hyperalgesic effect, indicative of increased sensitivity to painful thermal stimuli among male subjects (Fig. 6-14).

![Hot Plate Test](image-url)

**Figure 6-14.** *Ulk4*+/– mice show no difference from WT mice in thermal response. *ULK*+/– and WT mice were subjected to the hot plate test for thermal nociceptive behavior. The latency to withdraw from the 52°C hot plate was quantified. Data are expressed as mean ± SEM; n= 13-20 per group; a, ANOVA revealed overall effect of sex (but not genotype) in males vs. females.

6.3.13. *Ulk4*+/– mice show significant reduction of Gad67 cells in amygdala and hippocampus

GABAergic interneurons are the inhibitory major inhibitory neurons in the brain, and Glutamate decarboxylase 1 (*Gadl*) gene encodes an enzyme Gad67 to synthesize GABA. The amygdale region is well-known for anxiety and fear related behavior. We therefore investigate the Gad67 cell in the amygdale region of 5 WT and 5 *Ulk4*+/– mice at 2-month of age (Table 6-7 and Fig. 6-15).
We counted Gad67 positive cells in WT amygdala of 0.68 ± 0.05 mm², detected 72.9 ±6.3 cells with a cell density of 109.8 ±13.3. However, the Gad67 positive cells were significantly reduced (p=0.01) in the equivalent region (0.64±0.04 mm²) of Ulk4+/− mice, with an average of 50.9±1.7 observed on the Ulk4+/− brain sections, and a cell density of 58.7±11.1 (Table 6-7 and Fig. 6-15 A-D).

Table 6-7. Gad67 positive cells are reduced in the amygdala and hippocampus of Ulk4+/− mice

<table>
<thead>
<tr>
<th></th>
<th>WT (M±SEM)</th>
<th>HET (M±SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Amygdala area (mm²)</td>
<td>0.68 ± 0.05</td>
<td>0.64±0.04</td>
<td>0.450</td>
</tr>
<tr>
<td>Gad67-positive cells in amygdala</td>
<td>72.9 ±6.3</td>
<td>50.9±1.7</td>
<td>0.010</td>
</tr>
<tr>
<td>Gad67 cell density in amygdala</td>
<td>109.8 ±13.3</td>
<td>58.7±11.1</td>
<td>0.018</td>
</tr>
<tr>
<td>Gad67 cells in hippocampus</td>
<td>190.0 ± 3.0</td>
<td>153.0±7.6</td>
<td>0.002</td>
</tr>
</tbody>
</table>

One of the most prominent cellular features of schizophrenia that has emerged from postmortem studies is a consistent reduction of parvalbumin neurons (Hashimoto, Volk et al. 2003). Parvalbumin cells belong to a subgroup of GABAergic inhibitory interneurons, which are vital for neuronal synchronization. Along with the GABA-synthesizing enzyme GAD67, parvalbumin expression is consistently reduced in schizophrenic brains. Neuropathological studies have demonstrated deficits of GABAergic interneurons in the hippocampus of postmortem schizophrenic patients. 

GADI encoding an enzyme GAD67 for synthesizing GABA is strikingly downregulated in the hippocampus of schizophrenia and bipolar patients (Benes, Lim et al. 2007). Independently, a profound deficit in the relative density of parvalbumin-immunoreactive neurons was found in all subfields of schizophrenic hippocampus (Zhang and Reynolds 2002), and GABAergic neuronal reduction was also detected in animal model of psychosis(Shen, Lang et al. 2008) (Hikida, Jaaro-Peled et al. 2007; Pletnikov, Ayhan et al. 2008).
Figure 6-15 Reduced Gad67 positive cells in the amygdala and hippocampus of Ulk4+/− mice. Immunohistochemistry was carried out on brain section of 5 WT and 5 Ulk4+/− mice at 2-month of age. The Gad67 cells were counted from the equivalent regions of Amygdala (A,B) and hippocampus (G,H) and statistically analyzed (C-F), showing significant reduction of Gad67 cells in Ulk4+/− mice (p<0.05). Bar=200 µm in A,B,G,H.
We previously showed that ULK4 is deleted in 1/1000 schizophrenia patients and ULK4 was highly expressed in the GABAergic neurons in mouse and human brain (Lang, Pu et al. 2014). Here we carried out Gad67 immunohistochemistry to compare GABAergic neurons in WT and Ulk4<sup>+/−</sup> mice (table 6-7 and Fig. 6-15). We quantified Gad67 positive cells in 5 WT and 5 Ulk4<sup>+/−</sup> mice at 2-month of age. An average of 190.0 Gad67 cells was found on WT hippocampus sections, however, this was reduced to an average of 153.0 cells in the Ulk4<sup>+/−</sup> mice (Table 6-7 and Fig. 6-15 F,G,H), with a p value of <0.01. Therefore, reduced GABAergic neurons in the Ulk4<sup>+/−</sup> brain sections provide a cellular mechanism for stereotyped/ repetitive behavior and altered anxiety-like behavior in Ulk4<sup>+/−</sup> mice.

### 6.4 Discussion

Previously, ULK4 heterozygous deletion was shown to be associated with heterogeneous clinical symptoms including schizophrenia, autism and major depression, and ULK4 is expressed in GABAergic neurons in mouse and human brains (Schurov, Handford et al. 2004). In this Chapter, we carried out a series of behavioral tests on the Ulk4<sup>+/−</sup> mice, and showed anxiety-like behavioral phenotype. We investigated Gad67<sup>+</sup> expressing neurons and demonstrated a significant reduction in hippocampus and amygdala of the Ulk4<sup>+/−</sup> mice, which may underlie the anxiety-related phenotype observed in the heterozygous mice. This is the first systematic behavioral analyses showed that Ulk4 deletion or mutation can be a rare genetic risk factor for anxiety-related disorders.

#### 6.4.1 Ulk4 heterozygous mice were largely normal

In Chapter 3 we have showed that Ulk4<sup>+/−</sup> mice exhibited congenital hydrocephalus. They were born at a Mendelian rate and displayed growth retardation after birth, but the majority of them died postnatally within the first 3 weeks. All heterozygous mice however presented normal appearance and there was no growth retardation compared with the WT littermate controls. In this study, our further histological analysis suggested that there were no significantly hydrocephalus or schizophrenia-
related phenotypes such as dilation of ventricular system or thinning corpus callosum in the $\text{Ulk4}^{+/\text{-}}$ mice. Also the morphology and cells numbers of SCO were not altered, which was consistent with the observation in the P12 $\text{Ulk4}^{-/-}$ mice. In this study, Het animals did not show any alteration in brain morphology, normal survivability. However, effects of a single copy of $\text{Ulk4}$ gene deletion on brain function are unknown until then.

**6.4.2 $\text{Ulk4}$ heterozygous mice exhibit increased anxiety-like behaviour**

Behavioral tests were very useful tool for functional measures in animals. For example, behavioral abnormalities such as impaired social interaction and communication are associated with neurodevelopmental and neuropsychiatric disorders including schizophrenia, autism and depression (Cantilena, Pastorino et al. 2011). Whether the single deletion of the $\text{Ulk4}$ gene has influences on the general behavior has not been determined. Here a comprehensive battery of behavioral tests were applied for these mice, including tests for the assessment of behavior in a novel environment, anxiety and depression related behavior, social aspects, and motor coordination.

In this study, anxiety related behavior was concluded based on three independent tests and in both female and male of Ulk4 Het mice. In the elevated plus maze for anxiety-related phenotype, both $\text{Ulk4}^{+/\text{-}}$ male and female mice spent less time on the open arms and more time in the closed arms when compared to WT counterparts. In the Marble burying test for repetitive behavior, interestingly $\text{Ulk4}^{+/\text{-}}$ female buried more marbles when compared to WT counterparts. There was no significant difference of marbles number between $\text{Ulk4}^{+/\text{-}}$ male and WT male. In open field test for anxiety-related grooming activity in a novel environment, there was no significant difference of locomotor activity between $\text{Ulk4}^{+/\text{-}}$ mice and sex-matched WT mice. However, male $\text{Ulk4}^{+/\text{-}}$ mice spend significantly more time engaged in grooming behavior, when compared to female counterparts, an effect not observed in WT animals. The $\text{Ulk4}^{+/\text{-}}$ female would be expected to increase grooming behavior during this test, and this failure to acclimatize to the arena indicates possible anxiety-related behavior.
The social behavior of Ulk4 Het mice was mainly assessed by Three-Chamber apparatus test. First, mice showed no chamber preference during the habituation period, and there was no statistical significance among the four groups in frequency of contacting the novel animal. Social novelty recognition test showed that $\text{Ulk4}^{+/\cdot}$ male mice spent significantly less time interacting with the novel stimulus animal than WT counterparts, suggesting $\text{Ulk4}^{+/\cdot}$ male mice are less interested in novel social interactions. Meanwhile $\text{Ulk4}^{+/\cdot}$ female mice exhibited an attenuated interest in male urine. Taken together, the findings indicate single deletion of $\text{Ulk4}$ gene may affect different sex on different social interaction tasks.

The depressive behavioral tests showed that $\text{Ulk4}^{+/\cdot}$ mice are largely normal under these conditions. The forced swim test at 2-3 month of age showed no sex- or genotype-specific effect on the duration of immobility in the FST among the 4 mouse groups. Also at the age of 7-10 months, which correlated with the second wave of psychosis in human subjects, accumulated immobility time was not changed in $\text{Ulk4}^{+/\cdot}$ mice in either tail suspension test or forced swim test. Together, a single copy of $\text{Ulk4}$ gene deletion did not induce despair- or depression-like behavior in mice.

In addition, we examine the possibility of attention-related deficits by using the prepulse inhibition (PPI) and latent inhibition (LI) tests, as schizophrenic patients often have defects in prepulse inhibition (Bräff, Geyer et al. 2001) and latent inhibition (Rascle, Mazas et al. 2001). The data showed that there was no alteration in difference in Acoustic Startle reactivity (ASR) or Prepulse inhibition (PPI) between $\text{Ulk4}^{+/\cdot}$ mice and littermate controls. Also we did not observe significant difference in Thermal Nociceptive Behavioral test. Therefore one copy of Ulk4 gene deletion neither affects schizophrenia positive symptom-related behavior, nor pain threshold in the $\text{Ulk4}^{+/\cdot}$ mice.

In this study, we observed interesting sex-related behavioral changes in several tests. For examples, $\text{Ulk4}^{+/\cdot}$ female mice showed attenuated interest in male urine, spent less time interacting with novel animal despite visiting the novel animal equal frequently, buried more marbles and hot plate test. On the other hand, $\text{Ulk4}^{+/\cdot}$ male...
mice spent less time interacting with the novel animal than WT mice, were less interested in direct novel social interactions, and interacted less with novel co-specific, and spent more time on self-grooming. It is worth to note that in *Xenopus*, *Ulk4* was shown to be expressed in forebrain regions involving the homeostatic control of the brain, including the preoptic region, the hypothalamic territory and some neurosecretory circumventricular organs (Dominguez, Schlosser et al. 2015). Currently, it is an open question if *Ulk4* heterozygous deletion will alter the production of sex hormones and/or expression of the receptors in mediating the hormonal actions.

**6.4.3. Cellular basis of the behavioral phenotype of the *Ulk4* mutants**

Overwhelming evidence suggests that alterations in normal GABA transmission contribute to the pathophysiology of anxiety disorders in humans and in animals. For instance, various studies using nuclear imaging techniques have revealed diminished central GABA and GABA receptor levels in patients suffering from anxiety disorders, including panic disorder, generalized anxiety disorder and posttraumatic stress disorder (Millan 2003). Also, benzodiazepines are the most widely prescribed anxiolytic drugs, because they can act through GABA receptors (Nemeroff 2003). In mammals, GABA transmission in the amygdala is particularly important for controlling levels of fear and anxiety.

The levels of circulating GABA in the CNS is determined by the Gad67+ cells populations within the brain, as Gad67 is the major GABA synthesizing enzyme. Gad67 (and Gad65) regulate GABA synthesis in the brain and readily influence cellular and vesicular GABA content. Evidence from knockout mice indicates that Gad67 provides the majority of basal GABA for the inhibitory neurotransmission. Deletion of the *Gad67* gene in mice results in >90% reduction in basal GABA levels in the brain. Gad65 deleted homozygous mice express normal levels of GABA. GAD67, as the essential precursor of GABA must be produced and released from the Gad67 neurons in sufficient quantities to ensure maintenance of this detrimental balance between activation and inhibition (check this sentence), essentially between health and disease. Therefore our findings of decreased BLA Gad67 neurons in the
*Ulk4*+/- mice may significantly contribute to a pathophysiological phenotype, in which there is a disruption in this balance, such as anxiety disorders. Such disruptions have been correlated to male *Gad1*+/- mice, displaying disturbed social behaviors characteristic of neuropsychiatric disorders, such as depression and anxiety (Sandhu, Lang et al. 2014). Strikingly, *Gad67*+/- mice exhibit an enhanced susceptibility to fetal and maternal stress (Uchida, Oki et al. 2011), which are both significant determinants for neuropsychiatric disorders (Markham and Koenig 2011).

The tight links between genetic variation (Addington, Gornick et al. 2005) and reduced expression of Gad67 associated with neuropsychiatric diseases, such as bipolar and schizophrenia, are irrevocable (Heckers, Stone et al. 2002; Hashimoto, Volk et al. 2003). In parallel to this, genetic alterations are well-established as susceptibility factors in anxiety spectrum disorders. Studies have confirmed alterations of experiences during early developments, such as the quality of maternal care, to generate modifications in gene transcription which prevail to adulthood and induce changes in physiological and behavioral functioning (Moriceau, Raineki et al. 2009; Roth and Sweatt 2011). As such, data have depicted several candidates, such as gamma-aminobutyric acid receptor subunit aplpha-2 (Gabra2) (Nelson, Agrawal et al. 2009), opioid receptor-mu1 (Oprm1) (Liberzon, Taylor et al. 2007), and neurotrophic tyrosine kinase (TrkB)(Soliman, Glatt et al. 2010) as risk factor genes involved in PTSD. Of particularly significant relevance to this study, findings have revealed polymorphisms in *GAD2* gene (encoding GAD67) as a risk factor for anxiety disorders (Unschuld, Ising et al. 2009). Considering the significant decreases in Gad67 neurons in the *ULK4*+/- gene, and the correlating functions of GAD2 in humans, similar findings are likely in defining polymorphisms in the *ULK4* gene as a susceptibility factor in anxiety disorders.

It is known that a decrease in GABA results in a decrease in neuronal inhibition, thus increased excitatory firing. In this study we have exposed a decrease in Gad67+ neurons (thus presumably a decrease in GABA) accompanying an anxiety-like phenotype in *Ulk4*+/- mice. Intriguingly, studies have confirmed Gad+ neurons, i.e. GABAergic interneurons, to possess the highest postsynaptic GABA<sub>A</sub>-R expression
levels. This means stimulation of those GABA\textsubscript{A}-Rs would result in an inhibition on GABAergic transmission. Subsequent to this inhibition, anxiolysis must only occur through indirect mechanisms, such as disinhibition of the GABAergic projection neuronal element, enhancing the inhibition effect exerted on pyramidal target neurons. Considering this, it is not known whether these decreased Gad67\textsuperscript{+} cell populations in the Ulk4\textsuperscript{+/-} mice are those GABA neurons which normally project to and directly inhibit glutamate neurons, or GABAergic neurons projecting to other GABAergic neurons acting to dampen excitation. Therefore, further studies should use techniques investigating the electrophysiology within these Ulk4\textsuperscript{+/-} mice to clarify precisely which neuronal group is most affected. Such techniques could include \textit{in-vivo} magnetic resonance spectroscopy (MRS) which allows measurement and synthesis rate of both GABA and glutamate (Rothman, Sibson et al. 1999).

Decreased Gad67\textsuperscript{+} cells may not be the only population held accountable for the Ulk4\textsuperscript{+/-} anxiety-like phenotype, thus further studies should investigate populations of other GABAergic cells to further evaluate the extent to which they may contribute. For example, further consideration should be given to the cells responsible for the concentration and localization of various peptides which play an influential role on anxiety- such as the anxiogenic neuropeptide cholecystokinin (CCK) (Harro, Vasar et al. 1993) It is known that CCK and parvalbumin (PV) are co-localized with GABAergic neurons (Kawaguchi and Kubota 1997) so perhaps there is a subset of GABAergic neurons also decreased (such as only CCK expressing cells) which have not been assessed in this study. Additionally, although there is a decrease in Gad67 neurons, it is not known how much GABA the remaining Gad67 cells release, consequently indicating further studies should measure GABA release from these neurons. As such, previous studies have used \textit{in vivo} microdialysis to measure GABA levels in the BLA of pregnant rats under stress conditions.(Young and Cook 2006). Following such investigations, one could definitively conclude that a decrease in Gad67 neurons correlates with a decrease in GABA release.

In this study, Ulk4\textsuperscript{+/-} mice showed increased anxiety-related phenotype and decreased numbers of Gad67 neurons in both hippocampus and amygdala. This
study suggests that ULK4 may be involved in maintaining the excitation/inhibition balance, which is commonly disturbed in neurodevelopmental disorders. Therefore ULK4 is likely to play important roles in brain function, and ULK4 lesions are likely to lead to neurodevelopmental and neuropsychiatric illness.
CHAPTER 7

CONCLUSIONS
In PhD this project, I have investigated the roles of Ulk4 in brain development and function using Ulk4−/− mice. I provided anatomical evidence, functional analysis and whole genome Ulk4 targets for the discoveries that Ulk4 is critical for various fundamental aspects of normal brain development and function.

Roles of Ulk4 uncovered in this PhD project include:

Using multidisciplinary approaches, I discovered that **ULK4 gene is crucial for cilia development and CSF flow.** I showed that Ulk4 is predominantly expressed in the ventricular ependymal layer, SCO and aqueduct, and Ulk4−/− ependymal cells display reduced/disorganized cilia with abnormal axoneme. Ulk4−/− mice exhibited dysfunctional SCO, obstructive aqueduct and impaired CSF flow. The analyses of whole genome RNA sequencing data revealed that Ulk4 specifically regulates the Foxj1, a master regulator of ciliogenesis, and an array of other ciliogenesis molecules. This provides the first comprehensive report that Ulk4 is crucial for mouse survival, postnatal growth and ciliogenesis and CSF circulation, which support a wide implication of the ULK4 gene in human conditions.

Appropriate white matter development is essential for brain function. I showed that **Ulk4 regulates white matter integrity and myelination.** Ulk4−/− mice exhibited comprised integrity of the corpus callosum with reduced myelination, in association with decreased production of oligodendrocyte precursors and delayed maturation of myelin-forming cells. This was accompanied with aberrant gliosis and increased neuroinflammation. Ulk4 deficiency disturbed expression of the Olig2 and a series of other genes involved in oligodendrocyte development and myelination. Regulation of the ULK4 may therefore become helpful to neurodevelopmental, neuropsychiatric, as well as to neurodegenerative diseases.

**ULK4 regulates cell cycle and neurogenesis.** Abnormal neurogenesis is associated with neurodevelopmental and neuropsychiatric illness, in which ULK4 was previously linked to. We provide further genetic evidence that ULK4 is deleted in approximately 1.2/1000 patients with developmental disorders of pleiotropic clinical
symptoms including severe language delay, speech delay and learning difficulties. Analyses of the cerebral cortex in the Ulk4+/− mice showed a significant reduction, in particular in layers II-IV and VI. Ulk4 is shown to express in a cell cycle-dependent manner and peaked in the G2 and M phases. Targeted disruption of the Ulk4 results in a dramatic reduction of neural stem cell pool in the subventricular zone of newborn mice, which will undoubtedly also lead to reduction in adult neurogenesis. Analyses of the 618 dysregulated ULK4 targets showed that Ulk4 may act as a scaffold protein regulating neural progenitor proliferation and cell cycle partially through Wnt signaling pathway.

**ULK4 regulates excitation and inhibition balance and anxiety-related behavior.** We performed a series of behavioral tests on the Ulk4+/− mice, in collaboration with Dr Michelle Roche in the NUI Galway and Dr Steve Clapcote in the University of Leeds. Our data showed that Ulk4+/− mice display an anxiety-related phenotypes. GABAergic neurons are the major inhibitory neurons in the central nervous system, and I also provided the evidence for a reduction of GABAergic neurons in the Ulk4+/− mice, which may partially account for the mechanisms of the anxiety-related behavior in the Ulk4+/− mice.

**Compensational changes**

During evolution through natural selection, organisms have developed various buffer systems to overcome the challenges from the environmental pressure and/or internal mutations. A key question is, will Ulk4 knockout result in compensational changes in the mutant mice during development?

To address this question, I have examined the quantitative whole genome RNA sequencing data for the expression of other Ulk4 family members. The expression of Ulk1, Ulk2, Ulk3, Stk36 in the mutant mice were 1.09-fold (p=0.23), 0.92-fold (p=0.14), 1.03-fold (p=0.35), and 1.00-fold (p=0.49) of that in the WT mouse, respectively. None of the changes has reached statistical significance (Table 7-1).
Therefore, I conclude that there is no obvious compensational change in the *Ulk4* mutant mice.

**Table 7-1.** No compensational changes in the mRNA expression of the Ulk4 family members in the Ulk4 mutants

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<th>Gene ID</th>
<th>Transcript ID</th>
<th>KO1</th>
<th>KO2</th>
<th>KO3</th>
<th>KO4</th>
<th>Mean KO</th>
<th>SEM KO</th>
<th>KO/WT</th>
<th>p value</th>
<th>WT1</th>
<th>WT2</th>
<th>WT3</th>
<th>Mean WT</th>
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<td>0.14</td>
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<td>34.5</td>
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<td>0.35</td>
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**Key follow up studies**

**Ulk4 isoforms, expression and function**

Surprisingly, in the quantitative whole genome RNA sequencing data, the homozygous knockouts still expressed 66% of *Ulk4* mRNA of that in the WT mice. The *Ulk4* mRNA was decreased only by 34% in the mutants, despite this the reduction was statistically significant with a *p*=0.03. The question is where is the *Ulk4* mRNA coming from?

The first 6 exons of *Ulk4* mRNA shall still be expressed from both knockout alleles in a fusion mRNA together with *SA-ires-LacZ-PA*. Therefore, if the whole genome RNA sequencing data for the *Ulk4* only derived from the exons 1-6 region, we expect no reduction at all for the *Ulk4* mRNA in the knockout brain.

If the RNA sequencing purely covered the exon 7 (deleted in the knockout) and/or the downstream of the exon 7 (depleted splicing by the introduction of a transcription termination signal PA at the 3’ of the *SA-ires-LacZ* cassestte, then, there should be no *Ulk4* mRNA detected in the Ulk4 knockout mice by sequencing.
If Ulk4 gene possessed alternative promoter(s) downstream of the exon 7, and if the activity of alternative promoter(s) was not disrupted by the insertion of the knockout cassette, then, RNA sequencing primers covering the alternative splicing isoforms shall detect the same abundance of RNA expression in the knockout and WT mice.

Therefore, we believe:

(1) that the quantitative data of the whole genome RNA sequencing represent a sum of RNA abundance from different regions of any particular gene ID;

(2) that there can be alternative Ulk4 splicing variants which are not disrupted by exon 7 knockout,

(3) that it is unknown if the alternative isoforms encode functional proteins and/or purely function at the RNA level; therefore a 34% reduction at the RNA level does not simply imply a 34% decrease at the protein level; and

(4) that meanwhile, this further raises the importance of the Ulk4 gene, because there were already striking influence on the neurogenesis, ciliogenesis, oligodendrocytes, behaviour and survival, even in non-null Ulk4 knockout mice,

However, all of these will require validation by future experiments, including cloning of different Ulk4 isoforms, characterization of the isoform function, and/or isoform-specific targeting.

**Roles of Ulk4 in peripheral systems**

In addition, we have not studied effects of Ulk4 deficiency on the peripheral organs. The follow up studies may investigate related peripheral organs for vision, cardiovascular system, respiratory system and renal system as they all require proper ciliary function, as well as tumorigenesis as Ulk4 is cell cycle –related.
**Ulk4 interactome and substrates**

The differentially expressed genes detected by the whole geneome RNA sequencing are most likely the secondary targets of the Ulk4, because Ulk4 is not a transcriptional factor, but, a member of Ser/Thr kinase family member with protein-protein interaction domains – 3 ARM structure. Therefore, I hypothesize that Ulk4 protein may exert its functions through interacting with other proteins, forming interaction complexes, thereby activating and/or inactivating post-translational modifications including phosphorylation.

To test this hypothesis, a few key experiments will need to be carried out, which may include:

1. Searching for Ulk4 interaction partners. This may be done by Ulk4 immunoprecipitation and proteomics, which will identify Ulk4 interactome;
2. Searching for Ulk4 substrates. This may be performed by Phospho-proteomics to identify proteins, whose phosphorylation levels are altered by Ulk4 depletion;
3. Ulk4 pathway analyses and experimental validation including pharmacological validation of the related pathways.

These follow up studies shall significantly enhance our understanding of the Ulk4-related disease pathology, molecular mechanisms and potential therapeutic interventions.

In summary, through this PhD project, I demonstrate that *Ulk4* is critical for proper brain development and function. Deletion, mutation and/or polymorphism of the *Ulk4* gene may have implications in a wide range of neurodevelopmental/neuropsychiatric disorders as well as neurodegenerative diseases.
REFERENCES


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