Generation and characterisation of human embryonic stem cells deficient in ZDHHC8, a gene deleted in 22q11.2 deletion syndrome

Thesis submitted for the degree of Doctor of Philosophy
School of Medicine, Cardiff University
2017

Matthieu TRIGANO
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Je tiens à dédier cette thèse à mon grand-père qui nous a quitté quelque temps seulement après la soumission de ce travail. Merci beaucoup pour ton soutien et tes précieux conseils.
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First and foremost, I would like to thank my supervisors, Prof Meng Li and Prof Jeremy Hall, for giving me the opportunity and providing me with support and guidance to complete my project in good condition.

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I also would like to thank the core team and specifically Joanne Morgan who performed the transcriptomic experiment.

I would like to thank my family who has always supported me and without whom I wouldn’t be here. To my partner, Audrey, I know it hasn’t been easy for you with my mood swings and all my issue with the thesis, but you always supported me, and I am very grateful for that.
Abstract

The 22q11.2 Deletion Syndrome is caused by a deletion on the chromosome 22q11.2. Individuals carrying 22q11.2 deletion have an increased risk to develop schizophrenia and Parkinson disease. However, how this deletion leads to the development of these diseases and the specific role of the individual 22q11.2 genes remains largely unknown. In order to understand the neuronal cell types and developmental stages in which the 22q11.2 genes may function, we investigated the temporal and spatial expression profile of the genes located in the 22q11.2DS by RT-PCR. Human embryonic stem cells (hESCs) was used to generate excitatory projection neurons, cortical interneurons, GABAergic medium spiny neurons (MSN) and dopaminergic neurons. This study revealed that several genes appear to exhibit a specific temporal expression profile. Moreover, another group of genes were found to be preferentially expressed in dopaminergic neural lineage.

Within the 22q11.2 deletion region, ZDHHC8 is an interesting candidate due to its implication in the physiology and morphology of the neurons. I generated a hESCs cellular model carrying a heterozygous deletion of ZDHHC8. These cells can be induced toward cortical projection neuron fate in a comparable temporal kinetics to that of the parental control cells. However, phenotypic characterisation revealed that ZDHHC8 mutation altered the motility and the spontaneous calcium activity in ZDHHC8+-neurons. Interestingly, transcriptomic analysis of excitatory progenitors identified altered expression of genes regulating calcium activity and axonal growth (motility). Furthermore, this study suggests that ZDHHC8 may also be involved in neuronal development, patterning and synaptic signalling.

In conclusion, this thesis provides further knowledges regarding the expression of the 22q11.2DS genes which would be valuable to guide future studies either in cellular or animal models. Furthermore, this study indicates that ZDHHC8 function is involved in several aspects of neuron development that potentially plays a role in the aetiology of 22q11.2DS.
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AP5</td>
<td>2R-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>ARVCF</td>
<td>Armadillo repeat gene deleted in Velocardiofacial syndrome</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism spectrum disorder</td>
</tr>
<tr>
<td>BCL11B</td>
<td>B-Cell CLL/Lymphoma 11B</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>CDC42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLDN5</td>
<td>Claudin 5</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variant</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O- methyltransferase</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>DGCR</td>
<td>DiGeorge Syndrome Critical Region</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopfl</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>DRD</td>
<td>Dopamine receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FGF8</td>
<td>Fibroblast growth factor 8</td>
</tr>
<tr>
<td>FRZ</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GE</td>
<td>Ganglionic eminence</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>GRIA</td>
<td>Glutamate Ionotropic Receptor AMPA Type</td>
</tr>
<tr>
<td>GRIN</td>
<td>Glutamate ionotropic receptor NMDA type</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cells</td>
</tr>
<tr>
<td>HIRA</td>
<td>Histone cell cycle regulator</td>
</tr>
<tr>
<td>iCas9 hESCs</td>
<td>doxycycline-inducible expression of Cas9</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IP</td>
<td>Intermediate progenitor</td>
</tr>
<tr>
<td>IPC</td>
<td>Intermediate progenitor cells</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>ISI</td>
<td>Interspike interval</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>LCR</td>
<td>Low copy repeats</td>
</tr>
<tr>
<td>LGE</td>
<td>Lateral Ganglionic Eminence</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>mESC</td>
<td>Mouse embryonic stem cells</td>
</tr>
<tr>
<td>MGE</td>
<td>Medial Ganglionic Eminence</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neurons</td>
</tr>
<tr>
<td>NEC</td>
<td>Neuroepithelial cells</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NPC</td>
<td>Neuronal progenitor cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRODH</td>
<td>Proline dehydrogenase</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>RANBP1</td>
<td>RAN Binding Protein 1</td>
</tr>
<tr>
<td>RGC</td>
<td>Radial glial cells</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>TXNRD2</td>
<td>Thioredoxin reductase 2</td>
</tr>
<tr>
<td>UFD1L</td>
<td>Ubiquitin recognition factor in ER associated degradation 1</td>
</tr>
<tr>
<td>UQCRCL</td>
<td>Ubiquinol-Cytochrome C Reductase Core Protein I</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage gated calcium channel</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricle zone</td>
</tr>
<tr>
<td>ZDHHC8</td>
<td>Zinc Finger DHHC-Type Containing 8</td>
</tr>
</tbody>
</table>
1. General Introduction

1.1. The 22q11.2 deletion syndrome

22q11.2 Deletion Syndrome (22q11.2DS) is a genetic disorder caused by a hemizygous deletion on the long arm (q) of the chromosome 22 (Rump et al. 2014). 22q11.2DS is the most common microdeletion in human and the second most common chromosomal disorder after down syndrome (Bassett et al. 1999; Swillen et al. 2015). Due to the wide range of disorders that arise from this mutation, 22q11.2DS is also known as DiGeorge Syndrome (DGS), Velocardiofacial Syndrome (VCFS) or CATCH-22. Table 1.1 presents a historical background associated with 22q11.2DS.

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital thymic hypoplasia associated with hypocalcaemia</td>
<td>1959</td>
</tr>
<tr>
<td>DiGeorge syndrome described</td>
<td>1992</td>
</tr>
<tr>
<td>Takao syndrome described (conotruncal anomaly face syndrome)</td>
<td>1996</td>
</tr>
<tr>
<td>Velocardiofacial syndrome described (Shprintzen syndrome)</td>
<td>1978</td>
</tr>
<tr>
<td>DiGeorge syndrome speculatively linked to chromosome 22</td>
<td>1981</td>
</tr>
<tr>
<td>Partial monosomy of chromosome 22 described</td>
<td>1982</td>
</tr>
<tr>
<td>“CATCH-22 syndrome” described</td>
<td>1989</td>
</tr>
<tr>
<td>Cayler syndrome associated with del22q11.2</td>
<td>1994</td>
</tr>
</tbody>
</table>

1.1.1. Genetics of 22q11.2DS

1.1.1.1. Prevalence of 22q11.2DS

This disorder is associated with a high prevalence with a range from 1 in 3000 to 1 in 6000 live births, which was estimated by the diagnosis of infants with major birth defects (Driscoll et al. 1993; Goodship et al. 1998). The disease occurs as a de novo mutation in 90% of the patients while it is inherited in an autosomal fashion in the remaining 22q11.2DS individuals (Carlson et al. 1997; Karayiorgou et al. 1995).

1.1.1.2. Causes of 22q11.2DS

The 22q11.2 region is unstable due to the presence of low copy repeats (LCRs). Sequence analysis of the chromosome 22 has identified four LCRs in the 3Mb deletion from LCRs A-D (Shaikh et al. 2000). These LCRs have high similarity with DNA sequences that
span other regions in the chromosome 22 which result in non-allelic crossing also called non-allelic homologous recombination (NAHR) (Colnaghi et al. 2011; L Edelmann, Pandita, and Morrow 1999). NAHRs occur during meiosis and in the case of 22q11.2 results in the appearance of copy number variant (CNV).

It was estimated that around 90% of the 22q11.2 deletion carriers have a 3Mb deletion between LCRA and D, 8% are carrying a 1.5Mb deletion between LCRA and B while the remaining 2% are carrying rare deletion that overlap the typical region (Shaikh et al. 2000).

The 3Mb deletion region contains around 46 proteins coding genes and about 90 known or predicted genes for pseudogenes and non-coding RNAs, including 7 microRNAs, MIR185, MIR1306, MIR1286, MIR3618, MIR 649, MIR4761 and MIR6816. Conversely, the 1.5Mb deletion contains around 29 proteins coding genes (McDonald-McGinn et al. 2015). Strikingly, it was demonstrated that the 1.5Mb deletion was able to recapitulate the phenotype observed in patients with the 3Mb deletion.

### 1.1.2. Clinical manifestations of 22q11.2DS

The clinical expression of the 22q11.2DS is extremely wide, more than 180 phenotypic representation have already been found (Demily et al. 2015) (Figure 1.1). The classical features include congenital heart defects, facial anomalies such as cleft palate, speech and learning difficulties and many others (Digilio et al. 2005). Interestingly, while the prevalence of schizophrenia in the general population is about 1%, it was shown to be about 30% in patients with 22q11.2DS, which makes this disorder the most frequent known cause of schizophrenia (Chow et al. 2011; Gambini 2016; Schneider et al. 2014). Other neuropsychiatric pathologies associated with 22q11.2DS are ASD, attention deficit hyperactivity disorder (ADHD), affective anxiety and obsessive-compulsive disorders.

In addition, it was recently reported that, at 37.10%, ADHD was the most frequent diagnosis in children with 22q11.2DS followed by ASD and schizophrenia at 12.77% and 1.97%, respectively. Conversely, at adulthood the proportion of patients with ADHD drop at 15.59% while it remains relatively constant for 22q11.2 patients with ASD at 16.10%. However, 22q11DS patients with schizophrenia drastically increase in adulthood to reach in some study 42% (Schneider et al. 2014).
Although these patients have higher susceptibility to develop psychosis, the mechanism by which the 22q11.2DS elevates the risk for the psychotic disorders mentioned above remains largely unknown. Nevertheless, several hypotheses might be able to explain these genetic variabilities such as 1) the breakpoint heterogeneity which corresponds to the region of the LCRs, 2) the allelic variation, 3) the multiple hit model (Jonas et al. 2014). The multiple hit model suggests that patients with 22q11.2DS will develop schizophrenia due to the presence of additional CNVs at other locations on the genome (Bassett et al. 2017; Hywel et al. 2013).

**Figure 1-1 Organs affected in 22q11.2 deletion syndrome.** In orange are the organs commonly affected in 22q11.2DS. In blue are the organs less affected and in grey are the minor malformation observed in patients. This schematic shows that the brain, palate, parathyroid gland, heart, kidney, gastrointestinal system, thymus and immune system are the organs commonly affected in individuals with 22q11.2DS. Less frequently, organs such as the thyroid gland, the haematological system, the eyes, the ear nose and throat and bone are affected. While minor malformation can affect the craniofacial features and also result to polydactyly in patients with 22q11.2DS. Figure adapted with permission from Springer: Nature reviews. Disease primers, McDonald-McGinn 2015 (4271450447971) Copyright (2015)
Schizophrenia

Schizophrenia is a neuropsychiatric disorder with an onset at late adolescence and early adulthood. Clinically, schizophrenia is characterized by symptoms usually classified as positive, negative and cognitive. The positive symptoms are typically associated with the psychotic symptoms (delusion, hallucination) and tend to relapse and remit. These symptoms result from an increase in the dopamine D2 receptor activation (Walter et al. 2009). The negative symptoms are generally chronic and associated with a decrease in motivation and social withdrawal and conversely to the positive symptoms, are due to a reduction in the activation of the dopamine D1 receptor (Walter et al. 2009). On the other hand, cognitive symptoms include an impairment in memory performance, attention as well as poor language ability (Moyer, Shelton, and Sweet 2015).

The dopamine hypothesis arose from the study of Jean Dealy and Pierre Deiner in 1952 in which they studied the chlorpromazine and its antipsychotic effects (Delay et al., 1952). Therefore, the dopamine hypothesis stated a hyperactive dopamine transmission as the pathophysiology of schizophrenia. Because the antipsychotic drugs act on the dopamine D2 receptor, they are able to alleviate partly the positive and negative symptoms. However, post mortem examination were unable to provide evidence of an alteration of the dopamine levels or dopamine receptors, therefore it was proposed that the dopaminergic overactivity was secondary to the change of other neuronal systems (Coyle 2004; Knable et al. 1994). A new hypothesis, the glutamatergic hypothesis is based on the ability of anaesthetics phencyclidine (PCP) but also the ketamine to bind to N-methyl-D-aspartate (NMDA) receptors and induces schizophrenia like symptoms in healthy subjects while it worsen the positive symptoms in schizophrenic patients (Lahti et al. 1995; LUBY et al. 1962).

Thereby, the glutamate hypothesis asserts that in the cortico-striatal projection, the glutamate hypofunction produces an increase in the sensory input through facilitation of the thalamocortical circuit and thus increases the dopaminergic input (Lang et al. 2007). In addition to this assumption, there are the global disinhibition of the cerebral cortex which imply the loss of the tonic regulation by the GABAergic neurons (Lisman et al. 2008). Many studies therefore focused on the glutamate hypofunction and more particularly on the decrease inhibition by the GABAergic neurons which were observed to be related to a loss of a specific types of neurons, the parvalbumin interneurons.
1.1.3. Neurobiology of 22q11.2DS

1.1.3.1. Cognitive impairments

Children carrying 22q11.2 mutation are reported to have by average 30 points lower IQ compared to their siblings (Niarchou et al. 2014). Children with 22q11.2 mutation usually achieve higher scores in verbal than non-verbal task. These children have mathematical disabilities and have difficulties to compare numbers or to perform calculations (De Smedt et al. 2008).

Furthermore, while the ability to understand a speech is usually strong in pre-school, general language delays appears by school age (Gerdes et al. 1999; Scherer et al. 1999). Studies suggest that children have problems at finding and interpreting salient spatial and temporal information, suggesting that they are less able in integrating goal relevant information (Bish et al. 2007; Corbetta et al. 2002).

Because the attention is thought to be crucial for inhibiting irrelevant information, children with 22q11.2DS are reported to have impaired sensorimotor gating (Brocki et al. 2004; Sobin et al. 2005). In addition, patients with 22q11.2DS have difficulties in identifying novel auditory stimulus when using the mismatch negativity paradigm (Cheour et al. 1997). Finally, children with 22q11.2DS have impaired working memory performance (Wong et al. 2014).

1.1.3.2. Neuroanatomical deficits

Given the impairment in cognitive function in patients with 22q11.2DS, there are good reasons to expect anatomical change in these patients. Brain of children with this deletion have a 8-10% reduction in the volume of the posterior and inferior brain regions (Simon et al. 2005). These reductions appear to affect white matter to greater extent than grey matter (Simon et al. 2005). Furthermore, it is also demonstrated that the total surface area is decreased in 22q11.2DS. On the other hand, the cortical thickness as well as the volume of the corpus callosum is increased in 22q11.2 individuals (Lin et al. 2017).

1.1.4. Genes located in the 1.5Mb deletion region

1.1.4.1. DGCR6

Located at the proximal part within the 22q11.2 deletion, DiGeorge Syndrome Critical Region Gene 6 (DGCR6) is thought to contribute to the apparition of 22q11.2DS,
however its function is still unclear (Gao et al. 2015). *DGCR6* was shown to have similarity with *Gonadal (Gdl)*, a drosophila melanogaster gene of unknown function. In addition, this gene was shown to be expressed in brain, the neural tube, pharyngeal arches and the nasal process at E11.5 (Edelmann et al. 2001; Lindsay et al. 1997). *DGCR6* protein is a nuclear phosphoprotein expressed in the liver, heart and skeletal muscle (Pfuhl et al. 2005). Interestingly, *DGCR6* was suggested to regulate *TBX1*, another gene located within the 1.5Mb deletion. Therefore *DGCR6* is postulated to be responsible for abnormalities such as heart defect which is one of the outcome of *TBX1* dysregulation (Gao et al. 2015). Within the brain, a study showed that *DGCR6* may interact with GABA$_B1$, a receptor modulating excitatory and inhibitory synapses therefore, *DGCR6* mutation might alter synaptic transmission (Zunner et al. 2010).

1.1.4.2. PRODH

The proline dehydrogenase (*PRODH*) is an enzyme located on the inner mitochondrial membrane that catalyses the first step of the proline degradation (Bender et al. 2005). In turn the product of this catabolic reaction the Δ-1-pyrroline-5-carboxylate (P5C) is a precursor of glutamate and γ-aminobutyric acid (GABA) (Jacquet et al. 2002). In addition to its role in the metabolism of glutamate, proline was also demonstrated to act as a co-agonist for the NMDA receptor (Zwarts et al. 2017). *Prodh* was further suggested to potentiate excitatory transmission of pyramidal neurons between different regions of the hippocampus (Cohen et al. 1997). *PRODH* deletion has been associated with an increased risk of developing schizophrenia (Willis et al. 2008). However a recent study failed to replicate this result and found no association between *PRODH* and the risk to develop schizophrenia (Williams et al. 2003). It was demonstrated that patients with 22q11.2DS have an elevated serum level of proline which can be implicated in learning impairment as well as epilepsy and schizoaffective disorders (Karayiorgou et al. 2004). Homozygous *Prodh* mice were observed to be viable with normal brain morphology. However behavioural assessment revealed that these mice have a sensory gating deficits (Gogos et al. 1999). Finally, it was demonstrated that there is an epistatic interaction between *Prodh* and *catechol-O- methyltransferase (Comt)*.

1.1.4.3. DGCR2

*DGCR2* encodes for a putative adhesion receptor. This gene is also found to be associated with schizophrenia although this result is still controversial has other study found no association with schizophrenia (Shifman et al. 2006; Skowronek et al. 2006). *DGCR2*
expression is increased in the DLPC of patients with schizophrenia relative to matched controls (Shifman et al. 2006). DGCR2 protein is also observed to be weakly expressed in myelinating Schwann cells but strongly expressed in dorsal root ganglia (Spiegel et al. 2006).

1.1.4.4. DGCR14

*DGCR14* was identified as essential for early embryonic development (Wang et al. 2006). In situ hybridization revealed high expression in the embryo from E7 onwards with relatively higher expression in the central nervous system specifically in a sub-region of the pons (Lindsay et al. 1998). *DGCR14* is also associated with higher risk to develop schizophrenia in the Chinese Han population (Wang et al. 2006).

1.1.4.5. TSSK2

The *Testis-specific serine/threonine protein kinase 2* (*TSSK2*) is predominantly expressed in the testis. This gene was shown to be important for the formation and the function of the sperm cells. Mutation of *Tssk2* was demonstrated to cause infertility which was attributed to an impair of spermatogenesis in humans (Zhang et al. 2010). Real-time polymerase chain reaction (RT-PCR) carried out on different human tissue revealed that TSSK2 is also expressed in the fetal brain (Hao et al. 2004).

1.1.4.6. GSC2

*Goosecoid Homeobox 2* (*gsc2*) was observed to be expressed in the mouse embryo during development (Wakamiya et al. 1998). This gene is also suggested to be expressed while neural crest are maturing and differentiating into in the pharyngeal arches (Saint-Jore et al. 1998). *Gsc2* was shown to be present during gastrulation at E6.5 therefore suggesting its involvement in axis formation. However knockout for *gsc2* was unable to demonstrate the presence of abnormal axis formation (Wakamiya et al. 1998). In addition, at E11 and postnatal day 7 (P7), *Gsc2* was shown to be robustly expressed in the interpeduncular nucleus, a region important for the generation of the hippocampal theta rhythm and also for the control of rapid eye movement (REM) (Funato et al. 2010). Although *Gsc2* is suggested to be present during the development its exact role is still unclear in both mouse and human.

1.1.4.7. SLC25A1

The *solute carrier family 25 member 1* (*SLC25A1*) is part of a family of nuclear encoded transporters which are encored in the inner membrane of the mitochondria. SLC25A1
protein promotes the export of citrate from the mitochondria to the cytoplasm which is thought to be crucial for the energy balance in the cell. Recessive SLC25A1 mutation from unrelated family was demonstrated to be responsible of severe clinical phenotype which in 61% of the cases led to the death of the individual shortly after birth (Chaouch et al. 2014). Individuals diagnosed with SLC25A1 mutation appeared to primarily present neuromuscular junction (NMJ) defect (Chaouch et al. 2014). Whole genome sequencing identified that heterozygote of two pathogenic variant of SLC2A1 might be responsible of agenesis of the corpus callosum due to the loss of activity of the citrate transporter (Edvardson et al. 2013). On the other hand, knockdown in the zebrafish of Slc25a1 was demonstrated to lead to characteristic features observed in 22q11.2DS such as proliferation defect (Napoli et al. 2015). Furthermore, SLC25A1 activity is influenced by two pro-inflammatory factors, the tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ), suggesting that SLC25A1 might be central for cytokine induced inflammation signals (Infantino et al. 2014).

1.1.4.8. CLTCL1
The clathrin heavy chain like 1 (CLTCL1) encodes for the protein CHC22 which was shown to be expressed at various level in fetal tissue. CHC22 was recently demonstrated to be required for endosomal sorting (Esk et al. 2010). Furthermore, it was observed that CLTCL1 was upregulated in the developing brain suggesting that it might be important early in the patterning of the brain, conversely, in postnatal brain the level of the CHC22 protein was observed to be downregulated (Nahorski et al. 2015). Interestingly, knockdown of CHC22 in hESCs derived neural crest cells induces neurites outgrowth in neurons precursor cells, therefore it was concluded that the loss of CHC22 is necessary for the development (Nahorski et al. 2015).

1.1.4.9. HIRA
Histone cell cycle regulator (HIRA), also known as DGCRI, regulates cell cycle dependant histone transcription (Magnaghi et al. 1998). Hira was shown to be expressed in the neural crest during embryogenesis and also to interact with Pax3, a transcription factor important during embryonic development. Importantly, homozygous mutation of hira occurred to be lethal at E11 emphasizing its possible crucial function during embryogenesis (Roberts et al. 2002). Although the role of HIRA during development is thought to be important, its implication during early neurogenesis is still unknown. However, it was recently suggested that HIRA modulates the expression of β-catenin
which in turn was demonstrated to regulate neurogenesis through neuronal progenitors proliferation and differentiation (Li et al. 2017).

1.1.4.10. MRPL40

The mitochondrial ribosomal protein L40 (MRPL40) encodes for a protein responsible of protein synthesis in the mitochondria (Devaraju et al. 2017). Mitochondrial ribosomes are constituted of different subunits, a small 28S and large 39S subunits. Heterozygous mice for Mrpl40 were observed to alter the mitochondrial permeability transition pore (mPTP) which result in an abnormal control of the mitochondrial calcium at the presynaptic terminals which was shown to impair short-term plasticity. (Figure 1.2).

![Figure 1-2 Schematic of the calcium dysregulation due to Mrpl40+/-.](image)

In control, the presynaptic neurons mitochondrial calcium buffering is controlled by mitochondrial uniport, the sodium-calcium and proton-calcium exchanger and also through slow extrusion from the mitochondrial permeability transition pore. Instead, in the mutant, the slow extrusion from the mitochondrial pore is altered resulting in accumulation of calcium in the mitochondria and the presynaptic neurons, therefore creating an increase release of vesicle of neurotransmitter. The trace represents the calcium transient in the cytosol and the mitochondria in the control and mutant. Figure adapted with permission from Springer: BioEssays: news and reviews in molecular, cellular and developmental biology, Devaraju 2017 (4296970486895) Copyright (2017).
1.1.4.11. C22orf39

*Chromosome 22 Open Reading Frame 39 (C22orf39)* is a protein coding gene however its function remains unknown.

1.1.4.12. Ufd1l

*Ubiquitin recognition factor in ER associated degradation 1 (Ufd1l)* is expressed by several tissues during embryogenesis, it encodes for a component involved in the degradation of ubiquitin fusion proteins (Mohamed et al. 2005). In yeast, UFD1L protein was observed to be important for the ubiquitin dependent proteolytic degradation pathway. Interestingly, mice lacking this gene was shown to die before organogenesis therefore implies an important role for this gene during embryogenesis (Yamagishi et al. 2003). *UFD1L* was also observed to be important for the development of the neural crest cells.

1.1.4.13. Cdc45

*Cell division cycle 45 (Cdc45)* is necessary for DNA synthesis during genome duplication as it forms the replicative DNA helicase in eukaryotes. CDC45 protein was shown to be expressed in neuronal precursors during brain development (Meechan et al. 2015). Although its role in 22q11.2DS appeared to be important especially during cortical development, no study was performed to investigate the function of this gene alone during brain development.

1.1.4.14. Cldn5

*Claudin 5 (CLDN5)* is part of the claudin family composed of about 26 members which are localized at the level of the tight junction (Günzel et al. 2013). CLDN5 protein is the most expressed claudin in endothelial tight junction in the vascular endothelium of the brain. Indeed, its defect was responsible for the alteration of the blood brain barrier (BBB), a structure particularly important to restrict the diffusion of molecules to the brain (Irudayanathan et al. 2016). Evidences also suggest that this gene may be involved in the vulnerability to schizophrenia (Omidinia et al. 2014; Sun et al. 2004; Wu et al. 2010). Mice with homozygous for *cldn5* were observed to die at birth. While the BBB of these mice were shown to be unaffected, the selectivity of molecule inferior to 800 Dalton but not larger was affected (Nitta et al. 2003).
1.1.4.15. SEPT5

SEPT5 encodes for the protein SEPTIN5, which was originally identify as a GTPases required for cytokinesis. However, it was later demonstrated that SEPTIN5 is robustly expressed in the central nervous system suggesting it might have an additional role than its involvement in the cell cycle (Beites et al. 1999). In the mouse brain, SEPT5 was found to be localized at the presynaptic neurons where it binds to syntaxin, a SNARE (soluble NSF attachment protein receptor) protein and thus involved in the exocytosis of neurotransmitter vesicles (Asada et al. 2010; Beites et al. 1999). Furthermore, SEPT5 was observed to be a substrate of the protein PARKIN, a ring finger protein that is an E3 ubiquitin protein ligase (Choi et al. 2003). Mutation in PARKIN has been associated with familial Parkinson disease.

1.1.4.16. GP1BB

This gene was demonstrated to be part of the GPIb-V-IX system which composed the receptor for von Willebrand that mediates platelet adhesion in the blood circulation. While the main transcript of this gene is expressed in platelets and megakaryocytes, a smaller and less abundant transcript was observed to be expressed in other tissues such as the brain.

1.1.4.17. TBX1

The T-box transcription factor (TBX1) is a member of the family that share a common DNA binding domain, the T-box. TBX1 was shown to be expressed during embryogenesis primarily in the pharyngeal arches which coincides with the neural crest cells migration (Chieffo et al. 1997). TBX1 homozygous displays important cardiovascular, thymic, craniofacial and parathyroid defects (Ghosh et al. 2017; Kispert 2017). Mouse model for TBX1 exhibits several pathologies related to the heart and facial structure abnormalities. This gene was demonstrated to be responsible of the development of the pharyngeal arch artery during development. Interestingly, the expression of Tbx1 in the pharyngeal arches was demonstrated to overlap with Shh which was shown to regulate Tbx1 (Garg et al. 2001). Surprisingly, a mouse model of Trp53 which encodes for the protein p53, was demonstrated to be able to rescue the phenotype due to Tbx1 haploinsufficiency in mice heterozygous for both genes. It was shown that the Trp53+/- restored the level of expression of Gbx2, a gene requires for aortic arch outflow in Trp53+/-, Tbx1+/- . It was also recently observed that Tbx1+/- mice disrupt corticogenesis due to an early
differentiation of the region that give rise to the somatosensory cortex. In addition, mouse model of Tbx1 +/- were demonstrated to have PPI alteration.

1.1.4.18. GNB1L

*GNB1L* or *G Protein Subunit Beta 1 Like* encodes for a protein which contains six WD40 repeats which are known to enable the formation of heterotrimeric or multiprotein complex. This gene was shown to be expressed in the embryonic brain but in less extend in the adult brain (Sun et al. 2015). *GNB1L* was suggested to be associated with schizophrenia (Li et al. 2011; Williams et al. 2008), This gene is also thought to be involved in cell cycle progression, signal transduction, apoptosis and gene regulation.

1.1.4.19. C22ORF29

Chromosome 22 Open Reading Frame 29 (C22ORF39) is a gene coding protein of unknown function.

1.1.4.20. TXNRD2

Thioredoxin reductase 2 (*TXNRD2*) is part of the thioredoxin reductase family which is thought to regulate the cellular redox balance (Soerensen et al. 2008). *Txnrd2* encodes for a mitochondrial protein which was observed to be ubiquitously expressed in cells and tissues. The reductase activity depends on the availability of selenium due to the presence of selenocysteine residue onto the TXNRD2 protein. Furthermore, selenium was shown to be strongly retained in the brain thus its deprivation in transgenic mice was responsible to developmental and degenerative damages (Burk et al. 2007; Pillai et al. 2014). Mice specifically missing *Txnrd2* in the nervous system develop normally and do not display any histopathology alteration and have a similar lifetime than control mice. While its role in the brain is suggested to not be critical, it was demonstrated to be crucial for heart development and function (Conrad et al. 2004).

1.1.4.21. COMT

*COMT* is a key enzyme for modulating the dopamine level in the prefrontal cortex. As dopamine was demonstrated to be important for cognitive function, deficit in *comt* has been associated with psychiatric disorders (J. Chen et al. 2004; Tunbridge et al. 2007). COMT is a cytosolic enzyme present either in a soluble or membrane bound form, S-COMT or MB-COMT respectively (Harrison et al. 2008). Despite homozygous mouse for *Comt* showed normal basal level of dopamine, its clearance was slower suggesting a prolonged phasic activation of dopaminergic receptors. COMT has a functional
polymorphism, a valine substituted by a methionine at the codon 108 in S-COMT and 158 in for the transcript MB-COMT (Lachman et al. 1996). This is resulting to an alteration of the activity of both COMT isoforms.

1.1.4.22. ARVCF

ARVCF encodes for the ARVCF protein (Armadillo repeat gene deleted in Velocardiofacial syndrome). It is a member of the catenin family and was demonstrated to be the closest relative to the p120, a catenin (Mariner, Wang, and Reynolds 2000). The catenin family plays important role in the formation of adherents’ junction by facilitating the communication between the inside and outside of cell (Sirotkin et al. 1997). Despite ARVCF is widely expressed, it was demonstrated to be present at very low amount compared to other catenins therefore suggesting that high expression is not necessary for its function or else ARVCF expression is temporally or spatially expressed (Kausalya, Phua, and Hunziker 2004). In addition, ARVCF was observed to be expressed in the ganglionic eminence (GE) during the development suggesting its involvement in cell rearrangment and migration from the GE but also a role in tangential migration towards the cortex (Ulfig and Chan 2004).

1.1.4.23. C22ORF25

Chromosome 22 open reading frame 25 is also known has TANGO2 (Transport and Golgi organization 2). This protein was suggested to be responsible of the redistribution of the Golgi membrane into the endoplasmic reticulum in drosophila while in mice it was found to have a mitochondrial function (Kremer et al. 2016). Although its function is still unclear it was speculated that TANGO2 deficiency might be associated with an alteration of electron transfer from the FAD-dependant dehydrogenases of the respiratory chain of the mitochondria (Kremer et al. 2016).

1.1.4.24. DGCR8

DGCR8 is an RNA binding protein which is part of the microprocessor complex with Drosha in order to process long primary microRNAs (miRNA) (Petri et al. 2014). Furthermore, miRNA alteration was observed in post-mortem brain samples from individuals with schizophrenia and 22q11.2DS (Fénelon et al. 2011; Moreau et al. 2011). Analysis of heterozygous mice for Dgcr8 resulted in perturbation of miRNAs expression which were usually reduced by 20-70%. In addition, these mice resulted in smaller
dendritic spines and tree but also exhibited working memory impairment, all of which are features also observed in patients carrying 22q11.2DS (Stark et al. 2008).

1.1.4.25. TRMT2A

TRNA Methyltransferase 2 Homolog A (TRMT2A) encodes for a protein of unknown function. However, it was suggested to act as a cell cycle regulated protein. Furthermore, this gene was associated with clinical outcome in subset of breast cancer (HER2+) (Hicks et al. 2010; Zhou et al. 2002).

1.1.4.26. RANBP1

RAN Binding Protein 1 (RANBP1) encodes for a protein responsible of nucleocytoplasmic transport. RANBP1 messenger RNA (mRNA) was shown to be present throughout the embryo during development (Mangos et al. 2001). This protein is part of the Ran family, together with RANBP1, Ran and RCC1 (Regulator of chromosome exchange factor 1) form a complex important for the guidance of the mitotic spindle assembly. Indeed, RANPB1 was demonstrated to stimulate the activity of Ran GTP production which is important for the formation of this trimeric complex. Depletion of RANPB1 exhibited hyperstable spindle microtubules, disorganized microtubule asters and also defective chromosome segregation (Zhang et al. 2014). Furthermore, the expression of RANBP1 was shown to be misregulated in cancers (Rensen et al. 2008). In term of influence on the brain, RANBP1 was established as being important during the process of axonal injury. Indeed, upon injury, there is a local translation of RANBP1 creating a retrograde injury signalling that promotes axonal protein synthesis. Thereby RANBP1 perturbation reduces the conditioning of the neurons in response to the lesion (Pathak et al. 2016; Yudin et al. 2008). Following observation using a 22q11.2 mouse model, Paronett et al., generates a homozygous knockout for ranbp1. Although it was demonstrated that 60% of the embryos were encephalic, the remaining embryos showed alteration in neuronal migration. Indeed, authors observed a decrease of the layer 2/3 of the cortex (Paronett et al. 2015). Finally, RANBP1 was also suggested to play a role in the development of autistic spectrum disorder (ASD) (Wenger et al. 2016).

1.1.4.27. ZDHHC8

ZDHHC8 encodes for a palmitoyl transferase that share a DHHC domain, the Zinc Finger DHHC-Type Containing 8 protein (ZDHHC8). This enzyme is responsible of the addition of a 16 carbons fatty acid onto a cysteine residue also known as palmitoylation. This gene
was found to palmitoylate the post synaptic density 95 PSD-95 which is an adaptor protein localised at the postsynaptic neurons (Mukai et al. 2015). PSD-95 is the member of the membrane associated guanylate kinase family (MAGUK). This protein is particularly important for neurons as it was demonstrated to be involved in excitatory neurons maturation (El-Husseini et al. 2000). Primary culture from both homozygous and heterozygous knockout for Zdhhc8 were shown to affect dendritic arborisation (Mukai et al. 2008). This deficit was demonstrated to also alter the density of glutamatergic synapses which were visualized by a reduction of PSD95 and VGLUT1 puncta. ZDHHC8 deficit was shown to alter both the axonal complexity and the regulation of spine density which was established to be due to a decrease palmitoylation of cdc42palm, a CDC42 variant only expressed in the brain (Moutin et al. 2017; Mukai et al. 2015). Interestingly, by reintroducing back the full-length ZDHHC8 protein into neurons was able to recover the axonal arborisation and length in Zdhhc8/- (Mukai et al. 2015). Furthermore, the lack of dendritic complexity and spine density was also recover upon reintroduction of ZDHHC8 in a mouse model of 22q11.2DS (Mukai et al. 2008).

In addition to its role in the morphology of the neurons, ZDHHC8 was also demonstrated to interact with proteins responsible of synaptic transmission. Indeed, AMPA receptor trafficking which are mediated by GRIP1b and PICK1, was altered by ZDHHC5/8 and ZDHHC8 alone respectively (Thomas et al. 2012, 2013). Thereby, decrease palmitoylation of PICK1 was demonstrated to impair the induction of the cerebellar long term synaptic depression (LTD) while decrease GRIP1b was observed to alter AMPA receptor trafficking.

Despite, several substrates have already been shown to be associated with ZDHHC8, it remains unknown whether they are targeted by other palmitoyl transferase. However, the Parelemmin-1 protein encoded by the gene PALM gene was demonstrated to be only palmitoylated by ZDHHC8 (Huang et al. 2009). Furthermore, ankyrin G, a protein highly enriched at the axon initial segment was observed to require to be palmitoylated by ZDHHC5/8. In an experiment, He et al., reported that palmitoylation of ankyrin G by ZDHHC5/8 was necessary to its appropriate targeting to the lateral membrane of MDCK cells (He et al. 2014).

Recently, ZDHHC8 was observed to be localized in synaptic mitochondria of glutamatergic neurons (Maynard et al. 2008). This study by Maynard et al., also showed the association between ZDHHC8 and Ubiquinol-Cytochrome C Reductase Core Protein I (UQCRC1) using immunofluorescence. UQCRC1 is part of the complex III of the
respiratory chain whose expression was found to be decreased in brain of schizophrenic subjects but increased in individuals with Rett-syndrome and mood disorders (Johnston-Wilson et al. 2000; Kriaucionis et al. 2006; Prabakaran et al. 2004). Furthermore, their study also revealed using immunocytochemistry that ZDHHC8 colocalized with Synaptophysin, a protein localized at the presynaptic site of neuron, although few puncta colocalized with parvalbumin (PV) expressing neurons they preferentially found that ZDHHC8 colocalized in glutamatergic neurons. Surprisingly, the same authors discovered that overexpression of ZDHHC8 in fibroblast was toxic upon transfection therefore leading to cell death which was shown to be likely through apoptosis. This effect was thought to be responsible by the DHHC domain of the protein (Maynard et al. 2008).

In addition to its palmitoyl transferase, ZDHHC8 was also identified as a modulator of G2/M phase of the cell cycle in response to DNA damage (Sudo et al. 2012).

Mice deficient for Zdhhc8 were observed to have alteration in sensory gating and also open field behaviour (Mukai et al. 2008; Swerdlow et al. 2008). Furthermore, this mice were observed to have an alteration in the hippocampal medial prefrontal synchrony which is thought to also be altered in individual with 22q11.2DS (Mukai et al. 2015).

This thesis aims to evaluate the function of ZDHHC8+/- in glutamatergic neurons at the phenotypic level, and the analysis of the transcriptome of neuronal progenitors heterozygous for ZDHHC8.

1.1.4.28. RTN4R

Reticulon 4 receptor is also known as Nogo-66 receptor, a protein encoded by the RTN4R gene. RTN4R is a glycosylphosphatidylinositol (GPI)-linked protein highly expressed in the frontal cortex and also localized on the axonal membrane of the pre and post synaptic neurons (Josephson et al. 2002; Wang et al. 2002). This receptor is a crucial regulator of neurite outgrowth, indeed, the activation of RTN4R leads to the inhibition of a signalling cascade activating RhoA which ultimately inhibits axonal growth (Hsu et al. 2007; Sinibaldi et al. 2004). In addition, it was also demonstrated as being important for axon regeneration following injury (Hsu et al. 2007; Hunt et al. 2002).

1.1.4.29. DGCR6L

DGCR6L is a duplicate copy of DGCR6, they share 97% similarity, they only differ on only 220 residues (Chakraborty et al. 2012). DGCR6L encodes a homologous functional
copy of DGCR6, expression study identified that this gene was also expressed in fetal and adult tissue (Lisa Edelmann et al. 2001).

1.1.5. Animals model of 22q11.2DS

Several mouse models were generated to study the 22q11.2DS. The chromosome 16 in mouse harbour a syntenic region of the chromosome 22 except CLTCL1 (Karayiorgou et al. 2010). As presented in Figure 1.3, these mice models of 22q11.2DS harbour different deletions which span various region (Kimber et al. 1999; Lindsay et al. 2001; Lindsay et al. 1999; Merscher et al. 2001; Mukai et al. 2008; Noritake et al. 2009; Stark et al. 2008). These models gave a valuable insight into the different aspects of 22q11.2DS. Indeed, they exhibited similar behavioural impairment than those observed in 22q11.2 carriers such as decrease of sensorimotor gating or prepulse inhibition (PPI) (Paylor et al. 2006). In addition to behavioural studies, these mice models were found to alter cortical development such as the alteration of the tangential migration of interneurons or the cortical layering which was demonstrated to be altered in 22q11.2DS (Meechan et al. 2015).

Figure 1.3 Human 1.5Mb deletion of the 22q11.2 region and the syntenic region in mouse (MMU16). Legend on next page
1.1.6. 22q11.2 Duplication

Because the 22q11.2 region is predisposed to rearrangement due to the presence of multiple LCRs, both deletion and duplication can occur and may be expected in equal proportion. Only few duplications were detected however (Ensenauer et al. 2003). Moreover, individuals with 22q11.2 duplication have far less distinct phenotypic characteristics than those carrying the deletion. Therefore, 22q11.2 duplication are usually underdiagnosed (Yobb et al. 2005). Surprisingly, in a study that comprised 47005 individuals, in which they were 6882 schizophrenia case and 11255 controls, the CNV analysis revealed that 22q11.2 duplication have a protective effect against the risk to develop schizophrenia (Rees et al. 2014). Furthermore, 22q11.2 duplication was absent amongst the schizophrenic cases while they were 10% amongst the controls to have this CNV.

1.2. Brain development

1.2.1. Induction and patterning of the nervous system

During embryonic development, gastrulation results from the formation of the three-layered organization named, endoderm, ectoderm and mesoderm. The inner layer referred as endoderm was observed to form cells such as the lung and pancreatic cells. The middle layer, the endoderm becomes cells such as muscle, cartilage or bone cells. The outer layer also named ectoderm becomes tissues such as the epidermis as well as the nervous system. Experiments from Spemann and Hilde Mangold in which they transplanted the blastopore from an embryo to another resulted to the generation of an entire second axis including brain. Later, they discovered that this region have the capacity to promote neural induction and named the Spemann organizer or organiser (Spemann et al. 1924). By transplanting smaller pool of complementary DNA (cDNA) at each of its experiment, Harland managed to isolate the first morphogen NOGGIN (Smith et al. 1992). Indeed, in an experiment, embryos treated with UV irradiation therefore making them unable to
develop dorsal part, the injection of NOGGIN mRNA was able to restore the development of the dorsal part of the embryo (Sasai et al. 1994). It was later demonstrated that NOGGIN binds with high affinity to BMP2 and BMP4 therefore preventing their interaction with their receptors (Zimmerman et al. 1996). CHORDIN, which was isolated by DeRobertis, was demonstrated to have similar characteristics than NOGGIN (Sasai et al. 1994). Indeed, CHORDIN binds to BMPs therefore antagonizing the BMP signalling by preventing activation of their receptors (Piccolo et al. 1996). Finally, FOLLISTATIN known as activin-binding protein, was shown to be able to bind and inhibit activin therefore is involved in mesoderm differentiation. However, experiments from Hemmati-Brivanlou and Melton showed that a truncated activin receptor which is part of the TGF-β/BMP signalling was able to induce the generation of neurons at the expense of mesodermal tissue (Hemmati-Brivanlou et al. 1994). Therefore, FOLLISTATIN was considered as the third neural inducer. Experiments shows that without signal from the organiser, cells secret BMPs and therefore promote ectodermal cells formation. Conversely, the action of these three morphogens promotes the signalling that initiates the formation of the neural plate by inhibiting BMP and TGF-β signalling (Zimmerman et al. 1996).

The organiser homologous structure in mammals is the node. Both structures are responsible of the simultaneous formation of the neural plate that overlie the notochord, a structure that elongates along the rostrocaudal axis and produces secreted factors in its surrounding therefore providing fate and position information. Soon after these structures form, the neural plate fold to form the neural tube in a process known as neurulation. Along the rostrocaudal and dorsoventral part of the embryo, cells are exposed to different levels of morphogens which are important for their fates. While the rostral part of the neural tube forms the telencephalon, midbrain and hindbrain, the caudal part is responsible of the formation of the spinal cord.

1.2.1.1. WNT signalling

Wnt is part of a family of secreted glycoproteins regulating several cellular behaviours during embryogenesis. Interestingly, several WNT proteins require to be palmitoylated for their function (Willert et al. 2003). During the development of the brain, Wnt was shown to be particularly important, the secretion of DKK1 by the organiser is crucial for the patterning of the rostrocaudal axis and the telencephalon. Indeed, DKK1 inhibits the gradient of Wnt at the rostral part of the embryo (Yamaguchi 2001). Experiments in which DKK1 is deleted result to a loss of the rostral structure, more specifically these mice lack
head and brain structure anterior to the hindbrain. Conversely, ectopic expression of DKK1 is observed to be responsible of an enlargement of the head (Mukhopadhyay et al. 2001). FrzB another inhibitor of Wnt contains a domain similar to the putative Wnt binding domain of the frizzled receptors. FrzB protein, that is secreted by the organiser, binds to the WNT proteins preventing them from binding to their receptors. It was later demonstrated that FrzB inhibits β-catenin signalling by biding to Wnt1 and Wnt8 which led to the formation of head larger than normal (Leyns et al. 1997; S. Wang et al. 1997). While these two inhibitors demonstrate the importance of Wnt signalling for the patterning of the rostrocaudal axis, Wnt is also associated with the establishment of the dorsoventral axis. In an experiment, Kelly et al., demonstrated that an ectopic expression of β-catenin, a component of Wnt signalling, was associated with the formation of dorsal axis duplication in zebrafish (Ungar et al. 1995). In addition, Wnt signalling is also associated with the patterning of the dorsal telencephalon. Thus, overexpression of β-catenin in mouse disrupts the expression of several genes along the dorsoventral axis. Therefore, dorsal genes such as Pax6 and Pax7 saw their expression to be expended ventrally at the expense of progenitors with a ventral identity such as Olig2 or Nkx6.1. In addition, inactivating β-catenin demonstrated that Wnt signalling was necessary to preserve dorsal identity by suppressing the expression of ventral genes (Alvarez-Medina et al. 2008; Backman et al. 2005).

Cerberus is a secreted protein expressed in the anterior endoderm. This protein was shown to inhibit both the Wnt and BMP signalling. Experiment using Xenopus demonstrated that Cerberus mRNA injection differs from the three neural inducers in that it induces the formation of an ectopic head but also suppresses posterior mesoderm formation (Shawlot ety al. 1998).

While at the neural tube stage Wnt is required for proliferation of progenitors, Wnt genes start to be expressed when the neural tube fold. Once the neural tube is closed, several Wnt can be detected: Wnt1, Wnt3, Wnt3A, Wnt4 and Wnt7b are expressed in the dorsal region while Wnt5a and Wnt7a are in the ventral region (Gunhaga et al. 2003; Harrison-Uy et al. 2012).

Wnt signalling is composed of three main intracellular signalling, the canonical or WNT/β-catenin pathway, the planar cell polarity pathway and the WNT/Calcium pathway. The canonical WNT pathway involves WNT to bind its receptor Frizzled a seven-transmembrane protein that interacts with its coreceptors the low-density lipoprotein receptor related protein 5 and 6 (LRP5, 6). These coreceptors are crucial for
the canonical pathway signalling. Upon activation by WNT, the Dishevelled (DVL) is activated and induced the disassembly of the destruction complex composed of AXIN, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 β (GSK3β) therefore allowing the β-catenin to be translocated into the cytoplasm and activate the target of WNT. On the other hand, in absence of WNT, β-catenin is phosphorylated by GSK3-β and therefore results to the degradation of β-catenin by the proteasome. The planar cell polarity pathway, activation of DVL induces Rho GTPases and JNK which are responsible of tissue polarity and dendritogenesis through cytoskeleton changes (Habas, Dawid et al. 2003). Finally, upon binding of WNT to frizzled, DVL induces calcium influx from the endoplasmic reticulum that activates the protein kinase C (PKC) as well as the calcium/calmodulin dependant kinase II (CaMKII).

1.2.1.2. Sonic Hedgehog (SHH) signalling

SHH is a protein part the Hedgehog family that is secreted by the notochord (Placzek et al. 1991). SHH expression is concomitant with the notochord and therefore extend from the caudal part to the rostral part of the head. While the expression of SHH in mouse and fish was observed to precede its initial expression by the floor plate, its expression in the chick coincides in the floor plate and notochord. In vivo, SHH was demonstrated to be important for telencephalic patterning and more specifically for the ventral patterning of the telencephalon. While embryos lacking SHH do not develop ventral telencephalon, ectopic expression results in the expression of floor plate markers, thus markers of ventral identity (Gaiano et al. 1999; Johnson et al. 1994). An SHH signalling gradient is necessary for the generation of different neurons type along the dorsoventral axis. Thereby, SHH along the dorsoventral axis is capable to direct different cell fates at different concentration thresholds.

Upon release from the notochord, SHH binds to its receptor Pached (PTC) and relieves the repression of the receptor on Smoothened (SMO) therefore avoids GLI truncation by inhibiting the protein kinase A. GLI proteins can then promote or repress the transcription of specific target genes (Le Dréau and Martí 2012). Conversely, in absence of SHH, the activity of SMO is inhibited by PTC. The GLI family is downstream of the SHH signalling. The GLI family is composed of three members, GLI1, GLI2 and GLI3 that are expressed in the neural tube. An incorrect expression of GLI1 mimics SHH signalling such as the induction of the floor plate but also dopaminergic and serotonergic markers in the dorsal midbrain and hindbrain (Hynes et al. 1997). GLI2, that is expressed along the dorsoventral axis in the neural tube, is suggested to cooperate with GLI1 to control
cell fate (Hynes et al. 1997). Interestingly, GLI3 is repressed by SHH. Indeed, the gradient of GLI3 is higher in the dorsal telencephalon where the SHH gradient is the weakest (Rallu et al. 2002).

1.2.1.3. The transforming growth factor beta (TGF-β) signalling

TGF-β is a large family of proteins composed of two subfamilies, TGF-β, Activin and Nodal family and the bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) family. These proteins were demonstrated to be responsible of cell proliferation but also differentiation and cell migration.

In addition to its antagonizing role on Wnt signalling, Cerberus also inhibits nodal. Nodal is important for the formation of the floor plate thus its loss result to Cyclop mutant that involves an absence of the floor plate therefore resulting to important alteration of ventral forebrain development.

As previously mentioned, inhibition of the BMP signalling, mainly BMP4 by the three neural inducers is crucial for neural induction (Piccolo et al. 1996; Zimmerman et al. 1996). Experiments aiming to silence BMP2, BMP4 and BMP7 identified that the strongest dorsalization phenotype was achieved by BMP4. Furthermore, complete inactivation of these three BMPs causes complete neuralization of the ectoderm in Xenopus, interestingly, their reintroductions in the same embryo result to restoration of epidermal patterning (Reversade et al. 2005). Early during the development, the expression of NOGGIN and CHORDIN by the organiser is regulated by β-catenin which reduces BMP4 expression.

The TGF-β receptors family is composed of type I and II receptors serine threonine kinase. Upon binding of TGF-β onto its receptors, this one is phosphorylated which in turn induces the phosphorylation of the receptors activated smads (R-SMADs). Subsequently, R-SMADs form a complex with SMAD4 which is translocated into the nucleus to regulate the transcription of target genes (Derynck et al. 2003). R-SMADs activation through type I receptors kinase were observed to be inhibited by SMAD6 or SMAD7. SMAD6 and 7 acts by binding to the receptor therefore preventing its phosphorylation and subsequent recruitment of R-SMADs.

BMPs are secreted by the roof plate, they are important for the dorsoventral patterning of the neural tube. BMP concentration dependant mechanism is high at the dorsal part and
weak in the ventral neural tube which is in opposition with SHH thus the two-signalling pathways antagonised one another.

In vitro, it was demonstrated that the complex, TGF-β/Nodal/activin signalling was important for the pluripotency in hESCs. Thus, its inhibition was shown to induce neuroectodermal fate. Thereby in undifferentiated cells, TGF-β/Nodal/Activin are activated through SMAD2 and SMAD3 (James et al. 2005).

In this thesis, the action of NOGGIN, CHORDIN and FOLLISTATIN is mimicked in vitro by the action of the small molecule LDN193189 and SB431542 that allow the neuronal conversion of our hESCs and iPSCs culture.

1.2.2. Cerebral cortex development

During embryogenesis, the developing forebrain is divided into prosomeres along the rostrocaudal axis. The prosomere 1 is next to the mesencephalon, prosomere 2 and 3 divide the diencephalon while prosomere 4, 5 and 6 divide the telencephalon (Puelles et al. 2003).

The vertebrate forebrain is divided along the dorsoventral axis into the subpallial and pallial. While the pallium generates the cerebral cortex, the subpallium is composed of the GE, lateral, medial and caudal ganglionic eminence (LGE, MGE and CGE). Therefore, the pallial and subpallial boundary, at the level of the LGE, coincides with the expression of Pax6 dorsally and Dlx1/2 as well as Gsx2 ventrally. Pax6 and Dlx1/2 were shown to have complementary effect for the patterning of the forebrain (Yun et al. 2001). Due to the dorsoventral gradient of SHH, it was suggested that SHH could be responsible of the distinction between the LGE and MGE. The transcription factor, NKX2.1 is highly expressed in the MGE and give rise to cortical interneurons and striatal interneurons.

The brain is constituted of different neuronal population which are dispersed in different brain area. The most documented has been the cortical neurogenesis. The mammalian neocortex is composed of two classes of cortical neurons, the interneurons and the glutamatergic neurons which respectively makes local connection and extend axons either intracortical or to other part of the brain (Molyneaux et al. 2007).

1.2.2.1. Cortical Interneurons generation
Cortical interneurons release as neurotransmitter GABA, they are originated from the MGE in the subpallium (Molyneaux et al. 2007). Upon generation, these neurons expressed the transcriptional marker NKX2.1 whose expression is activated by SHH. However, reduction of SHH signalling was shown to reduce the proportion of NKX2.1 progenitors. In addition, mutant mice for Nkx2.1 exhibit a 50% reduction of cortical interneurons. Furthermore, it was observed that the MGE transforms into LGE. Interestingly, in interneurons migrating to the striatum the expression of NKX2.1 is maintained while it is downregulated for interneurons of the cortex.

The MGE primarily gives rise to PV and somatostatin interneurons (SST) whose fate depend upon the level of Shh, indeed, it was demonstrated that high level of SHH preferentially give rise to SST expressing neurons (Kelsom et al. 2013; Xu et al. 2005). These cells are then migrating tangentially in order to populate the pallium. During tangential migration, the interneurons precursors are expressing LHX6 (Figure 1.4). In mouse deficient for LHX6, interneurons were still shown to properly populate the pallium, however they did not express either PV or SST, while on the other hand, an increase of interneurons expressing neuropeptide Y (NPY) was observed. NPY expressing interneurons is another class of cells originating from the MGE. Once interneurons progenitors achieve their tangential migration in the pallium, they switched to radial migration and move towards the appropriate cortical layer within the cortical plate.

Another transcription factor, SOX6 which is expressed by the MGE was shown to be important together with LHX6 for the specification of PV and SST neurons. Mice deficient for sox6 exhibit a strong reduction of SST neurons while PV neurons were absent (Azim et al. 2009; Batista-Brito et al. 2009).
1.2.2.2. Cortical excitatory neurons generation

Cortical projection neurons originate from the lumen of the pallium, a region called the ventricular zone (VZ) and also from an additional proliferative layer above the VZ called the subventricular zone (SVZ). There are three different types of progenitor’s present in the developing cortex, the neuroepithelial cells (NEC), the radial glial cells (RGC) and the intermediate progenitors (IP) (Figure 1.5). Initially, division is symmetrical, one NEC give rise to two NEC, this step is important to expand the proportion of multipotent cells. A transition from NEC into RGC appears following the expression of bHLH (basic helix loop helix) transcription factor form Hes (hairy/enhancer of split) family together with Fgf10 (Hatakeyama et al. 2004; Sahara et al. 2009). This transition was also observed to be dependant of Notch signalling, indeed upon binding of delta localized on a neighbouring cell to the Notch receptors, it leads to the release of the intracellular part of Notch, named Notch-ICD which once translocated into the nucleus promotes the transcription of the Hes genes (Pierfelice et al. 2011). The RGC have long process that extend from the ventricular wall to the pial surface. In addition to their role in the neurogenesis, RGC were also demonstrated to be important to guide neurons into their final cortical layers. At the beginning of the corticogenesis, RGCs from the VZ divide symmetrically in order to expand their population. On the other hand, at the onset of the neurogenesis, the RGCs divide asymmetrical following different modes which can be 1)
a neurogenic division which results in a self-renewing RGC and a neuron, 2) an asymmetric progenitor division of a RGC into an IP or basal radial glial cells (bRGC) and a self-renewing RGC and finally 3) gliogenic division that corresponds to the division of a RGC into a neuron and an astroglia which will be translocated away from the VZ (Kriegstein et al. 2006; Noctor et al. 2004). Additionally, another subdivision of the pallium called the outer SVZ which is absent from mouse is populated by the bRGC (Hansen et al. 2010). During the asymmetrical division, the bRGC can divide into a self-renewing bRGC and an IP.

Neurons constituting the cortex were shown to radially migrate in the developing cortex. The earliest neurons constitute the preplate and then further neurons constitute the cortical plate which split the preplate into the marginal zone also known as the layer I and the subplate which is localized below the layer VI. The cortex is composed of 6 layers which are generated in an inside-out manner. Thus, the early neurons are populated the deep layer (layer VI and V) while the late neurons are migrating to the upper layer (layer IV and II/III).

Figure 1-5 Overview of the cortical neurogenesis. The principal types of NPCs with the progeny they produce are indicated by different colors. Additional NPC types that are typically found in mammalian neocortex are indicated in the box; note that only some of the possible daughter cell outcomes are depicted. Figure adapted with permission from Springer: EMBO reports, Paridaen 2014 (4296970210648) Copyright (2014).
1.3. **Disease modelling using pluripotent stem cells**

The majority of studies about disease modelling have been performed using animal models or primary tissues. Although rodent primary tissues can be used routinely in laboratory, human primary tissues are limited and do not allow to study early developmental stage of a disease.

Embryonic stem cells (ESCs) have the ability to differentiate into any type of tissues of the human body. Our laboratory has the expertise to differentiate ESCs into the four-main neurons population which are excitatory projection neurons, cortical interneurons, MSN and dopaminergic neurons.

Disease modelling can be achieved either by using the novel CRISPR/Cas9 technique described in detail below or taking advantage of the novel technique of induced pluripotent stem cells (iPSCs) to study polygenic disorder which would be more difficult to generate in vitro.

1.3.1. **Pluripotent stem cells**

Embryonic stem cells (ESCs) are derived from the inner mass of the embryo. They have the characteristic to possess unlimited self-renewal capabilities and can thus be maintained in culture indefinitely under the right conditions. The maintenance of pluripotency is controlled by essential genes such as Nanog, Oct4 (Pou5f1) and Sox2

Mouse embryonic stem cells (mESCs) were first used to explore the characteristics of these cells. In culture, mESCs require the addition of myeloid leukaemia inhibitory factor (LIF) to allow their self-renewal and proliferation. Indeed, experiments by Martins *et al.* demonstrated that upon transfer of these cells from feeder layer to gelatin, differentiation occurs (Martin 1981). Later, it was demonstrated that the addition of the recombinant LIF factor to gelatin coated mESC culture was able to maintain the cells into an undifferentiated state (Smith *et al.* 1987). It was also shown that the self-renewal property of LIF factor results from the activation of the transcription factor STAT3 (Niwa *et al.* 1998).

While human embryonic stem cells (hESCs) also originate from the inner mass of the embryo, these cells do not require LIF for self-renewal; instead, support by Fgf2, Nodal and activin is necessary for their self-renewal (Vallier *et al.* 2009). The SMAD2/3 signalling pathway is particularly important for hESC self-renewal. Indeed, SB531542

inhibit SMAD2/3 phosphorylation resulting in decrease expression of oct3/4 (Laping et al. 2002).

Another type of pluripotent stem cells, the induced pluripotent stem cells (iPSCs) were discovered by Yamanaka et al. in 2006. They demonstrated that mouse fibroblasts could be reprogrammed into iPSCs by viral transduction of four transcriptional factors, Oct3/4, Sox2, c-Myc and Klf4 (K. Takahashi et al. 2006). The following year the same authors reported the reprograming of human adult dermal fibroblast into iPSCs using the same four transcriptional factors (K. Takahashi et al. 2007). Figure 1.6 presents the ability of ESCs to differentiation into any types of tissues.

![Figure 1-6 ESCs differentiation](image)

**Figure 1-6 ESCs differentiation.** Schematic of the potential of embryonic stem cell lines. ESCs are extracted from the inner mass of the blastocyst. Following various treatment, they can give rise to any cell types such as muscle cells, blood cells, neurons, intestinal cells, pancreatic cells or liver cells. Picture from Meregalli et al. (K. Takahashi and Yamanaka 2006)

### 1.3.2. Genome editing using CRISPR/Cas9

The recent discovery of the CRISPR/Cas9 technology make it possible to efficiently edit the genome of hESCs. The RNA guided clustered regularly interspaced short palindromic
repeats (CRISPR) contain several short repeats conferring immunity to bacteria and archaea. In addition, the Cas9 in an RNA guided endonuclease cleaving DNA upon recognition of the foreign DNA/RNA therefore confer protection against further infection. The CRISPR/Cas system can be classified into two mains categories which are further classified into six different categories. This classification is based respectively on the presence of multiple or a single protein effector (Cas) which carried out the interference.

The defence mechanism that convey the protection against further posterior infection is divided in three stages, 1) adaptation, 2) crRNA biogenesis and 3) target degradation. The adaptation also called protospacer acquisition enables the host to memorize the unknown genetic material that is subsequently transcribed into a crRNA which is a small guide RNAs. A duplex formed between the crRNA and a tracrRNA (trans activating CRISPR RNA) is then processed by RNase III yielding to the mature small guide RNA that once hybridized to the Cas will confer interference to avoid further infection.

The CRISPR-Cas system as we use it in the laboratory is part of the second family and derived from the bacteria *Streptococcus pyogenes*. The only requirement is a 20 nucleotides guide sequence complementary to the targeted DNA which is immediately preceded by a 5’NGG protospacer adjacent motif (PAM). These three nucleotides sequence are responsible of triggering the transition between target binding and cutting conformation of the Cas9. Upon cleavage by the Cas9, the targeted sequence undergoes one of the two pathways responsible of DNA damage repair, respectively, the error-prone Non-Homologous End Joining (NHEJ) or the high fidelity Homologous Direct Repair (HDR). NHEJ has a higher capacity of repair and cause the introduction of unpredicted insertion or deletion (INDELs) in one or two alleles that can cause frameshift in the protein coding region by the introduction of a premature STOP codon, leading to loss of function mutation. Unlike NHEJ, HDR uses a donor template similar to the targeted regions allowing integration of an exogenous DNA sequence. Although this method is used worldwide as a tool for genome editing, a major drawback is the possibility for the nuclease to cleave off-target DNA targets called off-target mutagenesis. However, the addition of two extra base pair, a guanine, was demonstrated to reduce the off-target effect (Kim et al. 2015). This technique was demonstrated to efficiently edit the genome to generate KO in our laboratory. Furthermore, as an example, report demonstrated the generation of hESCs line deficient for DNMT3B (Horii et al. 2013). More recently it was also used to correct disease such as Huntington disease or Parkinson disease (Chung et al. 2015).
2013; X. Xu et al. 2017). Finally, it was reported that CRISPR/Cas9 could also be used for generating microdeletion and microduplication (Tai et al. 2016).

1.3.3. HESCs and or iPSCs to study the development of neurons

Due to their ability to give rise to any type of tissue, hESCs and iPSCs (hPSCs) offer a powerful tool to study neuronal development. In vitro differentiation of hPSCs into neurons can be achieved either through embryoid body formation or monolayer differentiation. Cells differentiating as monolayer are exposed to the various exogenous factors relatively uniformly and exhibit more synchronous differentiation than those differentiating in embryoid bodies. Therefore, unless for addressing question that require a 3D structure, such as cortical layer specification, studies in our laboratory use monolayer differentiation as a routine.

Monolayer differentiation was first described by Chambers et al., in which TGF-β and BMP inhibitor was used to convert hPSCs into neurons fate (Chambers et al. 2009). The molecule SB431542 (SB) was used to block the phosphorylation of ALK4, ALK5 and ALK7, therefore inhibit SMAD2/3 phosphorylation and thus inhibit the TGFβ/Activin/Nodal pathway. Thereby, dual smad inhibition by noggin and SB was demonstrated to be able to convert hPSCs into neurons. Later, noggin was replaced by LDN193189 (LDN), this molecule inhibits the BMP signalling pathway and was demonstrated in combination with SB to efficiently give rise to PAX6 positive cells, a neuroectodermal marker. Compared to dorsomorphin which is another BMP antagonist, LDN was demonstrated to be less toxic (Surmacz et al. 2012). Interestingly, this protocol was demonstrated by default to give rise to neurons from the dorsal telencephalon.

While neuronal conversion of hPSCs preferentially give rise to neurons from the dorsal telencephalon and therefore excitatory projection neurons, it is necessary to add further molecules to promote the generation of others neuronal population.

To generate cortical interneurons, an additional factor is necessary during the neural conversion, XAV939. This small molecule that substitute DKK1 is a WNT signalling inhibitor that simulates the degradation of β-catenin, therefore strongly increases telencephalic commitment and thereby increases the proportion of neural progenitors expressing FOXG1. Time window expression of SHH and its agonist purmorphamine promotes the induction of cells with a ventral identity expressing NKX2.1. Therefore,
progenitors with a MGE identity are expected to express protein such as NKX2.1 and FOXG1 but also OLIG2, a marker of cells originating from the MGE.

Medium spiny neurons (MSN) are primarily GABAergic projection neurons that form the striatum which is one of the nuclei of the basal ganglia. MSN originates from the LGE, a structure of the forebrain. The location of the LGE below the pallium requires the precise concentration of SHH with or without WNT inhibitor. Thereby several experiments over the years investigated the time window and concentration of WNT inhibitor and SHH that would lead to the highest proportion of dopamine and cAMP-regulated neuronal phosphoprotein (DARPP32) positive cells, a MSN marker (Aubry et al. 2008; Delli Carri et al. 2013; Ma et al. 2012; Nicoleau et al. 2013). A recent publication from our laboratory reported the generation of MSN using activin A which was demonstrated to promote the generation of neurons with an LGE identity (Arber et al. 2015). Therefore, following conversion into neurons, cells require the addition of activin A to obtain an LGE identity which is known to express the transcription factor GSX2 as well as CTIP2.

Compared to the other three neuronal populations previously presented, dopaminergic arise from the ventral midbrain. During development, the midbrain hindbrain boundary (MHB) is formed through the repression of the transcription factor orthodenticle homolog 2 (OTX2) and gastrulation brain homeobox 2 (GBX2). The patterning of the MHB is regulated by a gradient of WNT1 and FGF8 respectively from the midbrain and hindbrain. It was demonstrated that brain explants at distance from the source of FGF8 was able to generate midbrain dopamine neurons (Arenas et al. 2015). Therefore, midbrain dopaminergic neurons induction employs both SHH and FGF8.

In this thesis, all these four neuronal types were generated in order to evaluate whether the expression of specific genes differ depending on the neuronal population.

1.3.4. Disease Modelling using iPSC

Since the discovery of iPSCs, it is now possible to study the development of neurons from individuals with genetic background associated with monogenic neurological disorders such as Huntington disease or Amyotrophic lateral sclerosis. In addition, iPSCs are particularly valuable for the study of polygenic disorders such as 22q11.2DS which
results of the haploinsufficiency of more than 30 genes or also diseases with a genetic background not yet identified.

Thereby, Brennand et al., report the first study modelling schizophrenia using iPSCs. They demonstrated that neurons from individuals with schizophrenia had an impaired neuronal connectivity using a modified rabies virus (Brennand et al. 2011; Wickersham et al. 2007). They also observed an alteration of the overall morphology of neurons and identified using transcriptomic analysis novel signaling pathways such as NOTCH signaling that were not previously linked to schizophrenia. Physiological assessment was performed using electrophysiology and calcium imaging, the last one will be further discussion later. This study was the first to report the use of iPSCs to study schizophrenia. However, this study uses iPSCs from patients with idiopathic schizophrenia therefore an heterogenous genetic background, because schizophrenia is a multigenic disorder the results observed in this study might differ between individuals.

Several other studies reported the use of iPSCs from Parkinson disease patients. Schöndorf et al., reported the use of iPSCs from individuals with glucocerebrosidase ($GBA1$) mutation. While homozygous mutation is responsible of Gaucher syndrome, a lysosomal storage disease, heterozygous mutation of $GBA1$ is associated with higher risk to develop Parkinson disease. In this study, iPSCs were differentiated into midbrain dopaminergic neurons. The dopaminergic population was further enriched using fluorescence-activated cell sorting. They examined the overall expression of key markers of midbrain dopaminergic neurons differentiation, they also observed lysosomal and autophagic defect in mutant neurons therefore emphasizing that iPSCs can replicate in vivo pathology. In addition, they also observed an impairment of the calcium homeostasis in the neurons with $GBA1$ mutation.

Another study from Pașca et al., used fibroblasts from individuals with Timothy syndrome which were reprogramed to obtain iPSCs. This pathology was demonstrated to cause a missense mutation in the L-type calcium channel, $CACNA1C$ gene. The study investigates the development of the iPSCs into a protocol of cortical neurons precursors and neurons. They evaluated the calcium activity using Fura 2AM, a radiometric dye able to measure intracellular calcium which allow them identified calcium activity defect in neurons with Timothy syndrome mutation. Furthermore, they also performed single cell q-PCR which identified a decrease expression of lower cortical layer genes.
Several studies also evaluated neurons derived iPSCs from individuals with 22q11.2DS. In their study, Belinsky et al., used two hPSC from individual with 22q11.2DS. Their study investigated the electrophysiological and calcium imaging activity in neurons with a forebrain identify which was compared to a healthy individual. Electrophysiology and calcium imaging activity were performed simultaneously several times from day 13 to 88. No important differences were observed regarding action potential property and frequencies.

However, most study investigating iPSCs from 22q11.2DS primarily focused on transcriptomic analysis such as study from Toyoshima et al, as well as Lin et al, which investigated the transcriptome of neurons derived from individuals with 22q11.2DS.

Instead of studying the entire 22q11.2 deletion, we aimed to understand the function of one specific gene within this deletion. Therefore, we investigated its role during neuronal development using assays such as calcium imaging to determine whether the physiology of the neurons was altered in our mutant neurons.

1.3.5. Calcium Imaging a technique to assess neuronal activity in neurons derived stem cells

Calcium ion is responsible of many function in cells, such as muscle contraction but it is also involved in several steps of the cell cycles. In neurons, calcium is essential for several functions such as propagation of action potential and acts as second messenger through G protein couple receptors (GPCRs). In resting neurons, calcium concentration varies from 50 to 100nM. However, during electrical activity, this concentration can transiently rise ten or even hundred times higher (Berridge, et al. 2000). Overall, the calcium concentration is maintained lower enough at rest to allow a significant change without extensive energy cost during electrical activity. The calcium homeostasis is determined by the calcium efflux and influx (Figure 1.7). Calcium efflux is mediated by the plasma membrane ATPase, the sodium calcium exchanger and the endo/sarcoplasmic reticulum calcium ATPase (SERCA). Instead, the calcium influx is composed of the voltage gated calcium channel (VGCC), the ionotropic receptors (NMDA, AChR, AMPA). Finally, internal storage of calcium can be released through the ryanodine receptor or the inositol 1,4,5-trisphosphate receptor (IP₃).
The process of neurotransmitter release requires the calcium to have a dual role. First the calcium enters axons terminal through the VGCC following depolarization of the plasma membrane. Then the calcium binds to the calcium sensor synaptotagmin which is localized at the surface of the synaptic vesicle. Upon binding, synaptotagmin interact with SNAP25 and synaptobrevin (SNARE protein) which are anchored at the presynaptic membrane and allow the release of the neurotransmitter within the synaptic cleft (Mohrmann et al. 2013).

The plasma membrane calcium channel is comprised of four different groups which are composed of calcium influx, the VGCC, the NMDA receptors, the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-sensitive receptors (AMPARs). Calcium signalling can further be mediated through GCRPs located on the neurons, the metabotropic glutamate receptors.

Upon depolarization, the VGCCs are important to initiate the calcium signalling cascade. The VGCCs are composed of two main classes based on their thresholds of activation, the high and low voltage activated channel respectively, HVA and LVA (Catterall 2000).
The HVA can be further subdivided into four categories, the L-type composed of the Ca\textsubscript{v}1.1 to Ca\textsubscript{v}1.4, the P/Q type (Ca\textsubscript{v}2.1), the N type (Ca\textsubscript{v}2.2) and the R type (Ca\textsubscript{v}2.3). The LVA instead is only composed of the T type (Ca\textsubscript{v}3.1 to Ca\textsubscript{v}3.3).

Amongst the L-type calcium channel, the Ca\textsubscript{v}1.2 is encoded by the \textit{CACNA1C} gene which was shown to be expressed in neuronal cell body and dendrites. This channel permits large influx of calcium that activates pathways necessary for long term potentiation (Benarroch 2010). The Ca\textsubscript{v}1.3 (\textit{CACNA1D}) is expressed by auditory hair cells (Kollmar et al. 1997). Overall the Ca\textsubscript{v}1 family is important for synaptic transmission, neurotransmitter release and generation of calcium transient in dendrites (Brini et al. 2014).

The Ca\textsubscript{v}2 family are present at the presynaptic membrane but also on cell body and dendrites where they are responsible for the fast neurotransmitter releases. The P/Q channel type (Ca\textsubscript{v}2.1), encoded by \textit{CACNA1A}, is expressed at the excitatory synapses but also by the Purkinje cell from the cerebellum. The N-type channel (Ca\textsubscript{v}2.2), encoded by \textit{CACNA1B}, is involved in the neurotransmission at the inhibitory neurons, but also noradrenergic and the dorsal root ganglia. Both Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 were observed to be regulated through G protein coupled (Hille 1994).

The T type channel corresponding to the LVA channel were demonstrated to be located at the soma as well as the dendrites in most brain region. It was observed that gain of function of the Ca\textsubscript{v}3 channel was responsible of epilepsy in human and genetic mouse model (Khosravani et al. 2006). This family composed of three channels, Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 encoded respectively by \textit{CACNA1G}, \textit{CANAIH} and \textit{CANAIL}.

The calcium permeable AMPA receptor is anchored to the postsynaptic membrane. Upon binding of glutamate, different types of ions can enter into the cell through the channels pore. This receptor is responsible of the fast-synaptic transmission. AMPA receptors are permeable to sodium Na\textsuperscript{+}, potassium K\textsuperscript{+} and also in some case calcium and zinc et al 2007). The AMPA receptor is an heterotetramer composed of GluA1, GluA2, GluA3 and GluA4 which are encoded by different genes known respectively as, \textit{GRIA1}, \textit{GRIA2}, \textit{GRIA3} and \textit{GRIA4}. It was reported that the AMPA receptor in the brain is mainly in the form of an heteromeric complex composed between GluA1 with GluA2 or GluA2 and GluA3 subunits (Lu et al. 2009). Instead, the GluA4 subunit is developmentally regulated and expressed at adult synapse (Lu et al. 2009). There are two forms of GluA2 subunit, a
permeable to calcium which can be either GluA2 lacking or unedited GluA2-containing and a calcium impermeable named edited GluA2-containing. The difference between the unedited and edited is the presence of a glutamine or an arginine respectively on the GluA2 subunit at the 607 amino acids.

The NMDA receptor is localized at the postsynaptic membrane of neurons. The receptor is an heteromeric complex which is composed of either GluN1, GluN2 or GluN3 subunits. Each NMDA receptor contain at least a GluN1 subunit. The GluN2 subunit is subdivided into four, GluN2A, GluN2B, GluN2C and GluN2D. During development, the majority of NMDA receptors are composed of GluN1 and GluN2B subunits, however during postnatal development, there is a switch in subunit composition from GluN2B to GluN2A (Berridge, Lipp, and Bootman 2000). Compared to the AMPA receptor, the NMDA receptor has a high permeability for calcium which usually occur upon triggering of long term potentiation (LTP) or LTD.

Upon release of glutamate by the presynaptic neurons, the neurotransmitter binds to the AMPA receptor. Thus, the channel of the AMPA receptor opens provoking an influx of Na§ into the postsynaptic neurons which in turn induce depolarization of the postsynaptic membrane. If the depolarization if strong enough, it removes the magnesium block on the NMDA receptor and therefore allow an influx of calcium in the postsynaptic neurons which lead to activation of several molecular pathways (Brini et al. 2014).

The metabotropic glutamate receptors (mGluRs) are composed of 7 transmembrane G coupled receptors. The mGluRs are classified into three categories. The group I includes mGluRs 1 and 5 and are mostly expressed at the postsynaptic membrane than the presynaptic membrane of glutamatergic neurons. The group I is couple to G_q/G_11, which are responsible of downstream activation of the phospholipase C and therefore induces calcium inside the cells. Often, the function of mGluR1 and mGluR5 are different depending of the neuronal population. The group I also plays an important role in fast synaptic transmission conveyed by glutamate, it also involves in the induction of LTP and LTD (Bellone et al. 2008; Kullmann et al. 2008). The group II includes the mGluR2 and 3. Conversely to the group I, the group II is couple to G_i/o which results in the inhibition of the adenylate cyclase and thus calcium channel. The mGluR2 and mGluR3 are usually found on presynaptic membrane and inhibit neurotransmitter release at both glutamatergic and GABAergic synapses. In addition, the group II of metabotropic receptor can also be localized at the postsynaptic membrane where it induces
hyperpolarization (Muly et al. 2007). Also, the mGluR3 was found to be expressed by glial cells.

Finally, the group III of metabotropic receptors is composed of mGluR4, mGluR6, mGluR7 and mGluR8. Similarly to the metabotropic receptors of the group II, the group III is coupled with G_{i/o} and thus is also inhibiting calcium channel but also regulates potassium channels. However, it was demonstrated that group III is coupled with other signalling pathways than the adenylate cyclase, such as the MAPK or the phosphatidylinositol 3-kinase (Iacovelli et al. 2002). The metabotropic group III was observed to be in similar location than the group II.

Calcium indicator

Several techniques exist to record and evaluate the calcium activity in vivo and in vitro. While most of these techniques involved the loading of the calcium indicator using sharp micropipette during whole cell patch clamp recording. It is currently possible to load bulk of neurons either in vivo and in vitro using dye conjugated with acetoxyethyl ester (AM). Once loaded, cells are able to remove the ester because of the presence of intracellular esterase. In addition, new generation of genetically encoded calcium indicators (GCaMP) enable to evaluate calcium activity without injection of a dye. Furthermore, they can be used to target specific neuronal population in vivo.

In this thesis, we used Fluo-4-AM, a dye derived from the Fluo-3. Fluo-4 exhibits brighter dynamic range than Fluo-3 therefore is able to detect smaller change in the calcium activity. The excitation emission of this dye ranges from 494/506 nm respectively and therefore do not necessarily require the use of a confocal microscope.

1.4. Aims

The overall aims of this thesis are:

- To investigate the temporal and spatial expression profile of the genes between LCR A and B of the 22q11.2DS in different neuronal population
- To examine the role of ZDHHC8 during cortical excitatory projection neurons development.

The thesis is then subdivided into the three following results chapters:
1. The 22q11.2 mutation is composed of a multitude of genes whose function during brain development is not clear yet. Therefore, this study aims to explore whether genes within this mutation revealed a specific temporal and spatial expression profile through the examination of gene expression at different time points and/in different neuronal population (cortical interneurons, glutamatergic projection neurons, dopaminergic neurons and medium spiny neurons). The results would be an indication of the potential underlying function of those genes during brain development which therefore might guide which gene to study in future study.

2. In the chapter 4, I have used the novel CRISPR/Cas9 technique to generate a hESCs line deficient for ZDHHC8 whose function will further be studied in a protocol of glutamatergic neurons differentiation. The chapter 4 also aims to investigate whether ZDHHC8 haploinsufficiency alters the kinetics of glutamatergic neuronal differentiation and alters the expression of several key genes necessary for proper neuronal maturation. Moreover, both to elucidate its physiological role during neurons differentiation and due to its association with reduced dendritic and axonal arborisation, the motility and the spontaneous calcium activity was also investigated in neurons derived from hESC heterozygous for ZDHHC8.

3. Lastly, the chapter 5 investigates the transcriptome of ZDHHC8+- at the neuronal progenitor level in order to determine whether heterozygous deletion of ZDHHC8 affects signalling pathways responsible of neurons differentiation and maturation. These results were then compared to the transcriptome of progenitor cells from 22q11.2 derived hiPSC to identify if similar pathways are altered.
2. Materials and Methods

2.1. Cell Reprogramming

2.1.1. 22q11.2 patient

The 22q11.2 hiPSC line analysed in this PhD was recruited via clinic assessment at the National Centre for Mental Health by Professor Jeremy Hall. The individual consented to the DEFINE/ECHO and NCMH consent form. The patient is a female of 27 years old who was diagnosed with 22q11.2DS at the age of 14. She is thought to have inherited the 22q11.2 CNV from her mother who also carries a 22q11.2 mutation. She had speech and language delay during her childhood and was diagnosed with dyslexia. She also experienced delayed motor skills development. Her current medication is Aripiprazole 10mg and Fluoxetine 20mg twice a day. In terms of psychiatric disorders, the patient was diagnosed with ASD, intellectual disability as well as schizophrenia.

2.1.2. Control subject

The subject is a 27 years male who has been recruited and agreed to the NCMH participant consent form. The patient did not report any psychiatric disorders. His medical history did not mention any known disease.

2.1.3. Fibroblast reprogramming

A skin biopsy from individuals consenting to take part in the study was performed by a dermatologist and further processed by Craig Joyce. Directly after collection, the biopsy was kept in fibroblast media prior to being plated. The fibroblast media was composed of 90% DMEM (GIBCO), 10% of Fetal Bovine Serum (FBS, GIBCO), 1% of MEM Non-Essential Amino Acids Solution (Life technologies), 10mM and 0.1% of β-mercaptoethanol 55mM (Life technologies). After plating, fibroblasts were grown for a couple of passages and either frozen down or processed for reprogramming.

The CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Life technologies) was used to reprogram the fibroblasts. Two days prior the transduction, the fibroblasts were replated onto two wells of a 6-well plate and kept in the fibroblast media. The day of the transduction, fibroblasts were usually at 50-80% confluency. A volume of fibroblast
medium was pre-warmed prior to harvesting cells. Cells were harvested in 0.5ml of TrypLE™ per 1 well of a 6-well plate and incubated at room temperature until cells started to detach, at which point a double volume of fibroblast medium was added to the cells to inactivate the TrypLE. A portion of cells was taken out to perform cell counts using Trypan Blue exclusion criteria. The volume of virus was calculated using the formula below.

\[
\text{Volume of virus [µL]} = \frac{\text{MOI [CIU/cell] \times number of cells}}{\text{titer of virus [CIU/mL] \times 10^{-3} [µL/mL]}}
\]

The MOI for each virus were as follow, hKOS = 5, hcMyc = 5, hKLF4 = 3. The appropriate volume of virus was added to 1ml of fibroblast medium and subsequently added into the wells. The following day, the medium was replaced with fresh solution and then changed every day until the sixth day post transduction. At day 7, a second run of transduction was performed with the hKLF4 virus using similar concentration to that in the first transduction. Fresh medium was replaced every day for the next seven days. On the fourteenth day, iPSCs colonies were picked and replated onto Matrigel coated plates in mTeSR™1 (STEMCELL). After a couple of passages, mTeSR1 was switched to TeSR™-E8™ (STEMCELL).

2.2. Cell culture

All cell cultures were maintained in an incubator (Galaxy 170R, New Brunswick) at 37°C and 5% CO₂. All cell work was performed under a laminar flow hood (Maxisafe 2020, Thermo Scientific). The cells were grown on treated plastic 6-, 12- or 24-well plates (Thermo Scientific) or glass coverslips (VWR).

2.2.1. hESCs and hiPSCs culture

Experiments were carried out using an H7 hESC, iCas9 hESC, 22q11.2 iPSC, and 900 iPSC lines. Cell culture dishes were coated with hESC qualified Matrigel (Corning). An aliquot of Matrigel was resuspended into 6ml of Nutrient Mixture F-12 (DMEM/F12, GIBCO) media then 1ml/well of the solution were added and incubated for at least 1h at 37°C. Cells were maintained in TeSR-E8 medium except where mentioned otherwise. While media was changed every day, cells were passaged as follows once reached 60 to
80% confluence. Media was aspirated and cells were washed with Dulbecco's phosphate-buffered saline (DPBS, GIBCO) and incubated with Ethylenediaminetetraacetic acid (EDTA) 0.02% (Sigma) at 37°C for up to 5 minutes. Once the EDTA was removed, the cells were manually scratched to dissociate colonies into small clusters using a serological pipette. Cell suspension was gently centrifuged at 1150 rotation per minute (rpm) for 3 minutes. Cell pellet was then resuspended in fresh medium and seeded at 1:1, 1:2 and 1:5 ratios.

2.2.2. Freezing and thawing of stem cells

Cells were passaged as mentioned in Section 2.2.1. Once centrifuged, the pellet was resuspended in a solution of TeSR™-E8™ medium containing 10% of Dimethyl sulfoxide (DMSO, Sigma) for cryoprotection. Cell suspension was transferred into a 1.5mL cryovial (Thermo Scientific) and placed into a cell freezing container which allows a slow freezing rate of -1°C/min (Bioscission) when placed at -80°C. Cells were ready to be placed in liquid nitrogen after at least 3 hours at -80°C.

Thawing cells compared to freezing needs to occur quickly to stop ice crystals from damaging the cells. Cells were transferred from liquid nitrogen straight to 37°C in a water bath. They were then resuspended in 5 volumes of DMEM-F12 and centrifuged at 1150rpm for 3 minutes in order to dilute and remove DMSO. Cells were resuspended in TeSR™-E8™ medium and seeded into a well of a 6-well plate.

2.2.3. Neuronal differentiation

To begin with neuronal differentiation, cells reaching 70-80% confluence were replated into an appropriate number of wells coated with reduced growth factor Matrigel (Corning). Cells were let to grow in stem cell media for up to two days until they reached 80% confluence. Then cells were treated accordingly depending on the protocol.

A basal neuronal medium was prepared as follow, 100 ml of DMEM-F12 + 1ml of B27 supplement 50X w/o Vitamin A (Life technologies) with 50 ml of Neurobasal (Life technologies) + 1 ml of N2 supplement 100X (Life technologies), the total volume was supplemented with 2mM of L-glutamine (Life technologies) and 1:500 MycoZap™ Plus-CL (Lonza) and 0.1mM of β-mercaptoethanol (Gibco).
2.2.3.1. Cortical projection neurons

To start cortical excitatory neuron differentiation, TeSR™-E8™ media was removed and cells were washed with DPBS. Media was replaced with the basal neuronal media supplemented with 10μM SB431542 (Tocris) and 100nM LDN (STEMCELL), a modified dual-smad inhibition protocol (Arber et al. 2015; Chambers et al. 2009) to promote neuroinduction. Every other day, half of the media was changed. Between days 9 and 10, cells were passaged onto fibronectin coated plates (15μg/ml, Millipore). In brief, manual dissociation of the cells was performed using 0.02% EDTA in a similar way to that used for stem cells (see Section 2.2.1). Cells were collected and gently homogenised in neuronal media supplemented with 10μM ROCK inhibitor (Tocris) to promote cell survival. Cells were then replated at a 2:3 ratio and half of the media was replaced every other day. At day 12, LDN and SB were removed from the basal neuronal media and media was changed every other day. About 10 days after the first passage, cells were incubated in StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific) for up to 10 minutes, collected and triturate with a P1000 (Eppendorf) in order to obtain a quasi-single cell solution. Cells were replated onto wells coated with a solution of poly-D-lysine (Sigma) at 0.01mg/ml and laminin (Sigma) at 10μg/ml at a density of 75000 cells/cm² and 125000 cells/cm² for calcium imaging/immunofluorescence and RNA extraction, respectively. At day 20, B27 w/o Vitamin A in the basal neuronal medium was replaced with B27 with Vitamin A and cells were every few days as needed by changing half of the media.

2.2.3.2. Cortical interneurons

For cortical interneuron differentiation, neuronal conversion was performed using basal neuronal media supplemented with 10μM SB431542, 100nM LDN, and 2μMXAV939 (Tocris) until day 9. From day 10 to day 18, 200ng/ml of Sonic Hedgehog (SHH, R&D) and 1μM purmorphamine (STEMCELLS) were added to the basal neuronal media. During the second passage, cells were split at a ratio of 1:5. From day 20, the media was supplemented with 10ng/ml of BDNF (PeproTech) and 10ng/ml of GDNF (PeproTech).

2.2.3.3. MSNs (Dr Marija Fjodorova)

For MSN differentiation, the neuronal conversion was performed in the same way as for cortical projection neurons. SB and LDN were stopped and basal neuronal media was supplemented with 25ng/ml of Activin A from day 10 onwards. During the second
passage, cells were split at a ratio of 1:1 and BDNF and GDNF were added at the same concentration as in cortical interneuron differentiation.

2.2.3.4. Dopaminergic neurons (Dr Marija Fjodorova)

Finally, for dopaminergic neuron differentiation, neuronal conversion was performed with 10μM SB431542 and 100nM LDN from day 0. From day 3 to 7, neuronal conversion media was supplemented with 1μM PD0325901 (Sigma). At day 7, LDN, SB, and PD were removed and 200ng/ml of SHH and 1μM purmorphamine were added to the basal neuronal media until day 16. During the first passage at day 9, 100ng/ml of FGF8 was also added to the neuronal media until day 16. Following the second passage at day 20, BDNF and GDNF were added to the basal neuronal media at the same concentration as in the cortical interneuron differentiation.

2.2.4. Human primary astrocyte culture

Human primary fibroblasts (Lonza) were plated onto 75ml flask without coating. Primary medium was prepared with 50ml of neurobasal supplemented with 10% FCS, 1ml of B-27, 1:500 Mycozap, and 2mM L-glutamine.

Astrocytes were passaged once confluent at a ratio of 1:3. Cells were incubated in Trypsin for 5 to 10 minutes followed by the inactivation of the enzyme with a double volume of media. Cells were gently trititated and dispensed accordingly.

2.3. Gene expression analysis

2.3.1. RNA isolation and RT-PCR from cell culture

Total RNA was isolated from cultured cells according to the protocol supplied with TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA).

Concentration and purity of RNA samples were determined using a spectrophotometer (Eppendorf) which scans in the ultraviolet (UV). The absorbance ratio 260/280 was measured, a value above 2.0 was considered as pure.

To prevent DNA contamination, RNA samples were treated with DNase, PerfeCTa DNase I (Quanta bioscience). The reaction was designed as follow:
Reagents were combined in 0.2ml tubes sitting on ice. Each reaction was gently vortexed followed by a centrifugation to collect the solution at the bottom of the reaction tube. Samples were incubated at 37°C for 30minutes, then 1µl of 10X Stop buffer was added to each sample. Reactions were gently mixed and then centrifuged to collect samples at the bottom of the tube. Samples were incubated 10 minutes at 65°C. The entire reaction volume was then used as a template for the first-strand cDNA synthesis. Total RNA was reverse transcribed with the qScript cDNA superMix (Quanta Bioscience). The reaction was performed as follow:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qScript cDNA Supermix</td>
<td>4µl</td>
</tr>
<tr>
<td>RNA template</td>
<td>2µl</td>
</tr>
<tr>
<td>RNase/DNase free water</td>
<td>14µl</td>
</tr>
<tr>
<td><strong>Total Volume (µl)</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Reagents were added to 0.2ml tubes sitting on ice. The reactions were vortexed and subsequently centrifuged in order to collect the solution at the bottom of the tube. Tubes were placed in a thermocycler, which was set up as follow:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
</tr>
</tbody>
</table>

Samples were then kept at -20°C until processed for RT-PCR.

### 2.3.2. Pre-amplification

Prior to gene expression analysis, cDNA samples from the reverse transcription were pre-amplified in order to obtain optimal cDNA concentrations for gene expression.

In a 0.2ml tube, 1µl of each 100µM stock primer was added. Final volume was adjusted to 200 µl with the DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova, PN T0221), a full list of primers is presented in Table 2-1. When possible,
primers were designed to have a PCR product not exceeding 200bp. To prepare the reaction solution, reagents were added to a 1.5ml Eppendorf tube together with the mix of primers as presented below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol per reaction (µl)</th>
<th>Vol for 96 Reactions w/Overage (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preamp Master Mix (Fluidigm PN 100-5580)</td>
<td>1</td>
<td>105.6</td>
</tr>
<tr>
<td>Pooled Primers (500nM)</td>
<td>0.5</td>
<td>52.8</td>
</tr>
<tr>
<td>DNase free water</td>
<td>2.25</td>
<td>237.6</td>
</tr>
<tr>
<td>cDNA</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL Volume</strong></td>
<td><strong>5</strong></td>
<td><strong>-</strong></td>
</tr>
</tbody>
</table>

A 96-well plate was filled in by dispersing 3.75µl of the premix. Then 1.25µl of cDNA was added into individual wells. Samples were vortexed and centrifuged at 1000g for a minute. The pre-amplification reactions were loaded into a thermocycler using parameters presented below:

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>15 secs</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Following this, samples required incubation with exonuclease I (New England Biolabs PN M0293S) to remove unincorporated primers. In total, 2µl of diluted Exonuclease I at (4U/ µl) were added to each reaction and incubated at 37°C for 30 minutes followed by 15 minutes at 80°C. The final product was diluted 1:5 in TE buffer.

Table 2-1 List of primers

<p>| GAPDH          | F: aagaccccttcattgacctcaact  |
| R: atattttcgttggtccacccat |
| MASH1          | F: gtcttgtegcaccacatetc     |
| R: cctccccacgcaetatgcac  |
| NGN2           | F: tcaagaagaccecttagctgaagg |
| R: gtgaatcgccacagatgttgg |
| B-ACTIN        | F: tccacacacggccagagcg     |
| R: tecccttcgacctcttg   |
| NESTIN         | F: agcaggagaaacagggctac    |
| R: ctctggggcttaggaattg  |
| OTX2           | F: tgccaanaagaagacatctcaca |
| R: aagctgggcctcagataagac  |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUJ1</td>
<td>F: acctcaaccaacctggtatgcg</td>
<td>R: tgcgttctctgctgagaag</td>
</tr>
<tr>
<td>FOXG1</td>
<td>F: tgtgccagaggttggagtcg</td>
<td>R: ttggcctctctgctgagaag</td>
</tr>
<tr>
<td>LDB1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>NNX2.1</td>
<td>F: tggccgcaaggttggagtcg</td>
<td>R: ttggcctctctgctgagaag</td>
</tr>
<tr>
<td>NNX6.1</td>
<td>F: acacgacaccacacacttccg</td>
<td>R: ttggcctctctgctgagaag</td>
</tr>
<tr>
<td>CALRET</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>REELIN</td>
<td>F: acacgacaccacacacttccg</td>
<td>R: ttggcctctctgctgagaag</td>
</tr>
<tr>
<td>EOMES</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>BRN4</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>EBF1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>ISL1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>ARPP21</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
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<tr>
<td>DARPP32</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>DRD1</td>
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<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>CRABP1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>LHX8</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
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</tr>
<tr>
<td>ZCCHC12</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>PRODH</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>DGC1R14</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>GSC2</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>CLTCL1</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>HIRA</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
<tr>
<td>UFD1L</td>
<td>F: gcgacgctgctgcgtgctgtc</td>
<td>R: tgccgctctctgctgagaag</td>
</tr>
</tbody>
</table>
### 2.3.3. Gene expression using Biomark HD

In a 0.2ml tube, the SsoFast™ EvaGreen® and 20X DNA binding dye (sample pre-mix) were combined with each sample as specified in the table below. In brief, the master mix of the sample pre-mix was vortexed and centrifuged at 1000x for 10 seconds. A total of 3.3μl of pre-mix was added into individual tubes together with 2.7μl of the final product of pre-amplification. To assure correct homogenisation, samples were mixed then centrifuged at 1000g for at least 30 seconds.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
<th>Gene</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
</tr>
</thead>
</table>
| CLDN5 | F: tctgtggttgccaaaca  
R: acgctgtactgacttcgca | SEPT5  | F: aggacttcaiagcagcaggac  
R: caccgtgtgcctataac |
| GP1BB | F: ccacagtgggaacagtcagtt 
R: ttggaggagacagtgacg | TBX1  | F: tggcaactcagagtctggtgag  
R: gctggtactgctcttgatag |
| GNB1L | F: gattcgtgccttgactttgac  
R: gctggtactgctcttgatag | C22orf29 | F: ttcgcgtagcctcagattgc  
R: gacagggatggctgaacagg |
| TXNRD2 | F: gcatagccacttgacttgacg 
R: cgggctgtttcctgtctcatca | ARVCF | F: gngtgctgcacaaaagaaaaa  
R: gctgtgaaagttctgcgccttc |
| COMT  | F: aggacttcaiagcagcaggac  
R: caccgtgtgcctataac | C22orf25 | F: ttcgcgtagcctcagattgc  
R: gacagggatggctgaacagg |
| DGCR8 | F: tggcgtgtgtcctgcacatca  
R: tcgctgatgctcaccatcc | TRMT2A | F: cactccgaagcccaacagaa  
R: tctgggctgtactgttctgcttc |
| RANBP1 | F: gacccctagtgccagacttgac  
R: ctcagaggcaaatcggaaca | ZDHHC8 | F: acgctgtcgtgcaactgtgta  
R: gatgctggaagctgccccagga |
| ZDHHC8-F | F: catggacccctggttttccc  
R: ecacacatccaggtttgta | ZDHHC8-I | F: cccagctgctgcaactgtgta  
R: gatgctggaagctgccccagga |
| RTN4R | F: gccaaccctcatgtagaa  
R: acgctgtcgtgcaactgtgta | SHANK1 | F: ggtctgcgttcagctggttgag  
R: cccctgtctccctgggccaat |
| NANO | F: gctggtggtcctgtctctgc | OCT4 | F: cctctgtcctccctccta  
R: gctggtggtcctgtctctgc |
| GLUN1 | F: acaagacatccacttgacttgac  
R: tctctttcctgctgtctctgc | GLUN2A | F: cccagctgctgcaactgtgta  
R: gatgctggaagctgccccagga |
| GLUN2B | F: tcttgaccggaagatccaggg  
R: tcagatgctgtcactacgcag | GAD1 | F: cgcagatcttgagccccaggg  
R: cgtgcgtagctgctcttgacg |
| CUX1 | F: tctgagggcgaaccagag  
R: acctctgtctgctcttcgaag | PALM | F: cggctgacccaagtccaggtc  
R: ccaccacatcagcctagcttc |
| GLI1 | F: tggcctgactccttacagc  
R: gatcagatgtgctgctcttc | PV | F: aacaggtgggctggttgag  
R: acccagatcagcctagcttc |
| TH | F: tggcctgattggcagacttgg  
R: tgaggcggagccaaagt | SST | F: gctgtgctggtcaaagaagcc  
R: cgttcagcccccttcaggg |

2.3.3. Gene expression using Biomark HD

In a 0.2ml tube, the SsoFast™ EvaGreen® and 20X DNA binding dye (sample pre-mix) were combined with each sample as specified in the table below. In brief, the master mix of the sample pre-mix was vortexed and centrifuged at 1000x for 10 seconds. A total of 3.3μl of pre-mix was added into individual tubes together with 2.7μl of the final product of pre-amplification. To assure correct homogenisation, samples were mixed then centrifuged at 1000g for at least 30 seconds.
### Component Volume per inlet with overage (μl) Vol per 96.96 IFC overage

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol for 96.96 IFC overage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SsoFast EvaGreen supermix with low ROX</td>
<td>3</td>
</tr>
<tr>
<td>20X DNA binding Dye</td>
<td>0.3</td>
</tr>
<tr>
<td>Pre-amplified cDNA</td>
<td>2.7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>6</td>
</tr>
</tbody>
</table>

In a 0.2ml tubes, the forward and reverse primers at 100μM were combined and mixed as follows:

### Component Vol per inlet with overage (μl) Vol for 50 μl Stock (μl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol per inlet with overage (μl)</th>
<th>Vol for 50 μl Stock (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Assay Loading Reagent (PN 100-761)</td>
<td>3.0</td>
<td>25</td>
</tr>
<tr>
<td>IX DNA TE Buffer</td>
<td>2.7</td>
<td>22.5</td>
</tr>
<tr>
<td>100μM combined primers</td>
<td>0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>6.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Gene expression was carried out using the 96.96 IFC (BMK-M-96.96). Each IFC contains two accumulators that need to be filled in with a viscous fluid provided in a syringe. They were then placed into the Controller HX (Fluidigm) and the program Prime (136x) was run. Samples and primers were then loaded into their respective inlets on the IFC. Once performed, the IFC was placed back into the Controller HX and the program load mix (136x) was run. Then the IFC was inserted into the Biomark HD and was run using the program GE 96x96 PCR+Melt v2.

Data were collected with the software Biomark HD Data Collection (Fluidigm) and then processed with the Real time PCR Analysis software (Fluidigm). Once the software opened, gene expression data were loaded and a test report was generated. This test summarized the name of the chip, it also mentioned the program used for the real-time PCR and finally a screenshot of a heatmap with Ct values was generated. Each sample and primer were then manually annotated according to their position within the IFC. The quality threshold was set up as recommended by the company at 0.65. Although value below 0.65 are considered to have failed they were individually checked to determine whether or not the sample failed. Data were exported into an excel file in order to be analysed.
All qPCR data presented in this thesis are the result of one experiment with n = 3 or 2 biological replicates for cortical interneurons, glutamatergic neurons and medium spiny neurons, dopaminergic neurons respectively. Data were normalised to the reference gene β-ACTIN – and relative mRNA expression was compared to control basal conditions using the 2^{-ΔΔCT} method.

2.4. Genome Editing

2.4.1. DNA extraction

DNA was extracted from cultured cells with a solution of lysis buffer containing; 10nM Tris, pH8.0; 50mM EDTA; 100mM NaCl; 0.5% SDS. A solution of proteinase K at 20mg/ml stock was diluted 1:100 in lysis buffer. Once the culture media was removed, cells were washed with DPBS. A volume of 500μl of lysis buffer was added into the well and left to incubate overnight at 37°C. The next day, the solution was retrieved into a 1.5ml tube together with an equal volume of 100% isopropanol. The solution was briefly vortexed and centrifuged at 12,000g for 20 minutes at 4°C. The supernatant was aspirated and the white pellet was resuspended in a solution of 70% ethanol. Again, the supernatant was removed after centrifugation, the pellet was air dried for 10 minutes and dissolved in 50μl of ddH₂O (double distilled water).

2.4.2. Estimating nucleic acid concentration

DNA concentration was estimated with the spectrophotometer using the appropriate program. The optical density was taken at 260nm, the ratio 260/280 was used to estimate the purity of the sample. A ratio between 1.8 and 2.0 was being considered as pure.

2.4.3. Detection of nucleic acids

The polymerase chain reaction (PCR) reaction was used in order to amplify specific DNA fragment. Primers were designed to match specific DNA sequence. Using a Taq polymerase I, the DNA fragment between a pair of primers was amplified.

PCR reaction was performed in a PCR reaction tube using the MyTaq™ DNA polymerase (BIO-21107) as described below:
PCR reaction set-up

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x MyTaq Reaction Buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>Template</td>
<td>200ng</td>
</tr>
<tr>
<td>Primers 20μM each</td>
<td>1μl</td>
</tr>
<tr>
<td>MyTaq DNA polymerase</td>
<td>1μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Up to 50μl</td>
</tr>
</tbody>
</table>

Samples were then transferred into a thermocycler and processed according to the following protocol:

<table>
<thead>
<tr>
<th>PCR cycling conditions</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>1min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C ramp down</td>
<td>15s</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>-1°C/cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>10s</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15s</td>
<td>25</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>15s</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>10 minutes</td>
<td>-</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4.4. Agarose gel electrophoresis

Visualization of PCR fragments was performed using agarose gel electrophoresis. A range between 1 to 5μg of agarose (UltraPURE, Gibco) was dissolved in 1x TAE buffer. Once melted, Safeview (G108, ABM) 1/10000 was added to allow the visualization of nucleic acids when excited at 470nm. Electrophoresis was performed in 1x TAE solution with a voltage ranging from 60 to 100 volts until desired separation of different amplicons was achieved, usually 1h. DNA was visualized using a transilluminator and pictures were acquired for further analysis.

2.4.5. Cloning

2.4.5.1. Oligo annealing

Different gRNAs were designed using the CRISPR MIT website from the Zhang lab. A list with the resulting gRNAs is presented below. The first two gRNA and two last were targeting the exon 3 and 4 respectively of the ZDHHC8.
Below is presented the DNA sequence which were targeted by our different gRNA together with the primers used to check for INDELs and the set of primer to evaluate the mRNA level of *ZDHHC8*. The intronic and exonic sequence were respectively represented in lower case and upper case/bold. Both sequence highlighted in grey represent the pair of primers used to check for INDELs located in intron 2 and 4. The sequence highlighted in green are representing the different gRNAs with their PAM sequence in red. Finally, the sequence in yellow represents the primers used to assess the mRNA level of *ZDHHC8*. It was noteworthy that the third gRNA and the reverse primer were partly sharing the same sequence.

The complementary sequence of each gRNAs was synthetized together with the specific overhangs corresponding to the BbsI restriction enzyme thus allowing their cloning within the host plasmid. The nucleotides sequence is presented in the table below with in red the overhangs. In addition, two guanine bases were added, in green. The first guanine was necessary for the U6 promoter, while the second guanine was demonstrated to reduce the off-target effect.

<table>
<thead>
<tr>
<th>Target Exon</th>
<th>Forward 5’→3’</th>
<th>Reverse 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 3</td>
<td>CACCGGCGGGCGTGAAGTGACCGGACG</td>
<td>AAACCGTGCCACTTCTACCCGCCGCGCC</td>
</tr>
<tr>
<td>Exon 3</td>
<td>CACCGGCGGTTCTTGATACAGCGGACG</td>
<td>AAACCGTCGGCTGTACAAGAACG</td>
</tr>
<tr>
<td>Exon 4</td>
<td>CACCGGCGGTCGTGAAGTGACCGGACG</td>
<td>AAACCGGCTCAAACACTGCCGGGG</td>
</tr>
<tr>
<td>Exon 4</td>
<td>CACCGGCGGTTCTTGATACAGCGGACG</td>
<td>AAACCGGTCTACGTGCTGAAACCAGC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>gRNAs sequence (3’→5’)</th>
<th>PAM sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTTCTTTGACAGCGGACC</td>
<td>CGG</td>
</tr>
<tr>
<td>CGGCCGCGTGAAGTGACCGGAG</td>
<td>TGG</td>
</tr>
<tr>
<td>CCCGATGCCAGTTGTGACACC</td>
<td>AGG</td>
</tr>
<tr>
<td>CTTGGTTCCAGCAGTAGACCC</td>
<td>AGG</td>
</tr>
</tbody>
</table>
Each pair of oligos were annealed to form a dimer. In a 0.2ml tube, 1μl of each oligo at 100μM, 1μl of 10X T4 Ligation Buffer (NEB), 6.5μl of ddH2O and 0.5μl of T4 PNK (NEB) were added and incubated in a thermocycler with the protocol presented below.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>30 min</td>
</tr>
<tr>
<td>95°C</td>
<td>5min</td>
</tr>
<tr>
<td>95°C ramp down to 25°C at 5°C/min</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4.5.2. Multiplex CRISPR/Cas9 Construction

Two steps were necessary for the construction of the all-in-one multiplex CRISPR/Cas9. The CRISPR kit used to generate the multiplex CRISPR/Cas9 vectors(Sakuma et al. 2014) was a gift from Takashi Yamamoto (Addgene kit # 1000000054). The first step involved the cloning of the annealed oligos into four different plasmids, one pX330 Ampicillin (pX330A 1-4) resistant plasmid and three pX330 (pX330S 2, 3 and 4) resistant to spectinomycin.

Ligation of the oligonucleotides was performed in a 0.2ml PCR tubes. In four tubes, we premixed 0.2μl of 10X T4 DNA ligase buffer (NEB), 0.1μl of BbsI (NEB), 0.8μl of Quick ligase (NEB) and 0.8μl of ddH2O. Then 0.3μl of the circular plasmid at 25ng/μl together with 0.5μl of 10μM of annealed oligonucleotides were added into their respective tubes. The tubes were added to a thermocycler and 3 cycles at 37°C for 5 minutes and 16°C for 10 minutes were performed and followed by an additional BbsI digestion at 37°C for an hour and inactivation at 80°C for 5 minutes.

Digestion by BbsI results to the appearance of sticky ends allowing the integration of our different gRNAs therefore our gRNAs were generated with the specific overhang sequence allowing their specific integration into the host plasmid A schematic of this process is presented below with the example of the first gRNA. The gRNA sequence with the specific overhangs is presented at the top. The bottom sequence corresponds to the region where the BbsI enzyme is cutting with the letter in red corresponding to the sticky end sequence. Once BbsI is cutting, the fragment will be removed therefore allowing the integration of our donor sequence. Interestingly, due to the specificity sequence of the overhangs, there is a unique orientation in which the donor plasmids can be ligated.
It was noteworthy that once integrated, the BbsI recognition site isn’t present anymore therefore preventing it to be cut again by the enzyme. As a result, the last step of the protocol which consisted of the additional digestion was used to linearize all remaining plasmids that did not contain the donor plasmid. As only circular plasmid can be introduced into bacterial cells it increased the likelihood of obtaining a positive clone.

![Cloning map of the gRNA into the recipient plasmid](image)

**Figure 2-1 Cloning map of the gRNA into the recipient plasmid.** The gRNA sequence on top in black, contain the sequence of the overhangs corresponding to the BbsI restriction enzyme. Once the BbsI enzyme cut the plasmid, we can observe that the gRNA is able to be ligated within the plasmid. Furthermore, it was noteworthy that the BbsI sequence disappeared once ligated into the recipient plasmid.

The second step of the multiplex CRISPR/Cas9 consisted of golden gate reaction which was responsible of cutting the fragment of interest and inserting them into one recipient plasmid using the BsaI enzyme. Once cutting, the enzyme induces specific overhangs sequence which are different for each plasmid (Figure 2-2). Therefore, it ensures the specificity of insertion. The golden gate reaction was performed as follow, in one tube, a solution was prepared with 100ng/µl of each spectinomycin plasmid, 50ng/µl of the ampicillin plasmid together with 2µl of 10X T4 DNA ligase buffer, 1µl of BsaI enzyme and 1µl of quick ligase. The reaction was then run using a thermocycler with the following parameters.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>37°C</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>16°C</td>
<td>10</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

**Figure 2-2 below allows the visualisation of the cloning process during the step 2.**
Bacterial transformation which corresponds to the insertion of exogenous genetic material into bacterial cells was performed as follows: 1μl of each reaction were transferred to a corresponding vial of competent cells 5-alpha Flq Competent E. coli (C2292H, NEB) and carefully flicked to mix the bacteria with DNA. The vials were placed on ice for 30 minutes. A water bath was set up at 42°C and vials were heat shocked for exactly 30 seconds and placed on ice for 5 minutes. A volume of 950μl of SOC media provided by the manufacturer was added to each vial. They were further incubated for 1 hour at 37°C under constant agitation at 200 rotations per minutes (rpm). In the meantime, the agarose plates were warmed at 37°C to avoid condensation. Prior dispersion onto the agar plate, the vials were flicked thoroughly as well as inverted. Two different volumes, usually 50 and 75μl, were spread onto the plates and incubated overnight at 37°C.

Bacterial colonies were manually picked and transferred to a bacterial tube with 3ml of LB media and incubated overnight on the shaker at 37°C and 200rpm.
2.4.5.4. Genomic DNA extraction

2.4.5.4.1. Miniprep

From the previous step, 1.5ml of the bacterial solution was transferred into a 2ml Eppendorf tube and centrifuged at 12000g for a minute. The supernatant was removed and replaced by 200µl of solution A (25mM Tris HCl, pH8, 10mM EDTA) which was previously prepared by adding 3µl of RNase A per ml. In addition, 200µl of lysis buffer (200mM NaOH, 1% SDS) was incubated for no longer than 5 minutes. To neutralise the lysis buffer, 240µl of solution K (5M potassium acetate, pH5.5) was added and mixed gently by inverting 5-6 times. The bacterial solution was further incubated at room temperature for 3 minutes. The bacterial solution was centrifuged at 12000g for 2 minutes allowing the protein and debris to pellet. The supernatant was carefully collected into a 1.5ml Eppendorf tube and 400µl of 100% isopropanol were added to the tubes which were subsequently vortexed in order to precipitate the DNA and left to incubate for a minute at room temperature. The DNA was pelleted by centrifugation at 14000g for a minute. Supernatant was immediately removed and replaced by 1ml of 70% ethanol and vortexed to clean the DNA pellet. Tubes were centrifuged at 14000g for a minute, the supernatant was removed and centrifuged a second time to remove the remaining ethanol. Tubes were air dried for 10 minutes. Finally, 50µl of ddH₂O was added to resuspend the DNA.

2.4.5.4.2. Maxiprep

In order to perform a Maxiprep, 150ml of bacterial solution was incubated overnight at 37°C under continuous agitation. DNA extraction was performed using the HiSpeed Plasmids Kit (Qiagen, 12662) following the instructions provided by the manufacturer.

2.4.5.4.3. Plasmid Digestion

Plasmid digestion was performed using the appropriate enzymes and buffers which was suggested by the manufacturer. The usual protocol is described below and particular restriction enzymes are stated in relevant results chapters.

<table>
<thead>
<tr>
<th>NEB digestion Reaction</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X</td>
<td>5µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>1µg</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>0.5µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Up to 50µl</td>
</tr>
</tbody>
</table>
The solution was mixed and centrifuged to collect the solution at the bottom of the tube. Tubes were run using a thermocycler for 1h at 37°C. The digestion product was loaded to a 1% agarose gel and run for 1h at 80V.

2.4.5.4.4. Nucleofection

In order to integrate the all in one CRISPR/Cas9 complex, electroporation was performed using the Amaxa NucleoFector™ system (Lonza). This system was designed for the transfection of primary cells. It consists of the application of an electric pulse generating momentary pores in cell membrane thereby allowing the integration of an exogenous plasmid.

Prior to nucleofection, cells were harvested onto another well in order for them to be in their exponential growth phase. This step increases the number of cells surviving the nucleofection process.

An aliquot with 3.5µg of a neomycin resistant plasmid was mixed with 1.5µg of the multiplex CRISPR/Cas9 complex. A volume of 68µl of the P3 Primary cell 4D-nucleofector® X solution was mixed with 12µl of supplement. This solution was mixed with the plasmids and left in the incubator at 37°C.

Cells were harvested as described above (chapter 2.2.1). The cell suspension was centrifuged at 1150rpm for 3 minutes. The supernatant was discarded while the pellet was resuspended with the pre-warmed nucleofection solution and passed through a cells scrapper to obtain a quasi-single cell solution. The solution was added to the Nucleocuvette® and quickly processed in the Nucleofector using the CB-150 program. Cell suspension was divided into 6 wells of a 6-well plate in E8 medium supplemented with 1/100 of RevitaCell (Life technologies).

2.4.5.4.5. pGEM-T easy and sequencing

PCR product from the screening of positive clones were cleaned using the PCR Purification Kit (QIAquick, Qiagen) following the manufacturer protocol. Clean PCR products were ligated into the pGEM®-T Easy vector (Promega). This plasmid contains a 3’-T overhangs at the insertion site which greatly improves the ligation efficiency. Due to the presence of a restriction site and the RNA promoter T7 and SP6 it can respectively be digested or amplified. Figure 2-3 represents a map of the vector with all the different enzymes that can be used to screen for insert or for sequencing.
The pGEM-T-easy vector was used firstly to visualize single allele amplicon secondly to send those selected plasmids for sequencing.

Several plasmids for each clone were sent for sequencing. The T7 promoter was used to amplify the different ligated PCR products.

![Map of the pGEM-T Easy vector](image)

**Figure 2-3 Map of the pGEM-T Easy vector.** Several enzymes can be used to screen for fragment insertion. To determine whether or PCR product were inserted, we digested the plasmid with EcoRI. For sequencing, fragments were amplified using the T7 promoter.

### 2.5 Immunocytochemical staining

Cells in culture were washed once with PBS and subsequently fixed in a solution of 3.7% PFA for up to 15 minutes at room temperature. After two washes in PBS, fixed cells were incubated in PBS-NH₄Cl to quench the remaining aldehyde residues for 20 minutes. Cells were stored for up to three weeks at 4°C or stained immediately after the quench. For nuclear stains, cells further underwent 5 minutes of ascending and descending methanol washes in PBS (33%, 50%, 66% at 4°C, then at -20°C in 100% methanol). Fixed cells were permeabilized in a solution of PBS-T (PBS + 0.3% Triton-X100, Sigma) for 4 minutes at room temperature. The cells were incubated overnight at 4°C in PBS with 5% donkey serum (Gentaur) in which the primary antibodies were added. The following day, the cells were washed three times for 10 minutes. Secondary antibodies raised against species of the primary antibody were then added to PBS (1/200, Alexa Fluor 488, 594,
647, Life technologies) and incubated for 40 minutes at room temperature under constant agitation. Cells were counterstained with DAPI (1/3000, Life technologies) in PBS for 5 minutes at room temperature followed by three 10-minute PBS washes. Either the stained cells were covered in the culture plate with a coverslip or coverslips were transferred onto a glass slide (VWR) and mount respectively with a 20µl drop of DAKO (Life technologies). A list of primary and secondary antibodies is presented in Table 2-2.

Table 2-2 List of primary antibodies used for immunostaining

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Supplier</th>
<th>Code</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTIP2</td>
<td>rat</td>
<td>Abcam</td>
<td>ab18465</td>
<td>1/500</td>
</tr>
<tr>
<td>FOXA2</td>
<td>goat</td>
<td>Santa cruz</td>
<td>Sc6554</td>
<td>1/200</td>
</tr>
<tr>
<td>FOXG1</td>
<td>rabbit</td>
<td>Abcam</td>
<td></td>
<td>1/250</td>
</tr>
<tr>
<td>Ki67</td>
<td>mouse</td>
<td>BD bioscience</td>
<td>550609</td>
<td>1/200</td>
</tr>
<tr>
<td>LMX1A</td>
<td>Guinea Pig</td>
<td>Custom made</td>
<td></td>
<td>1/1000</td>
</tr>
<tr>
<td>MAP2</td>
<td>rabbit</td>
<td>Millipore</td>
<td>AB5622</td>
<td>1/1000</td>
</tr>
<tr>
<td>N-CADHERIN</td>
<td>mouse</td>
<td>Invitrogen</td>
<td>18-0224</td>
<td>1/1000</td>
</tr>
<tr>
<td>NKX2.1</td>
<td>rabbit</td>
<td>Abcam</td>
<td>Ab40880</td>
<td>1/1000</td>
</tr>
<tr>
<td>OCT4</td>
<td>goat</td>
<td>Santa cruz</td>
<td>Sc8628</td>
<td>1/500</td>
</tr>
<tr>
<td>PAX6</td>
<td>mouse</td>
<td>DSHB</td>
<td></td>
<td>1/1000</td>
</tr>
<tr>
<td>PSD-95</td>
<td>mouse</td>
<td>Neuromab</td>
<td></td>
<td>1/50</td>
</tr>
<tr>
<td>SSEA3</td>
<td>rat</td>
<td>Chemicon</td>
<td>MAB4303</td>
<td>1/500</td>
</tr>
<tr>
<td>TRA1-81</td>
<td>mouse</td>
<td>Chemicon</td>
<td>MAB4381</td>
<td>1/500</td>
</tr>
</tbody>
</table>

Cells were imaged on a Leica DMI6000 inverted fluorescence microscope. A stack of images was acquired to obtain information from all planes. For quantification, we used a 10x objective while for synaptic proteins a 100x oil immersion objective was used.

Images were post processed in ICY (open source software for bioimage informatics). One slice from each stack was extracted for each channel then processed using histogram equalization, a plugin in ICY which allow to obtain an equal histogram. This step is crucial for the subsequent quantification performed in CellProfiler (Cell image analysis software). To count the nuclear staining, CellProfiler was setup on DAPI then the marker of interest.

2.6. Motility Assay

Motility assay was performed on neurons plated on glass coverslip. The experiment was performed at day 20 with excitatory progenitor cells. The coverslip was transferred into
a recording chamber filled with neuronal media. The chamber was temperature, CO₂ and humidity controlled (Ibidi). The recording chamber was placed under a ZEISS Axio Observer Z1 microscope with a 10x objective. Five to eight random fields of view were selected on the same coverslip. Pictures were acquired every 5 minutes for 12 hours. The recording chamber was setup at 37°C, with 90% humidity and 5% CO₂.

The images were analysed using a manual tracking plugin in Fiji. Data were then transferred to excel and analysed.

2.7. Calcium Imaging

In order to accelerate neuronal maturation of our cortical excitatory projection neurons, at day 20 - 26 of differentiation, basal neuronal media was supplemented with β-secretase inhibitors, 10µM DAPT (Sigma) and 2µM PD0332991 (Selleckchem) to synchronise cell cycles and push progenitors to a post-mitotic stage to mature. After this treatment, half of media was replaced with a mix (at 1:1 ratio) of the basal neuronal media and astrocyte-conditioned medium (ACM). At the same time, neurons were transferred to an incubator monitored at 2% oxygen. Until the end of the differentiation, the media was replaced once a week.

Neurons were loaded with 5µM Fluo-4AM (Life technologies), 0.02% Pluronic F-127 (20% in DMSO, Life technologies) and 0.01% Cremophor EL (10% in DMSO, Sigma) and incubated at 37°C in fresh media for 30 minutes. Neurons were quickly washed twice and transferred into a solution of artificial cerebrospinal fluid (aCSF), which contained (in mM): 142 NaCl, 5 KCl, 2.5 MgCl₂, 1 CaCl₂, 30 D-glucose, 10 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) (Sigma), pH 7.4. Neurons were further incubated in the aCSF for 30 minutes at 37°C. Prior to recordings, the aCSF media was replaced with a fresh recording solution.

Calcium activity was acquired using an LED system (Rapp OptoElectronic and Lumencor) at 10Hz for 10 minutes with a final resolution of 1024*1024 pixel. From each time frame, we extracted random regions of interest of 308*308 pixels. The segmentation was performed using NeuroCa, standalone MATLAB package (Jang et al. 2015) with the following parameters: the type of raw image, TIF; the image size, 308*308; the radius, 2-7; sensitivity, 0.9 and the frame rate at 10Hz. The segmentation files were retrieved and
run in FluoroSNNAP, a MATLAB script for the analysis of calcium activity (Patel et al. 2015).

The analysis generates a summary text file with values corresponding to the amplitude ($\Delta F/F_0$), the inter-spike-interval (ISI), the number of events, the rise and fall time of the spikes. All cells exhibiting no events were discarded from the analysis.

### 2.8. RNA sequencing

#### 2.8.1. Sample preparation and sequencing

Heterozygous cell line for ZDHHC8 and its isogenic control (iCas9?) as well as an iPSC line (one from each) from a 22q11.2 carriers and a healthy individual were used for this experiment. Cells were differentiated into cortical excitatory projection neurons.

At day 15, RNA samples were extracted by pooling two wells together in order to reduce the variability of the samples. In total, we extracted six RNA samples per line using the mirVana™ miRNA Isolation Kit following the protocol provided by the manufacturer. The concentration and the purity of each sample were determined using the spectrophotometer.

To accurately assess the purity of our RNA samples, they were run on a bioanalyser (CBS Cardiff University) which evaluates the integrity of the 28S and 18S ratio using the Agilent RNA 6000 Nano Kit (Agilent). RNA samples were quantified again using the Qubit RNA High Sensitivity Kit (Life technologies). To extract only the mRNA from the total RNA, the KAPA mRNA HyperPrep Kit was used and aimed to capture the poly(A) RNA. The protocol was followed according to the manufacturer recommendation and using a total of 1µg of RNA.

Prior to RNA sequencing, the library preparation was quantified using the Qubit DNA high sensitivity kit (Life technologies). The clustering and sequencing was carried out as per standard Illumina protocols, with 2 times 75bp paired-end reads on HiSeq 4000.

Except for the neuronal differentiation and RNA extraction, the sample preparation and sequencing were performed by Joanne Morgan.

#### 2.8.2. RNA sequencing analysis
Prior to alignment of the reads, data were processed with the Trimmomatic software to remove the adaptor sequences and also base pairs with a Phred lower than 30 (Bolger et al. 2014). FastQC was performed before and after the trimming as a quality control.

The RNAs-seq reads alignment was performed by Daniel Cabezas De la Fuente in our laboratory using a script developed by Dr Robert Andrews and Daniel Cabezas De la Fuente. Data were aligned to the reference genome hg38 with the STAR aligner software (Dobin et al. 2013).

The average read depth for present samples is summarised in table 2-3 below

<table>
<thead>
<tr>
<th>Samples</th>
<th>Read depth in millions reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas9 (Control)</td>
<td>42.82</td>
</tr>
<tr>
<td>541 (ZDHHC8+/−)</td>
<td>47.13</td>
</tr>
<tr>
<td>900 Control</td>
<td>43.32</td>
</tr>
<tr>
<td>E11 (22q11.2)</td>
<td>46.83</td>
</tr>
</tbody>
</table>

After alignment, the RNA-seq data were processed in R using a script developed and tested by Daniel Cabezas De la Fuenta and Dr Robert Andrews. The script used the DEseq2 package from the Bioconductor (Love et al. 2014).

2.9. Statistical analysis

For cell counting analysis, the proportion of cells was calculated using different field of view from one experiment. Normality of the data was first examined. If data passed normality test, we performed a parametric t-test, unpaired t test. Conversely if they did not pass normality, we performed a nonparametric t-test, Mann-Whitney test. Statistical analysis was performed using Prism Graphpad.

Similarly, for motility assay and calcium imaging, data were first checked for normality and statistical analysis was performed accordingly.

For gene expression data, a one-way and a two-way ANOVA was conducted to test for significant differences in our samples with a post-hoc Bonferroni correction for multiple pairwise comparisons.
3. Temporal and spatial expression of the 22q11.2 genes during neuronal differentiation of hESCs.

3.1. Introduction

The 22q11.2DS is a highly penetrant mutation that affects 1/4000 live births making it the most common microdeletion in humans (Karayiorgou et al. 2010). Most deletions are either 3Mb or 1.5Mb, which encompass around 60 genes and 28 genes, respectively (Williams 2011). 90% of the 22q11.2 deletion cases arise by de novo mutation while 10% are inherited in an autosomal dominant fashion (Ellegood et al. 2014). Patients with 22q11.2DS are more likely to develop psychiatric disorders than the general population (Schreiner et al. 2013). Recent evidences have also linked 22q11.2DS with Parkinson’s disease (Dufournet et al. 2017; Retnakaran et al. 2010). Although most patients commonly carry a 3Mb deletion, it has been demonstrated that the 1.5Mb deletion was responsible for the underlying pathologies, hence many studies are focusing on this specific region (Jonas et al. 2014).

Whereas most studies on 22q11.2DS focused on the role of selected genes such as Dgcr8, Tbx1, Comt or Prodh in mouse model, the study from Maynard et al. 2003 investigated the expression profiling of all 22q11.2 genes in WT mouse. Thus, the group addressed whether genes within the 1.5Mb deletion were temporally and spatially expressed during mouse embryonic development and adulthood. Samples from either mouse brain or other organs extracted at different time points were screened against genes from the 22q11.2 small deletion. The study identified six genes that were primarily located in neurons including forebrain regions which are known to be altered in schizophrenia. Amongst the twenty-two genes tested, twelve exhibited a similar expression across the different time points from embryonic stage to adulthood. Tmvf, also known as Cldn5, displayed an expression pattern that increased over time. Conversely, Cdc45l (Cdc45) exhibited a brain expression profile that peaked at E12 and E14. The PCR result of Prodh2 (Prodh) was negative in brains at embryonic stage but increased postnatally and declined in the adult. Tbx1 and Wdvcf (Gnb1l) exhibited expression in other tissues but not in the brain. Three genes: Gscl (also known as Gsc2), Gpibb (Gp1bb) and Stk22b (Tssk2) were found not expressed. Finally, authors examined the expression of a subset of these genes in human fetal brain, different adult brain regions and several other human tissues with similar observations to that in mice (Maynard et al. 2003).
Although studies have already shed light on the expression profiling and brain region specific expression of genes in the 1.5Mb deletion, the data presented lacked temporal and spatial resolution. Furthermore, microdissected brain tissue does not allow to assess differentially expressed genes in specific neuronal populations.

Neurons derived from hESCs offer a unique opportunity to understand the expression of these genes during human neural development. It is currently possible to differentiate hESCs into the major neuronal lineages present in the brain allowing the examination of the spatial and temporal expression profile of the genes present in the 1.5Mb deletion.

Aims

The aims of this chapter were to determine the temporal and cell-type specific expression of twenty-eight genes located in the 1.5Mb deletion. Due to the transcript similarity between DGCR6 and DGCR6L, only DGCR6 was studied. The specific objectives are:

1. To differentiate hESCs into glutamatergic cortical neurons, GABAergic cortical interneurons, medium spiny neurons (MSN) and dopaminergic neurons and to extract RNA samples at different time points.
2. To investigate the temporal expression profile and determine whether any genes exhibit a cell type specific expression using a high-throughput Biomark HD PCR machine.

3.2. Results

The hESCs iCas9 and H7 were differentiated using previously establish protocols into cortical excitatory projection neurons (Espuny-Camacho et al. 2013), GABAergic MSN (Arber et al. 2015), cortical interneurons (Maroof et al. 2013) and midbrain dopaminergic neurons (Jaeger et al. 2011). All protocols were based on dual SMAD inhibition that block BMP signalling. The newly derived naïve neuroepithelial cells, largely of forebrain characteristics by default, can then be directed towards specific regional fate by appropriate morphogens. For example, SHH and/or SHH agonist purmorphamine are normally applied to ventralize forebrain progenitors into medial ganglionic fate for generating cortical interneurons. Instead addition of activin A is necessary to the differentiation into LGE progenitors that gives rise to MSNs. Finally, a MEK blocker PD0325901, SHH and FGF8 were used for generating midbrain dopaminergic neurons.
All different protocols are presented in the schematic timeline in Figure 3-1 moreover, details of the concentration for each compound are presented in 2.2.3.

![Figure 3-1 Schematic timeline of the four differentiation protocols.](image)

Figure 3-1 Schematic timeline of the four differentiation protocols. A, cortical excitatory projection neurons differentiation. B, cortical interneurons. C, MSN. D, dopaminergic neurons. Time points for RNA extraction are also represented in blue.

RT-PCRs were performed on 3 or 2 biological samples from one experiment using the qScript cDNA superMix as explained in chapter 2 and processed using the Biomark HD to assess the variation level of these different genes. To identify potential regulated expression, all data were firstly normalized to the housekeeping gene β-actin and compared to one common reference usually time point at day 0 when possible.

### 3.2.1. Neuronal Subtype differentiation from hESC

#### 3.2.1.1. Glutamatergic neuron differentiation

To verify glutamatergic fate induction, cultures at day 15 were stained with antibody against FOXG1, a transcription factor expressed exclusively in the developing telencephalon. The majority of cells were positive for FOXG1 (Figure 3-2A), suggesting highly efficient induction of glutamatergic precursors. VGLUT1 (SLC17A7), PSD-95
(DLG4) and GRIN1 (GLUN1) are proteins essential for excitatory synapses and hence can be used as markers for mature neurons (Figure 3-2B). As neurons matured morphologically from day 30 to 80, an increase in transcript levels of these marker genes was observed. Furthermore, we observed that during the course of differentiation the expression of these genes were significantly higher when compared to day 30 for GRIN1 and SLC17A7 and day 0 for DLG4.

In keeping with the RT-PCR observation, immunofluorescence staining confirmed the expression of the postsynaptic protein PSD-95 and microtubule associated protein 2 (MAP2) in neuronal processes (Figure 3-2C).

Together these data support the generation of glutamatergic neurons derived from hESCs.

Figure 3-2 Glutamatergic precursors and mature neurons Legend on the next page.
Cortical interneuron differentiation was characterized at day 20 by immunostaining for NKX2.1, a transcription factor expressed in medial ganglionic eminence (MGE), a region in the ventral forebrain that gives rise to major types of cortical interneurons. As illustrated in Figure 3-3A, a large proportion of cells were NKX2.1 positive. To further characterize those cells, the expression of key transcripts was analysed by RT-PCR (Figure 3-3B). LHX6 is a transcription factor responsible for the tangential migration of MGE cells towards the cortex (Alifragis et al. 2004). An increase of this crucial gene could be detected from day 30. SST and PV are two major classes of inhibitory interneurons, generation of which was confirmed by increased expression of these two genes in the present experiment.

For this experiment, day 30 interneurons progenitors were provided by Maria Cruz Santos and re-plated onto poly-D-lysine laminin and kept in neuronal media until day 80. RNA samples were extracted every 10 days from day 30. Pictures presented below are a courtesy of Maria Cruz Santos. The above data together suggests an efficient conversion of hESCs into cortical interneurons.
3.2.1.3. Dopaminergic neuron differentiation

Immunofluorescence using FOXA2 and LMX1A antibodies was performed at day 20 (Figure 3-4). Together, these early markers are key determinants for dopaminergic neuron identity. As illustrated in Figure 3-4A, an important proportion of cells were positive for both FOXA2 and LMX1A suggesting an appropriate regionalisation towards a dopaminergic fate. While maturing, these neurons express the enzyme tyrosine hydroxylase (TH), responsible for the conversion of tyrosine into L-DOPA, a precursor of the neurotransmitter dopamine. This gene started to be expressed from day 20 and increased 11-fold at day 50 when compared to day 20 (Figure 3-4B).

RNA samples and immunofluorescences were a courtesy of Dr Marija Fjodorova.
3.2.1.4. MSN differentiation

GABAergic medium spiny projection neurons were characterized at day 20 using an antibody against CTIP2 (BCL11B) protein, a transcription factor known to be expressed by striatal progenitors in the LGE. A high proportion of cells positive for CTIP2 could be observed in day 20 cell cultures (Figure 3-5). While this protein is also expressed by glutamatergic neurons, CTIP2 and the transcription factor GSX2 are two genes which can be used to assess the cellular identity of the LGE. Indeed, a peak of expression of GSX2 was detected at day 20 corresponding to the progenitor stage. CTIP2 expression increased over the course of differentiation. During development, these progenitors are morphologically and physiologically maturing thus marker like DRD1 can be used to evaluate the maturation stage. Dopamine receptors D1 are expressed by mature neurons from the direct pathway and an increase in expression of this gene was observed over the course of differentiation.

RNA samples and immunofluorescence were a courtesy of Dr Marija Fjodorova.

Figure 3-4 Dopaminergic neuron differentiation. A, Immunofluorescence of dopaminergic precursors at day 20 with DAPI (blue), FOXA2 (red) and LMX1A (green). Images were acquired using a Leica inverted microscope with a 20x objective, scale bar = 50µm. B, Temporal expression profiling of gene expressed by dopaminergic neurons. The expression of TH was normalized to the housekeeping gene β-actin and mRNA expression level was compared to day 20. Error bars represent mean±/SEM. No statistical analysis was performed due to sample size.
Taken together, these data demonstrate that four different neuronal precursor populations were properly differentiated from hESCs stage. Given the high proportion of relevant progenitor markers, cells were directed to mature glutamatergic, cortical GABAergic, striatal medium spiny and midbrain dopamine neurons.

### 3.2.2. Temporal expression of the 22q11 genes

First, genes were categorized according to their Ct value from high to low and not expressed. These data are presented in table 3-1 and an average Ct value of all cell lineages for each gene is shown.
### Table 3-1 Average Ct value across cell lineages

<table>
<thead>
<tr>
<th>Ct &lt; 15</th>
<th>15 ≤ Ct ≤ 22</th>
<th>Ct &gt; 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGCR6, DGCR2, DGCR14, SLC25A1, CLTCL1, HIRA, MRPL40, CDC45, SEPT5, COMT, COMT, DGCR8, TRMT2A, RANBP1, ZDHHC8</td>
<td>PRODH, TSSK2, UFD1L, CLDN5, GP1BB, TBX1, ARVCF, RTN4R</td>
<td>GSC2, C22orf39, GNBI1, TXNRD2</td>
</tr>
</tbody>
</table>

Amongst the 28 genes in the 1.5Mb deletion, four had a Ct value higher than 22, eight had a Ct value between 15 and 22, while sixteen genes had a Ct value below 15.

According to the manufacturer, when samples are processed on the Biomark HD, due to the high sensibility of the equipment, they advised to discard genes with a Ct value of 22 and above. Thereby, in our experiment genes with a Ct value of 22 and above were also discarded.

Subsequent analysis investigated whether these genes exhibited a temporal expression profile and the twenty-four genes were grouped into three categories: early expression, later expression and constitutive expression.

#### 3.2.2.1. Early expression

The expression of four genes, *HIRA, CDC45, RANBP1* and *UFD1L* showed a higher level at early stage of differentiation compared to the later stages (Figure 3-6). These genes are thought to regulate the cell cycle (Meechan et al. 2009). *Hira* is only weakly present in the mouse developing cortex; however, this gene is considered to be an important candidate responsible for the pathophysiology of 22q11.2DS (Maynard et al. 2003). *Cdc45* has been shown to be expressed in the VZ and SVZ of the mouse developing brain and is essential for the initiation of DNA replication (Maynard et al. 2003). Likewise, *Ranbp1* is also highly expressed in the VZ/SVZ where it controls the nucleocytoplasmic transport of proteins and nucleic acids (Maynard et al. 2003). Finally, *Ufd1l* has demonstrated to be restricted to the intermediate zone of the mouse developing cortex (Maynard et al. 2003).

Because we did not collect RNA samples prior day 30 in cortical differentiations, data were plotted on two different graphs. The first one displayed dopaminergic and MSN while the other displayed glutamatergic and cortical interneurons. The relative expression
of *CDC45* and *RANBP1* were significantly higher at day 10 and 20 in MSN and dopaminergic differentiation cultures. Although, it appeared that the relative expression of *HIRA* at day 10 and day 10-20 in MSN and dopaminergic neurons respectively was higher, data failed to reach significance. Similar result was obtained for *UFDIL* although the expression was higher in dopaminergic cultures than MSN.

The level of *HIRA*, *CDC45* and *RANBP1* transcript showed little change in the time windows analysed. The Ct value for these RT-QPCRs were low in both the cortical glutamatergic and interneurons samples, suggesting a high level of expression of these genes in these cells. However, *UFDIL* expression appeared to increase in cortical interneurons and decrease in glutamatergic cells as the cultures matured.

Table 3-2 below presents the results of the two-way ANOVA to compare the expression between the different cell type. Only significant data were displayed.

### Table 3-2 Results of two-way ANOVA that compare difference between cell lineages

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Cell types</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HIRA</em></td>
<td>40</td>
<td>Cortical Interneurons vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td><em>CDC45</em></td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Cortical interneurons vs MSN</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>**</td>
</tr>
<tr>
<td><em>RANBP1</em></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td><em>UFDIL</em></td>
<td>30</td>
<td>Cortical interneurons</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>80</td>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Glutamatergic vs MSN</td>
<td>***</td>
</tr>
</tbody>
</table>
3.2.2.2. Late expression

*SEPT5, GP1BB, DGCR6* and *ARVCF* are four genes which displayed a later stage expression pattern (Figure 3-7). *Sept5* has been shown to be involved in the process of

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**Figure 3-6 Expression profile of a subset of the 22q11.2 genes displaying an early expression pattern.**

A small subset of four genes in the 22q11.2 genes displayed an early expression profile. Cortical interneurons (black), glutamatergic neurons (red), dopaminergic neurons (green) and MSN (blue). Data were normalised to the housekeeping gene β-actin and compared to day 0. Error bars represent mean±/SEM. The data presented correspond to one experiment with n = 3 biological replicates for cortical interneurons, glutamatergic neurons and n = 2 for dopaminergic and medium spiny neurons. Error bars represent ±/SEM. Statistical analysis was performed on the ΔCT value with a one-way ANOVA with Bonferroni post-hoc to compared within cell lineage the expression of each time point to day 0 with * (cortical interneurons), # (glutamatergic neurons), ¬ (dopaminergic neurons) and ~ (medium spiny neurons). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.
exocytosis and cell division (Choi et al. 2003), while Gp1bb has a role in the formation of the platelet plugs (Kunishima et al. 2013). Dgcr6 is part of the family of the DiGeorge syndrome Critical Region regrouping genes implicated in DiGeorge Syndrome. Dgcr6 is suggested to play a role in neural crest migration (Hierck et al. 2004), finally Arvcf was shown to be involved in protein-protein interaction at adherens junction (Ulfig et al. 2004).

Due to high Ct value, data for GP1BB were normalized from day 16 for MSN and dopaminergic neurons whereas cortical interneurons and glutamatergic neurons were normalized from day 30. ARVCF was normalized at day 8 for dopaminergic neurons. Thus, comparison between these two groups was not possible.

As shown in Figure 3-7, transcript levels of this group increased over the course of neuronal maturation in all four differentiation cultures. All genes in this group showed their highest expression at the end of differentiation for most of the cell lineages, with the exception of DGCR6, which peaked at day 30 in dopaminergic neuron cultures.

Although the expression profile of GP1BB in glutamatergic and cortical interneurons was not obvious, an increase from day 30 to 80 was observed. A 4-fold change increase was observed in glutamatergic neurons while it was 2-fold in cortical interneurons.

Similarly to the early expression profiling, statistical analysis was performed on our data in order to determine whether different existed between the different cell type at a given time point. These data are presented in table 3.3.

**Table 3-3 Results of two-way ANOVA that compare difference between cell lineages.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Cell type</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEPT5</td>
<td>40</td>
<td>Cortical interneurons vs Glutameric</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Dopaminic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Glutameric vs MSN</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>**</td>
</tr>
<tr>
<td>DGCR6</td>
<td>80</td>
<td>Cortical interneurons vs Glutameric</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Dopaminic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Glutameric vs Dopaminic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Glutameric vs MSN</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Dopaminergic vs MSN</td>
<td>**</td>
</tr>
<tr>
<td>ARVCF</td>
<td>30</td>
<td>Cortical interneurons vs Dopaminic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Glutameric vs Dopaminic</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 3-7 Expression profile of a subset of the 22q11.2 genes displaying a later expression pattern. Four of the 22q11.2 genes displayed an expression profile that increased along the course of differentiation. Black, red, green and blue lines represent cortical interneurons, glutamatergic neurons, dopaminergic neurons and MSNs, respectively. Data were normalised to the housekeeping gene β-actin and compared to day 0 for SEPT5, DGCR6 and ARVCF while GP1BB was compared to day 16 for dopaminergic, MSN and day 30 for cortical interneurons and glutamatergic neurons. The data presented correspond to one experiment with n = 3 biological replicates for cortical interneurons, glutamatergic neurons and n = 2 for dopaminergic and medium spiny neurons. Error bars represent +/- SEM. Statistical analysis was performed on the ΔCT value with a one-way ANOVA with Bonferroni post-hoc to compared within cell lineage the expression of each time point to day 0 with * (cortical interneurons), # (glutamatergic neurons), ¬ (dopaminergic neurons) and ~ (medium spiny neurons). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.
3.2.2.3. Genes with no apparent temporal profile

The profiles of *PRODH*, *DGCR2*, *TSSK2*, *TBX1* and *COMT* did not exhibit an obvious trend. (Figure 3-8).

*PRODH* expression decreased during the course of MSN and dopaminergic neuron differentiation when compared to day 0. On the other hand, the transcript level remained constant at all the time points analysed for the two cortical cultures. However, the average Ct value was 16 suggesting that the transcript was present in enough quantity to be detected in these samples. *COMT* followed a similar pattern to that of *PRODH* except a very small increase during glutamatergic differentiation at day 80. *DGCR2* encodes for an activity dependent adhesion protein (Kajiwara et al. 1996). As shown in Figure 3-8, the expression level of *DGCR2* increased slightly in all four differentiation paradigms. *TSSK2* is part of a family of serine threonine kinase that is mostly expressed in testis (Hao et al. 2004). Surprisingly, as illustrated in Figure 3-8, *TSSK2* expression was detected in all four lineages. The Ct value was classified as high with an average Ct of 16 across the different neuronal populations. The expression profile rose in dopaminergic neurons from day 0 until day 10 then remained steady. In MSNs, the expression kept increasing until day 50. A similar pattern was observed in cortical interneurons where the transcript levels increased from day 30 to 80. However, during glutamatergic differentiation, *TSSK2* expression appeared to drop first before it increased again from day 50 to 80.

Table 3-4 below is the result of the statistical analysis which aimed to compare difference between cell lineages at a given time point.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Cell type</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGCR2</td>
<td>40</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>***</td>
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<tr>
<td></td>
<td>50</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Cortical interneurons vs MSN</td>
<td>***</td>
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<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Dopaminergic vs MSN</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Activity (%)</td>
<td>Comparison</td>
<td></td>
</tr>
<tr>
<td>-------</td>
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<td>-----------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>COMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>****</td>
</tr>
<tr>
<td>40</td>
<td></td>
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<td>****</td>
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<td>80</td>
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<tr>
<td>30</td>
<td></td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>**</td>
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<td>40</td>
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<td>**</td>
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<tr>
<td>40</td>
<td></td>
<td>Glutamatergic vs Dopaminergic</td>
<td>****</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td>****</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>Cortical interneurons vs MSN</td>
<td>****</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>Dopaminergic vs MSN</td>
<td>*</td>
</tr>
<tr>
<td>TSSK2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td></td>
<td>Cortical interneurons vs Glutamatergic</td>
<td>**</td>
</tr>
<tr>
<td>70</td>
<td></td>
<td></td>
<td>*</td>
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<tr>
<td>40</td>
<td></td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>*</td>
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<tr>
<td>30</td>
<td></td>
<td>Glutamatergic vs Dopaminergic</td>
<td>**</td>
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<tr>
<td>50</td>
<td></td>
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<td>*</td>
</tr>
</tbody>
</table>
In addition to temporally regulated expression, it is particularly interesting to investigate whether any of the 22q11 genes show a cell type-specific expression. Therefore, the data was next analysed from this perspective.

**3.2.3. Subtype specific expression**

In addition to temporally regulated expression, it is particularly interesting to investigate whether any of the 22q11 genes show a cell type-specific expression. Therefore, the data was next analysed from this perspective.
As shown in Figure 3-9, ten genes were identified as being preferentially expressed in a specific cell lineage. This group including *DGCR6*, *CLTCL1*, *MRPL40*, *CLDN5*, *TBX1*, *C22ORF29*, *C22ORF25*, *DGCR8*, *ZDHHC8* and *RTN4R* were selected due to their expression profile that suggested an enrichment in dopaminergic neurons. Similar to the data presented earlier, the classification was based on first a visual assessment, then confirmed by statistical analysis. Reported functions of these genes are summarized in table 3.-5.

### Table 3-5 Function of the gene exhibiting cell type specific expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGCR6</td>
<td>Suggested to play a role in neural crest migration</td>
</tr>
<tr>
<td>CLTCL1</td>
<td>Encodes for CH22 protein thought to be involved in intracellular endosomal trafficking</td>
</tr>
<tr>
<td>MRPL40</td>
<td>Implicated in mitochondrial translation</td>
</tr>
<tr>
<td>CLDN5</td>
<td>Plays a role in tight junction at the blood brain barrier</td>
</tr>
<tr>
<td>TBX1</td>
<td>Transcriptional regulator involved in developing process</td>
</tr>
<tr>
<td>C22ORF29</td>
<td>May induce apoptosis in a BH3 domain</td>
</tr>
<tr>
<td>C22ORF25</td>
<td>Predicted to play roles in secretory protein in the endoplasmic reticulum</td>
</tr>
<tr>
<td>DGCR8</td>
<td>Component of the microprocessor complex that is required to process micro RNA</td>
</tr>
<tr>
<td>ZDHHC8</td>
<td>Palmitoyltransferase responsible of posttranslational modification</td>
</tr>
<tr>
<td>RTN4R</td>
<td>Mediates axonal growth inhibition, may be involved in axonal regeneration</td>
</tr>
</tbody>
</table>

In order to directly compare the relative level of expression, data of all four cell types from day 20 (committed neuronal progenitors) to 50 (neurons) were included in Figure 3-9.

*DGCR6* was already described above as a late expression gene, and indeed its transcript levels increased in cortical interneurons and glutamatergic neurons from day 40 onward. In addition, this gene demonstrated preferential expression in dopaminergic neurons at days 30 and 40. *TBX1* exhibited a peak of expression in dopaminergic neurons at day 30 and although it decreased thereafter, the fold change was still higher in these cultures compared to the other cell lineages. *CLTCL1*, *MRPL40*, *CLDN5*, *C22ORF29*, *C22ORF25*, *DGCR8*, *ZDHHC8* exhibited a constant expression in MSN, cortical glutamatergic and interneurons while peaking at day 30 in dopaminergic neurons. *RTN4R* followed a similar pattern of expression to the aforementioned group of genes in dopaminergic neurons, while its expression in the other lineages exhibited only a slight increase at day 50. Such enrichment of these particular genes in dopaminergic neurons may suggest that at this stage they are more important for the development of this specific category of neurons.
The result of the statistical analysis which aimed to compare whether we had different in expression between cell lineage at a given time point is presented below in table 3-6. Only significant data were presented.

### Table 3-6 Results of two-way ANOVA that compare difference between cell lineages

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Cell Type</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGCR6</td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>****</td>
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<td></td>
<td>40</td>
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<td>****</td>
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<tr>
<td></td>
<td>40</td>
<td>Dopaminergic vs MSN</td>
<td>**</td>
</tr>
<tr>
<td>CLCTL1</td>
<td>30</td>
<td>Cortical Interneurons vs Dopaminergic</td>
<td>**</td>
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<tr>
<td></td>
<td>40</td>
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<td>****</td>
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<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
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<td>50</td>
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<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
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<td></td>
<td>40</td>
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<td>****</td>
</tr>
<tr>
<td>MRPL40</td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>**</td>
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<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
<td>****</td>
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<td></td>
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<td>Dopaminergic vs MSN</td>
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<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
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<td>20</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td>CLDN5</td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
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<td>****</td>
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<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
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<td>****</td>
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<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
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<td>40</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>TBX1</td>
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<td>Glutamatergic vs Dopaminergic</td>
<td>*</td>
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<tr>
<td></td>
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<td></td>
<td>***</td>
</tr>
<tr>
<td>C22orf29</td>
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</tr>
<tr>
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<td>40</td>
<td></td>
<td>**</td>
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<tr>
<td></td>
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<td>Glutamatergic vs Dopaminergic</td>
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<td></td>
<td>****</td>
</tr>
<tr>
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<tr>
<td></td>
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<td>Glutamatergic vs Dopaminergic</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>DGCR8</td>
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<td>Glutamatergic vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>***</td>
</tr>
<tr>
<td>ZDHHC8</td>
<td>30</td>
<td>Cortical interneurons vs Dopaminergic</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Glutamatergic vs Dopaminergic</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>RTN4R</td>
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<td>****</td>
</tr>
<tr>
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<td>40</td>
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<td>****</td>
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<tr>
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<td>Glutamatergic vs Dopaminergic</td>
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<td>40</td>
<td></td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Dopaminergic vs MSN</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>40</td>
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</tr>
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</table>
Finally, table 3-7 shows a summary of above analyses, specifying at what stage of differentiation 22q11.2DS genes were expressed and whether they were enriched in a specific neuronal population.
Table 3-7 Summary of the findings on the expression of 22q11.2DS gene expression. Genes were classified according to their order in the genome.

<table>
<thead>
<tr>
<th>GENE LIST</th>
<th>Temporal profile</th>
<th>Cell type specific expression</th>
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<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Late</td>
</tr>
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<td>DGCR6</td>
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<tr>
<td>TSSK2</td>
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</tr>
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<td>CLTCL1</td>
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<td>SEPT5</td>
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<tr>
<td>GP1BB</td>
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<td>X</td>
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<td>TBX1</td>
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<tr>
<td>ARVCF</td>
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<td>C22orf25</td>
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<tr>
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<td>RANBP1</td>
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<td>ZDHHC8</td>
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<tr>
<td>RTN4R</td>
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</tbody>
</table>

3.3. Discussion

Previous studies in mouse brain have suggested that some of the 22q11 genes exhibited a temporally regulated expression fashion. However, little is known about the cellular identity in which specific 22q11 genes are expressed, particularly in human neurons. This chapter investigated the temporal expression profile of the genes located in the 1.5Mb deletion of the 22q11.2DS and identified genes whose expression was enriched in a specific neuronal population. Over the twenty-eight genes present in this region, twenty-two displayed identifiable expression features. Present data suggest that HIRA, CDC45, RANBP1 and UFD1L, are expressed early during hESCs neuroectodermal differentiation. Conversely, DGCR6, SEPT5, GP1BB and ARVCF are expressed later during neuronal development. Interestingly, DGCR6, CLTCL1, MRPL40, CLDN5, TBX1, C22orf29, C22orf25, DGCR8, ZDHHC8 and RTN4R showed preferential (or enriched) expression in dopaminergic neurons.

Variation in the expression of PRODH and COMT has been shown to cause brain abnormalities and increases vulnerability to schizophrenia (Zinkstok et al. 2008). Furthermore, due to their implication in neuronal physiology, these two genes were
intensively studied. PRODH is a mitochondrial protein responsible for the first step of the proline catabolism that is subsequently converted into glutamate (Willis et al. 2008). COMT is an enzyme that degrades catecholamines like dopamine or norepinephrine (Craddock et al. 2006). However, as presented in the current study, expression of COMT and PRODH did not show convincing results. Except in dopaminergic neurons where levels of COMT decreased, it remained constant in other neuronal populations. Furthermore, PRODH expression was similar to that of COMT in glutamatergic and cortical interneurons while it decreased in MSN and dopaminergic neurons. Despite the level of maturity of neurons in this study, they still may not be mature enough at these stages for these two specific genes to be expressed. It was demonstrated that PRODH as well as COMT showed enrichment in adolescent brain but not in embryonic brain supporting their role later in life (Lin et al. 2016).

Previous studies using mouse model of 22q11.2DS identified genes in the 1.5Mb deletion which have crucial role in early stages of brain development, namely, Cdc45, Hira, Ranbp1, Ufd1l (Meechan et al. 2015; Meechan et al. 2009). Thus, present experiment was in accordance with these previous findings as the same genes were identified as having their peak of expression early in the process of differentiation.

The role of RANBP1 at the first stage of neuronal differentiation is coherent with a recent study which revealed that Ranbp1 homozygous mice showed cortical growth alteration (Paronett et al. 2015). Authors discovered that it was due to a disruption of the basal progenitors which in turn are responsible for the generation of the layer 2/3 projection neurons (Paronett et al. 2015).

Due to the function of Ranbp1 early in cortical neurogenesis, it is very likely that despite the absence of RNA samples before day 30 in glutamatergic and cortical interneurons, one should expect to observe a similar profile to that observed in dopaminergic and MSN differentiation.

Moreover, individual homozygous knock-out of these four genes, Cdc45, Ranbp1, Ufd1l and Hira was demonstrated to be lethal, emphasizing their importance during development (Meechan et al. 2015).

With novel tools available for gene editing, such as CRIPSR/Cas9, it would be interesting to generate hESCs lines deficient for these genes in order to study their role over the course of human neuronal differentiation.
Tssk2 and Gp1bb are two genes whose expression was not detected in the mouse brain in previous studies (Maynard et al. 2003). Tssk2 was shown to be mainly expressed in testis while the Gp1bb has been associated with platelet adhesion. However, present findings suggest that these two genes might be expressed in the brain. Indeed, GP1BB exhibited a profile of expression that increased along the course of differentiation in all four neuronal populations. TSSK2 transcripts were also observed in all four neuronal lineages whereas this gene was previously shown to be absent or only weakly expressed in the brain (Li et al. 2011). To conclude, current data demonstrated the presence of two novels genes in differentiating human neurons, GP1BB and TSSK2. While the function of TSSK2 in the brain is still largely unknown, present study suggested that this gene may have a function in mature neurons. On the other hand, as GP1BB is important for platelet formation which involves secretion of granules, a recent study from Goubau et al. identified common molecular mechanism between granule trafficking in platelets and neurons (Goubau et al. 2013). Thus, it would be interesting to study the role of GP1BB in mature neurons to identify whether it has a role in neuronal trafficking.

All genes that were classified as being expressed later during development in current analysis have been implicated in neuronal physiology. Septin5 has been shown to be expressed exclusively in non-dividing cells and its role in neurons has been linked to vesicular exocytosis, an important process in neurons. Dgcr6 has been found to interact with GABA_B1, a metabotropic receptor subunit part of the GABA_B metabotropic receptor (Zunner et al. 2010). On the other hand, Arvcf has been suggested to be expressed in the MGE during development.

Thus, it can be concluded that the increase of expression of these different genes is consistent with neuronal maturation. During this process, vesicle exocytosis increases, more receptors are either secreted or sent to synapses and neurons in turn are making more connections giving rise to the network complexity.

Present analysis discovered that a subset of 10 genes from the 1.5Mb deletion exhibited an enrichment in dopaminergic neurons when compared to other neuronal populations. Although these results have not been replicated yet, it is the first time that a study revealed an enrichment of these genes in a specific neuronal population.

Dopaminergic alteration is known to be implicated in the occurrence of both Parkinson’s disease and schizophrenia. Indeed, dopaminergic neurons were observed to be involved in cognitive deficits in individuals with schizophrenia. This alteration of dopamine
transmission occurs primarily in the mesolimbic brain area, but also in the amygdala and prefrontal cortex. On the other hand, Parkinson’s disease occurs due to a loss of dopamine neurons from the substantia nigra pars compacta. It would thus be interesting to investigate the function of these genes in dopaminergic neurons.

In addition to the study presented in this chapter and due to the availability in our institute of iPSCs derived from individuals carrying a 22q11.2 mutation, it would be interesting to perform a similar experiment than the one presented in this chapter. This study would aim to investigate whether hemizygous deletion of the 22q11.2 genes alter their temporal profile but also determine if we would obtain similar genes enrichment than the one observed in the control experiment. This experiment therefore might explain the appearance of the subsequent neurological alteration observed in patients with 22q11.2DS.

Finally, our experiments revealed limitations regarding neurons differentiation. Indeed, genes such as COMT or PRODH are usually expressed in adolescent neurons. However, neurons derived stem cells in vitro never mature past the second trimester. Therefore, progress would be necessary to obtain neurons in vitro that would mimic older neurons in human. Recently a new media was developed by Telezhkin et al. 2016, named the synaptoJuice (Telezhkin et al. 2016). This media consists of different steps and different media composition during the course of the differentiation. Authors demonstrated that it improves the maturation of hESCs derived GABAergic neurons in vitro. Others have also investigated the composition in ions of neuronal media. The study demonstrated that the culture media usually used for neuronal differentiation is altering the electrical activity thus synaptic communication of neurons. Therefore, they designed a new media in which the ions concentration was adjusted in order to mimic physiological condition. Future studies should also consider the use of different medias when examining genes whose function appear later during development.

To summarize, the data presented in this chapter, we first confirmed that neurons derived stem cells are able to recapitulate expression features observed in mouse model of 22q11.2DS. Secondly, our analysis successfully identified genes that exhibit a specific expression profile across lineages. Indeed, our classification revealed genes with a preferential expression early or late during the development while others showed a gene specific enrichment in dopaminergic neurons when compared to the other cell lineages.
Although, the classification was exclusively based on visual assessment at first, statistical analysis was enable us to confirm our categories. To conclude, this study gives valuable insight on the potential genes associated with early and late neuronal differentiation which therefore might be responsible of the underlying pathologies in 22q11.2 carriers. In addition to these genes, we demonstrated that the cell type is also important when considering studying a gene.
4. Generation and characterization of ZDHHC8-deficient hESCs lines

4.1. Introduction

ZDHHC8 is a particularly interesting candidate within the 1.5Mb of 22q11.2DS due to its suggested physiological role in neurons. It is a palmitoyl acyltransferase (PAT) protein belonging to a family of 23 members that share a conserved cysteine rich domain referred to as the DHHC domain (Korycka et al. 2012). Protein palmitoylation (protein S-acylation) is a process which consists of 16-carbons fatty acids attached onto a cysteine residue (Chamberlain et al. 2015). Compared to the other lipid modification such as myristoylation or geranylgeranylation, palmitoylation is the only posttranslational lipid modification that is reversible which thus has a significant role in the control of cellular protein dynamic. Palmitoylation enhances the hydrophobicity of protein thus contributes to their membrane association, protein-protein interaction as well as their subcellular trafficking (Globa et al. 2017; Holland et al. 2017; Huang et al. 2005).

Figure 4-1 Palmitoylation of protein regulates synaptic transmission. This figure shows that several key regulators of synaptic transmission located at the pre and post-synaptic neurons require to be palmitoylated. On the presynaptic side, palmitoylation is necessary for protein regulating synaptic vesicle fusion and neurotransmitter synthesis and release. We can also found ions channel. On the postsynaptic side, protein palmitoylation include protein involved in the scaffolding of receptors but also ionotropic receptors. Figure adapted with permission from Springer: Nature reviews. Neuroscience, El-Husseini 2002 (4296960901940) Copyright (2002).
Growing lines of evidence suggest that protein palmitoylation plays a central role in synaptic physiology. As illustrated in Figure 4-1 (Fukata et al. 2010), protein palmitoylation is suggested to be essential for the proper function of the majority of the pre- and post- synaptic proteins, as well as proteins involved in neurons development (el-Husseini et al. 2002).

Studies of Zdhhc8 homozygous deficient mice have demonstrated interaction between ZDHHC8 and PSD-95, a key component of the postsynaptic density responsible for the anchoring of postsynaptic receptors as well as protein that mediates the formation and maintenance of synapses (Irie et al. 1997; Sheng 2001; Sheng et al. 2011). Previous studies have also shown that ZDHHC8 is the only palmitoylation enzyme for Paralemmin-1, a regulator of filopodia (small protrusion on dendrites that serves as precursor of spines) (Gauthier-Campbell et al. 2004). Thus, reduction of Paralemmin-1 palmitoylation was suggested to result in a decrease of functional synapses. Similarly, CDC42, a protein regulating actin cytoskeleton has been demonstrated to be palmitoylated by ZDHHC8. Thus, impairment of CDC42 palmitoylation results in a decrease of dendritic and axonal arborisation (Mukai et al. 2008, 2015; Nishimura et al. 2013; Wirth et al. 2013).

ZDHHC8 has been shown to be primarily localized in the vicinity of the nucleus and in vesicle-like clusters in the dendritic shafts (Mukai et al. 2004). However, a study from Maynard et al. 2008 observed that ZDHHC8 might also be localized in the mitochondria, which was demonstrated by its colocalization with a mitochondria specific protein UQCRC1 (Maynard et al. 2008; D. W. Meechan et al. 2011).

It is known that 30% of individuals carrying the 3Mb or 1.5Mb 22q11.2 deletion develop schizophrenia (Liu et al. 2002). Recently, it was also demonstrated that individuals with 22q11.2DS have an increased risk to develop Parkinson disease (Dufournet et al. 2017). Furthermore, several studies identified single nucleotide polymorphism (SNP) which were shown to increase the risk to develop schizophrenia, one of which is rs175174 A/G in ZDHHC8 locus (Chen et al. 2004; Mukai et al. 2004). This specific SNP located in the middle of the intron 4 of ZDHHC8 gene is expected to introduce a STOP codon which is suggested to lead to a premature stop of translation. However, several other studies revealed no association with schizophrenia for this SNP (Demily et al. 2007; Glaser et al. 2006; Otani et al. 2005).
Neuronal migration defect was observed in patients carrying 22q11.2 mutation but also in patients developing schizophrenia (Castro et al. 2011; Nadarajah et al. 2003). Therefore, it would be interested to identify whether ZDHHC8 alter the migration process in culture of neurons.

Dysregulated neuronal electrical activity has been identified in mouse models of 22q11.2DS (Earls et al. 2010). These mice exhibited an increase in their short- and long-term plasticity. Moreover, neurons presented an increase in presynaptic neurotransmitter release whilst the postsynaptic function was unaltered. This increase of neurotransmitters release was suggested to be linked to the altered calcium kinetics and upregulation of the sarco-endoplasmic reticulum calcium ATPase type 2 (SERCA2) which was observed in CA3 presynaptic terminal.

Aims

In this chapter, we investigate the role of ZDHHC8 in cortical neuronal development using hESCs as a model. The specific objectives are:

- To generate and validate hESCs deficient for ZDHHC8 using the CRIPSR/Cas9 technology
- To evaluate the effect of ZDHHC8-deficiency in cortical excitatory neuron differentiation (kinetics and expression)
- To investigate the role of ZDHHC8 in glutamatergic progenitors and neurons using motility assay as well as calcium imaging

4.2. Results

4.2.1. Generation of ZDHHC8 KO hESCs using CRISPR/Cas9

The all in one multiplex CRISPR/Cas9 employed to target and edit ZDHHC8. This method is believed to reduce the stress caused by multiple plasmids insertion and increase the cutting efficiency. Indeed, in order to efficiently target and maximize the likelihood of at least one gRNA inducing INDELs in ZDHHC8, we designed four gRNAs, with two targeting exon 3 and two targeting exon 4 respectively named 1, 2, 3 and 4 (Figure 4-4). The gRNAs were chosen according to their off-target score; high score closed to 100
indicated a low probability of off-target of the specific gRNA. Our gRNAs 1, 2, 3 and 4 had respectively a score of 76, 91, 85 and 88.

4.2.1.1. Construction of the multiplex CRISPR/Cas9 plasmid

To generate a stem cell lines deficient for ZDHHC8, I used the all-in-one multiplex CRISPR/Cas9 system. As illustrated in Figure 4-2, two steps were necessary for its construction. The first one consisted of the insertion of each gRNA into individual plasmid whereas the second step involved the assembly of each individual gRNA into the final recipient plasmid by golden gate assembly (Sakuma et al. 2014).

Figure 4-2 Illustration of the construction of the multiplex CRISPR/Cas9. Two plasmids are necessary for the construction of the all-in-one CRISPR/Cas9, one ampicillin resistant plasmid in grey and three others spectinomycin resistant plasmids in purple. The Cas9 protein is represented in red. The U6 promoter in green is necessary to drive the expression of the gRNAs. The BbsI enzyme in black was used to clone the gRNAs. The presence of overhangs specific for BbsI (see 2.4.5.2) ensured the correct orientation of the gRNAs into their recipient plasmids. Moreover, once ligated, the BbsI site disappeared therefore the enzyme can be used to screen for positive clone. Instead, BsaI enzyme in pink was used for the golden gate assembly. BsaI first opens the ampicillin plasmid then cut at the restriction site on the spectinomycin plasmids (see Figure 2-2). Finally, the AflIII and KpnI enzymes in grey and orange respectively was used to control the total number of gRNA cloned following golden gate assembly. Figure adapted from Tetsushi Sakuma et al. 2014.
In order to determine whether each individual gRNA in step 1 were properly inserted, a double digestion using BbsI and EcoRI was performed (Figure 4-3A-D). Figure 4-3A-D illustrates the digestion pattern of the four-different gRNAs from which we have five samples for the 1st, 2nd and 4th gRNAs and four samples for the 3rd gRNA. The positive control (C+) corresponded to the expected pattern for an empty plasmid with two bands at 5255 and 3421bp.

Due to the presence of a difference resistance cassette in the 1st plasmid, ampicillin instead of spectinomycin for the remaining plasmids, the size of the 1st plasmid was larger than the others (Figure 4-3A).

Figure 4-3A, B and D, we can observe that the sample 1 of the 1st, 2nd and 4th gRNAs have a digestion product similar to the C+ thereby the gRNAs are not inserted. On the other hand, as all other samples have one digestion product at 8702bp, we concluded that they were cut by EcoRI only and therefore the gRNAs were inserted. To conclude, the first step resulted in an 80% of efficiency of insertion.

The step 2 is illustrated in Figure 4-2 and 2-2 and consisted to the golden gate reaction using the BsaI restriction enzyme that enabled to cut and insert the gRNAs of each spectinomycin plasmid into the final recipient, the ampicillin resistant plasmid. As illustrated in Figure 4-2, the BsaI site of the ampicillin plasmid is located downstream of the gRNA insertion site therefore resulting to the opening of the plasmid. On the other hand, the remaining three spectinomycin plasmids have two sites for BsaI respectively upstream and downstream of the gRNAs and gRNA scaffold which resulted to small fragments of 455bp which were then inserted into the recipient ampicillin plasmid. The reaction uses the sample 2 from each gRNA (step 1). The restriction enzymes AflIII and KpnI were used to screen and identify the number of gRNAs inserted. While an empty vector (C+) had a digestion product of 8063bp, a plasmid with 4, 3 or 2 gRNA had an additional digested product at 1790bp, 1335bp or 880bp respectively. From the 13 plasmids screened, 61% appeared to have correctly integrated the four gRNAs.

To generate the hESCs line deficient for ZDHHC8, we used the plasmid number 2 from the second step, Figure 4-3E.
4.2.1.2. Generation of ZDHHC8-deficient hESCs

The iCas9 hESCs line was used to generate the ZDHHC8 KO. Cells were nucleofected as described in the methods with the multiplex CRISPR/Cas9 construct along with a plasmid containing a neomycin resistant gene. Cells were cultured for five days to form individual colonies before being picked.

As shown in Figure 4-4, exon 3 and 4 of ZDHHC8 were targeted by two gRNAs each. This particular region was chosen because it encodes the DHHC domain responsible for the enzymatic activity of ZDHHC8. Thus, frame shift mutations in this region is expected to disrupt the enzymatic activity of the protein.

reaction without plasmid. E, step 2 of the multiplex CRISPR/Cas, the picture represents a gel electrophoresis preceded by a double digestion by BbsI and EcoRI to confirm the construction of the all-in-one CRIPSR/Cas9 by golden gate assembly. These two-restriction enzymes are flanking the region of the gRNAs. Thus, as shown, a band at 880, 1335 or 1790bp respectively correspond to a plasmid which contained 2, 3 and 4 gRNAs.
Genomic PCR was employed to examine whether INDELs were generated. A pair of primers was designed to encompass the four gRNAs which gave rise to a predicted 470 nucleotides amplicon in wild type allele (Figure 4-4).

Figure 4-5 illustrates an example of gel electrophoresis of the amplified PCR fragments from the parental isogenic iCas9 hESCs (C+) and four independent clones (297, 510, 539 and 541) carrying a mutation. The control amplified only one band corresponding to the predicted 470bp PCR product. On the other hand, in addition to a 470bp band, an additional amplicon of about 300bp in length was also present in clones named 297, 510, 539 and 541. This pattern suggested that these clones might be heterozygous for ZDHHC8. Of the 100 clones screen, 12 clones yielded an additional PCR product, all of which are around 300bp.
PCR fragment from clones presumably heterozygous for ZDHHC8 were ligated into the pGEM-T-easy vector. Due to the presence of two different restriction sites for EcoRI flanking the cloning region, a gel electrophoresis was performed to distinguish the wildtype and mutated amplicon. The plasmids containing candidate mutant amplicons were sent for sequencing with selected wildtype controls. The result is illustrated in Figure 4-6. We observed that amongst the four-gRNAs used to target exon 3 and 4, only gRNA 2 and 3 successfully cut the DNA sequence and yielded to a deletion of 166bp length. Interestingly, the deletion was almost 100% identical between the different mutant amplicons. Furthermore, except one amplicon which had a guanine instead of an adenosine, the sequences of the WT mutant amplicons were 100% identical. The WT amplicons were also almost 100% identical except for couple of bases pairs difference at random location. In addition, in mutants and controls the WT amplicons were also similar. Overall the difference in several bases pairs was attributed to error in the sequencing process rather than mutation.

While the gDNA sequence was deleted of 166bp, the resulting mRNA sequence which contains only exons had a deletion of 83 nucleotides. This deletion was suggested to result in the appearance of a premature STOP codon and therefore a truncated protein (Figure 4-7). Figure 4-7A represents the gDNA sequence of the WT ZDHHC8 which is composed of 11 exons. On the other hand, Figure 4-7B shows the resulting gDNA of the mutated allele of ZDHHC8 on which we observed the disappearance of the intron 3 as well as the truncation of the exon 3 and 4. While WT full length ZDHHC8 protein is 765aa, prediction of the resulting heterozygous protein showed a truncated protein of 147aa. In addition, it appeared that the enzymatic domain of the protein, the DHHC domain is missing. Therefore, it would suggest that the palmitoylation activity would be reduced by half in the mutant.
Figure 4-6 Sequencing results of different clones. Twenty sequences were sent for sequencing, four controls together with four clones, 297, 510, 539 and 541. The sequences highlighted in yellow represent the position of the pair of primers used for the screening while in green are the location of the four different gRNAs used to edit the ZDHHC8 sequence. In total, we sequenced four PCR fragment for each of the clones.
4.2.2. General phenotypic characterisation of ZDHHC8 deficient hESCs

4.2.2.1. Stem cell properties

To determine whether ZDHHC8 heterozygous cells maintain key pluripotency properties, we examined the expression of OCT3/4, TRA1-81 and SSEA3 by immunostaining on two heterozygous (clones 297 and 541) and the parental iCas9 hESCs (Figure 4-8). OCT3/4 is a transcription factor playing a pivotal role in controlling self-renewal of embryonic stem cell, while TRA1-1 and SSEA3 are surface markers expressed by undifferentiated hESCs. As shown in Figure 4-8A and B, the majority of cells expressed OCT4, TRA1-81 and SSEA3. The proportion of cells stain positive for the different markers are similar between the control iCas9 hESCs and the two ZDHHC8 heterozygous...
lines. Therefore, the major pluripotent characteristics is preserved in ZDHHC8-heterozygous hESCs.

Figure 4-8 Immunofluorescence of iCas9 hESCs and two ZDHHC8+/-, 297 and 541. (A), (B) represents pictures acquire using a LEICA DMI6000B Inverted Time-lapse microscope. Those pictures are a tile scan with a magnification of 40x in order to acquire the entire colony and stained by the nuclear marker OCT3/4 and the membrane marker TRA1-81 and SSEA3. In (A), TRA1-81 is co-stained with DAPI and expressed all over the cells in all different cell line. (B), SSEA3 and OCT3/4 are co-expressed with DAPI in all cells.

In order to determine if the expression level of ZDHHC8 was affected by the deletion, RNA was extracted from self-renewing ZDHHC8 KO clones. One set of primers targeting the deleted exonic region was designed as illustrated in Figure 4-9 below. Thus, it was possible to assess whether the deletion in the genomic DNA (gDNA) remained at the mRNA level.
Figure 4-9 Real-time PCR screening strategy for ZDHHC8. One set of primer in purple was targeting the exon 3 and 4 within the deletion respectively in red and green. In WT condition, primers are targeting both allele of ZDHHC8. On the other hand, in the mutant condition, because the primers are targeting the region deleted in the mutant allele of ZDHHC8, we are expecting to observe an expression of ZDHHC8 which is reduced by half when compare to the control.

Figure 4-10 illustrates the relative RNA level of ZDHHC8 in undifferentiated cells using a set of primer targeting the deleted region to assess if the deletion resulted to a decrease in the mRNA levels of ZDHHC8. The expression in cell line 297, 510 and 541 showed a 50% reduction of ZDHHC8 mRNA expression. Although the clone 539 carried a similar deletion than the cell line 297, 510 and 541, the mRNA expression was unchanged.

Figure 4-10 ZDHHC8 expression at the stem cells levels. Quantitative PCR at the stem cells levels using primers for ZDHHC8. The primers target the region deleted in ZDHHC8+. Data were normalized to the housekeeping gene β-actin. 297, 510, 539 and 541 are four cell type that demonstrated to have a deletion between the exon 3 and 4 of the ZDHHC8 gene. Statistical analysis using one-way ANOVA failed to demonstrated significance.
4.2.2.2. Expression of key class defining molecular markers during cortical differentiation of ZDHHC8 +/- hESCs

Normal brain development can be ‘visualised’ by dynamic temporal and spatial expression of region- and cell type-specific gene markers. During pluripotent stem cell in vitro differentiation, regulated expression of these markers can be used as readout to access whether neural development is in order. To investigate whether ZDHHC8 deletion affects the process of cortical neural differentiation, our hESCs were differentiate towards the neuroectoderm fate using dual SMAD inhibition with LDN and SB, the concentration of each compounds is shown in 2.2.3.1. Cells which are mainly of cortical excitatory neurons identity with this protocol were let to mature for the subsequent experiments. We performed a panel of immunofluorescence to evaluate the proportion of cells expressing specific markers. While we according to chapter 3, ZDHHC8 expression was suggested to be preferentially expressed in dopaminergic neurons, experiment in chapter 3 and 4 were performed in parallel therefore we were unaware of this result at the time.

PAX6 and FOXG1 were used as markers of cortical identity (Figure 4-11). PAX6 is expressed in the neuroepithelial cells in the VZ of the developing pallium. While FOXG1 is expressed by cells in the neural plate from which the telencephalon is originating. As shown in Figure 4-11, a large proportion of cells stain positive for FOXG1 and PAX6 (Figure 4-11A and B), suggesting efficient conversion of hESCs to the forebrain fate. Moreover, we detect no difference in the proportion of PAX6+ or FOXG1+ cells between the wild-type and ZDHHC8 +/- cultures (Figure 4-11.C-D).

Finally, Figure 4-11E represented the temporal expression of PAX6 in control and mutant ZDHHC8 +/- . The expression of PAX6 was absent from the stem cell stage. The expression profile of PAX6 was shown to increase at day 20-30 which corresponded to the neural progenitor’s cells (NPCs), while it decreased thereafter and became significantly reduced compared to day 10 from day 60 onwards. On the other hand, unlike to control, the expression of PAX6 in ZDHHC8 +/- showed no increases at the progenitor’s stage between day 20-30. However, PAX6 expression decreased from day 50 and become significantly reduced from day 60 onwards when compared to day 10 of ZDHHC8 +/- .
During differentiation, hESCs-derived neural epithelial cells often organize themselves in polarized structures referred to as neural rosettes. This structure has a radial arrangement that mimic the formation of the neural tube in vitro. To determine if
ZDHHC8+/- influenced the development of the neural rosette, the N-cadherin antibody which stains the apical structure of the neural rosette was used (Figure 4-12). A similar morphology and number of rosettes was observed in ZDHHC8+/- and control cultures, suggesting that reduced levels of ZDHHC8 do not compromise their formation (Figure 4-12A and B). However, analysis of their area demonstrated that ZDHHC8+/- mutant cells have a small but significant neural rosette area (p < 0.0001).

During the first ten days of differentiation, cells are transiting from pluripotent stem cells to neuroectodermal cells. At around days 10-12 of differentiation, the cultures are enriched with cycling neural progenitors. To determine whether progenitor proliferation was affected in ZDHHC8+/- cultures, an antibody staining for Ki67, a marker for cells in all phase of the cells cycle, was performed (Figure 4-13). Figure 4-13A displays the cells following immunostaining. Quantification of the number of positive cells demonstrated that the proportion of Ki67 positive cells in the WT was 45.71 ± 2.303 (mean ± SEM)
while it was 56.94 ± 1.679 in ZDHHC8+/- . Thus, these data suggested that proliferation of the neuronal progenitors were increased in ZDHHC8+/-.

At day 30 of differentiation, neurons start to express proteins of the different cortical identity which start by the deep layer 5 and 6 as cortex is generated in an inside out manner. CTIP2 antibody was used to evaluate the proportion of neurons from the layer 5 (Figure 4-14A). Both control and mutant expressed CTIP2 proteins. However, quantification revealed a significant decrease of CTIP2 positive neurons in the mutant line (Figure 4-14B). Indeed, the proportion of CTIP2 in ZDHHC8+/- was 35.58 ± 1.653 (mean ± SEM, P < 0.0042) while in the control it was 45.80 ± 3.046 (mean ± SEM). Finally, we observed that the temporal expression profile of CTIP2 (Figure 4-14) showed a peak of expression at day 30 and decreased thereafter. On the other hand, the expression of CTIP2 increased from day 30 but remained relatively constant until day 70 and then started to decrease at day 80.
4.2.2.3. Gene expression analysis of \textit{ZDHHC8+/-} hESCs derived cortical neurons

To investigate whether \textit{ZDHHC8} heterozygous deletion affects glutamatergic neuron differentiation, we examine the expression of a panel of cortically expressed genes during the course of differentiation. RNA samples were extracted and run with the Biomark HD. 

Ct value above 22 were considered as not expressed and thus discarded from the analysis. The data were presented to first assess the temporal profile and therefore compared to day 0. Next, we compared the expression of \textit{ZDHHC8+/-} to the control and
therefore $ZDHHC8^{+/-}$ expression was compared to the control of the same time point. In addition, similar to chapter 3, the statistical analysis performed in this chapter are the result of one experiment with $n=3$ biological replicate. It was noteworthy that some time point only exhibited two biological replicates due to the presence of outliers.

Although, $ZDHHC8^{+/-}$ do not affect the expression of the pluripotency markers at the protein levels, the expression of $OCT4$ ($POU5F1$) is examined to determine whether $OCT4$ extinction is delayed in $ZDHHC8^{+/-}$ (Figure 4-15). We observed that $OCT4$ mRNA expression stopped from being expressed in both control and mutant from day 10. Furthermore, the relative level of $OCT4$ at the stem cell levels was similar between $ZDHHC8^{+/-}$ and the control. Thereby, heterozygous mutation of $ZDHHC8$ do not seem to alter pluripotency.

4.2.2.3.1. Temporal expression profile of key markers of differentiation

In addition to the temporal expression of $PAX6$ and $CTIP2$, we also investigated the profile of other key molecular markers to evaluate whether the conversion of $ZDHHC8^{+/-}$-cells into glutamatergic neurons followed a similar kinetic than the control. (Figure 4-16). These data were used to visualize the expression profile compared to day 0. Thereby, data indicated whether the expression at a given time point was different to day 0, see Figure legend. In control condition, the expression of $ZDHHC8$ showed a slight increase for couple of time points while the expression in the heterozygous was constant along the course of the differentiation when compared to day 0. (Figure 4-16A). We examined the
EMX family of transcription factor, *EMX1* and *EMX2* (Figures 4.16B and C). Both genes are expressed by cells in the dorsal telencephalon however, *EMX2* is mostly expressed in the visual area of the cortex. While the *EMX1* expression rapidly decreased and remained relatively weakly expressed from day 20 to 80 when compared to day 0 in both the control and the ZDHHC8+-/- neurons, *EMX2* expression appeared to gradually increase from day 30 to day 80. TUJ1 is a beta tubulin protein encoded by the *TUBB3* gene which is primarily expressed in neurons (Figure 4-16D). The control and the mutant neurons exhibited a similar expression profile of *TUBB3* during the course of the differentiation.

The expression of genes involved in the patterning of the cerebral cortex such as *TBR1*, *CUTL1* (CUX1) and *FOXP2* were also examined (Figures 4-16E-G). These genes were demonstrated to be expressed by cells of a specific cortical layer. *TBR1* is specifically expressed by new-born neurons from the cortical layer 6. *FOXP2* is also expressed by deep layer neurons, usually layer 5-6. *CUX1* is an upper layer marker, expressed by layer II/IV neurons. Similarly, the profile of expression of ZDHHC8+-/- followed a similar pattern than the control for *TBR1*, *CUTL1* and *FOXP2*.

Next, we examined the temporal profile of genes that have a functional role for neurons such as genes coding for the NMDA receptors but also presynaptic genes involved in neurotransmitter transport such as VGLUTs (Figures 4-16.H-K).

In the heterozygous neurons for ZDHHC8, we can observe that the expression profile of *DGLA* and *GRIN2B* have a similar profile than the control (Figures 4-16H and I). The expression profile of *GRIN2A* and *SLC17A7* followed a similar trend which increase along the course of differentiation in both mutant and control. However, by day80, we observed a stronger expression of *GRIN2A* in control as well as a stronger expression of *SLC17A7* from day 50 and onwards in the control condition.

Thus, we conclude that overall, the temporal expression analysis indicated that the kinetics of the differentiation in the ZDHHC8+-/- followed a similar profile than the control.
4.2.2.3.2. Differential expression of key neuronal genes between control and mutant

In addition to evaluate the temporal expression, we also compared the expression of ZDHHC8+/− with its isogenic control to determine whether genes were found to be differentially expressed.

We investigated the expression of PAX6 and OTX2 (Figure 4-17B and C). We observed that the expression of PAX6 was particularly downregulated at day 10 and 30 which correspond to the progenitor’s stage (Figure 4-17B). The expression of PAX6 remained downregulated at day 60 and 80 but in less extend than at day 10 and 30. Conversely, OTX2 a gene expressed by forebrain and midbrain neuronal precursors, showed a strong...
Figure 4-17 Temporal expression of key expressed gene in ZDHHC8+/− and the parental line. The control and ZDHHC8+/− were respectively represented in grey and red. A, ZDHHC8. B, EMX1 expression was demonstrated to be significantly decreased from day 20 and onwards in both control and ZDHHC8+/−. At day 20 and 30 in the control and day 30, 50, 60 and 70 in ZDHHC8+/−, we only had one biological replicate. C, EMX2 expression profile was significantly increased from day 50 onwards in ZDHHC8+/− while only significantly increased at day 70 and 80 in the control. Data in control at day 40 had one biological replicate. D, TUBB3 (TUJ1) expression was observed to be significantly increased in ZDHHC8+/− from day 20 onwards when compared to day 0. Although the expression in the control appeared to be increased statistical analysis failed to reach significance. E, TBR1 expression was significantly higher at day 30 and 40 then decreased until day 80 in ZDHHC8+/− when compared to day 0. While similar trend was observed in the control, statistical analysis did not reach significance as data were compared to day 30 because data at day 20 only had one biological replicate. F, CUTL1 (CUX1) was observed to be relatively constant along the course of differentiation in control and ZDHHC8+/− when compared to their respective day 0. G, FOXP2 expression was observed in both conditions to be significantly higher at day 30 then started to decrease until day 80 although data remained significantly increased when compared to day 0. H, DLG4 (PSD-95) expression was shown to be significantly increase along the course of differentiation in both control and ZDHHC8+/−. I, GRIN2B expression was significantly increased from day 30 onwards in both control and ZDHHC8+/− when compared to value at day 0. J, GRIN2A expression showed an important and significant increase of its expression towards the end of the differentiation at day 80 in control and ZDHHC8+/− when compared to day 0. K, SLC17A7 (VGLUT1) expression was observed to significantly increase along the course of differentiation in control. However, while the expression in ZDHHC8+/− also appeared to increase the statistical analysis failed to reach significance. The data presented correspond to one experiment with n = 3 biological replicates. Data were normalised to the housekeeping gene β-actin and compared to day 0. Error bars represent +/-SEM. Statistical analysis was performed on the ΔCT value with a one-way ANOVA with Bonferroni post-hoc to compare whether for each differentiation (control and ZDHHC8+/−), the expression at each time point differs from day 0 (Control and ZDHHC8+/−). The significance is showed using * for the control and # for ZDHHC8+/−. The cell line used for the cortical neurons differentiation was iCas9 for the control and 541 for ZDHHC8+/−. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

upregulation from day 60 and day 80 while its expression was similar at earlier time point when compared to the control (Figure 4-17C). EMX1 expression was shown to be downregulated of more than 50% in the mutant ZDHHC8 at day 30 and 80 while similar at day 10 between control and mutant (Figure 4-17D). EOMES (TBR2), a transcription factor expressed by intermediate progenitor cell (IPC) also known as basal progenitors, is observed to have an expression comparable to control at day 10, however by day 30 its expression was significantly downregulated to be almost null (Figure 4-17E). Despite the strong downregulation of EOMES in ZDHHC8+/−, examination of the Ct values revealed that they were below 22 therefore the transcript of EOMES was present in enough proportion to be detected. FOXP2 expression was also observed to be downregulated of about 50% in ZDHHC8+/− (Figure 4-17H). The expression of TBR1 was shown to be increased at day 60 and 80 when compared to the control (Figure 4-17F). While the expression of BCL11B was downregulated of 50% in ZDHHC8+/−, at day 80 its expression was upregulated in ZDHHC8+/− compared to the control (Figure 4-17G). The expression of CUTL1 did not appear to be different in ZDHHC8+/− (Figure 4-17I). While our differentiation protocol is thought to give rise to excitatory glutamatergic neurons, an upregulation of GAD1 was observed at day 80 in ZDHHC8+/− (Figure 4-17J).
The NMDA receptor is located at postsynaptic site of the neurons. This receptor is an heterotetramer composed of four subunits. We were unable to detect the transcript of GRIN2C and GRIN2D in both control and mutant neurons. Despite the expression of GRIN2A was similar to the control in the mutant neurons at day 10 and 30, the expression was downregulated of almost 50% at day 60 and 80 in the mutant (Figure 4-17K).

SLC17A7 and SLC17A6 are two important genes encoding the proteins VGLUT1 and VGLU2 respectively (Figure 4-17L and 4.17M). The vesicular glutamate transporters family allows the loading of glutamate into vesicles. We observed a downregulation of SLC17A7 from day 10 which persisted until day 80 (Figure 4-17L). Conversely, examination of SLC17A6 mRNA expression in ZDHHC8+/- neurons was observed to be similar to the control at day 10 and 30 while it was downregulated at day 60 and 80 (Figure 4-17M).

DLG4 encodes for PSD-95, a protein expressed at the postsynaptic membrane of the neurons which is part of the postsynaptic density (Figure 4-17N). This scaffolding protein is responsible of the anchoring of different synaptic receptors and ions channels to the membrane. The data were unable to show an alteration of DLG4 in ZDHHC8 +/- differentiation at the mRNA levels except at day 80 which exhibited a downregulation of DLG4 expression in ZDHHC8+/- neurons.

To finish, I also examined whether the expression of ZDHHC8 was impaired during the differentiation using a similar set of primers than Figure 4-9 (Figure 4-17A). Overall, a 50% reduction of ZDHHC8 mRNA expression at each time point was observed suggesting that the deletion was still present while undergoing neuronal differentiation.
Motility defect in ZDHHC8+/- neuronal progenitors

Homozygous and heterozygous mouse model for Zdhhc8+/- showed alteration of axon growth and arborization in vitro due to a decrease of CDC42 palmitoylation by ZDHHC8.
In order to investigate whether ZDHHC8 is involved in the migration of excitatory neuronal progenitors, we investigated their motility using motility assay.

Excitatory neuronal progenitors at day 20 of differentiation from two independent experiments using the heterozygous mutant 541 and the control iCas9 were imaged over 12 hours and subsequently analysed. Different parameters were analysed such as the total distance, the displacement, the speed and the velocity (Figure 4-18). This analysis revealed that neuronal progenitors deficient for ZDHHC8 migrated less when compared to the control progenitors (Figure 4-18A). The displacement was also significantly decreased in the mutants compared to the control (Figure 4-18B). It also appeared that mutant neural progenitors were significantly slower as shown by the speed and the velocity (Figures 4.18C and D).

This data suggests that ZDHHC8+/- impairs migration and motility of the excitatory neuronal progenitors. Because cells were too close from each other we were unable to trace neurons in order to determine their length and branching number.

Figure 4-19 Glutamatergic progenitor cells motility. Cells were recorded for 12h in a recording chamber (Temperature, CO₂ and humidity monitored) under a light microscope. Images were acquired every 5min with a 10x objective at different location. Data were analysed and visualized using a bar plot which represent mean ± SEM. A, total migration in µm. of the WT and the KO ZDHHC8. Control = 83.92 ± 2.645 µm; KO = 51.42 ± 1.228 µm, P < 0.0001. B, displacement in µm, WT = 50.50 ± 3.428 µm; KO = 27.22 ± 2.411 µm, P < 0.0001. C, Migration speed in µm/min, WT = 0.1456 ±0.003627 µm/min; KO = 0.09055 ± 0.001964 µm/min, P < 0.0001. D, Velocity in µm/min, WT = 0.09155 ± 0.006285 µm/min; KO = 0.04955 ± 0.004178 µm/min, P < 0.0001. Statistical analysis was performed using a Mann-Whitney U test. N = 2 WT with 218 values and 2 KO with 180 values.

4.2.2.5. Calcium activity is enhanced in ZDHHC8+/-

Due to the important role of palmitoylation at the synapses (Figure 4-1). We investigated whether ZDHHC8 deficiency had an impact on spontaneous neuronal calcium activity in excitatory projection neurons. Indeed, as explained in chapter 1, calcium activity is responsible of several signalling pathways in neurons amongst them the release of
neurotransmitter from the presynaptic neurons. Thereby we used the calcium indicator Fluo-4AM in order to evaluate the calcium activity in vitro in neurons comparing ZDHHC8+/− with the control parental line. The calcium activity from excitatory projection neurons in a solution of aCSF was acquired using an inverted fluorescent microscope equipped with a LED setup. The questions we were interested were: 1) whether the calcium activity was different between the WT and ZDHHC8 mutant cultures; 2) how ZDHHC8 neural cells were maturing in the context of calcium activity during long time culture. Three different clones of ZDHHC8 heterozygous cells (297, 541 and 510) and a control (iCas9) were used in this study with neurons recorded at different time points as presented in the table 12.

Table 4-1 Time point analysed for each cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time points in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – iCas9</td>
<td>60, 70, 80, 110, 130, 150</td>
</tr>
<tr>
<td>ZDHHC8 +/- 541</td>
<td>60, 70, 80, 110, 130, 150</td>
</tr>
<tr>
<td>ZDHHC8 +/- 297</td>
<td>60, 70, 80, 110</td>
</tr>
<tr>
<td>ZDHHC8 +/- 510</td>
<td>60, 70</td>
</tr>
</tbody>
</table>

To determine the longitudinal calcium activity maturation, we first examined the calcium activity in the control cells line (iCas9) (Figure 4-19). The calcium amplitude represented the variation of the calcium intensity emitted by the Fluo-4 dye. The amplitude was represented as ΔF/F₀ from which ΔF represents the variation in the calcium intensity at a given time while F₀ is representing the fluorescence calcium intensity at the beginning of the experiment. We observed that the calcium amplitude remained similar from day 60 to 80 with a value of ΔF/F₀ around 0.06 in the control cell line. However, by day 110 the amplitude reached an average of 0.2438 ± 0.008220 but decreased by day 130 until day150 to be 0.06028 ± 0.002085 (Figure 4-19A).

The interspike interval (ISI) represented the interval between two spikes. Instead, the total number of events described the average number of spikes occurring at a given time point recorded from all the active cells in our different fields of view. During the course of the differentiation, an overall decrease of the ISI was observed in the control which was correlated by an increase number of spikes (Figure 4-19B and C). Except at day 110 which exhibited a high ISI and low number of spikes, the average ISI varied from 106 ± 5.926 seconds at day 60 to 41.64 ± 2.077 seconds at day 150 (Figure 4-19B). Similarly, the average number of events varied from 5.386 ±0.2636 at day 60 to 25.39 ± 0.785 at
day 150 (Figure 4-19 C). Thereby, when the ISI decreased, the number of events was instead increased.

The rise was defined as the time necessary to reach half of the maximal amplitude while the fall time corresponded to decay of the calcium event (Figures 4.19D and E). From day 60 to 80, the average control rise time was 1.30 ± 0.038 seconds. By day 110, it unexpectedly increased to reached 1.745 ± 0.017640 seconds however it decreased to reach 0.7563 ± 0.01207 seconds at day 150 (Figure 4-19D). Figure 4-19E illustrates the evolution of the calcium decay time. Overall, the average fall time in the control cell line kept increasing from 0.8093 ± 0.027410 seconds at day 60 to 1.56 ± 0.01349 seconds at day 150 (Figure 4-19E).

Therefore, we observed that despite the amplitude remained relatively similar during the course of the differentiation, the ISI and the number of events were observed to be decreased and increased respectively during the course of the differentiation. Therefore, these data demonstrated that in vitro excitatory projection neurons derived from embryonic stem cells have the ability to mature which was demonstrated by the increase in the number of events occurring during spontaneous calcium activity which was correlated with the decrease in the ISI.

Next, we decided to investigate whether the ZDHHC8 mutation altered the calcium activity in neurons and also compared its activity with the control neurons. Strikingly, the ZDHHC8 KO neurons exhibited an overall higher amplitude compared to the control from day 60 to 80 (day 70 P_{297} < 0.0001, P_{510} < 0.0001, P_{541} < 0.0001). Although the amplitude was higher in the control at day 110 and 130, at day 150 it was higher in the mutant clone 541.

The ISI in the mutant ZDHHC8 was observed to constantly decrease at each recording time. In the mutant, except at day 70 at which two mutant clones exhibited similar value of ISI than the control, all remaining recording time points displayed a significant decrease of the ISI in the mutant (day 80, P_{297} = 0.0002, P_{541} < 0.0001; d150, P_{541} < 0.0001). The total number of calcium events appeared to also constantly increase while ZDHHC8+/- neurons mature. At day 60 they were on average 9.5 ± 0.2813 events during the recording while it increased to reach 86.01 ± 1.343 events at day 150 in the mutant clone 541.
The rise time in the mutant \textit{ZDHHC8} was very heterogeneous from day 60 to 80. While the rise time was significantly slower (higher values, day 60 $P_{541} = 0.0015$, day 70 $P_{541} = 0.0008$) than the control for clone 541 at day 60 and 80, we observed that it was faster (lower values) than the control in the clone 297 and 510 (day 60, $P_{297} < 0.0001$, $P_{510} = 0.0001$; d70 $P_{297} < 0.0001$, $P_{510} = 0.0017$). From day 110 and onwards, the mutant \textit{ZDHHC8\/+} had values of rise time that were significantly lower than the control therefore the rise time were faster in the mutant neurons. Similarly to the control, the decay time of the calcium transient in the mutant kept increasing at each recording time point. From 0.8217 ± 0.01373 seconds at day 60 it was 1.763 ± 0.01396 seconds at day 150 for the clone 541. When compared to the control, we observed that the fall time was significantly higher in the mutant \textit{ZDHHC8}.

Table 13 depicts the number of cells analysed per cell lineages and time point.

Table 4-2 Number of cells analysed per cell lineages and time point

<table>
<thead>
<tr>
<th>Cell lineages</th>
<th>D60</th>
<th>D70</th>
<th>D80</th>
<th>D110</th>
<th>D130</th>
<th>D150</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{iCas9}</td>
<td>210</td>
<td>155</td>
<td>166</td>
<td>816</td>
<td>476</td>
<td>778</td>
</tr>
<tr>
<td>541</td>
<td>549</td>
<td>363</td>
<td>228</td>
<td>581</td>
<td>1347</td>
<td>768</td>
</tr>
<tr>
<td>297</td>
<td>301</td>
<td>334</td>
<td>212</td>
<td>639</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>510</td>
<td>267</td>
<td>104</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

To conclude, the control cells demonstrated a maturation of the neurons which was demonstrated by a decrease of ISI and increased of the number of events. A similar trend was also observed for the different KO for \textit{ZDHHC8}. However, the data suggested that the neurons heterozygous for \textit{ZDHHC8} mature faster than the control. Although we observed some variability between the different KO, the difference was usually in the same direction especially for day 110. However, while maturing, although the rise time of the calcium amplitude appeared to be faster, the fall time was observed to be slower.
Figure 4-20 Longitudinal calcium activity analysis in cortical excitatory projection neurons. The control isogenic line is represented by iCas9 while 541, 297 and 510 are three heterozygous cell lines for ZDHHC8. A. the bar plot represented the calcium amplitude ΔF/F0. B. Inter Spike Interval (ISI) in seconds. C. Total number of events at a given time point. The number of events corresponded to the total calcium event occurring for one neuron. The value is then average with all the cells analysed. D, rise time of the calcium spike in seconds. D, fall time represented the decay of the calcium spike. The data presented in the graphics are the results of the recordings of hundred of neurons across different field of view. Data are presented as the mean +/- SEM, however, they are very small therefore not visible. Statistical analysis was performed using a two-tailed unpaired Student’s t-test comparing each condition to the control. Calcium activity was analysed in Matlab, using the FluoroSNNAP package.

Figure 4-20 illustrated calcium traces recorded and their respective temporal raster plot from the control cell Cas9, KO 541 and 297 at day 110 and 150 for the control and the KO 541.

We observed that the control cells only displayed two calcium events (Figure 4-20A). However, while analysing different neurons, a similar pattern was observed (Figure 4-20B). Indeed, the raster plot of six random cell displayed similar spontaneous calcium events
Although the number of events was significantly higher for the KO 541, we observed a similar phenomenon than the control in the KO 541 and 297 (Figure 4-20A-B). Therefore, by day 110, we start to observe that the spontaneous calcium activity become synchronizes across our recordings. Furthermore, this synchronicity was also observed at day 130 (data not shown) and 150 (Figure 4-20C-D). It was noteworthy that the number of events also increased from day 110 to 150 (Figures 4-19C, 4-20C-D). Figure 4-20D represented the calcium activity of six cells randomly selected. Similar than day 110, most neurons were active together as a network.

While calcium is important for the physiology of the cells, in a preliminary experiment (data not shown), we examined whether the incubation of specific drugs would block the calcium signalling. The main purpose was to determine whether the signal we acquired was due to calcium activity mediated by action potential or by the movement of calcium from the different internal storage of the cells. We observed that upon incubation of the neurons in a solution of aCSF containing Tetrodotoxin (sodium channel blocker), AP5 and CNQX, the calcium signalling activity of the neurons become quiet. Therefore, we conclude that the calcium signal was mediated by synaptic activity as otherwise cells would have remained active.

To conclude, we found that is possible to assess neuronal maturation using calcium imaging. Furthermore, while maturing the spontaneous calcium transient evolve from an asynchronous to a synchronized activity.
Figure 4-21 Calcium trace and raster plot of control and ZDHHCS+/− glutamatergic neurons. 541 and 297 are two heterozygous line for ZDHHCS which were differentiated into glutamatergic excitatory neurons. A and C represented the calcium trace recorded with their respective spikes recorded for 10min or 6000s. Each traces represented a single cells. B and D, raster plot of couple of cells at day 110 and 150 respectively. Each bar on the raster plot represented a calcium event. The X-axis represents the time in milli-seconds.
4.3. Discussion

While animals carrying either heterozygous or homozygous Zdhhc8 deletions were previously generated, investigations of its role in hESCs derived excitatory projection neurons has yet to be reported. This study therefore aimed to investigate whether ZDHHC8 influenced human cortical projection neuron development.

While we successfully generated lines carrying a heterozygous deletion using the CRISPR/Cas9 technique, the different clones were genetically the same with disruption that occurred in the middle of exon 3 and 4. It resulted to the deletion of the intron 3 and the fusion of the upstream and downstream sequence of exon 3 and 4 respectively (Figure 4-7).

In order to understand why only gRNA 2 and 3 were able to cut the gDNA sequence, it would be interesting to sequence the plasmid used for the generation of our heterozygous line. Indeed, although we observed the integration of 4gRNA, it still remains possible that we had ligated two gRNA 2 and 3 instead of one of each gRNA.

Although the heterozygosity of these clones was confirmed by sequencing at the gDNA level, we were unable to confirm a change at the protein level. Several ZDHHC8 antibodies tested yield multiple bands in western blot in the control. Even if all these antibodies were expected to detect regions of the protein that is deleted in our heterozygous, we were unable to detect the wild-type protein in our control condition. Despite several attempts were made using a blocking peptide in order to determine which bands corresponded to the wild-type ZDHHC8 protein, it failed to give satisfactory result. Other attempts should be made aiming at varying the different incubation time during the western blot process, such as the blocking step or else the antibody incubation.

Several attempts were also made in order to generate a homozygous hESCs line for ZDHHC8. We used the same gRNAs that were shown to be efficient and anticipated that it would target the second allele. To overcome this issue, we decided to design a multiplex lentiviral vector (Alessandra Sperandeo) which was based on the publication from Kabadi et al. 2014 (Kabadi et al. 2014). Although we successfully managed to generate the lentiviral vector, at the moment we did not start the lentivirus production yet. Due to the overall higher efficiency of lentivirus compared to conventional plasmids, we would expect to generate a homozygous line based on the hemizygous deletion. Although 22q11.2DS is a hemizygous deletion, using a homozygous will allow us to study in more depth the function of ZDHHC8 during neuronal differentiation.
Study reporting a decrease of neural stem cells proliferation in patients with schizophrenia are thought to result in decrease of the cortical thickness in several brain areas in schizophrenic individuals, interestingly such observation was also found in 22q11.2DS carriers (Jalbrzikowski et al. 2013; Reif et al. 2006). Interestingly, our data suggested the opposite, we observed an increase in proliferation of the neural progenitor cells (Figure 4-13). As the area of the rosettes was also observed to be increase in ZDHHC8+/- (Figure 4-12). We suggested that the increase of neural rosettes area and the proliferation of the progenitors might be related. Thus, it would be interested to determine first the identity of the cells which displayed an increase proliferation, then determine whether these cells survived over the course of the differentiation.

Apart from Nissl staining which were used to evaluate the mouse brain morphology, which moreover appeared to be normal in both Zdhhc8+/- and Zdhhc8-/- no extensive analysis was performed (Mukai et al. 2004). Overall, although they were slight fluctuation in the temporal expression profile of ZDHHC8 in the control condition, the temporal profile was considered as being constant along the course of differentiation in both control and ZDHHC8+/- (Figure 4-16).

The temporal expression of key markers of neuronal differentiation was used to examine the kinetics of differentiation of the ZDHHC8+/- neurons, therefore to assess whether ZDHHC8+/- alter the process of differentiation. Our analysis did not reveal an alteration of the kinetics of differentiation in the ZDHHC8 KO neurons. On the other hand, we observed that several genes in ZDHHC8+/- are differentially expressed when compared to the control. Examination of the relative mRNA expression of crucial developmental and synaptic genes in ZDHHC8+/- differentiation revealed a significant downregulation of PAX6 mRNA at day 10 and 30 which corresponded to the peak of its expression (Figure 4-17B). Therefore, it would be interesting to examine whether we also observed a decrease of PAX6 protein by quantifying the protein in western blot.

We observed an overall significant downregulation of the mRNA expression of EMX1, EOMES and FOXP2. Emx1 is part of the EMX family, in mouse study, this gene was shown to be restricted to the dorsal telencephalon while Emx2 is expressed in the dorsal and ventral telencephalon. Our data suggested a downregulation of 55% of EMX1. Interestingly, while studies using homozygous knockout for Emx1 provide evidence regarding its role in cortical aeralization, others showed that Emx1 knockout reduced the migratory capacity of the neural stem cells (Kobeissy et al. 2016; Muzio et al. 2003). It would be interested to determine if this decrease at the mRNA level would result to a
decrease at the protein level using western blot. We could also perform immunocytochemistry to examine whether the proportion of cells positive for EMX1 are also decreased in ZDHHC8+/-). Similarly, Eomes is important for neurogenesis as it promotes the generation and proliferation of IPCs in vivo. Thereby, TBR2 depletion in the mouse cortex was demonstrated to decrease the proliferation of cells from the SVZ but also decrease the proportion of IPCs which overall led to a decrease of the cortical plate (Sessa et al. 2008). While we observed an increase in cell proliferation at day 15, it would be interesting to examine whether this alteration in proliferation still occurs at later time point when cells that express TBR2 are generated. FOXp2 encodes a protein which is mainly expressed by deep layer neurons in the cortex. Loss of FOXp2 has been associated with severe speech and language disorders. As patients with 22q11.2DS were also observed to have delay in the emergence of speech it would therefore be interesting to investigate whether it could be due to ZDHHC8 (Solot et al. 2001). Therefore, immunocytochemistry that would examine the proportion of cells positive for EMX1, FOXp2 and TBR2 but also the quantification of these proteins by western blot would enable us to determine whether these alterations at the mRNA level are associated with a difference at the protein level between ZDHHC8+/- and the control condition.

Interestingly, we observed an upregulation of GAD1 (Figure 4-17J). This gene is known to be enriched in inhibitory neurons and is responsible of the decarboxylation of glutamate to GABA (Erlander et al. 1991; Fenalti et al. 2007). Although small proportion of inhibitory neurons are always found in our excitatory projection neurons protocol, we observed an upregulation of GAD1 expression by day 80 in the KO for ZDHHC8 which might suggest a higher propensity of the remaining mitotic cells to differentiate towards inhibitory neurons. However, two genes are responsible of the decarboxylation of GABA, while GAD1 is localized throughout the cell, GAD2 (GAD65) was instead observed to be mostly localized at the synapses (Soghomonian et al. 1998). To determine if ZDHHC8 heterozygous alters the proportion of GABAergic cells it would be interesting to derived hESCs deficient for ZDHHC8 using a protocol of cortical interneurons. Furthermore, it would also be interesting to evaluate the proportion of both GAD65 and GAD67 positive cells in our culture.

The proportion of the synaptic protein PSD-95 and VGLUT1 were shown to be impaired in Zdhhc8+/- and Zdhhc8-/- mouse model (Mukai et al. 2008). In this study, we showed that both SLC17A7 and SLC17A6 mRNA were downregulated. VGLUT1 and VGLUT2 are mainly expressed by glutamatergic neurons but not exclusively. These proteins are
responsible for glutamate uptake into synaptic vesicles, thus their alteration could affect the glutamatergic synaptic transmission (Vigneault et al. 2015; Zander et al. 2010). Studies investigating the role of VGLUT1 identified that mice homozygous knockout despite not showing phenotypic change at birth, presented severe coordination impairment, learning and memory deficits (Takamori 2006). On the other hand, VGLUT2 is predominantly expressed during embryonic development and homozygous knockout is lethal immediately after birth (Wallén-Mackenzie et al. 2010). Although we observed a downregulation of both SLC17A7 and SLC17A6, we have no indication whether their reductions result in a decrease of their proteins level. It would therefore be interesting to address this question by quantifying the proportion of both VGLUT1 and VGLUT2 proteins in mature neurons but also to identify whether these proteins could be palmitoylated by ZDHHC8.

PSD-95 is a major scaffolding protein localized at the postsynaptic density of excitatory glutamatergic neurons (Gomperts 1996). It allows the clustering of the different synaptic proteins such as AMPA and NMDA receptors (Chen et al. 2015). PSD-95 alteration has been associated with neurodegenerative disorder but also psychiatric disorders such as schizophrenia and ASD (Catts et al. 2015; Shao et al. 2011; Xing et al. 2016). Despite our study did not reveal an alteration of the DLG4 expression it did not indicate whether ZDHHC8+/− protein is altering PSD-95 protein. Indeed, several studies demonstrated that in order to be correctly addressed to the postsynaptic membrane, PSD-95 required to be palmitoylated (El-Husseini et al. 2000). Interestingly, the palmitoylation enzyme was shown to be ZDHHC8 which upon phosphorylation by the brain specific PKC isoform protein kinase Mζ (PKMζ) is able to palmitoylate PSD-95 (Yoshii et al. 2011; Yoshii et al. 2014). In addition, mouse model of 22q11.2DS demonstrated a decrease proportion of PSD-95, interestingly a similar phenotype was observed in mouse deficient for Zdhhc8. Therefore it was postulated that the decrease of PSD-95 in mouse model of 22q11.2DS could be attributed to the loss or the alteration of the palmitoyl activity of ZDHHC8 (Mukai et al. 2008). Using acyl-biotin exchange in our ZDHHC8+/− heterozygous, we could identify whether PSD-95 requires to be palmitoylated by ZDHHC8 furthermore it would allow us to identify additional proteins that are targeted by ZDHHC8.

Neuronal migration occurred in two distinct mechanism in the forebrain, by radial or tangential migration (Nadarajah et al. 2002). While radial migration is thought to be the principal mode of migration of cortical excitatory neurons, the cortical interneurons are migrating from the MGE and LGE through tangential migration. Thus, the correct
position of neurons within the brain is essential for the correct neuronal circuitry. Alteration can therefore be responsible of several disorders such as schizophrenia (Rehn et al. 2005). Migrating neurons are known to be highly polarized in the direction of their movement (Marín et al. 2010). Neuronal migration which is also called locomotion is composed of three steps. First, cells extend a leading process which then lead to the translocation of the nucleus and finally, the cell retracts all its processes. The leading process usually acts as a compass by migration toward or avoiding chemotactic cues. Migration is dependent on different mechanisms which involved adhesion molecule such as N-cadherin or SEMA3A receptor, both were shown to be important for the regulation and stability of the processes (Chen et al. 2008; Jossin et al. 2011). On the other hand, regulation of intracellular signalling involves proteins like slit-robo or GTPase activating protein (srGAP2) which stimulates branching and thus slows the migration (Guerrier et al. 2009). Our data identified that neuronal progenitor cells deficient for ZDHHC8 showed indication of migration defect. Future work would be needed to identify which steps of the migration process was altered and also identified which genes/proteins is associated with this alteration. Neurons from primary neuronal culture of Zdhhc8+/- mice was shown to exhibit shorter axons and dendritic arborisation which were demonstrated to be due to a defect in CDC42 palmitoylation, a protein part of the Rho GTPase family. As Rho GTPases are a regulator of radial migration it might therefore be interesting to identify whether other members of this family are affected by ZDHHC8 mutation such as Rac1 which is known to affects lamellipodia and therefore important for axonal growth (Ng et al. 2004; Woo et al. 2006).

In order to determine whether ZDHHC8 mutation altered the physiology of the neurons, we decided to use calcium imaging as a readout for neuronal function. Here, we identified that glutamatergic neurons deficient for ZDHHC8 exhibited an increased calcium activity compared to control. Indeed, these neurons have a higher amplitude as well as an increase in the number of spontaneous calcium transients. We also identified that the calcium activity was first asynchronous but over the course of differentiation gradually become synchronous, suggesting that these neurons form connexion. This switch in their activity might explain the strong decrease in spontaneous calcium events occurring in the control at day 110. Indeed, by day 130, control neurons exhibited a large increase in the number of events which kept increasing until day 150 therefore the pattern at day 110 is likely to be due to the switch from an asynchronous to a synchronous activity. In addition, these data also showed that this switch might happen earlier in the ZDHHC8 heterozygous
mutant compared to control as we did not observe alteration of the number of calcium events in this population during our recordings. To determine whether synchronisation involves the appearance of more synaptic connexion, we could use the advantage of the modified rabies virus (Reardon et al. 2016). Indeed, this technique allow to map monosynaptic connexion through retrograde trans-synaptic transfer. Furthermore, during neuronal development, spontaneous calcium activity regulates many processes including neuronal migration, axonal outgrowth, dendritic patterning and synaptic receptors maturation (Komuro et al. 1998; Spitzer 2006; Wong et al. 2002). Calcium concentration is characterized by the balance between influx and efflux (Grienberger et al. 2012). Therefore, several studies focused on identifying the compartment or receptors from which the calcium activity arise (Kirwan et al. 2015). It would therefore be interesting to identify in a similar way which synaptic receptors are responsible of the spontaneous calcium activity. This could be achieved by using drugs which are known to block specifically a receptor and then record the subsequent calcium activity. Furthermore, it was demonstrated that ion channels as well as voltage gated calcium channels required to be palmitoylated to be correctly addressed to the pre and post-synaptic membrane (Shipston 2011). Furthermore, study have suggested that palmitoylation might regulate calcium channel inactivation (Hurley et al. 2000). As in our study we observed an overall increase in spontaneous calcium activity it might suggest that one of these channels require palmitoylation by ZDHHC8 and therefore might explain the increase in the calcium activity in ZDHHC8+/-.

Previous studies have demonstrated the utility of protein palmitoylation for their correct function and localization. While in this study we obtained a heterozygous mutant for ZDHHC8, we currently have no data assessing the resulting proteins level of potential target of ZDHHC8. However, the Figure below represents a schematic representation of what we should expect with our mutant ZDHHC8 and therefore might explain our motility and calcium data. While in normal condition, PSD-95 proteins receive the addition of a palmitate by ZDHHC8 and therefore are properly targeted to the postsynaptic membrane. In ZDHHC8+/- due to the expected reduction in the palmitoyl activity, we suggest, that ZDHHC8 protein would be unable to palmitoylate all PSD-95 proteins therefore would result to a PSD-95 impairment at the postsynaptic membrane. It was noteworthy that this example is expected to apply for other proteins than PSD-95. Indeed, the impaired motility observed in our NPCs heterozygous for ZDHHC8+/- could result from impaired
CDC42 palmitoylation which are thought to be a protein involved actin cytoskeleton. In addition, we also suggest that the increase of calcium activity in \textit{ZDHHC8}+/− could result from either a decrease palmitoylation of a protein responsible of calcium channel inactivation or else the decrease of a specific channel or voltage gated channel at the surface of neurons.

To summarize our data from this chapter, it is the first time a hESC line heterozygous for \textit{ZDHHC8} was generated. While no defect was observed at the embryonic stem cells level, we identified several genes (Figure below) whose expression was suggested to be downregulated in red and upregulated in green by the \textit{ZDHHC8} heterozygous mutation.
Furthermore, phenotypic characteristic of the motility and spontaneous calcium activity respectively identified decrease (red) and increase (green) in NPCs and neurons deficient for ZDHHC8.

While motility defect has been observed in 22q11.2 carriers, it’s too early to conclude whether the increase in spontaneous calcium activity resulted with an increase in neurotransmitter release as demonstrated in patient carrying a 22q11.2 deletion (Earls et al. 2010), however further study should consider to identify the proteins targeted by ZDHHC8 in order to elucidate its role during these processes.
5. Transcriptomic analysis of neural progenitors derived from

ZDHHC8 +/- hESCs and 22q11.2DS iPSCs

5.1. Introduction

Individuals with 22q11.2DS have higher probability to develop psychiatric disorders such as schizophrenia. Whereas the 22q11.2 pathology has already been intensively studied using engineered animal model, the possibility of reprogramming fibroblast into iPSCs allows investigations into 22q11.2DS in a human setting. Several studies already reported the successful reprogramming fibroblasts from 22q11.2 patients into iPSCs which were subsequently differentiated into neuronal fate (M. Lin et al. 2016; Pedrosa et al. 2011).

Lin et al. has previously performed a RNA sequencing (RNA-seq) study using iPSCs lines derived from eight 22q11.2 patients and seven control subjects (M. Lin et al. 2016). iPSCs were differentiated to a mix population of glutamatergic and GABAergic cortical neurons (Marchetto et al. 2010). The study revealed a 2-fold reduction of almost all of the transcripts from genes of the 22q11.2 region except CLDN5 and TBX1. Amongst the 12981-proteins coding transcripts identified, 782 were differentially expressed between the controls and 22q11.2 neurons. Gene ontology (GO) terms enrichment analysis identified dysregulated genes related to apoptosis as well as immune response while GO terms related to cell cycle, glutamate metabolic process and synaptic transmission were upregulated.

Despite the generation of mouse model homozygous and heterozygous for zdhhc8, the transcriptome of these mice remains unknown. In the previous chapter, we showed that hESCs heterozygous for ZDHHC8 had an increase neuronal rosette area. Neuronal rosettes express several markers of neurectoderm and form columnar structure that mimic the formation of the neural tube in vivo. Cells within the rosettes are capable of symmetrical division to constitute the pool of cells which will become neurons.

Furthermore, our study on neurons derived from hESC heterozygous for ZDHHC8 revealed an increase in cells proliferation which was assessed by Ki67 immunostaining. In addition, we discovered that excitatory neuronal progenitors were migrating slower and less when compared to neuronal progenitors from an isogenic control. Finally, evaluation of the neuronal calcium activity revealed alterations in neurons mutant for ZDHHC8. As several aspects of the development of neuronal progenitor was suggested
to be affected in ZDHHC8+/- progenitor cells, we decided to investigate whether specific neuronal pathways were affected in our model. To our knowledge, how ZDHHC8 deficiency affects gene expression has not been reported. Therefore, transcriptomic analysis would be able to shed light on molecular pathways regulated by ZDHHC8.

Due to the availably of fibroblasts from carrier of a 22q11.2DS, we generated our own 22q11.2 iPSCs. In a pilot experiment performed by Alessandro Sperandeo, both control and 22q11.2 iPSCs were differentiated using a protocol of cortical excitatory projection neurons. During neuronal rosettes formation that usually occurs at the NPCs stage, we observed an alteration of their morphologies exhibiting various shapes (unpublished data). While, immunofluorescences examination of the developing forebrain marker FOXG1 and the neuroectodermal marker PAX6 suggest that both cells line correctly convert to neuronal fate, the proportion of cells positive to PAX6 was observed to be significantly decreased in 22q11.2 NPCs. In addition, the proportion of SOX2 positive cells, was observed to be decreased in 22q11.2 NPCs. SOX2 is a transcriptional factor crucial for stem cells regulation, furthermore, this protein is also important for the proliferation and maintenance of dorsal telencephalic neuronal progenitor cells (Zappone et al. 2000).

Therefore, in this chapter we performed a genome wide transcriptomic analysis using excitatory neuronal progenitors at day 15 derived from a line of 22q11.2 iPSCs (E11), a line of control iPSCs (900), a hESCs line heterozygous for ZDHHC8 (541) and its isogenic control (cas9).

Aims

- To differentiation hESCs and iPSCs (iCas9’control’, 541’ZDHHC8+/-’, 900’control’ and E11’22q11.2’) in a protocol of excitatory projection neurons.
- To compare the transcriptome of ZDHHC8 mutant with the control
- To determine whether genes overlap between ZDHHC8+/- and 22q11.2 neuronal progenitors
5.2. Results

5.2.1. Experimental design and quality control of RNA samples

Four different cell lines were used for the purpose of this experiment a ZDHHC8 heterozygous line (541) and its isogenic control (iCas9), a control iPSCs from a healthy subject (900) and a 22q11.2 line. All four-cell line were differentiated using a protocol of cortical excitatory projection neurons (Chambers et al. 2009; Espuny-Camacho et al. 2013). The experimental procedure for neuronal differentiation was illustrated in Figure 5.1. At day 15 of differentiation, RNA samples from 6 biological replicates per cells line were extracted.

![Diagram of RNA extraction](image)

**Figure 5-1 Glutamatergic neurons differentiation.** Schematic of the cortical excitatory neuronal progenitor’s differentiation. The grey area is representing the timeline; the blue area represents the different coating composition while the red area is the media composition. At day 15 all RNA samples were extracted, 6 biological replicates per cell line. The concentration of LDN and SB are described in 2.2.3.1

5.2.1.1. Immunofluorescence of glutamatergic progenitors

To verify whether our different cells line properly commit to neural fate, we stained our cultures of cortical progenitors which were fixed on the same day of the RNA extraction at day 15 (Figure 5.2). PAX6 and FOXG1 antibody were used as markers of neuroepithelial and telencephalic cell markers respectively. A high proportion of PAX6 and FOXG1 positive cells were observed in all four lines which suggest a proper induction into cortical progenitors.
5.2.1.2. RNA and cDNA quality control

RNA samples were run on a bioanalyzer in order to control for the purity of the RNA. This technique allows to determine whether the two ribosomal RNA subunits 28S and 18S are intact. Over the 24 samples (6 biological replicate per cell line), all had a RNA integrity Number (RIN) over 8.70.

Samples were further processed for the library preparation and subsequently the quality of the cDNA was examined using the Qubit DNA High Sensitivity Kit (life technologies) while the size was controlled with the DNA High Sensitivity Kits (Agilent) The results indicated that all cDNA samples achieved an appropriate purity for being processed on the sequencer (HiSeq 4000, Illumina).
5.2.2. Analysis of the heterozygous ZDHHC8 transcriptome

To analyse the data collected by the Illumina HiSeq4000, the reads were first aligned on the human genome, hg38. To perform this step, the Spliced Transcripts Alignment to a Reference (STAR) protocol was used in Unix (Dobin et al. 2013). The outcome of the STAR analysis was a text file which contained all the different genes with their respective number of reads per samples. To determine the differentially expressed genes, data were processed using DESeq2 package in R (Love et al. 2014).

Genome wide RNA-Seq coverage of the ZDHHC8 sequences were visualized using IGV (Integrative Genomic viewer). Figure 5.3A illustrates an image encompassing the region of exon 3 and 4 of ZDHHC8 from 6 biological replicates for the mutants and controls. There was a decrease in the number of reads in the ZDHHC8 mutation region compared to the controls. It was noteworthy that IGV only displayed a random number of samples on the alignment, thereby, region with high reads were downsampled. Therefore, exons count would be more suitable to evaluate whether the number of reads in the region of ZDHHC8 deletion is reduced.

The Principal Component Analysis (PCA) is a useful tool to visualize genetic distance between populations and reveals which components of the data cause variability in the data set (Figure 5.3B). PC1 captures the direction with the most variation in gene expression while PC2 captures the direction with the second most variation in gene expression. Thereby, Figure 5.3B displayed that all samples for cas9 and 541 respectively controls and mutants are located on the left and right of the PCA. This graphic therefore indicated that 70% of the variation, which is the biggest variation in our samples, is attributed to the deletion. On the other hand, 13% of the variation which corresponded to the PC2 was attributed to the variation between our biological replicates.

DESeq2 analysis identified 23421 genes in total (Figure 5.3C). Of which, 64.24% of the genes (15046 genes) are protein coding. By applying a specific cut-off for the pvalue adjusted below 0.01, we found that 0.86% (130 genes) of the genes were downregulated while 0.41% (62 genes) were upregulated of an absolute FC superior to 1.5. As a result, the transcript of 98.73% (14854) of the genes did not show statistical significance between ZDHHC8 mutants and controls.

Together, these data demonstrated that a total of 192 genes were found to be differentially expressed in ZDHHC8+/-.
Figure 5-3 Variability due to ZDHHC8 mutation. A, RNA seq coverage of the exon 3 and 4 of ZDHHC8 which represents the number of reads. B, PCA of the biological replicates of Cas9 (control) and 541 (ZDHHC8+/-) in blue and red respectively. C, Pie chart categorizing all the genes in our data set. FC strictly superior or inferior to 0 are in red and blue respectively. Genes with a p value adjusted inferior to 0.01 are in green while those with both a p value adjusted inferior at 0.01 and an absolute FC superior at 1.5 are in yellow.
Figure 5-4 Gene expression of the 22q11.2DS genes. All genes from the 3Mb deletion were displayed on this plot. Control samples (cas9) were represented in grey while the mutant ZDHHC8+/− (541) were in red. The p-value for each gene was written above each boxplot. Only significant p-value was written in red. Data for each gene represented the normalized count. Data are represented as the median ± SD.
5.2.2.1. Expression of 22q11.2 genes in ZDHHC8-deficient cells

The expression of the 22q11.2 genes in ZDHHC8 heterozygous knockout cells were analysed (Figure 5.4). Only 37 out of 48 genes in the 22q11DS region between LCRA and D were detected in our analysis. Amongst them only TXNRD2 and COMT exhibited statistical difference between the control and ZDHHC8+/- (P_{TXNRD2} = 0.00048, P_{COMT} = 0.000081). Indeed, these two genes were observed to be upregulated in ZDHHC8+/- NPCs. However, it was noteworthy that we had very low reads for these two genes in the control condition therefore only a small change could result in a significant change in the mutant condition.

5.2.2.2. Cross comparison of GO terms and KEGG pathways altered in ZDHHC8+/-

5.2.2.2.1. GO terms analysis

Gene ontology analysis revealed a total of 318 GO terms enriched in our data from which the top genes related to neuronal development are displayed in Table 5-1. Interestingly, the majority of the GO terms are related to neurons. The top four GO terms with the smallest p value relates to synaptic transmission. Furthermore, 18 of the top 30 GO terms directly relates to the brain suggesting that ZDHHC8 function is mostly involved in brain development and/or its physiology.

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<td>GO:0005578</td>
<td>Proteinaceous extracellular matrix</td>
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<td>GO:0061564</td>
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<td>106/1597</td>
</tr>
<tr>
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<td>GO:0007411</td>
<td>Axon guidance</td>
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</tr>
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<td>GO:0072001</td>
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Genes from three GO terms, axon development/migration, neurogenesis/brain development and synaptic signalling were identified (Figure 5.5A, B and C). Thereby, we investigated amongst the 192 differentially expressed genes those belonging to one of these categories (p < 0.001; -1.5 < FC > 1.5). Interestingly, when analysing the other genes from the different GO terms present in table 5-1, we found that it was the same gene. Therefore, we can say that the genes presented below are representative of the genes identified by GO terms.

Interestingly, most of the genes involved in axon development were upregulated. This include three members of the SLIT family, SLIT1, SLIT2 and SLIT3. Conversely, LRRC4C, SEMA5A and SLITRK3 are three of the top downregulated genes (Figure 5.5A).

Likewise, several genes involved in neurogenesis/brain development were upregulated in our data set (Figure 5.5B). Three genes from of the Wnt family were identified, WNT8B, WNT7B and WNT2A were shown to be upregulated in ZDHHC8+/- . On the other hand, FOXG1 expression was shown to be downregulated. Two members of the Wnt receptors family Frizzled, FZD5 and FZD8 were shown to be also downregulated.

Although, we were not expecting genes whose expression are only expressed in mature cells to be expressed in NPCs, we found, eleven differentially expressed genes part of GO terms related to synaptic signalling (Figure 5.5C). CHRM3 gene which encodes for the cholinergic/acetylcholine receptor M3 was shown to be downregulated. DRD2 and DRD4 which are dopaminergic receptors were also upregulated. In addition, GRIA2, a subunit of the AMPA receptor was also upregulated in ZDHHC8+/-.

To conclude, GO terms analysis revealed that several genes belonging to the brain development and its physiology were altered in ZDHHC8+/-.
## Neurogenesis/Brain development

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## Axon development

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## Synaptic signalling

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<th>Normalised counts</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZDHHC8+/−</td>
<td>Control</td>
</tr>
<tr>
<td>CBLN1</td>
<td>95.94</td>
<td>21.50</td>
</tr>
<tr>
<td>DRD2</td>
<td>74.18</td>
<td>28.43</td>
</tr>
<tr>
<td>P2RX3</td>
<td>702.31</td>
<td>360.70</td>
</tr>
<tr>
<td>DRD4</td>
<td>668.43</td>
<td>379.90</td>
</tr>
<tr>
<td>GRIA2</td>
<td>195.57</td>
<td>90.70</td>
</tr>
<tr>
<td>PCDHB5</td>
<td>353.06</td>
<td>207.13</td>
</tr>
<tr>
<td>SYT5</td>
<td>82.01</td>
<td>42.55</td>
</tr>
<tr>
<td>SLC6A1</td>
<td>54.39</td>
<td>27.33</td>
</tr>
<tr>
<td>P2Y5</td>
<td>16.99</td>
<td>4.25</td>
</tr>
<tr>
<td>GJD1</td>
<td>21.79</td>
<td>5.52</td>
</tr>
<tr>
<td>CHRM3</td>
<td>63.93</td>
<td>110.85</td>
</tr>
</tbody>
</table>
5.2.2.2.2. KEGG pathways analysis

In addition to GO enrichment analysis, data were processed to examine the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment which are predicted to be altered in ZDHHC8+/-. A total of 18 pathways were shown to be altered by ZDHHC8 mutation with several relating to the brain and heart pathologies (Table 15). For the purpose of this thesis, only pathways related to the brain will be presented. These pathways were associated to neuronal receptors, calcium signalling and axon guidance.

<table>
<thead>
<tr>
<th>KEGG ID</th>
<th>Description</th>
<th>p.adjust</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04080</td>
<td>Neuroactive ligand receptor interaction</td>
<td>0.00074532</td>
</tr>
<tr>
<td>hsa04020</td>
<td>Calcium signalling pathway</td>
<td>0.00074532</td>
</tr>
<tr>
<td>hsa05410</td>
<td>Hypertrophic cardiomyopathy (HCM)</td>
<td>0.00074532</td>
</tr>
<tr>
<td>hsa04512</td>
<td>ECM-receptor interaction</td>
<td>0.00074532</td>
</tr>
</tbody>
</table>

Figure 5-5 GO enrichment in ZDHHC8+-. The plots are representing genes belonging to three different categories identified in our GO analysis. Gene represented in red are those which were upregulated while those in blue were downregulated. A, genes part of the GO term axons development/neuronal migration. B, genes identified in the GO term forebrain development. C, gene identified in the GO term synaptic signalling. From the different GO terms which contains several hundreds of genes each, we applied a specific threshold in order to only identify genes in our dataset that had a p-value adjusted below 0.01 as well as an absolute FC higher than 1.5. The X-axis represents the fold change (FC) difference.
Figures 5.6 and 5.7 illustrate three neuronal pathways altered by ZDHHC8 mutation.

Pathway involved in synaptic receptors Figure 5.6A. This pathway represents a map of the different neuronal receptors together with their ligands altered in ZDHHC8+/-.

Pathway involved in calcium signalling (Figure 5.6B). As presented in the general introduction, extracellular calcium is the main source of Ca2+ for the cells and is involved in a multitude of physiological functions even in young NPCs. Many genes coding for receptors responsible of calcium uptake were upregulated, which include the voltage gate calcium channel CaV2 and CaV3 and also the receptor operated channels (ROC) which comprised most of the ionotropitic receptors such as AMPA and NMDA. GPCR were also observed to be downregulated, upon activation by an extracellular signal which can be a neurotransmitter, these receptors are involved in signal transduction either through cAMP or phosphatidylinositol signalling pathway. We also observed that genes such as the gene coding for the inositol 1,4,5-trisphosphate receptor, type 1 (ITPRI) and the calmodulin are also upregulated by ZDHHC8+/-.
Figure 5-6 KEGG enrichment pathways. A-B, the intensity which is represented in the top right corner of each schematic shows the FC of each gene, from green and red respectively a FC of -5 and 5. A, Neuroactive ligand receptor interaction. B, Calcium signalling pathway. The analysis had as parameter a p value cut off of 0.05. Figure can be found at higher magnification in annex.
Pathway involved in axon guidance (Figure 5.7). In addition to be one of the top GO term enriched in our analysis, the axon guidance pathway was also revealed by KEGG enrichment analysis. This pathway is primordial for the proper growth of axons which ultimately lead to novel synaptic connexion. As shown in Figure 5.7, the expression of genes of the SLIT family were upregulated. It also appeared that the expression of ROBO which encodes the SLIT receptors are likewise upregulated. Similarly, Plexin B, a receptor of semaphorins and EphA, an ephrin receptor, were both upregulated in ZDHHC8+/−. Conversely, a strong downregulation was observed for TRPC (Transient Receptor Potential Channel), which is responsible for axons attraction and outgrowth. Finally, NGL-1 also known as LRRC4C, was also downregulated. Together, KEGG pathways analysis established that several signalling pathways were found to be altered by heterozygous knockout of ZDHHC8.
5.2.3. Differentially expressed genes of a 22q11.2 hPSC derived NPCs

Analysis of the 22q11 iPSCs derived into excitatory NPCs revealed that a CNV might be located on the chromosome 17 in the iPSCs from 22q11.2 NPCs as 25 genes in the vicinity of TP53 were observed to be half expressed when compared to the control cells. The function of this gene was suggested to impact the viability of cells (Amir et al. 2017). Therefore, we decided to only investigate the expression of the of the 3Mb deletion in the 22q11 iPSC derived NPCs (Figure 5.8).
We observed that 37 out of 40 genes were expressed in the 22q11.2 NPCs. However, it appeared that not the same genes were present in 22q11.2 and ZDHHC8+/- NPCs. The analysis revealed that 24 genes were significantly different in 22q11.2 NPCs when compared to the control NPCs. In contrast to the assumption that these genes would show reduced expression, many (DGCR6, PRODH, CLTCL1, C22ORF39, C22ORF29, RTN4R, MEP15, AIFM3 and HIC2) were in fact upregulated in the 22q11.2 iPSCs when compared to the control.

While other studies did observe a reduction in the expression of the genes of the 22q11 mutation, our analysis were unable to find similar result. Therefore, it would be interested to examine the number of reads per exons which should provide us with more information regarding the expression of these genes.
Figure 5-8 Gene expression of the 22q11.2DS genes in the iPS cells carrying the 3Mb 22q11.2 deletion. All genes from the 3Mb deletion were displayed on this plot. Control samples from the cell line 900 were represented in grey while the mutant E11 (22q11.2 deletion) were in blue. The pvalue for each gene was written above each boxplot. Only significant pvalue was written in red. Data for each gene represented the normalized count. The box plot allows to visualize the spread of the data as each point is representing a biological replicate.
5.2.4. Comparison of the differentially expressed genes in 22q11.2 and ZDHHC8+/-.

To investigate whether ZDHHC8 deficiency and 22q11.2 deletion lead to the alteration of similar genes, we compared the overlap between the genes differentially expressed in ZDHHC8+/- and 22q11.2 iPSCs derived NPCs.

Instead of comparing the transcriptome of control and mutant as performed in 5.2.2 and 5.2.3, the DEseq2 analysis compared the transcriptome of the two mutants (22q11.2 and ZDHHC8+/-) and the two controls (900 and iCas9) with each other’s. In total, the analysis identified 24748 genes from which we performed a Venn diagram to identify the genes coding proteins overlapping between ZDHHC8+/- and 22q11 derived NPCs (Figure 5.9).

Figure 5.9A illustrates the number of significant genes (pvalue < 0.01) overlapping between ZDHHC8+/- and 22q11.2 NPCs. We observed that each group is composed of 7080 and 1176 for 22q11.2 and ZDHHC8 respectively. From these genes, it appeared that 978 overlapped. Figures 5.9B and C illustrate the number of genes with a pvalue inferior to 0.01 and either a FC higher or below 1.5 or -1.5 respectively. We observed that 36 genes with a FC superior to 1.5 appeared to overlap while only 15 genes with a FC below -1.5 overlapped between ZDHHC8 and 22q11.2 derived NPCs.

Using similar cut off than in 5.2.2.1, we examined whether amongst the genes significant in 22q11.2 derived NPCs, we found overlap with significant genes identified in ZDHHC8+/- Table 17 presents the genes identified in the GO terms neurogenesis and synaptic signalling that were significant in ZDHHC8+/- and 22q11.2 derived NPCs. While no genes from the axon development appeared to overlap, several genes from the neurogenesis and synaptic signalling GO terms was found in both populations.
Table 5-3 List of genes identified by GO terms in ZDHHC8+/- transcriptome analysis that overlap with 22q11.2

<table>
<thead>
<tr>
<th>Neurogenesis</th>
<th>FC</th>
<th>Average normalised counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>541 (ZDHHC8)</td>
<td>22q11.2</td>
</tr>
<tr>
<td>HES3</td>
<td>2.74</td>
<td>1.72</td>
</tr>
<tr>
<td>S1PR5</td>
<td>2.19</td>
<td>11.80</td>
</tr>
<tr>
<td>SOX10</td>
<td>1.90</td>
<td>2.13</td>
</tr>
<tr>
<td>FOXB1</td>
<td>1.84</td>
<td>1.67</td>
</tr>
<tr>
<td>SPOCK1</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>ATP8B1</td>
<td>1.83</td>
<td>1.84</td>
</tr>
<tr>
<td>GBX2</td>
<td>1.66</td>
<td>3.08</td>
</tr>
<tr>
<td>MMD2</td>
<td>1.66</td>
<td>2.00</td>
</tr>
<tr>
<td>IRX3</td>
<td>1.62</td>
<td>1.52</td>
</tr>
<tr>
<td>RELN</td>
<td>1.54</td>
<td>2.02</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Synaptic signalling</th>
<th>FC</th>
<th>Average normalised counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRA4</td>
<td>1.70</td>
<td>2.33</td>
</tr>
<tr>
<td>PCDHB5</td>
<td>1.64</td>
<td>11.27</td>
</tr>
</tbody>
</table>

To conclude, despite 978 genes overlapped between 22q11.2 and ZDHHC8 when we apply a cut off for those with a pvalue below 0.01. This number drastically decreased when they were also classified depending on their FC.
In this study, we performed a whole transcriptome shotgun sequencing (also known as RNA-seq) analysis of ZDHHC8+-/ glutamatergic progenitor cells with the aim 1) to analyse the transcriptome of ZDHHC8+- derived NPCs, 2) to determine whether similar group of genes overlapped between 22q11.2 and ZDHHC8. The first part of the study investigates if the 22q11.2 genes located in the region of the 3Mb deletion were altered by ZDHHC8+-/. Apart from COMT and TXNRD2, the expression of the remaining 22q11.2 genes were similar when compared to the control. COMT expression was observed to be altered in individual with schizophrenia. The function of COMT was demonstrated to be responsible of the inactivation of dopamine neurotransmitter following its releases in the synaptic cleft. Individuals with schizophrenia were reported to have an increase mRNA level of COMT in the dorsolateral prefrontal cortex which was shown to be detrimental for the dopaminergic innervation (Matsumoto et al. 2003). Therefore, we suggest that ZDHHC8+-/ might be responsible

**5.3. Discussion**

In this study, we performed a whole transcriptome shotgun sequencing (also known as RNA-seq) analysis of ZDHHC8+-/ glutamatergic progenitor cells with the aim 1) to analyse the transcriptome of ZDHHC8+- derived NPCs, 2) to determine whether similar group of genes overlapped between 22q11.2 and ZDHHC8+/-.
of the increase in COMT expression, which therefore could result in decrease dopaminergic innervation in individuals with 22q11.2DS. Further studies should consider investigating whether these two genes interact.

TXNRD2 is important for the development and function of the heart. Mice deficient for Txnrd2 die around E13.5 due to severe anaemia which resulted in part to an increase apoptosis in the liver that alter haematopoiesis but also due to a thinner ventricular walls of the heart and decrease of cardiomyocyte proliferation (Conrad et al. 2004; Kiermayer et al. 2015; Soerensen et al. 2008). Conversely, TXNRD2 overexpression was found to be associated with an increase lifespan in female Drosophila melanogaster, in addition overexpression of this gene was also found decrease the therapeutic effect of bortezomib, a proteasome inhibitor in multiple myeloma cells (Fink et al. 2016; Pickering et al. 2017). Although we identified several GO terms and KEGG pathways enrichment related to cardiac function such as Dilated cardiomyopathy (DCM), the relation between TXNRD2 and ZDHHC8 if one remains largely unknown.

The ZDHHC8+/- transcriptomic analysis identified 1203 proteins coding genes whose expression were significantly different between ZDHHC8+/- and its isogenic control (pvalue < 0.01), from which 192 genes had an absolute FC higher than 1.5. Interestingly, most of the GO terms identified were related to neurons development and physiology emphasising the role of ZDHHC8 during brain development.

Axon development was identified as a top GO enriched term. Several genes related to axon development were demonstrated to be upregulated in ZDHHC8 +/-, such as the SLIT family (SLIT1, SLIT2 and SLIT3), LRRC4C, SEMA5A and SLITRK3. Interestingly, KEGG pathway enrichment analysis also identified the axonal guidance pathway as altered in ZDHHC8+/-.. While axonal guidance defect was demonstrated to be responsible for neuronal migration disorder (NMD) such as lissencephaly, polymicrogyria or micropolygyria. Neuronal migration was also linked to schizophrenia and Parkinson disease (Gilman et al. 2012; Lesnick et al. 2007).

The SLIT1, SLIT2 and SLIT3 genes was observed to be upregulated in ZDHHC8+-. The SLITs proteins are implicated in a multitude of different physiological processes such as axon pathfinding as well as axonal and dendritic branching (Bagri et al. 2002; Wang et al. 1999; Whitford et al. 2002). While SLITs homozygous knockout are lethal, the data presented in this chapter indicate an upregulation of these three genes with a FC of at least 1.5. In drosophila, Slit overexpression appears to have a similar phenotype than
Roundabout (Robo) loss of function, the receptor of slit. Once crossing midline, high level of ROBO are expressed by commissural axons to avoid them to cross again. In the vertebrate brain, neurons require to cross the floor plate or midline (drosophila) in order to innervate the other part of the brain. Thus ZDHHC8+/- might alter this process resulting in an abnormal axons guidance which might result in abnormal axonal connexion. While the Slit family is shown to be upregulated in our study, previous report by Mukai et al. 2008, 2015 using heterozygous mouse model for Zdhhc8 demonstrated an alteration of the palmitoylation of CDC42 (Mukai et al. 2008, 2015). This protein is an actin cytoskeleton regulator, whose palmitoylation alteration resulted in shorter axons arborisation (Tapon et al. 1997). The SLIT genes are located in the same signalling pathway than CDC42 (Figure 5.7) (Huber et al. 2003). It was demonstrated that Slit increases the complex of Robo1-srGAP which in turn inactivate CDC42 (Huber et al. 2003). Although our data suggest that the expression of SLITs genes as well as their receptors ROBOs are increased, an increase in the expression of srGAP was not detected. Thus, it is possible that this overall upregulation of the SLIT/ROBO might have no effect on the expression of CDC42. Therefore, it would be interesting to evaluate the level of these proteins by western blot analysis in order to estimate if they influence the SLIT/ROBO/CDC42 pathway.

LRRC4C also known as NGL-1 is the receptor of the guidance cue netrin-G1 which is part of the netrin family and was shown to be downregulated in ZDHHC8+/- NGL-1 is thought to be highly expressed in the striatum and cerebral cortex (Lin et al. 2003). While its role was suggested to promote thalamic axons outgrowth, it was also demonstrated to interact with PSD-95 at the postsynaptic membrane (Lin et al. 2003; Ricciardi et al. 2012). While our data suggested a downregulation of the NGL-1, knockout experiment of this gene revealed that the signalling cue netrin-G1 was also significantly reduced (Matsukawa et al. 2014) therefore resulting to a decrease in synaptogenesis and disruption of the growth of thalamic axons. As thalamic reduction was also observed in children with 22q11.2DS, this phenotype could be attributed to the reduction of LRRC4C (Bish et al. 2004) due in part by ZDHHC8.

In addition, we also observed a downregulation of SEMA5A. Semaphorins are a group of proteins that were identified as axon guidance molecules. In the central nervous system, this class of proteins acts as both chemoattractant and chemorepellent for the growth of axons (Raper 2000). Interestingly, a de novo deletion of SEMA5A was recently identified as contributing to ASD and intellectual disability which are pathologies that also arise in
patients with 22q11.2DS (Angkustsiri et al. 2014; Radoeva et al. 2014; Subramanian et al. 2015).

SLITRK3 was also found to be downregulated in ZDHHC8+/- . This molecule was discovered as a synaptogenesis molecule responsible for inhibitory synapse development. Indeed, slitrk3-/- mice was observed to increase the risk for spontaneous epileptic seizure due to an unbalance between excitatory and inhibitory synapses (H. Takahashi et al. 2012). As patients with 22q11.2DS also exhibit spontaneous epileptic seizure it would be interested to investigate whether alteration of this gene is also present in 22q11.2DS which could therefore be due to ZDHHC8 (Kao et al. 2004; Strehlow et al. 2016).

While this study revealed migration defect due to ZDHHC8+/-, several studies in individuals with 22q11.2DS were also observed to have migration defect such as polymicrogyria or reduced corpus callosum volume (Castro et al. 2011; Robin et al. 2006). However, it is currently not known which genes or groups of genes are associated with this pathology in 22q11.2DS. Finally, to determine the implication of the gene listed above, it would be interested to perform experiment aiming to identify whether the proportion of these different proteins is altered at the growth cone of ZDHHC8+/- NPCs.

Amongst the other GO enrichment identified was the one related to neurogenesis/brain development. Three members of the WNT family, WNT8B, WNT7B and WNT2B were shown to be upregulated. The WNT family constitutes a major signalling pathway involved in neural development. These three genes were shown to be expressed in the forebrain during development suggesting their importance for this process (Abu-Khalil et al. 2004; Lako et al. 1998). Wnt7b was observed to be highly express throughout the cortex but more specifically in the cortical plate while absent from the ventricular zone during cortical neurogenesis (Chenn 2008; Funatsu et al. 2004). Wnt8b was shown to be mostly expressed in the cortical hem and hippocampus, similarly the expression of Wnt2b was shown to be restricted to the cortical hem (Harrison-Uy et al. 2012). While Wnt8b knockout was observed to be responsible of decrease Wnt2b expression, the expression of Wnt7b was unchanged (Fotaki et al. 2010). In addition, alteration of Wnt2b and Wnt7b was shown to affect neuronal development by altering the neural tube formation (Bai et al. 2016). Despite their implication in many others signalling pathways apart from the brain, these genes should be further investigated, such as quantifying their proteins level in ZDHHC8+/- neurons differentiation.
**FOXG1** is a gene which was demonstrated to be associated with development of Rett syndrome. In addition, this gene is also crucial for the development of the telencephalon during embryonic development. Indeed, experiments in which mice were deficient for Foxg1 were demonstrated to exhibit telencephalon and brain size defects. Instead Foxg1 overexpression resulted in telencephalon and mesencephalon overgrowth (Ahlgren et al. 2003). In chapter 4, we observed no difference in the proportion of FOXG1 positive cells in ZDHHC8+/− when compared to the control. Also, mice deficient for Zdhhc8+/− or Zdhhc8−/− did not have obvious brain morphological defect. Therefore, this decrease in the transcript of FOXG1 might not be correlated to a decrease of the subsequent FOXG1 protein in vitro.

**FZD5** and **FZD8** are two genes part of the frizzled family of G-protein coupled receptors (Schulte et al. 2007). Interestingly, it was demonstrated that the sequence of both FZD5 and FZD8 shared 70% identity (Huang et al. 2004). While Fzd8 was shown to be expressed throughout the ventricular zone in the dorsal and ventral telencephalon, the expression of Fzd5 was observed to be restricted to the ventral telencephalon (Fischer et al. 2007; Harrison-Uy et al. 2012). Knockdown of Fzd5 was shown to result in loss of neuronal polarity (Slater et al. 2013). While mice heterozygous knockout for Fzd5 are viable, homozygous knockout die in utero due to angiogenic defect (Fischer et al. 2007). Considering that we observed a decreased expression of both FZD5 and FZD8 it would therefore be interested to investigate the resulting phenotype to such a decrease by examining their respective proteins.

To conclude, in addition to the three WNT genes upregulated, we also found that ZDHHC8+/− had a decrease of the expression of two WNT receptors FZD5 and FZD8.

Alteration of signalling pathway related to synaptic receptors. KEGG pathway enrichment analysis identified that the “neuroactive ligand receptor interaction” pathways were altered in ZDHHC8+/−. While we might not expect these types of genes to have an important impact on young neurons, it might demonstrate that their alteration at early stage could result to alteration of their expression when expressed in mature neurons. Therefore, we decided to present these data in the present thesis. Amongst these genes, the DRD and GRI family, respectively dopaminergic and glutamatergic AMPA receptors. The dopamine receptor D2 was recently identified as being palmitoylated by ZDHHC8 (Ebersole et al. 2015). Furthermore, ZDHHC8 was demonstrated to palmitoylate PICK1 (protein interacting with C-kinase) and GRIP1 (glutamate receptor interacting protein), two proteins known to bind the GRIA2 receptors (Thomas et al. 2012, 2013). However,
our data are suggesting an upregulation of the GRIA2 receptor which might suggest a novel role for ZDHHC8 other than its implication at the protein levels. Thereby it would be interested to assess the protein level of GRIA2 to determine whether this upregulation is correlated with an increase of the protein translation. Similarly, to GRIA2, the DRD2 and DRD4 were upregulated. Antipsychotics medication are known to increase the level of DRD2 and DRD4 proteins. However it is noteworthy that an elevated level of DRD2 receptor was observed in patients with schizophrenia prior their psychotic medication (Seeman et al. 2000). Whereas it was not previously observed with the DRD4 protein. These data are thereby suggesting that ZDHHC8 might alter the expression level DRD2 and DRD4 thereby we would need to determine if this increase results to an increase translation of their respective proteins.

Lastly, the calcium signalling pathway was shown to be enriched by KEGG pathway analysis. Calcium signalling is particularly important for neurons (chapter 1). It was previously demonstrated that calcium signalling was altered in two different mouse models of 22q11.2DS, the Df(16)A/+ and the Df(16)5 (P Devaraju et al. 2017; Earls et al. 2010) as well as in schizophrenia patients. Although no report described calcium alteration with ZDHHC8, the data presented in this study are suggesting otherwise. Indeed, Figure 5.6B showed an increase of the voltage dependent calcium channel which might suggest an increase in calcium uptake within the neuron which would thereby result in an alteration of the neuronal calcium signalling. Furthermore, we observed a slight upregulation of SERCA which was observed to be associated with an alteration of calcium signalling through increase neurotransmitter release at the presynaptic neurons (Earls et al. 2010). Although more than one gene within the 22q11.2DS might be responsible of the calcium signalling alteration, our data suggested that ZDHHC8 might be an important contributor to this signalling pathway.

This study investigated for the first time the role of ZDHHC8 at the transcriptional level in excitatory neuronal progenitor cells. Therefore, these data will give valuable insight on the genes and pathways affected by ZDHHC8 mutation.

The second aim of this study was to examine whether similar genes in ZDHHC8+/- and 22q11 derived NPCs were found to overlap. Although we aimed to compare the RNA-seq data from 22q11.2 and ZDHHC8+/- derived NPCs, however, due to issue regarding
a potential CNV on the chromosome 17 we did not perform gene enrichment analysis on the 22q11.2 NPCs.

Genomic instabilities in ESCs have already identified several mutations which were observed to be mostly located on the chromosome 1, 12 and 17 and X (Lamm et al. 2016). Surprisingly, these mutations often correspond to observation made with embryo carcinoma (Nouspikel 2013). These observations also note a higher number of CNV in iPSCs than hESCs (Hussein et al. 2011; Laurent et al. 2011; Martins-Taylor et al. 2011). Thus, in order to avoid similar outcome for future experiments, it would be advised to test all new cells lines on SNP arrays. Furthermore, regular SNP array might be useful to check for spontaneous deletion or duplication that might be due to the culture condition as well as the number of passages.

Furthermore, it was noteworthy that the iPSCs from the 22q11.2 individual was a female while the control iPSCs (900) was a male. Therefore, we could not exclude that several genes overlapping between 22q11.2 and ZDHHC8+/- might be influenced by genes located on sexual chromosome.

However, our comparison of the genes overlapping between ZDHHC8+/- and 22q11.2 NPCs suggested that ZDHHC8 might only have small impact on the pathophysiology of 22q11.2DS. Indeed, only 36 and 15 genes were significantly different (p < 0.01) with a FC superior to 1.5 or below -1.5 respectively. However, because we only had one line for ZDHHC8+/- and one line for 22q11.2, it would be interested to test with more line.

To summarize, this chapter allowed us to identify that the expression of the region deleted in ZDHHC8+/- was reduced by half when compared to the control. In addition, the transcriptomic analysis of ZDHHC8+/- allowed us to identify that several genes/pathways involved in neurons development and function appeared to be altered by the mutation such as axonal guidance as well as calcium signalling. While it was already demonstrated that the function of ZDHHC8 was link to neuronal and axonal arborisation, it is the first time that its relation with neurons was shown at the transcriptomic level. The study also identified that only few genes deleted in the 22q11.2 mutation overlapped with gene deleted in ZDHHC8+/-.
Chapter 6. General discussion

This thesis was divided into two main part, the first part investigates the temporal and cell specific expression profile of the genes from the 1.5Mb deletion of the 22q11.2 mutation into four different cell lineages while the second and third part was focusing on elucidating the function of ZDHHC8 in cortical projection neurons and NPCs.

Many pathologies are associated with 22q11.2DS, several of which affect the central nervous system (Angkustsiri et al. 2014; Bassett et al. 2003; Cascella and Muzio 2015; Retnakaran et al. 2010). Although several mouse models have been generated to study either the full spectrum or individual genes (Gogos et al. 1998; Kimber et al. 1999; Mukai et al. 2008; Paronett et al. 2015; Paylor and Lindsay 2006), only few studies have been performed in a human setting. In addition, due to the number of genes present in this CNV, the role of individual genes and their relative contribution to multiple pathologies remains largely unknown.

The study from Meechan et al. 2003 performed a comprehensive analysis of the 22q11.2 genes, they found that most genes were expressed in the developing and adult mouse brain, however, the study lacks temporal and spatial resolution (Maynard et al. 2003). On the other hand, our study allows to address when and in which cell lineages cells the gene present in the 1.5Mb deletion of the 22q11.2 mutation are expressed. Our study identified genes with higher expression during period of neuronal progenitors proliferation which appears to be in accordance with previous report (D. W. Meechan et al. 2006). Amongst them, the loss of Ranbp1 has recently been studied in a mouse model which revealed a decrease of the cortical layer II/III due to alteration in the proportion of neuronal progenitors (Paronett et al. 2015). Interestingly, our study demonstrated consistent findings, indeed RANBP1 was found to be mostly expressed when neuronal progenitors are proliferating therefore suggesting similar function in vitro and in vivo. In vitro mouse model of 22q11.2DS also reveals that Cdc45 mRNA expression happen to follow the one of Ranbp1, however cdc45 appears to exhibit an even higher expression than Ranbp1 during cortical neurogenesis. Therefore, a loss of Cdc45 might be responsible to a greater loss of progenitor cells than in mouse model of Ranbp1-/-.. Interestingly, we also identified potential genes involved in neuronal maturation such as SEPT5. This gene has a role in neurotransmitters release and vesicular trafficking, as well as being a substrate of PARKIN. While knockout model of Septin5 did not reveal strong phenotypic alteration of the synaptic signalling, its overexpression in dopaminergic neurons was observed to be toxic for the cells thus leading to cell death (Son et al. 2005; Tsang et al. 2008).
addition, *Sept5* deficiency in mouse reveals behavioural deficit in social interaction but also increases of the prepulse inhibition therefore implying a potential underlying synaptic alteration that remain unknown. (Harper et al. 2012; Suzuki et al. 2009). Surprisingly, we identified novel genes that were not observed to be expressed by previous study such as *GP1BB*. Indeed, this gene is known for its role in platelet, however, because it increases over the course of the differentiation our data suggested that it might have a role in neuron maturation. While it was recently suggested that mechanisms responsible of granules exocytosis are conserved between different cell types (Goubau et al. 2013), it was noteworthy that the *SEPT5-GP1BB* locus can be transcribed due to an imperfect polyA signal in *SEPT5*. Although the resulting *SEPT5-GP1BB* transcript is candidate for nonsense-mediated mRNA decay, it might explain why we detect *GP1BB* in neurons and the reason why it follows similar profile than *SEPT5*.

While previous analysis from Maynard et al. in 2003 attempted to investigate the tissue specific expression of the 22q11.2 genes, their study 1) did not reveal specific candidates, 2) they used adult tissue 3) microdissected tissues contain different neuronal and non-neuronal population therefore is not specific. While the dopaminergic population is altered in both schizophrenia and Parkinson’s disease, our study reveals enrichment of several genes in dopaminergic neurons. Therefore, this data suggests that the neuronal population in which the gene is studied should be taken into consideration.

In addition, to discover that several of these genes exhibit a specific temporal profile, our study also emphasized that neurons derived stem cells can mimic some aspects of the development in vivo.

The major part of this thesis then focused at the same time on the phenotypic and transcriptomic characteristic of *ZDHHC8* in cortical excitatory neurons development.

The fourth chapter is the first to report the generation of a hESCs line heterozygous for *ZDHHC8* using the CRISPR/Cas9 technique. While most study on *ZDHHC8* used either brain primary culture from mouse model or cellular model (HEK cells), it is the first time that *ZDHHC8* is studied in the context of neuronal development in vitro. Despite the kinetics of differentiation appeared to be unaltered in *ZDHHC8*+/− when compared to control, the differential expression identified that several genes are altered in *ZDHHC8*+/−.
While several studies reported that individual’s carrier of 22q11.2DS and or affected with schizophrenia exhibited neuronal migration defect, a study from Mukai et al., 2005 demonstrated that in vivo, ZDHHC8 was not essential for radial migration as neurons were able to reach the cortical plate in mice Zdhhc8+/- and Zdhhc8-/- (Mukai et al. 2015). Conversely, our study reveals that in vitro, ZDHHC8+/- neurons progenitors appear to migrate less and slower, therefore we suggest that although the ability of neurons to reach the cortical plate is unaltered their ability to populate the appropriate cortical layer might be altered. Furthermore, the axonal connexion to other cortical or brain region might also be impaired in these neurons.

Calcium activity is crucial for neurons as it allows the fusion and release of neurotransmitters from the presynaptic neurons but it is also involved in mechanisms such as synaptic plasticity. Mouse model of 22q11.2DS were shown to display an increase in the amplitude of calcium activity, while our data shows similar effect on the amplitude in ZDHHC8+/- we also observed an overall increase in the calcium activity resulting to an increase in the number of events that had not been reported in the past. It would be interesting to examine if this observation is due to an increase in neuronal receptors population or proportion or else due to an excessive release of glutamate which therefore can result to an hyperexcitability of the neurons. Hyperexcitability has been associated with pathology such as Alzheimer disease in which an excessive release of glutamate become toxic for the neurons due to an over excitation of extrasynaptic glutamate receptors (Hardingham and Bading 2010). It would therefore be interested to examine the proportion of synaptic versus extrasynaptic receptors. Furthermore, it would also be interested to investigate the population of metabotropic receptors such as CACNA1C or else potassium or sodium channel that could also be altered and therefore responsible of the underlying phenotype. Due to the function of ZDHHC8 as a palmitoyl transferase and the lack of knowledges about the substrates of this protein. It would be interested to perform proteomic analysis in order to determine which proteins are palmitoylated by ZDHHC8.

It was also interested to note that our preliminary result using ZDHHC8+/- derived neurons also identified an alteration of the dendritic morphology in late progenitors/young neurons. Overall, the total length was shorter and neurons appears to have less intersections. These results are in accordance with the previous work from Mukai et al.2008 which identified that these neurons appear to have a less complex dendritic arborization (Mukai et al. 2008)
Lastly, we investigated the transcriptome of neuronal progenitors heterozygous for ZDHHC8. The analysis reveals that pathways related to axon guidance and calcium signalling were altered in ZDHHC8 mutant progenitors which therefore strengthen our findings in chapter 4. In addition, these results on axonal guidance complements studies performed in human reporting a disruption of the white matter in patient with 22q11.2DS which might be due to axon guidance alteration (Kikinis et al. 2012; Radoeva et al. 2012). Interestingly, ZDHHC8 mutation appears to alter several pathways and genes related to neurons Although our comparison between ZDHHC8+/- and 22q11.2 iPSCs derived NPCs reveal that only a small proportion of genes overlap between the two lines, the study of ZDHHC8 alone provide us with convincing information on its involvement for neurons development. Amongst the several genes associated with neuronal development, it was noteworthy that three genes from the WNT signalling pathway were upregulated while the two genes coding for the WNT receptors FZB were downregulated. Interestingly, Wnt8b overexpression was observed to promote hypothalamic markers. Additionally, Wnt7b overexpression was instead showed to impairs neuronal differentiation in neuronal progenitors and therefore forebrain development in mouse, interestingly, Wnt7b overexpression was also associated with a decrease in the expression of Tbr2 and Tbr1 without affecting pax6 (Papachristou et al. 2014). Wnt2b overexpression was shown to inhibit differentiation therefore induces growth of tissue in retinal explants (Papachristou et al. 2014). While it would be interested to examine whether this gene affect early patterning of neurons development in our cellular models, the study would also would also benefit to investigate early neuronal patterning in vivo using the mice models for Zdhhc8+/- and Zdhhc8-/- (Mukai et al. 2004). Additionally, transcriptomic analysis identified that the DRD2 and DRD4 was upregulated in ZDHHC8+/- NPCs, as dopaminergic neurotransmission was observed to be disrupted in 22q11.2DS it would be interested to investigate whether we observed difference at the protein level between the control and the mutant (Boot et al. 2008). ZDHHC8+/- has been associated with downregulation of other genes coding synaptic receptors such as GRIA2 and CHRM3 or DRD4. Thus, it would be interesting to investigate whether this downregulation lead to a decrease expression of specific neuronal receptors at the protein levels.

Therefore, this thesis in addition to provide valuable information regarding the expression of the 22q11.2 genes, demonstrates by the study of ZDHHC8 that a cellular model can be a valuable tool to unravel the function of genes and therefore can be used to generate
cellular model that would enable to decipher the function of other genes of the 22q11.2 mutation.

To summarize, our study identified that several genes of the 22q11.2DS have a specific expression profile during neuronal differentiation. In addition, some of these genes appeared to be preferentially expressed in dopaminergic neurons. The study of ZDHHC8 heterozygous mutation in hESCs derived cortical projection neurons addressed that the kinetics of neuronal differentiation was similar to control. However, the motility/migration of the cells lacking ZDHHC8 was abnormal compared to control. Furthermore, the calcium signalling activity of the neurons was also found to be altered by the lack of ZDHHC8. Lastly, transcriptomic analysis demonstrated that the role of ZDHHC8 appeared to be associated with the development of neurons and its physiology. Finally, although only few genes appeared to overlap with a hiPSCs derived from 22q11.2 patients, our study strongly suggest the importance of ZDHHC8 for neuronal development.

Limitations and future directions

Limitations

Although my RT-PCR analysis (Chapter 3) on 22q11.2DS genes offer potentially valuable insight on their temporal profile, the study remains preliminary and requires repeat with independent sets of samples. I had similar issues regarding the differential expression between ZDHHC8+/- and control neurons. Therefore, future experiments should consider having more samples to solve this issue.

Genome editing using CRISPR/Cas technique successfully allowed the generation of several cell lines heterozygous for ZDHHC8. Despite confirmation of heterozygosity by sequencing, we were unable to verify a change of protein expression. Despite being used in previous studies to evaluate the ZDHHC8 protein in human cells, I observed multiple unspecific bands in western blot and therefore were unable to evaluate the protein expression of ZDHHC8.

My preliminary phenotypic analysis of ZDHHC8 KO cells revealed an increase in cortical neural progenitor proliferation, accompanied by a decrease in the number of CTIP2 positive deep layer neurons. However, further investigation will be necessary to assess whether this increase in progenitor proliferation leads to a subsequent alteration in the
number of neurons expressing cortical layer-specific markers, such as CUX1, TBR1, TBR2.

The transcriptomic analysis of the 22q11.2 iPSCs revealed the presence of a potential CNV on chromosome 17. Therefore, we did not carry on with gene enrichment analysis as well as KEGG pathways enrichment. My observation points to the importance of performing SNP array on all PSC lines to detect spontaneous mutation that may accumulate.

**Future Experiments**

As already mentioned in chapter 3, in addition to the temporal expression performed using control cells, we aimed to investigate the temporal profile of the 22q11.2 genes using iPSCs from individual carrier of a 22q11.2 mutation.

Although we have identified some deficits using heterozygous ZDHHC8 cells, a ZDHHC8-null mutant model would provide a better model to decipher the functional role of ZDHHC8 in neurons. Towards this goal, we have constructed a lentiviral vector expressing ZDHHC8 gRNAs. Future work is needed to produce the virus in HEK cells, which can then be used to transduce either the ZDHHC8+/− or wildtype hESCs. In addition, the temporal expression analysis identified that ZDHHC8 is preferentially expressed in dopaminergic neurons, therefore future experiments will investigate the function of ZDHHC8 in this neuronal population.

My calcium activity assay suggests that heterozygous neurons for ZDHHC8 exhibit more spontaneous calcium events but also appear to mature faster than the control neurons. Future experiments should aim to investigate whether blocking synaptic receptors with specific antagonist alter spontaneous calcium activity differently in the ZDHHC8+/− compared to the control neurons. The drugs could be used for such studies include 2R-amino-5-phosphonovaleric acid (AP5), a selective NMDA antagonist and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a competitive AMPA antagonist. In addition, the administration of these drugs during calcium recording could determine whether mutant cells are maturing in a similar fashion to the controls. Other drugs targeting GABAergic receptors could also be tested such as bicuculine or picrotoxin which are a GABAa antagonist.
To further investigate whether the synaptic activity and electrical activity of neurons heterozygous for ZDHHC8 is altered we thought to use MEA. Indeed, our institute has the expertise and the equipment necessary for such experiment. MEA allows to study the network activity in group of neurons. We previously observed an increase calcium spontaneous activity in ZDHHC8 KO therefore we are interesting to determine if it also results in an increase of the electrical activity but also if it affects the overall network activity.

Finally, a rescue experiment in which we reintroduce ZDHHC8 back into our ZDHHC8+- line would be interested to determine whether we can reverse the phenotype due to heterozygous loss of ZDHHC8. In addition, due to the availability of 22q11.2 iPSCs, it would also be interested to perform similar experiment in which we would reintroduction the ZDHHC8 in 22q11.2 iPSCs. Therefore, it would enable us to determine whether the phenotype we would observe in 22q11.2 derived neurons can be rescue by ZDHHC8.
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Supplementary Figure 1. Neuroactive ligand receptor interaction
Supplementary Figure 2 Calcium Signalling pathway
Supplementary Figure 3 Axon guidance