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Analysis of a new mouse model of mental illness: Derived (1)

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Declaration

I declare that this thesis has been composed by myself. Unless mentioned otherwise, the work discussed in this thesis has been performed by myself or I contributed to it substantially. This work has not been submitted for any other degree or professional qualification except as specified.

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Acknowledgements

Abstract

In a Scottish family, a t(1;11) (q42;q14) translocation between chromosome 1 and 11, is linked with mental illness. People carrying this translocation are diagnosed with mental illnesses such as schizophrenia, schizoaffective disorder, bipolar disorder and recurrent major depressive disorder. The *DISC1* gene localised on the chromosome 1 is known to be disturbed by this translocation. The DISC1 protein is involved in neurite outgrowth, cortical development, cell proliferation, differentiation, migration, mitochondrial activity, and cytoskeletal organisation and function. Multiple animal models have been created to study the effect of disrupting *DISC1* on brain development and mental illness. Rodent models have shown critical changes in behaviour, brain function and structure, as well as alterations in various molecular pathways. These modifications are consistent with an involvement for *DISC1* in psychiatric disorders. However, none of these *Disc1* mouse models accurately model any known causal events.

This thesis aims to characterise a new mouse model of the derived chromosome 1 carrying a modified endogenous *Disc1* gene, in order to reproduce the human transcript obtained from the chromosome 1 after the translocation happens. While MRI analysis of its brain structure showed no gross changes, histological analysis of brain structure revealed an enlargement of the lateral ventricles and a trend towards smaller cortical layer and corpus callosum thickness in heterozygous mice. There was also increased apoptosis in the prefrontal cortex of these mice. Investigation of cell density, GABAergic neuron density and hippocampal neural precursor proliferation and migration showed no significant change. Cultured homozygous mutant cortical neurons showed impaired neuronal outgrowth and somal hypertrophy. Lastly, RNAseq analysis and gene ontology analysis revealed disturbed RNA expression of numerous genes including genes involved with transport mechanisms, vesicular trafficking, cell signalling and communication in the cortex as well as genes involved with transport mechanisms and the electrical activity of the neurons in the hippocampus. Moreover, in both these regions, these alterations are more prominent in the synapse.

Overall, the t(1;11) (q42;q14) translocation seems to lead to subtle structural changes in the brain, neuronal outgrowth impairment and major changes in RNA expression. The latest suggests critical alteration in molecular pathways such as signalling pathway and synaptic pathway indicating that Disc1 is necessary for neuronal signalling and synaptic activity. As those characteristics are known to be strongly affected in psychiatric disorders, this indicates that the Der1 mice model could be a great model to study the underlying genetic and molecular pathway leading to developing those disorders. These results support the hypothesis that *DISC1* is a susceptibility gene for the development of mental disorders.

Lay summary

A translocation between chromosomes 1 and 11 was found in a Scottish family which seems to be linked with mental disorders. A translocation happens when pieces of DNA are exchanged between two chromosomes of the genome where the broken ends of the two chromosomes are then reconstructed with the wrong chromosome. When this event happens, genes are disrupted, and their proteins can malfunction. Members of this family carrying the translocation t(1;11) appear to be predisposed to develop psychiatric illness when compared to unaffected individuals in the family. The *Disrupted in Schizophrenia 1 (DISC1)* gene, was discovered to be affected on chromosome 1 in these individuals. Numerous studies on *DISC1* have revealed its involvement with the development and physiological functioning of the brain.

Multiple animal models have been created to study the effect of disrupting *DISC1* on brain development and development of major mental illness. While these animal models have shown significant changes in behaviour, brain function and structure as well as in important molecular pathways, they do not completely recapitulate the pathology or behavioural changes seen in mental disorders. Therefore, to accurately model the genetic translocation seen in the Scottish family, a mouse model with the translocation between chromosomes 1 and 11 was created.

This thesis aims to characterise this new *DISC1* mouse model in the hope that it will prove to be a better mouse model for mental health disorders. The end of the gene containing an exposed hydroxyl group on the deoxyribose is named the 3' half of the gene. This end of the *DISC1* gene has been removed from a position corresponding approximately to that of the translocation breakpoint in humans and replaced with a segment of human chromosome 11 to mimic the translocation found in the Scottish family.

Studying this mouse model has given us a better understanding of DISC1 function and its involvement in psychiatric disorders. The structure of the brain in these mice showed subtle changes due to the translocation. Moreover, it seems that the translocation affects cell death in the brain. RNA expression was also found to be disturbed along with impaired neuronal outgrowth. Overall, the translocation seems to affect the normal functioning of the brain. These results support the hypothesis that impaired functioning of *DISC1* could be involved in the development of mental disorders. However, further investigation is required to completely understand this new mouse model.

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<u>List of Abbreviations</u>

Genes and proteins

6-PTPS	6-pyruvoyltetrahydropterin
AC	Adenylyl cyclase
ACHE	Acetyl cholinesterase gene
ADCY	Adenylate cyclase
AKT	Protein kinase B
ANK3	Ankyrin 3
APP	Amyloid precursor protein
ARC	Activity-regulated cytoskeleton-associated
ATF4/5	Activating transcription factor4/5
BBS	Bardet-Biedl syndrome protein
BDNF	Brain-derived neurotrophic factor
CACNA1C/2D2	Calcium voltage-gated channel subunit alpha 1C/2D2
CACNB2/3	Calcium voltage-gated channel auxiliary subunit beta 2
CACNG3	Calcium voltage-gated channel auxiliary subunit gamma 3
CEP63	Centrosomal protein 63
CHL1	Cell Adhesion Molecule L1 Like
CIT	Citron Rho-Interacting Serine/Threonine Kinase
CNTN4	Contactin 4
D2R	Dopamine receptor D2
DBZ	DISC1-binding zinc finger
DCX	Doublecortin
DISC1/2	Disrupted-in-schizophrenia 1/2
DISC1FP1	DISC1 Fusion Partner 1
DIXDC1	Dishevelled axin domain containing 1
DLG4	Disks large homolog 4
DRP1	Dynamin related protein 1
DYNC1H1	Dynein cytoplasmic 1 heavy chain 1
EIF2	Eukaryotic initiation factor-2
ERBB4	Erb-B2 receptor tyrosine kinase 4
FAK	Focal adhesion kinase
FEZ1	Fasciculation and elongation protein zeta 1
FOXG1/P1	Forkhead box G1/P1

GBP	Guanylate binding protein
GLNS	Glutamate-ammonia ligase
GNRH	Gonadotrophin releasing hormone
GRIA3	Glutamate ionotropic receptor AMPA type subunit 3
GRIK3	Glutamate ionotropic receptor kainate type subunit 3
GRIN2A	Glutamate ionotropic receptor NMDA type subunit 2A
GSK3(α/β)	Glycogen synthase kinase 3 (alpha/beta)
IMMT	Inner membrane mitochondrial protein
ITIH3/4	Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member ¾
JNK	c-Jun N-terminal kinase
KAL7	Kalirin 7
KIF5A	Kinesin family member 5A
KLC1/2	Kinesin light chain 1/2
LIS1	Lissencephaly protein 1
MAO	Mono-amine oxidase
MAP2/1a	Microtubule Associated Protein 2/1a
MEKK/ MLK	Mitogen-activated protein kinase kinase kinase
MKS	Meckel syndrome type 1
MTOR	Mammalian target of rapamycin
N-CoR	Muclear receptor co-repressor
Nde1	NudE Neurodevelopment Protein 1
NDEL1	NudE nuclear distribution E homolog 1-like 1
NLS	Nuclear localization signal
NMDA(R)	N-methyl-D-aspartate (receptor)
NPTX	Neuronal pentraxin 1
NRXN1	Neurexin
OSBPL3	Oxysterol binding Protein like 3
PCM1	Pericentriolar material 1
PCNT	Pericentrin
PDE4	Phosphodiesterase type 4
PKA	Protein kinase A
PLCβ	Phospholipase C, β isoform
PML	promyelocytic leukaemia
PP1	Protein phosphatase 1
PSD95	Post synaptic density 95

PSEN1/2	Presenilin 1/2
PV	Paravalbumin
RBFOX1	RNA binding fox-1 homolog 1
SCN	Sodium voltage-gated channels
SFRP	Secreted frizzled related protein 1
SOX	SRY-boxes
SRC	SRC Proto-oncogene, non-receptor tyrosine kinase
STAT2	Signal transducer and activator of transcription 3
TBR2	T-box brain 2
TCF2	Transcription Factor 2
TNF	Tumor necrosis factor
TNIK	TRAF2 And NCK Interacting Kinase
TRAK1/2	Trafficking Kinesin Protein 1/2
TSNAX	Translin associated factor X
VIPR2	Vasoactive Intestinal Peptide Receptor 2
ZNF804A	Zinc finger nuclear 804A

Chemical Compounds

BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CaCl2	Calcium chloride
DAB	3,3'-diaminobenzidine
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
dPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
ENU	N-Ethyl-N-nitrosourea
HCL	Hydrogen chloride
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IgG	Immunoglobulin G
IMS	Industrial Methylated Spirits
LF2000	Lipofectamine 2000
MES	2-(N-Morpholino) ethanesulfonic acid
MgCl2	Magnesium chloride

MOPS	3-(N-Morpholino) propanesulfonic acid
NaCL	Sodium chloride
NaH2PO4	Sodium dihydrogen phosphate
NaHPO4	Sodium phosphate
NAOH	Sodium hydroxide
NFB	Neutral Buffered Formalin
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde

Biomolecules

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
cAMP	Cylic adenosine monophosphate
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
GABA	Gamma-Aminobutyric acid
mRNA	Messenger ribonucleic acid
NAA	N-acetylaspartate
NMDA	*N*-methyl-D-aspartate
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid.
shRNA	Short hairpin RNA
siRNA	small interfering RNA
SNP(s)	Single nucleotide polymorphism(s)
SRR	Serine racemase

Units of measurement

$	United States Dollars
%	Percentage
°C	Degrees Celsius
DALYs	Disability adjusted life years
g	Gram
L	Litre
M	Molar (mols per liter)

mm	Millimetre
mol	Mols
µm	Micrometre
Bp	Base pair

Brain regions

CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
Cg	Cingulum
DG	Dentate gyrus
dlo	Dorsolateral orbital cortex
DLPFC	Dorsolateral prefrontal cortex
fra	Frontal association cortex
Hp	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
lo	Lateral orbital cortex
mo	Medial orbital cortex
mPFC	Medial prefrontal cortex
PFC	Prefrontal cortex
Pir	Piriform cortex
prl	Prelimbic cortex
SRLM	Stratum radiatum and lacunosum molecular layer
vo	Ventral orbital cortex

Other

3-D	3 dimensions
ANOVA	Analysis of variance
ANTs	Advanced normalization tools
BAC	Bacterial artificial chromosomes
CNV(s)	Copy number variant(s)
CP(1/60/69)	Chimeric protein (1/60/69)
CSF	Circulation of the cerebrospinal fluid
Der1	Derived chromosome 1 (in t(1;11))
DIV	Days in vitro
DSM-5	Diagnostic and statistical manual of mental disorders 5
E18	Embryonic day 18
FCS	Foetal calf serum

GLM	Generalized linear model
GO	Gene ontology
GWAS	Genome wide association study
HET	Heterozygote
HOM	Homozygote
IGMM	Institute of Genetics and Molecular Medicine
In vitro	Within the glass
IPA	Ingenuity Pathway Analysis
IPSC	Induced pluripotent stem cells
ITK	Insight segmentation and registration toolkit
Kb	Kilobase
LOD	Logarithm of the odds
Log2fc	Log 2fold change
MHC	Major histocompatibility complex
MIA	Maternal immune activation
NPC(s)	Neural precursor cell(s)
MRI	Magnetic resonance imaging
Padj	Adjusted p value
PCA	Principal-component analysis
PCR	Polymerase chain reaction
PGC	Psychiatric Genetics Consortium
Poly-A	Polyadenylation
PPI	Pre-pulse inhibition
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROI	Region of interest
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
t(1:11)	Translocation (1;11)
WHO	World Health Organisation
WT	Wild type

CHAPTER 1
-
INTRODUCTION

1.1 Global burden of mental disorders

Mental disorder is a worldwide burden that currently affects 450 million people (WHO, 2001). The latest Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has tried to re-define the characteristics describing mental disorders in order to obtain more accurate diagnosis for patients suffering from mental disorder. The DSM-5 defines mental disorder as a significant distress found in an individual which reflects an impairment of mental capabilities (American Psychiatric Association, 2013). The disorders considered as mental illness can be quite different from one another such as: major depression disorder, bipolar disorder, schizophrenia and other psychoses, dementia, intellectual disabilities as well as developmental disorders. A various range of symptoms characterizes these disorders. This heterogeneity of disorders and symptoms makes mental disorders a major economic burden for our society (Bloom *et al.*, 2011; Trautmann, Rehm and Wittchen, 2016). Indeed, some costs can be considered direct such as medical help and research on those disorders but also indirect such as economic loss due to incapability of patients to contribute to society (Trautmann, Rehm and Wittchen, 2016). The global costs of mental disorders were estimated at US$2.5 trillion in 2010 (Trautmann, Rehm and Wittchen, 2016). In 2011, the World Economic Forum defined three different approaches to quantify economical cost of mental disorders: a standard cost of illness, an economic approach, and the value of a statistical life (figure 1.1) (Bloom *et al.*, 2011).
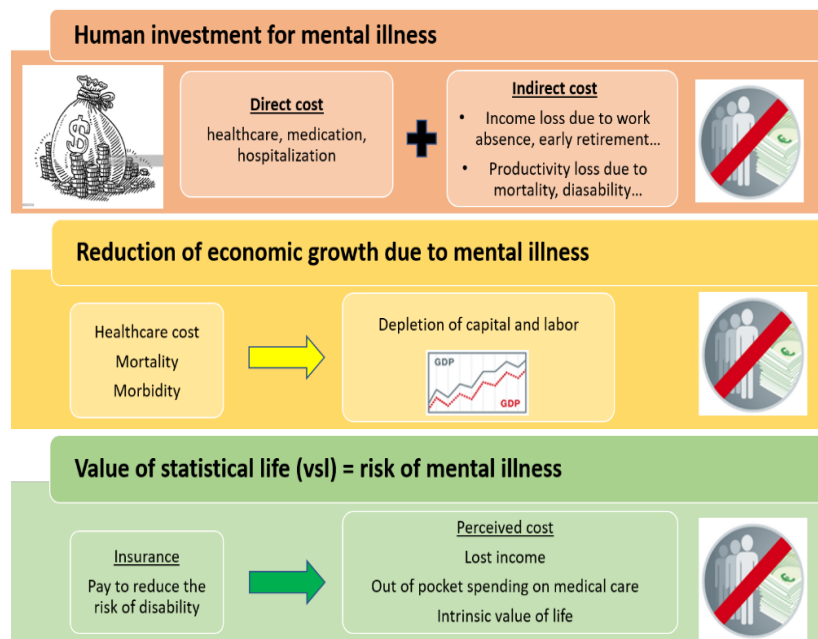
Figure 1.1: Schematic representation of the economic costs of mental disorders
Image adapted from EMBO report, The economic costs of mental disorders, Trautman et al. (2016).

Another way of assessing the burden caused by mental illness is by using the disability adjusted life years (DALYs) (WHO 2010). DALYs represents the total number of years lost to illness. Mental, behavioural and neurodevelopmental disorders are the third cause of global DALYs (WHO 2010). It has been estimated that 7.4 % of DALYs are due to mental and behavioural disorders from which 34.12 % are due to depression (WHO 2010, figure 1.2).

This shows that mental disorders are a real burden for the individuals affected and our society. The clinical presentations of these disorders or the biological mechanisms that underlie them are still not completely understood. Moreover, available treatments frequently produce side effects which make them difficult to support, and current treatments only provide limited benefits when suffering from these disorders but no curative therapies are yet available (Davis *et al.*, 1980; Wang, Demler and Kessler, 2002). Therefore, it is necessary to understand the origin of these illnesses and their biological mechanisms in order to improve treatments and maybe even prevent the development of psychiatric disorders.
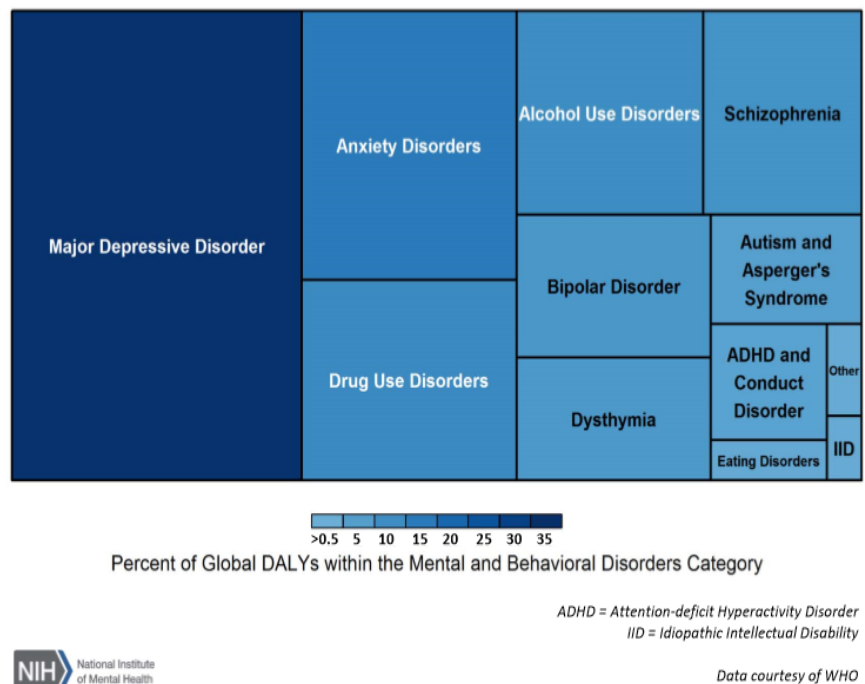
Figure 1.2: Representation of the global DALYs for each disorder comprised in mental and behavioural disorders. From the World Health Organisation (2010).

1.2 The origin of mental illness

Mental illnesses may be diagnosed as early as during childhood and adolescence. For example, schizophrenia can occur in childhood but is rarely observed before puberty, with its major onset happening in the mid-20s which correspond to early adulthood. Onset during late adulthood can also be observed in some cases (Howard et al. 2000; Thompson et al. 2001). Depression is a mental illness that can start during either childhood, adolescence, or adulthood (Frcpc & Frcpc 2001; Drachmann et al. 2011). While the cause of mental disorders has not yet been found, two factors are believed to be involved: genetics and environment.

1.2.1   Genetic factors lead to higher susceptibility to mental illness

Twin studies have been used to identify the heritability of mental health disorders, as monozygotic twins have the same chromosomal DNA sequence while dizygotic twins share 50% of the DNA (Martin, Boomsma and Machin, 1997; Wong, Gottesman and Petronis, 2005). Traits found to be shared by monozygotic twins, and not with dizygotic twins are believed to come from the genome. Thanks to these studies, a large range of heritable traits for mental disorders were discovered. This indicates that mental disorders can be a familial trait, due to a shared genetic background. However, since these disorders are complex their heritability is variable. For example, several twins studies have revealed that the heritability of schizophrenia is 80-85%, while for bipolar disorders it is 85% and for major depression disorders it is 40-50%   (Cardno and Gottesman, 2000; Malhi, Moore and McGuffin, 2000; McGuffin *et al.*, 2003; Craddock *et al.*, 2005). Adoption and family studies have also been used to study the link between genetic and mental illness (Jacobs *et al.*, 1970; Gershon *et al.*, 1988; Berrettini, 2000; Tsuang, 2000; Tully, Iacono and McGue, 2008).

So far, a combination of multiple genes has been hinted to be implicated in the development of psychiatric disorders (Craddock *et al*, 2005). Moreover, it seems that the same genes may also be associated with several mental disorders (Blackwood et al, 2001; Craddock et al, 2005). This brings to light the fact that mental disorders could share similar genetic factors and therefore similar biological mechanism impairments. Recently, genome wide association studies (GWAS) have been used to discover new genetic candidates for these disorders, based on the idea that a combination of several common risk alleles may act together leading to mental disorders (Lander, 1996). GWAS consist of observing a genome-wide set of specific genetic variants across a large number of individuals to see whether any variants are associated with a phenotypic trait (Witte, 2010). As sample sizes have increased, GWAS have generated increasingly clear evidence for common single nucleotide polymorphisms (SNPs)

contributing to mental illness (Ripke *et al.*, 2014; Ikeda *et al.*, 2017). SNPs happens quite frequently through the genome, being harmless most of the time. However, analysis of the genomes of patients suffering from mental illness revealed that the presence of specific SNPs is linked to the risk of developing these disorders (Szczepankiewicz, 2013; Falola *et al.*, 2017). Moreover, analysis of SNPs could help predict the severity of mental disorders (Jiao *et al.*, 2012). An initial GWAS for schizophrenia with 21,246 cases and 38,072 controls identified 22 genomic regions in total, of which 14 were novel risk loci (Ripke *et al.*, 2013). However, a follow up study with 36,989 cases and 113,075 controls identified 108 loci that meet genome-wide significance, of which 83 were novel (Ripke *et al.*, 2014). The results suggest dysregulation of the calcium pathway, the immune system, neuronal processes control and glutamatergic synaptic channel function (Ripke *et al.*, 2014).

Similarly, a GWAS with 1,461 patients with bipolar disorder and 2,008 controls established 36 associated loci (Sklar, 2013). A second GWAS of 9,784 patients suffering bipolar disorder replicated four of these known loci from the former study and identified two novel loci (Hou *et al.*, 2016). These findings suggest the implication of circadian rhythm, cell signalling and vesicle trafficking as well as the neuregulin pathways in bipolar disorders (Hou *et al.*, 2016). A recent GWAS which included 2964 patients and 61,887 control subjects from the Japanese population brought to light two novel loci significantly associated with bipolar disorders (Ikeda *et al.*, 2017). This study suggests that lipid abnormality may be involved in the pathophysiology of bipolar disorders as well as impaired cell cycle regulation, transcription and replication.

However, a GWAS for major depression disorder which has been done on a total of 18,759 individuals did not find any positive results but it was believed that the number of individuals studied was too low (Daly *et al.*, 2013). Recently, a study using data from 121,380 subjects affected by depression revealed 15 genetic loci linked to the risk of major depressive disorder in individuals of European descent, with some of these loci having already been found in GWAS of related psychiatric traits (Hyde *et al.*, 2016). The main results indicate that the regulation of synaptic function, developmental functions and neurogenesis could be impaired in major depression disorders (Hyde *et al.*, 2016). A GWAS study of 130,664 cases has further identified 44 independent loci that met criteria for statistical significance (Major Depressive Disorder Working Group of the PGC, 2017). The results indicated that several functions were implicated in the disease such as neurite outgrowth, synaptic plasticity and immune system as well as neuronal calcium signalling, dopaminergic neurotransmission, glutamate neurotransmission, and presynaptic vesicle trafficking (Major Depressive Disorder Working Group of the PGC, 2017).

The numerous loci, function and pathways found to be affected in these studies revealed that the identified candidate genes seem to be shared by the different mental disorders. For example, genes such as *CACNA1C*, *CACNB2*, *ZNF804A*, *ANK3*, *ITIH3-ITIH4* and *TCF2* seem to be implicated in both bipolar disorder and schizophrenia (Green *et al.*, 2010; Bhat *et al.*, 2012; Ripke *et al.*, 2013; Cardno and Owen, 2014). Some of these loci are also involved in autism, attention-deficit/hyperactivity disorder, and intellectual disabilities (Smoller *et al.*, 2013; Uher and Zwicker, 2017).

Copy number variants (CNVs) involve deletions or duplications of sections of chromosomes and can be either inherited or sporadic. While GWAS results show overlap between schizophrenia, major depression disorders and bipolar disorder, the study of CNVs does not always indicate similar results. Indeed, another line of thought is that psychiatric disorders are caused by CNVs (Joober and Boksa, 2009; Rees, O'Donovan and Owen, 2015). 13% of the human genome is believed to be composed of CNVs and numerous studies have revealed that CNVs increase the risk of developing mental disorders, moreover concentration of rare CNVs in individuals with schizophrenia is higher compared with controls, but a lesser quantity of very large CNVs in bipolar patients was found (St Clair, 2009; Stankiewicz and Lupski, 2010; Grozeva *et al.*, 2012, 2013). CNVs associated with mental disorders are numerous (Cardno and Owen, 2014; Kirov, 2015). However, they seem to be rarer than the variants that are the focus of GWAS, but they seem to have a superior effect on risk of schizophrenia when they occur. CNVs have been less well studied in bipolar disorder than schizophrenia and studies have not found a significant increase in the amount of rare CNVs in individuals with bipolar disorder compared with controls. Therefore, patients diagnosed with a mental disorder carrying large, rare deletions are more likely to be diagnosed as schizophrenics, and those without them are more likely to be diagnosed as having bipolar disorder (Grozeva *et al.*, 2015). CNVs occurrence is quite heterogeneous between the different mental disorders with some CNVs more common in some illness compared to others (Green *et al.*, 2016). A recent study analysed CNVs in the whole genome of patient affected by major depression and showed that deletion CNVs are more frequent in major depression compare to controls (Yu *et al.*, 2017).

Overall, psychiatric disorders are genetically heterogeneous. GWAS studies and CNVs analysis allowed to find multiple possible genetic risk factors for psychiatric disorders and pointed toward numerous similarities between the genetic of these disorders. However, genes modification is not the only feature which could lead to the development of psychiatric disorders.

1.2.2   Environmental factor could trigger mental disorders

As mentioned previously, the genome may hold a partial information indicating the risk-level for the development of mental disorder. However, the twin studies which revealed this also demonstrated that an individuals' environment is also a risk factor (Uher & Zwicker 2017; Polderman et al. 2015; Arseneault et al. 2011). Studies have also indicated a significant link between the environment and psychiatric disorders (figure 1.3) (Uher & Zwicker 2017; Ehrenreich 2017). Initially, research tried to link one aspect of the environment with one individual's mental disorder (Deater-Deckard et al. 1998; Rutter et al. 1975). To understand better the range of the environmental effect on psychiatric disorders, larger and more accurate studies were completed, which allowed the identification of multiple environmental risk factors for those disorders (Uher & Zwicker 2017; Brown 2011; van Os et al. 2010).

	Autism	Schizophrenia	Bipolar disorder	Depression
Pregnancy risk factors				
Infections	+	+++	++	+
Malnutrition		+++	++	++
Heavy metals	+++	++		
Perinatal risk factors				
Preterm birth	++	++	++	++
Season of birth	++	+++	++	+
Birth complications	+++	+++	0	
Childhood environment				
Urbanicity	+++	+++	+	+
Poverty	++	+++	+	+++
Maltreatment	N/A	++	++	+++
Bullying	N/A	++	+	+++
Drug use in adolescence				
Cannabis	N/A	+++	++	+
Stimulants	N/A	+++	++	0

The number of + marks the strength of evidence (+ means some evidence of association/single report; ++ means moderate replicated evidence of association/multiple reports; +++ means strong evidence of association/multiple replications or good meta-analysis). Evidence of no association is noted as 0. Empty cells reflect absence of evidence for or against association. No factor has been negatively associated with any of the disorders. Because of the early age at onset of autism, environmental factors occurring after age 3 cannot be reliably studied and are marked as not applicable (N/A).

Figure 1.3: Environmental factors associated with mental illness

Table from "Etiology in psychiatry: embracing the reality of poly-gene-environmental causation of mental illness", Uher et al. (2017)

It was shown that in urban area higher rates of psychiatric disorders were found compared to rural areas (Peen et al. 2010; Brown et al. 1975; Vassos et al. 2016). Urban life is believed to be associated with poverty, environmental degradation, and stress which could all be risk factors for mental illness. Bullying has also been linked to the development of mental disorders with the victims having a higher risk of developing mental disorders and this could even impact their own future children (Swartz & Bhattacharya 2017).

Numerous epidemiological studies suggest that maternal infection increased the risk of depression, schizophrenia and major affective disorders in children, especially if the infection happens during the second trimester of pregnancy (Mednick *et al.*, 1988; Boksa, 2008; Machon, Mednick and Huttunen, 2012). Maternal stress during pregnancy was also linked to depression in the offspring (Enayati *et al.*, 2012; Murphy *et al.*, 2017). The cumulative effect of both maternal stress and infection during gestation has been showed to augment even more the risk of developing depressive symptoms in the children during adolescence (Murphy *et al.*, 2017).

Substance abuse has also been identified as a risk of developing psychiatric disorders especially in adolescents (Center for Behavioral Health Statistics and Quality, 2015). For example, cannabis use has been discovered to lead to an earlier initiation of schizophrenia and a greater risk of psychosis (Manrique-Garcia *et al.*, 2012; Donoghue *et al.*, 2014).

Overall, the same kind of environmental exposure can increase the risk of developing different mental disorders, while the same disorders could be due to different sort of environments. However, many individuals seem to be resistant and don't develop any mental disorder even if they are exposed to multiple adverse environmental factors. This is due to the fact that each individual experience life in different ways. One event can make a person more vulnerable while this event would not affect another person possibly making her more resistant to future experiences (Collishaw *et al.*, 2007; Rutten *et al.*, 2013; Davis *et al.*, 2016).

No environmental factor is necessary or completely sufficient to cause any psychiatric disorder, in fact, the encounter of multiple environmental factors across the course of life and as well of multiple genetic factors are believed to act cumulatively to trigger mental disorders. Therefore, these gene-environment interactions seem to be the cause of the development of mental health disorders and it is now believed that environmental factors act on personal traits according to an individual's genetic predispositions (Hopwood and Donnellan, 2011; Uher and Zwicker, 2017). Using 16 environmental variables known to all be very strong risk factors for mental, an interaction between nine SNPs and those risk factors were identified, showing an enrichment of genes linked to serotonin neurotransmission and neurodevelopmental

processes (Bernardo *et al.*, 2016). More research is currently ongoing to fully understand the mechanism of gene-environment interaction which leads to psychiatric illness. This could lead to better therapeutic treatments and personalised medication (Halldorsdottir and Binder, 2017).

1.3 Common psychiatric disorders

Some of the most common psychiatric disorders are schizophrenia, bipolar disorder and major depression disorder. The biological mechanisms underlying those disorders is still not understood and diagnostic of patients affected by these disorders is difficult. Because of the lack of understanding of these illnesses, this can sometime lead to misdiagnose. Major depressive disorder is one of the main causes of disability worldwide with an estimated 300 million people are affected globally, affecting more women than men (Seedat *et al.*, 2009; Otte *et al.*, 2016). In comparison, bipolar affective disorder affects about 60 million people and schizophrenia about 21 million people worldwide (WHO, 2017). Since these disorders can be hard to differentiate clinically, as they sometimes share the same symptoms and clinical impairment, it is necessary to discover their underlying mechanisms in order to better define and treat them.

1.3.1   Characteristics of major depression disorder

Major depression affects approximatively 4.4% of the population worldwide, and more and more people are getting affected (World Health Organization, 2017). It is more frequent in women than in men (Otte *et al.*, 2016). It is a mood disorder that affect how the person feels, thinks and behaves and is characterised by persistent feeling of sadness, loss of interest, low self-esteem (World Health Organization, 2017). It sometimes drives patients to a point where they feel that life isn't worth living and may even lead to suicidal thoughts (World Health Organization, 2017). Major depression disorder is a real illness for which medical treatment is needed and it is not something you can just "get over" (US Department of Health and Human Services, NIH and NIMH, 2015). Unfortunately, proper treatment is still under investigation and no established mechanism has been found to explain all aspects of the disease.

Patients suffering from major depression have been found to have a smaller hippocampal volumes (Sheline *et al.*, 1996; Bremner *et al.*, 2000; Cole *et al.*, 2011). In a more recent study, magnetic resonance imaging (MRI) scans from 1728 major depressive disorder patients and 7199 controls from 15 research samples worldwide were analysed, showing lower hippocampal volume mainly in patients with recurrent major depression disorder and no difference in first episode patients (Schmaal *et al.*, 2016). Moreover, the volume reduction was

more pronounced in early onset patients, where the illness starts before 21 years of age. These results indicate a progression of the hippocampus volume loss correlating with the progression of the disorder (Schmaal *et al.*, 2016). A study also shows that the shape of the hippocampus could be modified in major depression disorder compare to controls (Posener *et al.*, 2003). However, these studies are not always consistent, and the sample sizes are often small. Adults suffering from major depression disorder were reported to additionally show cortical thinning in the frontal and temporal lobes whereas no difference in cortical thickness was found in depressed adolescent patients but left and right hemisphere total surface area was smaller (Schmaal *et al.*, 2017). Other than the volumetric reductions of the hippocampus and cortical thinning, modification of the amygdala, temporal and frontal lobes, basal ganglia and cingulate cortex anterior have been noticed in some major depressive disorder patients (Coffey *et al.*, 1993; Lacerda *et al.*, 2003; Ballmaier *et al.*, 2004; Hastings *et al.*, 2004; Lorenzetti *et al.*, 2009). However, similar to changes in the hippocampal volumes, these structural changes have not always been found consistently. Moreover, it seems that more persistent forms of depression are associated with greater change in regional brain volumes.

Hypothalamic-pituitary-adrenal (HPA) axis activation seems to be a known feature in depressed patients as shown by elevated concentration of cortisol and corticotropin-releasing hormone (Varghese and Brown, 2001; Borges, Gayer-Anderson and Mondelli, 2013). Additionally, impairment of the immune system in major depressive disorder patients has been revealed, with patients suffering from depression showing an increase of inflammatory response with elevated concentration of biomarkers of inflammation (Zorrilla *et al.*, 2001; Miller, Maletic and Raison, 2009; Schmidt, Shelton and Duman, 2011). It is believed that the peripheral changes in cortisol levels and inflammatory mechanisms might ultimately induce depressive symptoms by disrupting neuroplasticity and neurogenesis in the brain which are vital to its function. Additionally, lower levels of brain-derived neurotrophic factor (BDNF) and other regulators of neuroplasticity might affect behaviour through their control of neurogenesis and the inflammatory cytokines could lead to reduction of monoamine synthesis and imbalance of neurotransmitters having for consequences a modification in the brain function and therefore of the behaviour (Molendijk *et al.*, 2014; Otte *et al.*, 2016). Indeed, research focusing on serotonin involvement in depression indicate that serotonin level was decreased in patients as well as in animal models of depression (Paul-Savoie *et al.*, 2011). These mechanisms increase monoamine synaptic membrane reuptake and reveals neurotoxic effect (Paul-Savoie *et al.*, 2011). Lastly, it is possible that trauma could also lead to activation of the immune system, which in turn could cause depression. It seems that all mechanisms involved in depression have a very close interaction with each other and further understanding

of their entanglements is necessary to understand depression, with possibly a special focus on the inflammatory mechanisms of major depressive disorder (Otte *et al.*, 2016; Guo and Jiang, 2017).

1.3.2 Characteristics of Bipolar disorder

Bipolar disorder affects 60 million people worldwide (WHO, 2017) and is a brain disorder that usually causes extreme mood swings. It leads to patients feeling euphoric and full of energy wanting to do a lot of things at times, and extremely low and depressed wanting to stay secluded at some other times (NIMH, 2016). These distinct periods are called "mood episodes" (NIMH, 2016). Sometimes, low and high mood episodes can happen simultaneously, referred to as a "mixed feature episode" (NIMH, 2016). This inconstant mood state prevents patients from carrying out daily tasks and being actively part of the society. Bipolar disorder also affects patients' sleep, energy, behaviour and thought process.

There are four basic classes of bipolar disorder (NIMH, 2016), ranging from periods of manic episodes to depressive episodes: i) Bipolar I disorder is defined by extremely severe manic episodes. In this case, the episode last at least 7 days and immediate hospital care is needed. Moreover, a depressive phase of two weeks also occurs most of the time; ii) Bipolar II disorder is less intense than described previously but is also characterised by manic and depressive episodes; iii) Cyclothymic disorder is characterised by several periods of hypo-manic symptoms which are less severe manic episode and depressive symptoms lasting for at least 2 years; iv) Other specified and unspecified bipolar and related disorders which do not fit in the categories previously listed. Currently, treatment to help cope with bipolar disorder exists. It helps patient managing the mood swings but it is still difficult for them to have a "normal life" (US Department of Health and Human Services, NIH and NIMH, 2015). More research about the mechanistic of the disorder is needed to obtain better understanding of this disorder and better treatment.

Numerous studies have shown that the structure of the brain of bipolar disorder patients is different compared to healthy people, with each severe mood episode linked to the exacerbation of the brain volumetric change (McDonald *et al.*, 2004; Altshuler *et al.*, 2005). However, these studies haven't always shown the same outcome with regards to structural changes of the brain. In a meta-analysis of MRI, patients affected by bipolar disorder were discovered to have a mild enlargement of the right lateral ventricle, but no other volumetric changes were noticed (McDonald *et al.*, 2004). However, this study also revealed heterogeneity in the results of volumetric analysis between different studies for several other

regions such as the third ventricle, left sub-genual prefrontal cortex, bilateral amygdala and thalamus (McDonald *et al.*, 2004). This indicate the need to form homogeneous group within bipolar patients in order to study this disorder accurately. Another meta-analysis of MRI showed a reduction of total brain volume in bipolar patients compared to healthy controls, as well as enlarged ventricle and globus pallidus (Arnone *et al.*, 2009). Patients with bipolar disorder seem to also have significant reduced intracranial, whole brain, total grey and white matter volumes (De Peri *et al.*, 2012). However, another study indicated that bipolar disorder patients did not have a grey matter deficit compared to healthy control (Yüksel *et al.*, 2011). In an analysis which compared bipolar type I and II, it was found that patients had reduced total brain volumes (Maller *et al.*, 2014). They specifically showed that only bipolar disorder type I had reduced volume and thickness in the medial orbitofrontal region of the right hemisphere and reduced thickness of the superior temporal region of the left hemisphere. However, only patients with bipolar type II had lower white matter integrity. Moreover, it seems that bipolar disorder severity correlated with brain structure abnormality (Maller *et al.*, 2014). This might indicate that specific abnormalities exist for specific bipolar subtypes, and therefore, it could be an indication on why results between studies on bipolar disorder are so variable. Several studies indicate that grey matter density in frontal, temporal, parietal and occipital lobes is reduced in patients suffering from bipolar disorder (Strakowski, DelBello and Adler, 2005; He *et al.*, 2017; Hibar *et al.*, 2018). The temporal and occipital lobes are necessary to process sensory and visual information respectively, therefore impairment of these two regions could lead to difficulties to do everyday tasks and to mood swings and their volume reduction seemed to be associated with a longer duration of bipolar disorder (He *et al.*, 2017; Hibar *et al.*, 2018). Additionally, reduction of the volume of the hippocampus and thalamus in bipolar patients seem to be consistent findings while volumetric changes of the amygdala have been found sometime either reduced or increased (Blumberg, 2003; Cao *et al.*, 2016; Hibar *et al.*, 2016, 2018). Both the hippocampus and the amygdala are key regions of the limbic system, additionally there seem to be a decrease in white matter tracts between the prefrontal regions and the limbic structures might indicate that the limbic system function is possibly impaired (Strakowski, DelBello and Adler, 2005; Clark, Sahakian and Luke Clark, 2008). This indicate a disruption in the connection between the different brain regions involved in cognition therefore indicating that cognition might be affected.

The neurobiology of bipolar disorder is sometime quite similar to the one of major depression disorder and schizophrenia (Savitz and Drevets, 2009; Muneer, 2016). There are several hypotheses indicating that dysregulation of mitochondrial function could lead to bipolar disorder (Cataldo *et al.*, 2010; Morris *et al.*, 2017; Scaini *et al.*, 2017) which has also been

suggested for schizophrenia and depression (Rajasekaran *et al.*, 2015; Bansal and Kuhad, 2016). Additionally, decrease in neuronal and glial density as well as in neuronal size was also found in bipolar disorder, the latter of which is thought to be linked with dysregulation of the immune system (Rajkowska, 2000; Harrison, 2002; Bearden, Zandi and Freimer, 2016). Further, pyramidal neurons seem to be particularly affected in bipolar disorder (Benes, Vincent and Todtenkopf, 2001; Liu *et al.*, 2007). The activity of neurotransmitters, such as serotonin, GABA and dopamine, also appear to be altered in bipolar disorder (Maletic and Raison, 2014; Wang and Young, 2016).

1.3.3 Characteristics of Schizophrenia

According to the national institute of mental health (NIMH), schizophrenia is "a chronic and severe mental disorder that affects how a person feels, thinks and behave". Individuals affected by this disorder have trouble managing their emotions and making decisions and have a hard time distinguishing what is real or not. All this makes it very difficult for people suffering from schizophrenia to live in society. More than 21 million people in the world are affected by this disorder (WHO, 2017). Patients suffering from schizophrenia can express various symptoms: i) Positive psychotic symptoms such as auditory and visual hallucinations, paranoid delusions as well as distorted perceptions and beliefs; ii) Negative symptoms such as loss speech, inability to express emotion, to find happiness in everyday life and to be active through the day; iii) Disorganization symptoms which are characterised by confused thinking and speech, trouble with logical thinking and sometimes abnormal behaviour and movements; iv) Impaired cognition identifiable by difficulties to pay attention, to concentrate, to learn and to make decision and therefore indicating an impaired working memory (WHO, 2017).

Patients affected by schizophrenia are believed to have a different brain structure and neurobiology. A lot of work having been done and still ongoing to understand the mechanisms of this disorder (Ross *et al.*, 2006; Iritani, 2013). The hope is also to find better treatments for this disorder as current treatments are associated with numerous undesired secondary effect such as weight gain, metabolic changes and sexual dysfunction (Lally and MacCabe, 2015). Moreover, treatments available don't work on every patient (Lally and MacCabe, 2015). Patients affected by this disorder have been shown to have larger volumes of the ventricles and caudate nucleus compared to healthy controls and also showed signs of cerebral atrophy which were related to the severity of the symptoms as well as a reduction of the volumes of the amygdala, hippocampus, temporal gyrus, thalamus, prefrontal cortex and prefrontal white matter (Johnstone *et al.*, 1976; Young *et al.*, 1991; Breier *et al.*, 1992; Chua and McKenna, 1995; Henn and Braus, 1999; Wright *et al.*, 2000). The reduction of prefrontal white matter

correlated with the reduction of the amygdala and hippocampus which indicate abnormal connections between the limbic system and the cortex in schizophrenia (Breier *et al.*, 1992). A meta-analysis used the MRI results of 771 schizophrenic patients to study brain volume modification, revealing that drug-naïve patients had a reduction of intracranial volume as well as a reduction of the total brain volume, total grey and white matter volumes , hippocampal volume, thalamic and caudate nucleus volume along with an increase in cerebrospinal fluid volume compared to controls (Haijma *et al.*, 2013). A more recent meta-analysis of brain MRI scans from 2028 schizophrenia patients and 2540 controls was performed, similarly indicating that schizophrenic patients had smaller hippocampus, amygdala, thalamus, nucleus accumbens, and intracranial volumes, as well as larger globus pallidum and lateral ventricle volumes. The most significant changes were in the reduction of the hippocampus and the enlargement of the ventricles. Moreover, the putamen and globus pallidum were getting larger with the progression of the disorder (Van Erp *et al.*, 2016). Another large study of subcortical brain volume obtained similar results, in terms of regional volumes, but additionally indicated a leftward asymmetry for globus pallidum volume in schizophrenic patients. Therefore, this indicates that schizophrenic patients might expressed structural and connectivity differences between their two cerebral hemispheres (Okada *et al.*, 2016).

It is believed that the reduction of the white matter volume indicates an impaired communication between the different brain regions affected by the disorder and this problem of connectivity could lead to the cognitive impairments seen in schizophrenia (Kyriakopoulos *et al.*, 2008; Karlsgodt *et al.*, 2010; Klauser *et al.*, 2017; Levitt *et al.*, 2017). Cellular post mortem studies show that there was a reduction of cellular proliferation and GABAergic neurons (Lewis *et al.*, 2012; Allen, Fung and Shannon Weickert, 2016; De Jonge *et al.*, 2017). The dysregulation of the GABAergic pathway might be linked to the NMDA receptor hypofunction found in schizophrenia. Indeed, the hypothesis that NMDAR hypofunction is the principal mechanism behind this disease's pathophysiology is one of the most supported (Olney, Newcomer and Farber, 1999; Coyle, 2012; Snyder and Gao, 2013; Balu, 2016). The presence of NMDAR antagonist in the neuronal network leads to non-activated GABAergic neurons and therefore to a reduction of GABA release and disinhibition of excitatory neurons (Cohen *et al.*, 2015). Multiple neuronal pathways would be then altered. (Olney, Newcomer and Farber, 1999; Nakazawa, 2011; Coyle, 2012). As well as an imbalance in GABAergic pathways, neurotransmitters such as dopamine and glutamate have also been shown to be affected in schizophrenia. Indeed, antipsychotics were shown to act on dopaminergic D2 receptor to block their function (Crow *et al.*, 1976; Kapur and Mamo, 2003). Moreover, drugs acting through these dopaminergic pathways such as amphetamine and cocaine can exacerbate

psychosis in individuals with schizophrenia (Brady *et al.*, 1990; Howes and Kapur, 2009; Bramness *et al.*, 2012). It seems that multiple causes could lead to dopamine dysregulation and this dysregulation is not only at the D2 receptor level but also related to the regulation of presynaptic dopaminergic synthesis and function which has been found increased particularly in the striatum of schizophrenic patients (Howes and Kapur, 2009; Howes *et al.*, 2012). More research on the dopaminergic pathway and its activity in the synapse is necessary to obtain more insight on the role of dopamine in schizophrenia.

Overall, these three major mental disorders share a lot of similar characteristics, but it seems that research has only been able to target a few specific mechanisms for each one so far. Therefore, more research is needed to fully understand these disorders and to discover their underlying mechanisms. The research previously done indicates that it is possible that different psychiatric disorders share similar pathogenic mechanisms, their alteration being indicated by similar structural and molecular changes in the brains of patients affected by these disorders.

1.4 DISC 1: a risk factor of mental illness

1.4.1   Discovery of DISC 1

When *DISC1* was first discovered, researchers found a balanced translocation between chromosome 1 and chromosome 11, t(1:11)(q42.1;q14.3), in the karyotype of a boy in a Scottish detention centre who was presenting signs of psychological disorder (Jacobs *et al.*, 1970). Researchers karyotyped his complete family and found the same genetic alteration in several individuals (Jacobs *et al.*, 1970). A follow-up study of this family reported that the t(1:11) translocation was co-segregating with psychiatric disorders (David St Clair *et al.*, 1990). Indeed, 16 of the 34 individuals carrying the mutation were diagnosed with mental disorders such as schizophrenia, major depression or bipolar disorder. However, five of the 43 unaffected family members also had other psychological issues (David St Clair *et al.*, 1990). This Scottish family was investigated for four generations and the affected gene localised on chromosome 1 was finally named Disrupted In Schizophrenia 1 or *DISC1* (Millar et al., 2000). Further research revealed that of the 87 members of the family who were karyotyped, 37 carried the translocation, the clinical data of only 29 translocation carriers were available, 18 of whom were diagnosed with a major mental illness, such as schizophrenia, bipolar disorder or major depression (Blackwood et al. 2001, figure 1.4). A logarithm of Odds (LOD) score analysis was performed to observe the degree of linkage of this gene with mental illness. Findings revealed that in this family, the translocation is significantly associated with schizophrenia with a LOD score of 3.6 (Blackwood *et al.*, 2001). The association increases

when only affective disorder (i.e depression, bipolar disorder, anxiety disorder) is considered with a LOD of 4.5. When data from recurrent major depression, bipolar disorder or schizophrenia are considered together, the score reached a LOD of 7.1 (Blackwood *et al.*, 2001). This evidence for linkage is among the strongest reported for a psychiatric disorder, and therefore suggested a link between the t(1;11) translocation and psychiatric disorders (Blackwood *et al.*, 2001).
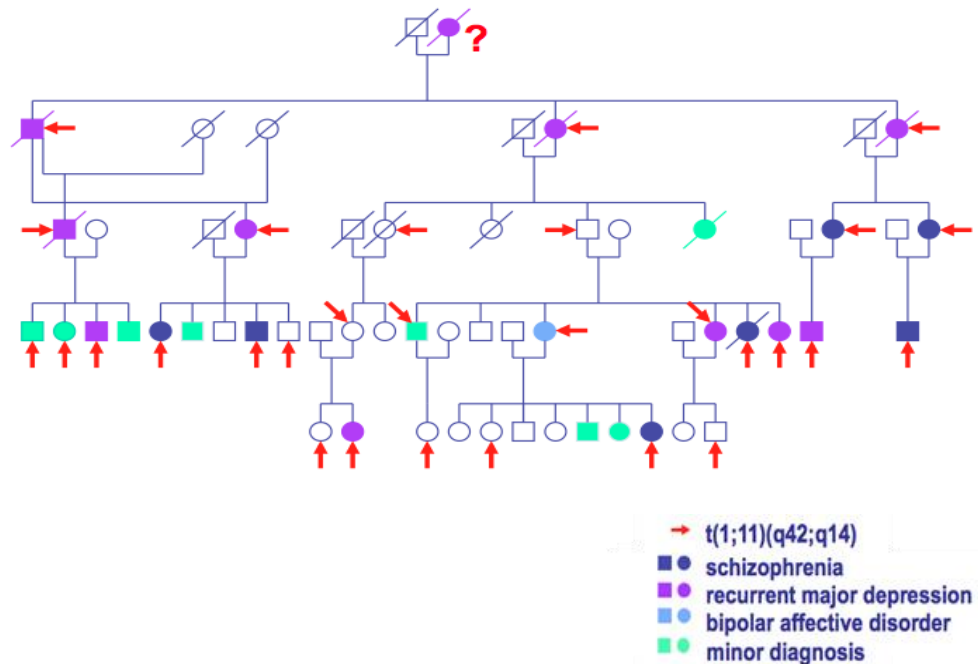
Figure 1.4: Representation of the family with a (1;11)(q42;q14.3) translocation
Karyotype analysis has been performed on 87 members of this family. Shown are 58 of the family members for whom carrier status is known (red arrow) and whose psychiatric phenotype has been defined through follow-up by direct interview, general-practice contact, or hospital case-note review. Adapted from Blackwood et al., (2001).

The translocation was not associated with any physical or neurological illnesses or developmental abnormalities. However, translocation carriers had prolonged latency and reduced amplitude of P300 event-related potential (ERP) (Blackwood *et al.*, 2001), which are features commonly seen in patients of schizophrenia and indicate deficits in the speed and the efficiency of the processing of information in short-term memory therefore indicating an impairment in working memory (Schreiber *et al.*, 1992; Blackwood, 2000). Thus, translocation carriers were similar to patients with schizophrenia and clearly different to the non-carriers from the family and to healthy individuals in measures of short-term memory. This indicated that the translocation alters brain function and have a role in the development of mental

disorders in the translocation carriers (Blackwood *et al.*, 2001). Similarly, a recent study on this family showed that the translocation was significantly linked with various psychiatric disorders which included schizophrenia, schizoaffective disorder, bipolar disorder, and recurrent major depressive disorder with a LOD score 6.1 (Thomson *et al.*, 2016).

Mapping of translocation breakpoints was therefore conducted to determine the genes that might be directly affected by the translocation (Evans et al. 1995; Millar et al. 1998; Millar et al. 2000b; Semple et al. 2001). The translocation was found to disrupt three genes located at 1q42.1 and 11q14.3: *Disrupted in schizophrenia 1 and 2 (DISC1* and *DISC2)* on chromosome 1 and *DISC1 Fusion Partner 1 (DISC1FP1)* on chromosome 11 (Eykelenboom et al. 2012; Millar et al. 2000b; Zhou et al. 2008). *DISC2* is a non-coding gene that is anti-sense to *DISC1* and is believed to regulate *DISC1* expression (Millar *et al.*, 2004) and *DISC1FP1*, is believed to be non-coding gene as well (Zhou, Geyer and Kelsoe, 2008; Zhou *et al.*, 2010; Eykelenboom *et al.*, 2012). The t(1;11) translocation has been shown to lead to the formation of aberrant fusion transcripts between *DISC1FP1* and *DISC1*, and the possible abnormal chimeric proteins encoded by these fusion transcripts seem to induce mitochondrial deficiencies in transfected cells (Eykelenboom *et al.*, 2012).

A GWAS analysis revealed that *DISCFP1* could be associated with schizophrenia (Debono et al. 2012), but this result has not been replicated. However, a recent study suggests that a small open reading frame in *DISC1FP1* is translated in the brain and might encode a small protein predominantly localized in mitochondria (Ji *et al.*, 2015). Moreover, *DISC1FP1* RNA expression was found increased in the post mortem brains of schizophrenic patients (Ji *et al.*, 2015). Therefore, *DISC1FP1* gene could be additionally be a susceptibility gene for major mental disorders, possibly specific to schizophrenia (Ji *et al.*, 2015). Additionally, if *DISC2* regulates *DISC1* expression, *DISC2* could then also be involved in the development of mental disorders and considered as a susceptibility gene (Thomson *et al.*, 2013).

1.4.2 DISC1 is associated with mental disorders

As of today, *DISC1* has been commonly cited as a candidate gene in psychiatric genetics research, with several studies providing evidence of *DISC1* involvement in several psychiatric disorders such as schizophrenia, bipolar disorder, major depression, schizoaffective disorder and autism (Chubb *et al.*, 2008; Thomson *et al.*, 2013). The first independent suggestion for the involvement of the *DISC* locus in psychiatric disorders came from studies based on the Finnish population which revealed evidence for the linkage of schizophrenia and schizoaffective disorder to the 1q32.2–q41 region of chromosome 1, proximal to the DISC1 gene. (Ekelund *et al.*, 2001, 2004; Hennah *et al.*, 2003). Additional linkage and association

studies analysing samples from populations in Taiwan, China, Scotland and Toronto have also revealed positive association between the *DISC* locus and mental illness (Hwu *et al.*, 2003; Thomson *et al.*, 2005; Qu *et al.*, 2007; Rastogi *et al.*, 2009). Further, there the *DISC* locus is known to interact with other candidate genes also linked to mental illness such as *NDE1*, *NDEL1* and *CIT* (Hennah *et al.*, 2007; Burdick *et al.*, 2008; Nicodemus *et al.*, 2010).

However, there are also studies that show negative associations between the *DISC* locus and mental illness studies and therefore it is difficult to estimate how well this locus id associated with psychiatric disorders (Sullivan, 2014; Niwa *et al.*, 2016). Indeed, some studies have failed to provide any convincing evidence of association between the *DISC* locus and mental disorders (Mathieson, Munafò and Flint, 2012; Fromer *et al.*, 2014; Kranz *et al.*, 2015). Therefore, further evaluation is required to understand the biological implications of this locus in the development of major mental illness.

Sequencing study where the genomic DNA of 288 schizophrenic patients were analysed led to the discovery of five novel ultra-rare *DISC1* variants R37W, S90L, T603I, G14A, and R418H and confirmed the association of two variant known variants Q264R and S704C with schizophrenia (Song *et al.*, 2008). Deep sequencing of the *DISC1* locus in 653 individuals affected by schizophrenia, bipolar disorders or major depressive disorders revealed the presence of 2010 rare variants of which approximatively 60% were novel, including the R37W variant seen in other studies such as the one previously mentioned (Thomson *et al.*, 2014). In another family study, two children diagnosed with schizophrenia and one child diagnosed with schizoaffective disorder, as well as their father whom did not express any symptoms, were found to carry a frameshift mutation in the *DISC1* gene which was predicted to encode a truncated protein with nine abnormal C-terminal amino acids (Sachs *et al.*, 2005). Some rare *DISC1* variants have also been found in individuals with autism spectrum disorder which suggests that *DISC1* might also be involved in autism as well (Girirajan *et al.*, 2013; Kenny *et al.*, 2014; Kanduri *et al.*, 2016). All these findings show a strong positive association between mutations in the *DISC1* gene and increased risk of schizophrenia and related psychiatric disorders.

While the genetic influence of common *DISC1* variants might not be strong enough to be detected, numerous studies emphasise the significance of rare variants with the psychiatric disorders. The studies conducted so far seem to indicate a strong association between rare *DISC1* variants and psychiatric illness. However, the debate of the importance of *DISC1* continues as long as no conclusive answer regarding DISC1 involvement in mental illness disorders is found (Porteous *et al.*, 2014; Sullivan, 2014).

1.4.3   DISC1 biology

a.   Structure and localisation

Initial characterisation of DISC1 predicted that the N-terminal region of the protein was composed of globular domains while the C-terminal region consisted of a helical domain (K. Millar *et al.*, 2000). *DISC1* has 13 major exons and produces a full-length transcript of around 7.5kb. Similarities between DISC1 protein and structural proteins involved in cellular or intracellular transport were discovered, suggesting that DISC1 might play a similar biological role (K. Millar *et al.*, 2000; Soares *et al.*, 2011). The mouse orthologue of *DISC1, Disc1,* was cloned and shared a 60% homology at the DNA level as well as 56% identity and 14% similarity at the protein level with the human gene (Ma *et al.*, 2002). However, the full-length 3-D structure of DISC1 is still unknown.

Research on short constructs and domain delineation was done based on sequence analysis (Soares *et al.*, 2011) and revealed that the N-terminal region, from amino acids 1-326, contains two particular regions that correspond to a nuclear localization signal sequence motif and a serine-phenylalanine-rich motif (Ma *et al.*, 2002; Taylor *et al.*, 2003; Soares *et al.*, 2011). The N-terminus of DISC1 is referred to as the "globular head domain" which suggests that the native state of DISC1 is represented as a folded 3-D structure with the N-terminal mainly containing excessive numbers of serine (consisting of 13-15% of residues), alanine, and glycine (together making up 16-23% of residues) residues (Taylor *et al.*, 2003; Soares *et al.*, 2011) (Figure 1.5). Moreover, it seems to be poorly conserved across species, as compared to the C-terminal (Taylor *et al.*, 2003; Chubb *et al.*, 2008).The C-terminal domain from amino acids 327-854 has a helical structure with some coiled-coils (Taylor et al., 2003) and is believed to contain at least four regions of coiled-coils, and five "regular" R-helices (Soares *et al.*, 2011). Overall, the C-terminal region seems to be composed of a series of helical packs and coiled-coil interactions (Leliveld *et al.*, 2009; Soares *et al.*, 2011) (Figure 1.5).

A more recent study identified four novel structured regions, named D (amino acids 257-383), I (amino acids 539-655), S (amino acids 635-738), and C(amino acids 691-836) (Yerabham *et al.*, 2017). They all incorporate coiled-coil or α- helical structures. The region D is located in the N-terminal section of DISC1. Further away from the N-terminal region, I and S regions are overlapping followed by the C domain. The first 3 regions are important for the formation of DISC1 oligomer, while the C domain is necessary for protein-protein interactions of DISC1 (Yerabham *et al.*, 2017). Moreover, the regions I, S and C should be lost or disrupted by the translocation t(1;11), and are therefore possibly linked to mental illness (Yerabham *et al.*, 2017).
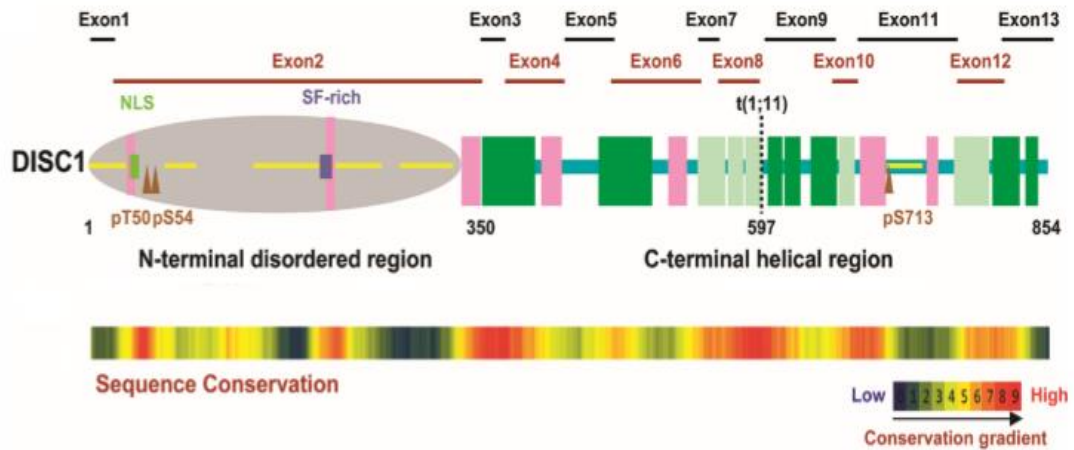
Figure 1.5: Structure of DISC1

Schematic depicting mapped structure: regular R-helices in pink; coiled-coil helices in green; ambiguous helix in light-green and extended consensus disorder predictions in thick yellow lines. Position of sequence motifs and features include the nuclear localization signal (NLS), serine phenylalanine-rich motif (SF-rich), phosphorylation sites (brown triangles) and translocation break point t(1;11) at amino acid position 597. Murine ortholog sequence conservation is shown mapped below the DISC1 schematic to represent the strength of conservation for each sequence position in DISC1 (gaps excluded). Adapted from Soares et al., 2011.

Several studies revealed that DISC1 could self-associate through the region between amino acids 403 and 504 as (Brandon *et al.*, 2004; Kamiya *et al.*, 2005). DISC1 can also form dimers, octamers, multimers and insoluble aggregates when expressed in physiological conditions (Leliveld *et al.*, 2008, 2009; Narayanan *et al.*, 2011). Indeed, in patients suffering of psychiatric disorder, DISC1 has been detected in the form of insoluble aggregates in 20% of post-mortem brains (Leliveld *et al.*, 2008). Two additional regions involved in DISC1 self-association were discovered: the 668-747 region, and the 765-854 region (Leliveld *et al.*, 2009). These results indicate that DISC1 could bind to itself in order to self-regulate. Therefore, DISC1 aggregation in the brain, could be an indication of the presence of mental disorders in individual (Leliveld *et al.*, 2008, 2009).

Alternative splicing of DISC1 leads to numerous alternative transcripts, however, their function is still not understood (Millar *et al.*, 2001; Ma *et al.*, 2002; Taylor *et al.*, 2003; James *et al.*, 2004; Nakata *et al.*, 2009). Four alternatively spliced human *DISC1* isoforms were initially described - i) the Long (L) isoform, which is the full-length transcript composed of 13 exons, ii) the Long variant (Lv) isoform, which lacks the 66 nucleotides at the end of exon

11, iii) the Short (S) isoform which utilises an alternate 3' untranslated region (UTR) in intron 9, and iv) the Extremely short (Es) isoform which contains an alternatively spliced exon 1a (Chubb *et al.*, 2008). Alternative splicing is particularly important in human brain and allows the regulation of gene expression (Yeo *et al.*, 2004; Pal *et al.*, 2011). Expression of different splice forms of *DISC1* is specific to human developmental phases. Therefore, it is possible that change in the expression and the ratio of those *DISC1* variants indicates that splicing mechanisms and splicing regulation play a role in the development of mental disorders, through the brain development. (Nakata *et al.*, 2009). Indeed, expression of several variants was increased in schizophrenia, as compared to healthy individuals (Nakata *et al.*, 2009). Additionally, intergenic splicing was discovered between *DISC1* and the translin-associated factor X gene (*TSNAX)*, producing low-abundance fusion transcripts from which no protein has yet been detected (J. K. Millar *et al.*, 2000).

DISC1 transcripts and the DISC1 protein have been detected in multiple tissues (Chubb *et al.*, 2008). *DISC1* transcripts were found in the brain, heart, placenta, kidney and pancreas of adult humans (K. Millar *et al.*, 2000; James *et al.*, 2004). Reports also indicated the presence of the DISC1 protein in brain, heart, limbs, kidney, liver and lungs in human foetal tissue (James *et al.*, 2004). Studies on adult human brain revealed the presence of *DISC1* transcript in various regions, such as the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, thalamus, cerebellum, cerebral cortex, medulla, and frontal lobe. (K. Millar *et al.*, 2000). DISC1 protein expression showed high levels in the brain during early childhood, while it reduced rapidly when entering adolescence and adulthood (Lipska *et al.*, 2006). Therefore, it seems that DISC1 expression varies with human brain development.

In the hippocampus, DISC1 protein expression has been found especially in the dentate gyrus (K. Millar *et al.*, 2000; James *et al.*, 2004; Lipska *et al.*, 2006; Chubb *et al.*, 2008), and is particularly expressed in a subset of granule cells and in the pyramidal cells in layers CA1-3 (James *et al.*, 2004). DISC1 is also localised in multiple subcellular compartments (Chubb *et al.*, 2008; Soares *et al.*, 2011) and appears to be highly present in the mitochondria (Ozeki *et al.*, 2003; Brandon *et al.*, 2004; James *et al.*, 2004; Millar, James, *et al.*, 2005; Thomson *et al.*, 2013), and it is also found in the nucleus (Millar, James, *et al.*, 2005; Malavasi *et al.*, 2012), centrosome (Kamiya *et al.*, 2005), Golgi and endoplasmic reticulum (Lee, Fadel, *et al.*, 2011; Lepagnol-Bestel *et al.*, 2013; Park *et al.*, 2015). The presence of DISC1 in so many different regions indicates that its function might be very diverse and may affect the general development of the brain.

b. DISC1 function through its interactors

To uncover the molecular function of DISC1, its interactome was studied. Yeast two-hybrid studies revealed a comprehensive set of such proteins using proteomic approach, which support the existence of over 200 DISC1 interactors (Millar, Christie and Porteous, 2003; Ozeki *et al.*, 2003; Camargo *et al.*, 2007; Soares *et al.*, 2011; Bradshaw and Porteous, 2012). Further research also defined various functions of DISC1. Indeed, DISC1 was found to be involved in the regulation of intracellular signalling by interacting with the phosphodiesterase 4B (Millar, Pickard, *et al.*, 2005; Brandon, 2007, 2016). More specifically, this interaction is believed to inhibits PDE4B activity by binding to it, which prevents the degradation of cyclic adenosine monophosphate (cAMP). When the concentration of cAMP is increasing, DISC1 and PDE4B dissociate allowing PDE4B to degrade cAMP and therefore the cells' cAMP concentration stays regulated (Millar, Pickard, *et al.*, 2005). This interaction between DISC1 and PDE4B is of particular interest due to the fact that PDE4 inhibitor, rolipram, is believed to have anti-depressant like effects (Zeller *et al.*, 1984; Zhang *et al.*, 2006). Moreover, studies on IPSC-derived neurons from schizophrenic patients showed that cAMP signalling was altered (Brennand *et al.*, 2011).

Moreover, full-length DISC1 interacts with the transcription factor Activating Transcription Factor 4 (ATF4) and the presence of the variants 37W and 607F prevents DISC1 to reach the nucleus and reduces interaction between DISC1 and cAMP-dependant ATF4 which lead to the dysregulation of the transcriptional activity of ATF4 and therefore impairment in the regulation of cellular stress responses, emotional behaviour and memory consolidation in *Disc1* mice model (Malavasi *et al.*, 2012; Soda *et al.*, 2013). Fasciculation and elongation protein zeta-1 (FEZ1) was also found to interact with DISC1 (Kang *et al.*, 2011). FEZ1 is known to be involved in axonal and neurite outgrowth and possibly in the regulation of the soma size (Bloom and Horvitz, 1997; Miyoshi *et al.*, 2003; Kang *et al.*, 2011). Several studies indicated that DISC1 was involved in the regulation of neurite outgrowth and soma size through its interaction with FEZ1 (Miyoshi *et al.*, 2003; Kang *et al.*, 2011).

It was also found that NudE-like (NUDEL), a protein involved in neuronal development, also interacts with DISC1 (Ozeki *et al.*, 2003; Soares *et al.*, 2011; Bradshaw and Porteous, 2012). Moreover, mutation in DISC1 prevent its binding to NUDEL and leads to the development of less and shorter neurites (Ozeki *et al.*, 2003). Additionally, the complex DISC1-NUDEL was also found to be important for neuronal migration (Kamiya *et al.*, 2005, 2006). These data show that the interaction of DISC1 and NUDEL is required for neurite outgrowth (Ozeki *et al.*, 2003; Kamiya *et al.*, 2005, 2006). Reduced interaction between DISC1-Binding Zinc-finger protein (DBZ) and DISC1 also reduced the neurite length in PC12 primary cultured

hippocampal neurons (Hattori *et al.*, 2007), and DISC1 interaction with the close homolog of CHL1, a member of the L1 family of neural cell adhesion molecules, also supports DISC1's involvement in neuronal outgrowth regulation (Ren *et al.*, 2016).

DISC1 protein has been found to also interact with several motor proteins such as kinesin, dynein and dynactin as well as with centrosome-associated proteins like NUDEL and Lis-1 and microtubule-associated protein (Niethammer *et al.*, 2000; Morris *et al.*, 2003; Taya *et al.*, 2007; Tsuboi *et al.*, 2015). This indicates that Disc1 might also be involved in the regulation of intracellular transport of various molecules through its action on those previously named proteins (figure 1.6).
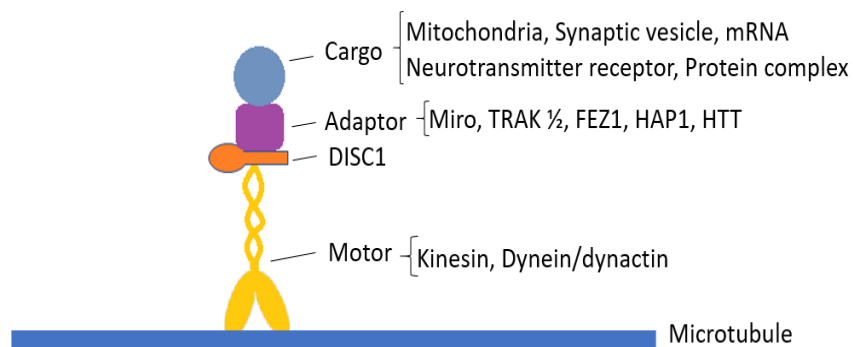
Figure 1.6: DISC1 neuronal trafficking machinery

Schematic view of the multiple components of trafficking machinery regulated by DISC1, which includes cargoes, adaptor molecules, motor proteins, and cytoskeletal components. Adapted from Tomoda et al.2017.

By regulating neuronal trafficking it is possible that DISC1 also regulates neuro-development and synapse maturation (Devine, Norkett and Kittler, 2016; Tomoda, Hikida and Sakurai, 2017). It has been shown that during embryonic development, DISC1 regulates neuronal migration and structural plasticity via the microtubules (Kamiya *et al.*, 2005). Neuronal migration is increased when DISC1 expression in hippocampal progenitors in adults impaired and it appears that DISC1 interaction with NUDEL also has a role in regulating adult neurogenesis (Duan *et al.*, 2007). Importantly, DISC1 interacts with GSK3β and inhibits its activity which reduces β-catenin phosphorylation and stabilizes β-catenin, which is involved in the regulation of neuronal progenitors (Mao *et al.*, 2009; Ming and Song, 2009). Moreover, as mentioned previously, Disc1 interacts with NUDEL and can then form a complex with Lis1 (Brandon *et al.*, 2004; Kamiya *et al.*, 2005). Lis1 is known to be very important for neuronal migration (Wynshaw, 2007) therefore the possibility of the formation of this complex including Lis1 suggests that DISC1 is involved in neuronal migration, indeed loss of DISC1 has been showed to lead to the inhibition of neuronal migration (Kamiya *et al.*, 2005; Duan *et al.*, 2007).

Another partner of DISC1 is KIAA1212 , their interaction seem to prevent AKT activation and disturb the AKT-mTOR signalling pathway (Kim *et al.*, 2009). This also indicates that DISC1 plays an important role in regulating neuronal development. Additionally, this interaction has been found to be involved in GABA signalling pathway. Indeed, it seems that interaction between extrinsic GABA and intrinsic DISC1 signalling is involved in the regulation of dendritic growth of new-born neurons (Kim *et al.*, 2012). Several molecular interactions with DISC1 are summarised in figure 1.7 (Brandon and Sawa, 2011).

The discovery of these numerous DISC1 interactors has allowed a more comprehensive idea of the DISC1's function, with it mainly implicated in neuro-development. However, DISC1 complexity has not been completely untangled and its mechanism of actions still need to be further elucidated.
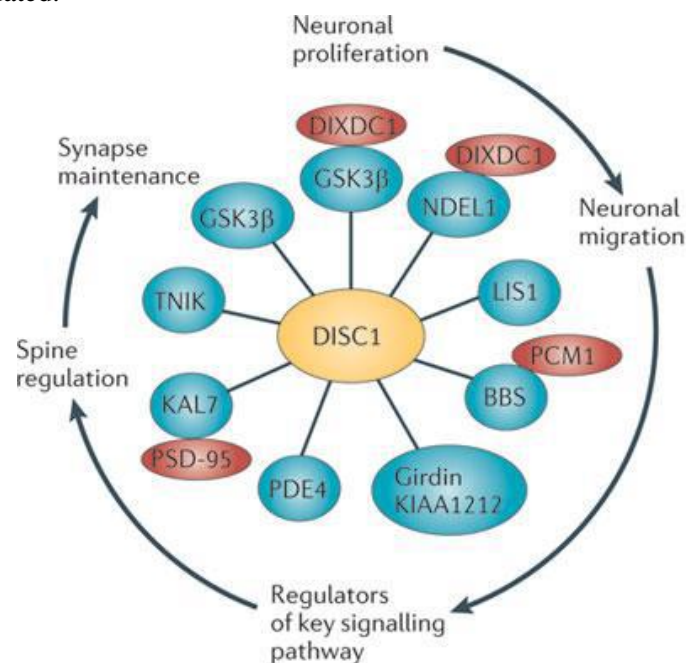
Figure 1.7: Representation of several interaction between DISC1 and several partners
The known functions of the various DISC1 complexes are shown in the perimeter of the figure, and the direction of the arrows indicates their chronological order during neuronal development. BBS, Bardet-Biedl syndrome protein; DIXDC1, dishevelled axin domain containing 1; GSK3β, glycogen synthase kinase 3β; KAL7, kalirin 7; LIS1, lissencephaly protein 1; NDEL1, nuclear distribution protein nudE-like 1; PCM1, pericentriolar material 1; PDE4, phosphodiesterase type 4; PSD95, postsynaptic density protein 95; TNIK, TRAF2- and NCK-interacting protein kinase. Taken from Brandon & Sawa, 2011

c. Consequences of the t(1,11) mutation

c.i The t(1:11) translocation

As previously discussed (Section 1.4.1), the t(1;11) translocation disrupts three genes: *DISC1* and *DISC2* on chromosome 1 and *DISC1FP1* on chromosome 11 (Millar et al. 2000; Zhou et al. 2008; Eykelenboom et al. 2012). However, several studies mentioned previously indicate that *DISC1FP1* gene could be a susceptibility gene for major psychiatric disorders. Similarly, as mentioned previously, *DISC2* was also suggested to play a role in DISC1 function and therefore in mental disorders (Millar *et al.*, 2004; Chubb *et al.*, 2008; Thomson *et al.*, 2013).

Amongst those three genes affected by the translocation, *DISC1* is the only disrupted gene that is known to encode a protein (Millar *et al.*, 2004). Gene fusion between *DISC1* and *DISC1FP1* occurs on both derived chromosomes, resulting in production of multiple aberrant chimeric transcripts in lymphoblastoids from translocation carriers (Eykelenboom *et al.*, 2012). More specifically, the translocation leads to at least three chimeric transcripts: CP69, CP60 and CP1 (figure 1.8) which add 69, 60 and 1 amino acids respectively to the truncated DISC1 protein (Eykelenboom *et al.*, 2012). The same transcripts are also detectable in skin fibroblasts and induced pluripotent stem cell (iPSC)-derived neural precursor cells and neurons from translocation carriers (Millar lab, unpublished). These transcripts encode abnormal protein species, some of which deregulate mitochondrial function when artificially overexpressed in COS7 cells or primary cultured mouse hippocampal neurons (Eykelenboom *et al.*, 2012). Although these chimeric transcripts are easily detectable in various cell lines, there is no evidence for the existence of the encoded aberrant proteins in tissue derived from the affected Scottish family. Overall, the translocation results in loss of normal DISC1 function from one allele (haplo-insufficiency) and has the potential to also produce aberrant DISC1 protein species with dominant-negative effects (Eykelenboom *et al.*, 2012).
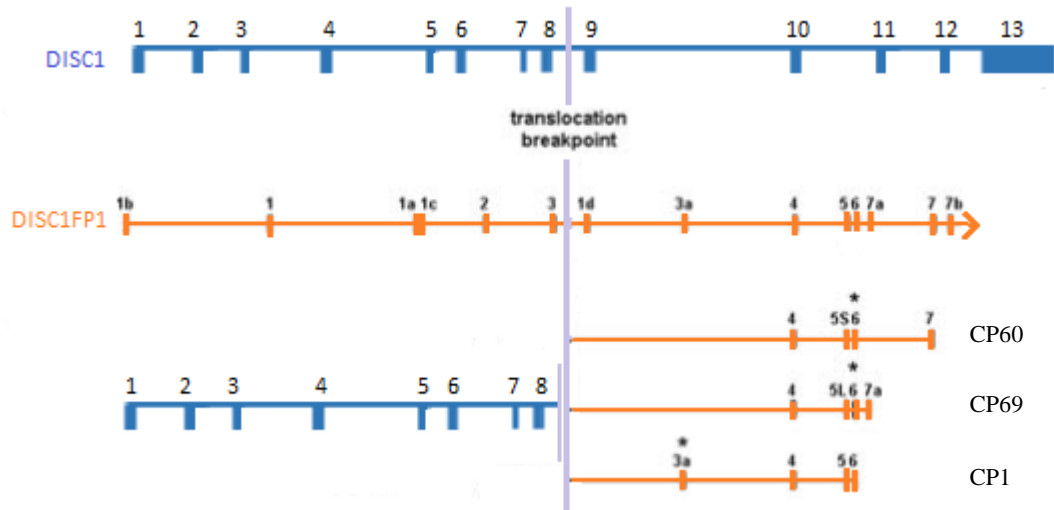
Figure 1.8: Schematic of DISC1 and DISC1FP1

Genomic structure showing all known exons and expressed fusion transcript between DISC1 (blue) andDISC1FP1 (orange). Asterisks mark positions of translation stop codons predicted to be utilized in chimeric transcripts. CP60 and CP69 both terminate within exon 6 and CP1 terminates within exon 3a. CP60 and CP69 both lack exons 1d and 3a while CP1 lacks exon 1d. Additionally, the short form of exon 5 is present in CP60 while the long form of exon 5 is present in CP69. Adapted from Eykelenboom et al. 2012.

Numerous studies have shown that this translocation leads to impairments in brain structure, brain development and molecular mechanism (Soares *et al.*, 2011; Bradshaw and Porteous, 2012; Niwa *et al.*, 2016) . Importantly, these modifications due to the translocation seem to be similar to those found in patients with psychiatric disorders (Ishizuka *et al.*, 2006; Soares *et al.*, 2011).

c.ii   Alteration in brain structure

The effect of the t(1;11) translocation on white matter was investigated in seven family members carrying the translocation and diagnosed with psychiatric disorders and in 13 family members without the translocation, along with 34 clinical control patients suffering from psychosis, and 33 healthy individuals (Whalley *et al.*, 2015). This analysis revealed decreased white matter integrity in the genu of the corpus callosum, the right inferior fronto-occipital fasciculus, the acoustic radiation and the fornix in patients carrying the t(1;11) (figure 1.9) as well as in patients suffering from psychosis (Whalley *et al.*, 2015). Therefore, this suggest that the t(1;11) translocation and DISC1 are involved in the structure of the white matter (Whalley *et al.*, 2015).
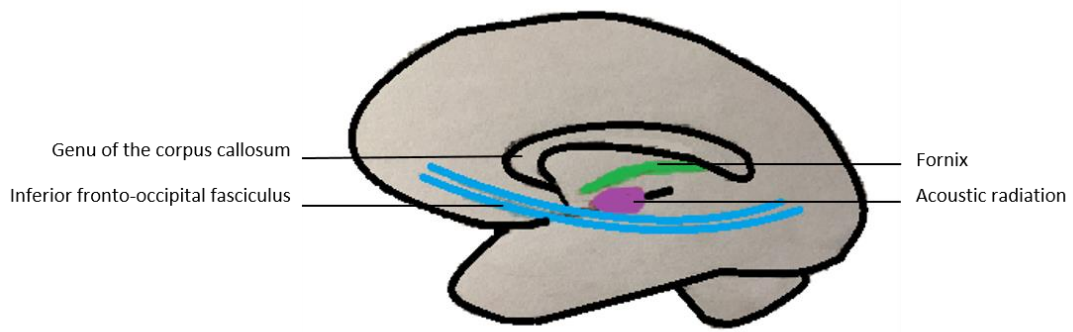
Figure 1.9: Brain schematic representing the areas affected by a decrease in white matter integrity, in the translocation carrier

Genu of the corpus callosum, Fornix, Inferior fronto-occipital fasciculus and acoustic radiation are indicated.

A recent study looked at structural MRI data from 12 t(1;11) translocation carriers and 18 non-carriers from the same family, and the carriers were found to have a reduced cortical thickness compared to the non-carriers (Thomson *et al.*, 2016). The study also revealed a reduced gyrification in the translocation carriers. Additionally, reduced left superior temporal sulcus cortical thickness in the temporal lobe and reduced right superior frontal sulcus local gyrification in the dorso-lateral prefrontal cortex were found in the translocation carriers. Overall this study showed that the translocation leads to reduced cortical thickness in the left temporal lobe and reduced gyrification in prefrontal cortex (Thomson *et al.*, 2016). Furthermore, fMRI data from eight family members carrying the t(1;11) translocation and 15 non-carriers revealed that there was no difference in working memory between the groups but increased activation of the left caudate nucleus was found in the translocation carriers. (Thomson *et al.*, 2016).

c.iii   Molecular mechanisms

From what is known of the structure of the DISC1 protein, it can be speculated that the t(1;11) translocation would prevent its interactors from binding to DISC1 and therefore lead to impairments across numerous molecular mechanisms.

A study of 12 t(1;11) translocation carriers and 16 non-carriers investigated the effect of the translocation on glutamate and N-acetylaspartate (NAA) concentrations in the brain and showed that in the right dorsolateral prefrontal cortex, translocation carriers were found to have a reduced level of glutamate(Thomson *et al.*, 2016). The different subgroups of clinical diagnosis within translocation carriers had no difference. Additionally, there was no convincing change in NAA levels in the translocation carriers. This study indicates that the

translocation leads to glutamate deficit in the dorsolateral prefrontal cortex, but NAA concentration is not affected (Thomson *et al.*, 2016). The use of human i-PSC from the translocation carriers will be necessary to discover the exact nature of the modification due the translocation on molecular mechanism.

These findings obtained directly from the translocation carriers overlap with those shown in patients suffering with mental illness mentioned in Section 1.3. Moreover, numerous DISC1 animal models were studied and revealed similar findings.

1.4.4   DISC 1 mouse model of mental disorder

Modelling of human psychiatric disorders in animals is very challenging when considering the nature of the symptoms, the lack of biomarkers and lack of objective diagnostic tests. However, it has become easier to create rodent models of these disorders using either genetic tools or environmental manipulations. These animal models are very useful for understanding the pathophysiology of mental disorders and to discover possible treatments. Multiple mouse models have been created to study the effect of disrupting DISC1 on brain development and the development of major mental illness (Tomoda *et al.*, 2016) (table 1.1) . Rodent models showed critical changes in brain function and structure, as well as in important molecular pathways and behaviour. However, results show great variability between models (Johnstone *et al.*, 2011; Lipina and Roder, 2014).

A natural *Disc1* mutation was found in several mouse strains such as the Swiss strain and 129 mouse strain, which carry a 25 base pair deletion mutation in exon 6 of *Disc1* (Clapcote and Roder, 2006; Ritchie and Clapcote, 2013). This deletion induces a frame shift which results in 13 novel amino acids, followed by a premature termination codon in exon 7. This mutation has been shown to block the production of the full-length protein and no truncated protein was found. Additionally, insertion of the premature stop codon at intron 8 has been showed to result in working memory impairment as well as cognitive and behavioural impairment (Clapcote & Roder, 2006; Koike et al. 2006). Further research on this mutation revealed that it was also leading to impaired proliferation, axonal targeting and dendritic growth in the developing dentate granule cells, as well as impaired short-term plasticity (Kvajo *et al.*, 2011; Lepagnol-Bestel *et al.*, 2013). Moreover, alterations in spontaneous locomotor activity, deficits in pre-pulse inhibition (PPI) and increased despair behaviour in *Disc1* heterozygous 129 strain mice were discovered (Gomez-Sintes *et al.*, 2014) as well as behavioural impairment, decreased dendritic diameters, reduction in spine density and neuronal excitability and increased transmitter release (Juan *et al.*, 2014). These characteristics are known to be

associated with psychiatric disorders, therefore, this suggest that DISC1 is involved in the development of psychiatric disorders.

Similar mouse models not expressing Disc1 have been generated. A mouse model lacking exons 2 and 3 of the *Disc1* gene was generated and showed a higher threshold for the induction of long-term potentiation, an amplified sensitivity to methamphetamine, a higher impulsivity as well as deficits of pre-pulse inhibition (Kuroda *et al.*, 2011). Another mouse model was generated with a large deletion of exons 1-3 of Disc1 and showed increased expression of APP at the cell surface along with decreased internalization, indicating an impairment in the protein transport (Shahani *et al.*, 2015). These models showed mainly cellular, molecular and behavioural impairments. Additionally, a *Disc1* knock down using a small interfering RNA (siRNA) showed the importance of DISC1 for NMDAR function and expression (Wei *et al.*, 2014). A *Disc1* hippocampal knock-down mouse model revealed somal hypertrophy in neurons, accelerated dendritic outgrowth with the appearance of ectopic dendrites, mis-positioning from overextended migration, enhanced intrinsic excitability, and accelerated synapse formation of new neurons (Duan *et al.*, 2007; Kim *et al.*, 2009).

A transgenic mouse model exogenously expressing the human dominant-negative truncated DISC1 (DN-DISC1) under the CaMKII promoter showed brain structural modifications such as enlarged ventricles and depression like behaviour, which correlate with characteristics of schizophrenic patients (Hikida *et al.*, 2007). CaMKII drives gene expression mainly in the pyramidal neurons of the cortex and hippocampus, which is also where endogenous DISC1 is preferentially expressed. In this model, the lateral ventricular volumes in these mice remained normal at 3 months but were enlarged at 6 months. The total brain volume in these mice was not affected, but an asymmetry between the left and right lateral ventricles was found in the 6-week-old mice. Additionally, MRI analysis revealed a change in the left/right ratio in the hippocampus and thalamus. This suggested asymmetrical structural changes in these DN-DISC1 mice. Reduction in parvalbumin and calretinin in DN-DISC1 mice was also noticed as well as impaired sensorimotor gating, hyperactivity, and an anhedonia/depression-like deficit (Hikida *et al.*, 2007). The study of another transgenic mouse model also generated with a dominant-negative human *DISC1* cDNA under expression control of the prion protein promoter showed that epigenetic and environmental factors interact to affect dopaminergic neurons (Niwa *et al.*, 2013). Prion protein promoter does not drive the expression in specific cells allowing a broader expression of the inserted gene. When this transgenic mouse is subjected to stress, pre-pulse inhibition, and locomotor activity are impaired. Moreover, meso-cortical projections of dopaminergic neurons are affected, and molecular changes were found. In this model, environmental factors enhance the alteration of neurotransmission caused by

the *Disc1* mutation (Niwa *et al.*, 2013). A more recent study using a conditional transgenic mouse expressing the human DISC1 C-terminal domain revealed that developmental disruption of DISC1 impairs cortical and synaptic plasticity (Greenhill *et al.*, 2015). Another transgenic mouse model expressing two copies of truncated *Disc1* using a Bacterial artificial chromosomes (BAC), showed behavioural impairments, structural modifications such as enlarged lateral ventricles and reduced cerebral cortex, and reduced numbers of GABAergic neurons in the hippocampus and the prefrontal cortex (Shen *et al.*, 2008). Several dominant-negative transgenic mutant DISC1 models revealed that the regulation of astrocytes was impaired as well as glutamate neurotransmission (Abazyan *et al.*, 2014); differentiation and function of oligodendrocytes was also perturbed (Katsel *et al.*, 2011) and CA1 pyramidal neurons properties were affected as well as long-term synaptic plasticity (Booth et *al*., 2014). These studies have revealed the involvement of specific cell types and show the importance of neuron-glia interactions in mental disorders. Other studies using *DISC1* transgenic mice revealed alterations in neurotransmitter systems, such as glutamatergic (Dawson *et al.*, 2015), cannabinoid (Ballinger *et al.*, 2015), dopaminergic, or nicotinic neurotransmission (Kim *et al.*, 2015). Transgenic mouse model studies have mainly lead to alterations of anatomical brain structure, cellular function, and neurotransmitter regulation which are relevant to human psychiatric conditions.

Mice carrying ethyl-nitroso urea-induced *Disc1* missense mutations were also found to exhibit characteristics of mental disorders. The first two mutations discovered were a glutamine change onto a leucine (Q31L) and a leucine changed into a proline (L100P) (Clapcote *et al.*, 2007). Q31L analysis revealed accentuated depressive-like behaviour such as a deficit in the forced swim test while L100P mutant mice exhibited schizophrenic-like behaviour such as impairment in pre-pulse inhibition and latent inhibition, decrease of social interaction as well as more immobility in forced swim test (Clapcote et al. 2007). However, in both mutants DISC1 interaction with PDE4B was reduced (Clapcote *et al.*, 2007). They also both displayed overall brain volume reductions with specific shrinkage of the entorhinal cortex, thalamus, and cerebellum (Clapcote *et al.*, 2007). Follow up on those models revealed a reduction in neuron number, decreased neurogenesis, and altered neuronal distribution as well as outgrowth impairment of pyramidal neuron (Lee, *et al.*, 2011). Additionally, L100P mice showed enhanced dopamine function (Lipina *et al.*, 2010), altered GSK3α activity in synapses and reduced dendritic spine length and surface areas (Lee *et al.*, 2011) and deficits in interneuron development (Lee *et al.*, 2013). Further analysis revealed that maternal immune activation of L100P mice leads to an increase of interleukins in the offspring, indicating that environmental factors may also exacerbate phenotypes linked to mental disorders (Lipina *et al.*, 2013). More

recently, another mutation was discovered by extending the ENU (N-ethyl-N-nitrosourea) mutagenesis screen to a different part of the *Disc1* locus to create a new mouse model where an aspartic acid was changed into a glycine (D453G) (Dachtler *et al.*, 2016). This model showed altered GSK3β signalling as well, and behavioural impairment such as hyperlocomotion, anxiety and reduced social interaction in the female while male express a deficit in passive avoidance behaviour.

A number of these mouse models have been designed after the t(1;11) translocation discovery, hoping to reproduce the effect seen by the translocation in the carriers. However, studies of the effect of truncated DISC1 protein expressed from the derived chromosome 1, encoded by exons 1-8 (K. Millar *et al.*, 2000) revealed that the presence of fusion transcripts CP 69, CP60 and CP1 (Eykelenboom *et al.*, 2012) might not be as similar as hoped to the translocation. Indeed, only CP1 encodes a truncated protein that contains one more amino acid from the translocation breakpoint belonging to *DISC1FP1* which happens to be the same amino acid which would appear after the breakpoint in wild type DISC1 protein. Therefore, it seems that mouse models created through bacterial artificial chromosome (BAC) (Shen *et al.*, 2008) or transgenics (Hikida *et al.*, 2007; Pletnikov *et al.*, 2008; Jaaro-Peled *et al.*, 2013; Greenhill *et al.*, 2015), express a truncated DISC1 protein and do not rigorously replicate the t(1, 11) translocation. A mouse model was therefore generated to model the t(1;11) translocation known as the DISC1-Boymaw mouse. This mouse has a bi-cistronic human transgene with both fusion transcripts - *DISC1-DISC1FP1* and *DISC1FP1-DISC1* (Ji *et al.*, 2015). The DISC1-DISC1FP1 protein seems to inhibit oxidoreductase activity, RNA expression, and protein synthesis. Moreover, reduction of the expression of Gad67, Nmdar1 and Psd95 was found in this model as well as expression of intermediate behavioural phenotypes related to major psychiatric disorders such as increased and prolonged responses to ketamine, abnormal information processing of acoustic startle and depressive-like behaviours displayed through significantly more immobility time when performing the tail suspension test (Ji *et al.*, 2015).

All these models are summarised in table 1.1 bellow

Model	Reference	Details	Reported findings
Swiss strain 129 strain	Clapcote et al., 2007 Ritchie et al., 2013	Deletion of 25bp in exon 6	↓ working memory
129 strain	Koike et al, 2006		↓ working memory cognitive and behavioural alteration
	Juan et al., 2014		behavioural impairment ↓ dendritic diameters ↓ in spine density and neuronal excitability ↑ transmitter release
	Gómez-Sintes et al., 2014		↓ in PPI, alterations in spontaneous locomotor activity, ↑ despair behaviour
Disc1tm1Kara	Kvajo et al., 2011	Truncated in exon 8 + deletion of 25bp in exon 6	changes in short-term plasticity altered axonal targeting and dendritic growth ↓ proliferation ↑ c-AMP level
	Lepagnol-Bestel et al., 2013		altered axonal and dendritic arborization Altered dendritic spines
Disc1 (△2-3)	Kuroda et al., 2011	Lack of exons 2 and 3	higher threshold for the induction of LTP, ↑ sensitivity to methamphetamine, ↑ impulsivity ↓ PPI
Disc1 (△1-3)	Shahani *et al.*, 2015	Lack of exons 1 to 3	↑ APP expression ↓ APP internalization Impaired protein transport

Disc1 KO	Wei *et al.*, 2014	short-hairpin RNA (shRNA)	↑ NMDAR currents ↑ GluN2A ↑ NMDAR-mediated synaptic response
Disc1 KD	Duan et al, 2007		aberrant morphological development mispositioning of new dentate granule cells ↑ excitability ↑dendritic development and synapse formation
	Kim et al, 2009		↑ AKT Signaling ↑ in soma size and number of primary dendrites ↑ dendritic growth
	Kim et al, 2015		Altered dopaminergic et nicotinic neurotransmission
Transgenic model DISC1 (DN-DISC1)	Hikida et al, 2007	C-terminally truncated DISC1 under αCAMKII promotor	↓ parvalbumin ↑ ventricle volume hyperactivity disturbance in sensorimotor gating impaired olfactory-associated behavior, anhedonia
	Ballinger et al, 2015		↓ CB1R expression ↓ fear associated response
	Jaaro-Peled *et al*		↓ D2R binding in striatum ↓ dopamine levels
Transgenic model DISC1-DN-Tg-PrP	Niwa et al., 2013	C-terminally truncated DISC1 under prion protein promotor. Model under stress	↓ PPI ↓ locomotion response ↓ immobility in forced swim test ↓ extracellular dopamine
Conditional transgenic mouse DISC1cc	Greenhill et al, 2015	Inducible, reversible induction of c-term DISC1 aa671-852 via calcium/calmodulin-dependent protein kinase II subunit a (aCaMKII) promoter controlled by tamoxifen	↓ synaptic plasticity lack of LTP ↓ of LTD

Transgenic mouse Disc1tr (Disc1tr Hemi mice)	Shen et al, 2008	Overexpression of Disc1 exons 1-8 using bacterial artificial chromosome	↑ ventricles volume ↓ cortical thickness ↓ neurogenesis ↓ GABAergic neurons ↓ latent inhibition ↑ immobility ↓ reduced vocalisation
	Booth et al, 2014		Altered CA1 property Altered synaptic plasticity
	Dawson et al, 2015		Altered metabolism in PFC Altered response to ketamine ↓ expression of GluN2A/B
Tet-off transgenic model (ΔhDISC1)	Abazyan et al, 2014	Tet-inducible C-terminally truncated DISC1 under αCAMKII promotor (forebrain restriced)	↑ alanine-serine-cysteine transporter 2 ↑ vesicular glutamate transporters 1 and 3 ↑ NR1 expression ↓ NR2 expression
	Katsel et al, 2011		Aberrant oligodendrocytes differenciation
	Pletnikov *et al.*, 2008		Altered gross brain structure ↑ lateral ventricles volumes abnormal neurite outgrowth
Q31L (glutamine change onto a leucine)	Clapcote et al, 2007	Mice carrying ethyl-nitroso urea-induced *Disc1* missense mutations	↓ immobility in forced swim test ↓ brain volume ↓ reduction PDE4B activity
	Lee et al, 2011		altered dendrite morphology ↓ neuron number ↓neurogenesis and proliferation
L100P (leucine changed into a proline)	Clapcote et al, 2007		↓ PPI and latent inhibition ↓ locomotion ↓ working memory ↓ brain volume
	Lipina et al, 2010		↓ locomotion ↓ PPI Enhanced dopamine function ↓ sensitivity to amphetamine

	Lee et al, 2011		↓ interneuron development
	Lee et al, 2013		altered GSK3α activity in synapses ↓ dendritic spine length and surface areas
	Lipina et al, 2013		↑ interleukins when subject to maternal immune activation ↓ PPI and latent inhibition
D453G (aspartic acid was changed into a glycine)	Dachtler *et al.*, 2016	extended ENU (N-ethyl-N-nitrosourea) mutagenesis screen	altered GSK3β hyperlocomotion anxiety ⎤ reduced social interaction ⎦ in female deficit in passive avoidance behaviour in male
DISC1-Boymaw Boymaw-DISC1	Ji et al., 2014	Insertion of a bi-cistronic human transgene with both fusion transcripts	↑ responses to ketamine abnormal information processing of acoustic startle depressive-like behaviour ↓ expression of Gad67, Nmdar1 and Psd95
Disc1 RNAi	Tomita et al, 2011	suppressed expression of Disc1 in the CA1 using the RNA interference (RNAi)	Impaired neuronal migration
DISC1△C	Maher et al, 2012	RNA1 KD	altered mEPSCs altered glutamatergic synapses

Table 1.1: Characteristics of published Disc1 mouse models

At present, none of the published *Disc1* mouse models accurately model any known sequence of events leading to psychiatric disorder as well as known phenotypes comparable to those characteristic of mental disorders, and very few causal events have been identified in analysis of the patients suffering from psychiatric illness. To facilitate a more complete understanding of the mechanisms involved in these disorders, more accurate animal models need to be designed to mimic known causal mutations, with which the sequential changes from early brain development to adult brain maturation leading to brain dysfunction could be investigated.

1.5 A new DISC1 mouse model: Der1

To accomplish the work detailed in this thesis, a novel mouse model of mental illness named Der1 was used, this mouse was gifted to Dr Kirsty Millar by Michel Didier from Sanofi.

As mentioned previously, the t(1;11) translocation shows a genetic link with mental disorders (Blackwood *et al.*, 2001; Thomson *et al.*, 2016). Indeed, the t(1;11) translocation seems to be involved in the increased risk of mental illness in carriers compared to non-carrier family members. A mouse model carrying this translocation might therefore help gain insights on the mechanisms involved in the development of mental illnesses. However, the mouse genome does not contain the orthologue sequences of *DISC2* and *DISC1FP1,* both of which are disrupted by the translocation (Millar et al. 2000; Taylor et al. 2003; Zhou et al. 2008). This makes it difficult to model the human translocation in mice. The Der1 mouse used in this thesis is a transgenic mouse model of the derived chromosome 1 that results from the t(1;11) translocation. This mouse carries a modified endogenous *Disc1* gene. The 3' half of the *Disc1* gene has been removed from intron 8 until the 5' end of the gene, which is a position corresponding approximately to that of the translocation breakpoint in humans. This has been replaced with a segment of human chromosome 11 from the translocation breakpoint to the end of the *Disc1fp1* gene, composed of exons 4-8 of *DISC1FP1* (figure 1.10). Insertion of the human partial gene was performed using the Cre-lox technic.
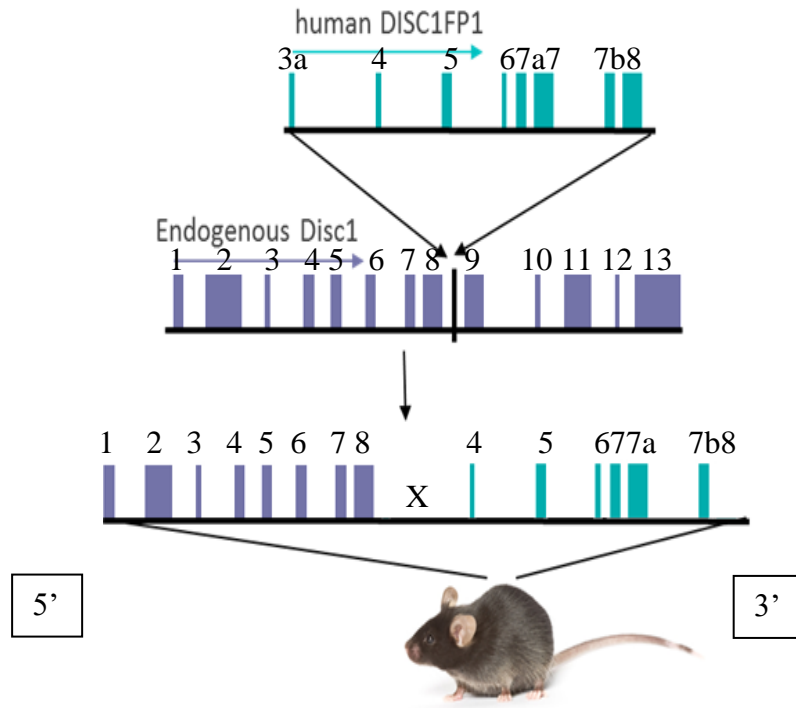
Figure 1.102: Representation of the construction of the new Der1 mouse model
Exon 3a of human DISC1FP1 is indicated and the cross (X) indicated that it is missing in the mice final construct as it was lost during the fusion of the genes. Exons after the breakpoint of *DISC1FP1* are fused to the pre-breakpoint exons of mice *Disc1.*

This modification has been done to mimic the derived chromosome 1 found in the affected Scottish family. In such a model, the endogenous *Disc1* gene should be expressed physiologically. Moreover, chimeric transcripts might be generated in this mouse as they were found in human cells carrying the translocation. The heterozygous Der1 mice carrying the modified *Disc1* allele therefore correspond to the human carriers of the t(1;11) translocation. They should express the full length Disc1 protein while homozygous Der1 mice should not express full length Disc1 at all. The genomic editing was carried out in mouse embryonic stem cells with a mixed 129/C57BL6 background. Mutant mice were crossed by Sanofi with C57BL/6 mice to obtain mice carrying only C57BL/6 *Disc1* alleles. Congenic breeding was then done at University of Edinburgh, and the Jackson Labs performed the genotyping in order to obtain mice with a >99.5% pure C57BL/6 background.

To confirm the mutation on *Disc1* transcription, reverse transcriptase PCR (RT-PCR) was used to assay the levels of mRNA from whole brains from the Der1 mice (n=8). The primers used were within exons 7 and 9 of Disc1, designed to pick up only wild type transcripts as they span the translocation breakpoint (section 2.2.2). No wild type transcripts were detected from homozygous Der1 mice and *Disc1* expression was reduced by around half in the heterozygous mice (figure 1.11 and figure 1.12).
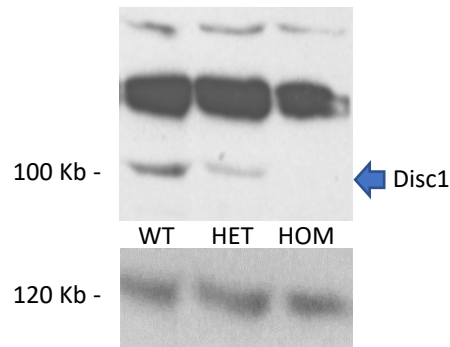
Figure 1.11: Disc1 protein expression in wild-type, heterozygous and homozygous Der1 mice

Loading control vimentin (120 Kb). Two additional non-specific bands can be seen above the band correspond to Disc1 expression.

Disc1 protein expression was then assessed using an in-house C-terminal Disc1 antibody raised to amino acids 666–852 of mouse Disc1 (Ogawa et al., 2016). The result shows that full length Disc1 expression is significantly lower in the heterozygous mice samples compared to wild type, and no Disc1 signal can be seen in samples from the homozygous mice (figure 1.12).
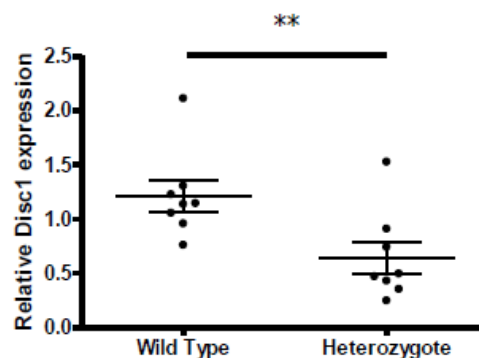
Figure 1.12: Expression of wild type Disc1 transcripts in Der1 mouse

Relative whole brain *Disc1* expression in heterozygous t(1;11) mouse model compared to wild type, n=8 for each group. Statistical test used was an unpaired students t-test, ** indicates $p<0.01$. Work performed by Helen Torrance.

To determine whether the predicted fusion transcripts CP60 and CP69 were produced by the modified *Disc1* allele, RT-PCR was performed on whole brain cDNA isolated from homozygous Der1 mice. CP1 was excluded as the exon 3a is absent in the mice model (figure 1.10). Transcripts CP60 and CP69 do not contain exon 3a. CP69 and CP60 were both detected in cDNA isolated from cortical and hippocampal tissue from homozygous Der1 mice. This work was performed by P. Makedonopoulou and Hazel Davidson-Smith. However, the presence of fusion proteins translated from CP69 and CP60 fusion transcripts has not been demonstrated in these mice. As anticipated, this novel mouse model reproduces the effects of the t(1;11) translocation on *DISC1* expression.

1.6 Aims of this PhD

The t(1;11) found in a Scottish family is linked with mental disorder. *Disrupted in Schizophrenia (DISC1)* is disturbed by this translocation and is an important gene involved in the development and function of the brain.

Multiple DISC1 animal models have showed important changes in behaviour, in brain function and structure, as well as in important molecular pathways and behaviour. These modifications due to DISC1 dysfunction are underlying the involvement of DISC1 in psychiatric disorders. However, none of the published Disc1 mouse models accurately model psychiatric disorders.

The aim of this PhD is to characterise this new Der1 mouse model in the hope to find a better mouse model for mental disorder.

With this model, I have explored the molecular mechanisms and the different pathways affected by the translocation t(1;11) and tried to learn more about DISC1 function and its involvement in psychiatric disorders.

The major questions addressed in this project are:

a) Does the Der1 mouse, which models the human t(1, 11) translocation in a slightly better way than done before, significantly differs from other animal or cell models.

b) Does the Der1 mouse exhibit any novel phenotypes at the structural, molecular or cellular level?

c) Based on these findings, could this novel mouse model be used to model psychiatric disorders?

To answer these questions, I studied the brain structure of Der1 mice, looked at the cellular level of the brain for changes and investigated the global RNA expression in the hippocampus and cortex of the Der1 homozygous and heterozygous mice.

CHAPTER 2

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MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Bovine Serum Albumin (Sigma)

Ethanol (Fischer)

Formaldehyde, 16%, methanol-free (ThermoFisher)

Formalin, 37-40% stock solution (Sigma-Aldrich)

IMS (Fisher)

Magnesium chloride (Fisher)

Methanol (Fischer)

Na2HPO4 dibasic/anhydrous (Sigma-Aldrich)

NaH2PO4 monobasic (Sigma-Aldrich)

PBS (Sigma)

Sodium chloride (Fischer),

Sodium bicarbonate (Sigma)

Sodium hydroxide (Fisher)

Sucrose (Sigma)

Tris (Fischer), pH adjusted using hydrochloric acid and/or sodium hydroxide

Tris-HCl (Fischer), pH adjusted using hydrochloric acid and/or sodium hydroxide

Tri-sodium citrate dihydrate (Fisher)

Triton X-100 (Sigma)

TWEEN-20 (Sigma)

β-glycerophosphate (Sigma)

2.1.2 Solutions and buffers

All cell culture reagents were purchased from Gibco Invitrogen.

Dissection buffer -used to dissect foetal mouse brains for primary neuron production:

   500 ml Hanks Balanced Salt Solution (HBSS) with $CaCl_2$ and $MgCl_2$

   5 ml 200mM Glutamax

   3.5 ml 1M HEPES

DMEM/FCS – used during primary mouse neuron culturing and for maintenance of cultured human cell lines: 500ml Dulbecco's Modified Eagle Medium

50ml foetal calf serum (prepared by in-house technical facility)

Glial medium – for astrocyte growth and maintenance:

500ml Dulbecco's Modified Eagle Medium with Glutamax

50ml foetal calf serum

Neurobasal medium– used for maintenance of primary neuron cultures:

500ml Neurobasal medium without phenol red

10ml B27 supplement

5 ml 200mM Glutamax

Trypsinisation buffer:

4 ml TrypLE Express

6 ml ml HBSS (without $CaCl_2$ and $MgCl_2$)

PBS (10X) from tablets (Invitrogen) containing:

10 mM Tris base

150 mM NaCL

Dissolved in up in dH_20, pH adjusted to 7.3-7.5 with 1M HCL

Autoclaved before used. Stored at 4°C

Sodium citrate buffer 10mM:

2.94 g Tri-sodium citrate dihydrate

Dissolved in 1000 ml of dH2O, pH adjusted to 6.0 with 1M NaOH

Autoclave before used

BrdU solution:

1 mg/ml of BrdU

1% sucrose, dissolved in drinking water

NFB buffer:

100 ml of Formalin

Diluted in 900 ml of dH2O

4 g/L NaH2PO4

6.5 g/L NaHPO4

Perfusion PFA:

4%PFA

0.1M PB)

pH 7.4

2.2 Mouse husbandry and genotyping

Once received from Sanofi the Der1 mice were held at the biological research facility on site which is a "dirty" animal facility. The mice were tested positive for Mouse norovirus and endoparasite Entamoeba sp, Syphacia obvelata and Tritrichomonas sp. The mice were bred by mating Heterozygous X Heterozygous in order to obtain WT, Heterozygotes and Homozygotes in the same litter. The mice were weaned at day 21 and kept in mixed genotype cages, 3 mice per cages. Breeders followed a RM3 diet of pellets containing a high level of nutrients while the stock followed a RM1 diet containing low nutrients and low proteins level. Regular genotyping of stock was performed by Helen S. Torrance and myself using a PCR assay on DNA extracted from ear notches. A forward primer targeted to mouse Disc1 was used in addition to reverse primers for either endogenous Disc1 or for the knocked in human sequence of DISC1FP1.

2.2.1   DNA extraction

Ear notches were collected in 1.5 ml Eppendorf tubes by technician at the animal facilities and passed on for genotyping. A DNeasy Blood and tissue kit was used to extract the DNA from those tissue samples. After adding180 µl of ATL buffer and 20 µl of proteinase K, the sample was vortexed and left to incubate at 56C overnight. The next day, the samples were vortexed for 15 seconds before adding 200 µl of buffer AL and vortexed again. Then, 200 µl of 100% ethanol was added, followed by a thorough mixing. The mixture was transferred to a DNeasy Mini spin column placed in a 2 ml collection tube. This was centrifuged at 8000 rpm for 1 minute and the flow through was discarded. The spin column was placed in a new 2 ml tube and 500 ul of AW1 buffer was added to each sample. The samples were centrifuged as before and the flow through discarded. The spin column was placed in a new 2 ml collection tube and 500ul of AW2 buffer was added to the mixture. The samples were then centrifuged for 3 minutes at 14.000 rpm and the flow through discarded. The spin column was transferred to a 1.5 ml micro-centrifuge tube. The DNA was eluted by adding 100 ul of AE buffer. After one-minute incubation at room temperature, the samples were centrifuged. The DNA samples were stored at 4^0C.

2.2.2   PCR

The basic PCR reaction was used to determine the genotype of the mice. A 'no template' control was always included in the experiment by replacing the DNA with RNase-free water. The reaction was set up as follows, the quantities being for one sample:

14.5 µl	H2O
2 µl	10x PCR reaction buffer (Sigma)
0.5 µl	10 M reverse human primer

5'ATAACGGTCCTAAGGTAGCGAGCTATTGCCAGGATrCCATCGCTCG

| 0.5 µl | 10 M reverse mouse primer |

5'CAGTAGTAAGAAAAGAGACAACCCCC

| 0.5 µl | 10M forward mouse primer |

5'CCTGCATCCACAGACGTGC

0.5 µl	10 mM dNTPs
0.5 µl	Taq DNA polymerase (Sigma)
1 µl	genomic DNA

The reaction conditions were set as follow:

95^0C 5 minutes

94^0C 45 seconds

60^0C 45 seconds ⎫ 35 cycles

72^0C 45 seconds

75^0C 10 minutes

Keep at 4 ^{0}C.

2.2.3   DNA electrophoresis: Preparation and running of agarose gels

DNA electrophoresis was used to assess the size of DNA fragments generated by PCR and therefore obtain the genotype corresponding to the samples used. A 2% low melting point (LMP) agarose gel was used for DNA electrophoresis. 5 µl of the solution obtained after PCR was added to 2.5 µl of DNA loading buffer and the mixture was pipetted into the wells. DNA marker (1 Kb Plus DNA Ladder, Invitrogen) was also pipetted into a well. The gel was then placed in an electrophoresis tank (Bioscience Service) filled with TBE buffer and completely submerged. To run the DNA gel, a current of 90V was applied for, 30 minutes to properly see separated bands on the gel corresponding to the DNA fragments of interest. The DNA fragments in the gel were stain with SYBR safe dye and visualised by UV light illumination using an Uvidoc Lightbox (Uvitec) and photographed with the built-in camera (figure 2.1).
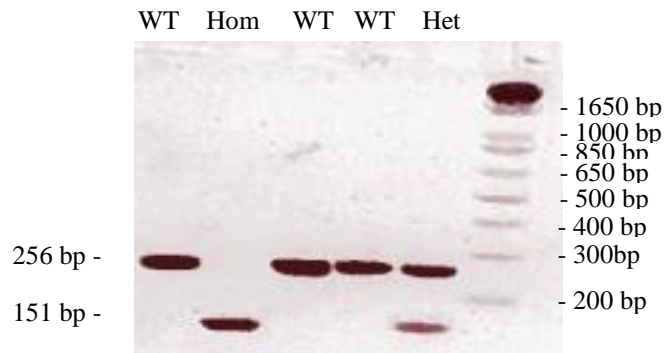
Figure 2.1: PCR results revealing the genotypes of the samples used

Homozygous (151 base pair (bp) band), heterozygous (256 bp band and 150 bp band) and wild type samples (151 bp band).

2.3 Sholl analysis

2.3.1 Co-culture of astrocytes with low density cortical neurons

The cell cultures were prepared by Elise Malavasi while astrocytes were prepared by Laura Murphy. To easily identify single neurons, low density neuronal cultures (10^5 neurons per well) were set up in a six well plate with astrocyte co-culture.

a. Astrocyte preparation

Wild-type pregnant mice were killed under the schedule 1 procedure by staff at the animal facility and the embryos removed at E18. The cortex was chopped with a sterile scalpel and transferred to 10ml of trypsinisation buffer and incubated at 37°C for 45 minutes. The tissue was dissociated by pipetting with a P1000, 50 times in 5ml DMEM/FCS, to create a single cell suspension, and then passed through a 70um cell strainer. The cell suspension was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed. The pellet was re-suspended in 5-10ml of glial medium and cells were counted using a haemocytometer. $7x10^6$ cells were transferred into a T75 flask with 15ml of glial medium. Then, medium was removed from the flask and replaced with 15ml fresh glial medium. When the flasks were confluent with astrocytes (at around 14 DIV) aliquots were stored in liquid nitrogen for long term storage. When frozen stocks were needed for co-culture with neurons, cells were thawed at 37°C for 1-2 mins in glial medium.

b. Plating of astrocytes

To grow healthy neurons at low density, wild-type astrocytes were plated on a well insert positioned above the neurons. The insert has a porous membrane upon which the astrocytes are maintained which allows trophic factors to diffuse into the shared media and help the growth of the neurons. Inserts were added to each well of a 6-well plastic plate and 600µl of solution of collagen mixed 1:4 with sterile 70% ethanol was added to each insert. The inserts were left to dry out overnight. The next day, 2ml of glial medium was added to the wells of the plate, underneath the insert. Astrocytes solution was thawed in 1 to 2 ml of glial medium and then diluted into 12ml of glial medium followed by the addition of 2ml of cell solution into each of the collagen-coated inserts, corresponding to $1x10^5$ astrocytes per insert. After 5-7 days, the inserts were transferred to plates containing Neurobasal medium in order to enrich the media for cortical neuron culture 1-2 days later.

c. Culture of low density cortical neurons

The cortical tissue was obtained from E18 embryos and immediately transferred to ice-cold dissection buffer. The cortices were dissected under a Leica MZ6 microscope with a Fiber-Lite MI-150 high intensity illuminator and stored in ice-cold dissection buffer. The tissue was finely triturated and placed in a 50 ml Falcon tube with 15 ml of trypsinisation buffer and incubated at 37 C for 30 to 40 minutes. Then the tissue was dissociated by pipetting 10-15 times with a Pastette. The resulting suspension was centrifuged for 5 minutes at 1500 revolutions per minute (RPM) in a MSE Mistral 1000 centrifuge and the supernatant carefully removed with a thin bore Pastette. The pellet was then resuspended in 20 ml DMEM/FCS and passed through a wide bore Pastette 10-15 times and centrifuged as previously. The pellet was resuspended in 10 ml DMEM/FCS by 20 passages through a wide bore Pastette followed by 10 passages through a thin bore Pastette, then centrifuged again and resuspended in 20 ml DMEM/FCS. The cortical neurons were then passed through a 40 µm cell strainer, collected in a 50 ml Falcon tube and counted. After centrifugation, the cell pellet was resuspended in Neurobasal medium supplemented with 200 mM GlutaMAX-1, 100 g/ml of penicillin and streptomycin and 20% of B-27 supplement and plated in 6 wells plates at a final concentration of $1x10^5$ cells/well. Neurons were maintained in an incubator at 37 C until they reached five days in vitro. The cells were then fixed and stained for imaging analysis.

2.3.2   Immunocytochemistry

Immunocytochemistry allows the detection of one or multiple proteins in structurally intact cells. This requires cells to be grown as a monolayer. Cells are then fixed, and the proteins of

interest are detected by sequentially incubating the cells with one or more protein-specific antibodies, followed by fluorescently labelled secondary antibodies. Stained cells can then be visualised using a light microscope equipped with the necessary excitation and emission filters.

a. Cell fixation and permeabilization

Fixing the cells blocks the activity of any biomolecule and kills the cells while preserving their structure and preventing deterioration. Membrane permeabilization allows immunodetection of antigens that are not exposed on the cell surface.

After five days in vitro, the media was taken out of each well and replaced with 1 ml of 4% paraformaldehyde (PFA) (diluted from 16% formaldehyde methanol-free, ThermoFisher). The cells were incubated for 10 min at room temperature. After removal of 4% PFA, the cells were washed once with ice-cold PBS and then incubated 3 times for 5 minutes in 1ml ice-cold PBS. For membrane permeabilization, the cells were then incubated in 1ml of PBS containing 0.1% Triton X-100 for 10 minutes at room temperature, then washed 1 times with room temperature PBS and incubated three times for 5 minutes in 1ml of room temperature PBS. The cells were then either immuno-stained immediately or wrapped in tin foil and stored in PBS at 4ºC for up to 2 days before immunostaining.

b. Immunostaining

Immunostaining was performed at room temperature. To block non-specific binding sites, the cells were incubated 1 hour in 1 ml of PBS containing 3% BSA. The primary antibody MAP2 (mouse IgG1, M9942 sigma) was diluted 1 in 2000 in this same blocking buffer. The blocking buffer was replaced by 1 ml of the primary antibody dilution, and the cells were incubated for 1hour. The cells were then washed once followed by 3 times 5 minutes' incubation with PBS. Then, the appropriate fluorescently labelled secondary antibody, in this case a Polyclonal Donkey anti-mouse IgG (Alexa Fluor 568 (H+L) A31570) was diluted 1 in 500 in blocking buffer. The cells were incubated with the secondary antibody for 1 hour. Nuclei were stained with Hoechst (Sigma-Aldrich), the dye was diluted 1 in 10000 and added to the cells along with the secondary antibody. From this moment forward, the cells were protected from light to prevent photobleaching of the fluorophores. After incubation with the secondary antibody, the cells were washed as before, then covered with a glass coverslip using mowiol (Sigma-Aldrich) mounting medium. The plates were wrapped in tin foil and kept at 4 C for at least 16 hours after mounting to allow the mounting medium to set completely.

c. Fluorescent microscopy

A fluorescence microscope uses a high intensity light source which excites a fluorescent molecule in a region of interest. This molecule emits a lower energy light of a longer wavelength. This produces a magnified image. Fluorescent microscopy is used to visually enhance cell structure, thanks to fluorescent tags to antibodies that attach to targeted molecules. This allows generation of images representing a horizontal plane of a stained cell or tissue. Fluorescent images of the MAP2 positive cells were acquired at 20X using a Photometrics Coolsnap HQ2 CCD camera and a Zeiss Zeiss Axioplan II fluorescence microscope with Plan-neofluar objectives (Carl Zeiss, Cambridge, UK), a Mercury Halide fluorescent light source (Exfo Excite 120, Excelitas Technologies) and Chroma #83000 triple band pass filter set (Chroma Technology Corp., Rockingham, VT) with the excitation filters installed in a motorised filter wheel (Ludl Electronic Products, Hawthorne, NY).

2.3.3   Outgrowth analysis

Cell cultures from wild-type and homozygous mice were analysed blind to genotype. The analysis was carried out on four independent cultures for each genotype. From the cultures, neurons which were distinct from each other were selected in order to assess the dendrites extending from cell bodies (figure 2.2, a). Each image analysis was done using ImageJ software and its "Neurite tracer" plug in. The centre of each cell body and the end of each dendrite was manually selected for the software to reconstruct the neuron carefully chosen. After applying the "sholl analysis" plug in, the number of crossings between the circle created by the plug in and the dendrites was obtained every 5 µm. The length of the dendrites, and the number of primary and secondary dendrites were also obtained (figure 2.2, b). To assess the soma area, imageJ was used and the soma outlines were drawn manually in order to obtain the measurement of its area (figure 2.2, c).
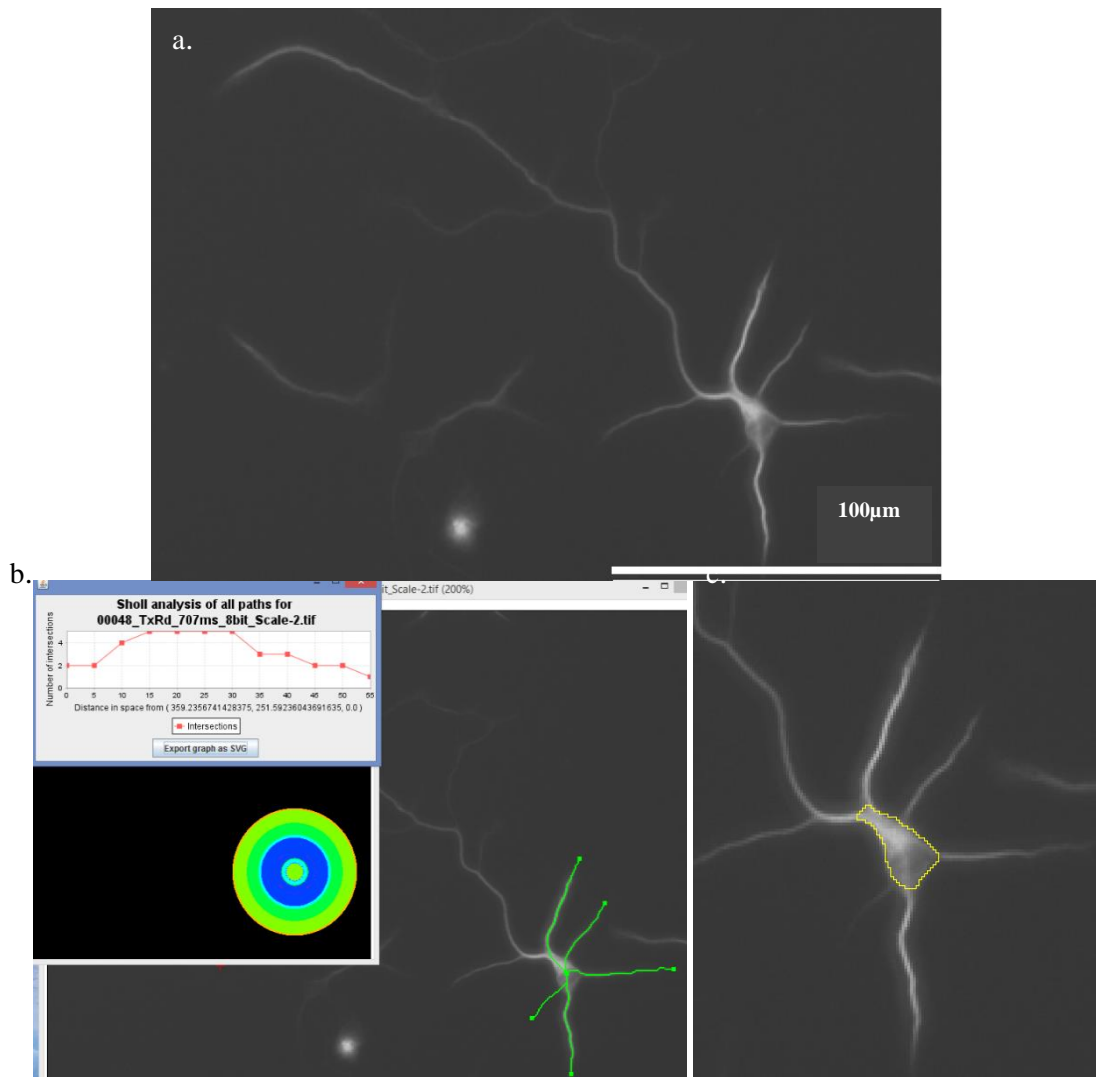
Figure 2.2: Cell growth analysis using imageJ

a. Representation of a neuron (x20) ready to be analyzed by imageJ software

b. Representation of the Sholl analysis. The different circles show where the crossings are estimated, and the graph gives the values and the trend of the crossings every 5 μm. The dendrites are represented in green. Here, the axon can sometime express MAP2 when neurons are immature (less than 7DIV). In this case the axon was recognized due to its excessive length and was therefore excluded from this analysis.

c. Analysis if the cell body area. The contour of the cell body is indicated by a yellow line.

2.3.4   Statistical analysis of the outgrowth analysis

To assess outgrowth changes, statistical analysis was performed using Graphpad Prism software (GraphPad Software Inc., La Jolla,CA). Normality of each data set was tested using the D'Agostino-Pearson omnibus test. If the data for the experiment in question was normally

distributed, an unpaired parametric student t-test (comparison of two groups) was used. If the data for the experiment in question was not normally distributed, a Mann-Whitney test (comparison of two groups) was used. Result of the outgrowth analysis are presented as mean ± standard error of the mean (SEM) and $p<0.05$ was considered statistically significant and is indicated with *, $p<0.01$ is indicated with **.

2.4 Histology

2.4.1   Tissue processing

Male littermate trios were selected for histology analysis. For each wild type control one homozygote and heterozygote were selected from the same litter. A subset of the mice underwent BrdU treatment starting at 7 weeks of age, until they were culled at 9 weeks. Mice were anaesthetized with a solution of Fentanyl/Fluanisone (Hypnorm®) and Midazolam (Hyponovel®) prepared by a technician from the animal facility. This was administered at a dose of 0.1ml/10g via the intraperitoneal route. Anaesthetic depth was ensured by measuring withdrawal reflexes. In rodents, withdrawal reflexes are tested by pinching the tail and the hind limbs to demonstrate the reflex is absent. The mice were then transcardially perfused. The chest cavity was opened by removing the patch of skin covering the ribs and sternum and making an incision under the sternum. Then the ribs were cut through and the diaphragm removed. A catheter was inserted into the left ventricle using a 10-14 gauge needle. To open the vascular system, the right atrium was cut. A syringe containing 20ml of PBS was connected to the catheter and depressed at approximatively 0.2-0.5 ml/second until the liver and internal organs cleared of blood and the atrial flow was clear. The syringe was replaced by a new syringe containing 4% PFA and the fixative was perfused at the same rate. For a strong fixation 50 ml of fixative was used. The head was then separated from the body with scissors, and the brain carefully dissected out. The olfactory bulbs and the cerebellum were removed from the rest of the brain. The brains were then left for 24h in 4% NFB after which they were transferred into 70% ethanol solution. The brains were then paraffin wax embedded. Sections were cut in three   different   zones   of   the   brain   (figure   2.3):

-Bregma ≈2.46; to see the prefrontal cortex (PFC)

-Bregma ≈ 0.75; to see the ventricles and the corpus callosum

-Bregma ≈ -1.94; to see the hippocampus.
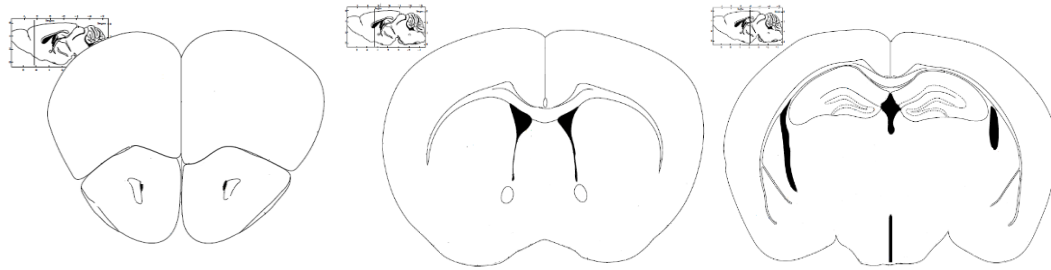
Figure 2.3: Depiction of mouse brain sections done at three different Bregma points derived from Paxinos *et al*, 2004.

a. 2.46 mm before Bregma; b. 0.75 mm before Bregma, c.-1.94 mm after Bregma

The brains were processed by the University of Edinburgh Shared University Research Facilities (SURF), using a Leica RM2235 base sledge microtome. Twenty coronal sections of 10μm were cut for each block. The sections were then floated out in a warm water bath and mounted on to Superfrost Plus slides (ThermoFisher Scientific) and dried in an oven. Two successive sections are used for each location for each staining.

2.4.2   Staining

a.   Cresyl Fast Violet (Nissl) staining

To visualize neuronal cytoarchitecture, two 10um sections on Superfrost Plus (ThermoFisher Scientific) slides per block were dewaxed in xylene (Fisher) in which they were incubated 2 times 15 minutes. Then the sections were rehydrated through graded alcohols: 2 times in IMS for twice 10 minutes, once 95% IMS for 5 minutes, once in 90% IMS for 5 minutes and once in 70 % IMS for 10 minutes. Then, the slides were rinsed in tap water and incubated for 2 minutes 0.2% cresyl fast violet solution (R.A.Lamb -Cresyl fast violet) containing 10 drops of acetic acid per 100 ml. Stained sections were then rinsed in tap water and dehydrated through graded alcohols: 70% IMS, 90% IMS, 95% IMS for 2 minutes each and 100% IMS twice for 5 minutes. Then the slides were cleared in xylene and cover-slipped with DPX (Fisher), a xylene-based mounting solution.

b.   Immunohistochemistry

Paraffin embedded sections were stained for Parvalbumin and Caspase 3 (table 2.1). The sections were dewaxed as previously mentioned and endogenous peroxidase activity quenched by incubating the sections in methanol containing 1% H_2O_2 for 30 minutes. Sections were rinsed in PBS and antigen retrieval performed. The sections were incubated in 10 mM of sodium citrate buffer at room temperature and slides were then microwaved for 20 minutes at

high power. To ensure constant coverage of the slides, distilled water was poured every 5 minutes. The slides were cooled on ice for 20 minutes and rinsed with PBS-Tx for 10 minutes. The sections were blocked in a 20% normal goat serum (Vector) diluted in PBS-Tx for 1 hour. The species of normal serum was directed against the host species of the secondary antibody. After blocking, sections were incubated overnight at 4°C in primary antibody in 20% goat serum in PBS-T. Sections were then washed 4 times 5 minutes in PBS-Tx and incubated at room temperature in biotinylated secondary antibody at a 1:200 dilution for 1 hours (table 2.1), followed by 4 washes of 5 minutes in PBS-Tx and incubation in Vectastain Elite ABC kit (1 drop A and 1 drop B in 2.5 ml of PBS-Tx, Vector Laboratories) for 30 minutes before visualization by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Kit, Sigma) containing 0.001% H_2O_2. Sections were washed with distilled water and dehydrated through graded alcohol (see 1.3.2.1) and then cleared in xylene and cover slipped with DPX. For Caspase-3 staining, nickel was added to DAB to obtain black positive staining. The slides were then rinsed in dH_2O and incubated in Nuclear Fast Red (Vector) in order to stain the nuclei pink. The sections were then rinsed in dH_2O and cover-slipped as before.

c.   Immunofluorescence

Mouse brain sections were stained to visualize expression of doublecortin and BrdU in the dentate gyrus. Sections were dewaxed and incubated in 10 mM of sodium citrate buffer at room temperature and slides were then microwaved for 10 minutes on simmer followed by 10 minutes at high power. To ensure constant coverage of the slides, distilled water was poured every 5 minutes. The slides were cool down on ice for 20 minutes and rinsed with PBS-Tx for 5 minutes. The sections were blocked in a 20% normal goat serum (Vector) solution in PBS-Tx for 1 hours and incubated overnight at 4°C in a cocktail of primary antibody BrdU and DCX (table 2.1) diluted in blocking serum. Sections were then washed 4 time 5 minutes in PBS-Tx and incubated at room temperature in secondary antibody at a 1:200 dilution for 1 hours (table 2.1). Sections were washed with PBS-Tx for 10 minutes. To stain the nuclei, sections were incubated in DAPI diluted at 1:1000 in dH_2O for 2 minutes. The slides were then rinsed in dH_2O and cover-slipped with Vectashield hard set.

Primary Antibody	Supplier	Dilution
Anti-Caspase 3, active (cleaved) form (Mooney *et al.*, 2018)	Millipore, AB3623	1:70
Monoclonal Anti-Parvalbumin antibody produced in mouse clone PARV-19, ascites fluid (Menegola *et al.*, 2008)	Sigma-Aldrich, P3088	1:400
Anti-BrdU antibody, Mouse monoclonal. Clone BU-33, purified from hybridoma cell culture (Wang *et al.*, 2011)	Sigma-Aldrich, B8434	1:200
Doublecortin antibody (Kim et al., 2017)	Abcam ab18723	1:1000
Secondary Antibody	**Supplier**	**Dilution**
Goat anti mouse biotinylated	Sigma	1:200
Goat anti rabbit biotinylated	Sigma	1:200
Goat anti mouse Alexa Fluor conjugates 568	Invitrogen	1:200
Goat anti rabbit Alexa Fluor conjugates 488	Invitrogen	1:200
DAPI	Invitrogen	1:50000

Table 2.1: Detail of antibodies used for immunohistochemistry (IHC) and immunofluorescence (IF)

2.4.3 Regional area Measurements

Regional areas were measured using Fiji and its region of interest (ROI) manager. The area of the region of interest was measured using two sections per animals. In this study, the area of the cortex, corpus callosum and lateral ventricles were measured (figure 2.4). The appropriate contours of each region of interest were drawn manually on each section using Fiji to assess areas of interest. The values of the area in μm^2 for each hemisphere would be obtained separately by using the ROI manager and extracting the measurements. Then the hippocampus was investigated, more specifically, at the area of the dentate gyrus (figure 2.5, a, b). The contour of the dentate gyrus was drawn manually using Fiji and a line was drawn to measure its length (figure 2.5, b). Additionally, the thickness of the CA1 and CA2 was evaluated, respectively four lines and three lines were drawn, and their length measured with Fiji (figure 2.5, c, d).
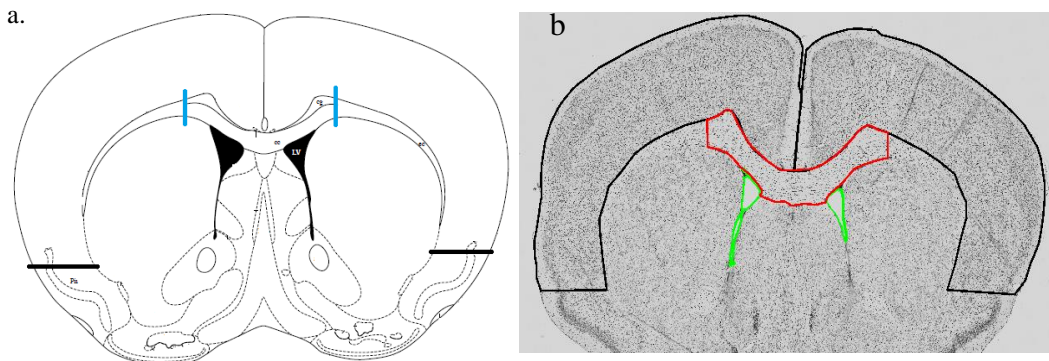
Figure 2.4: Delimitation of the area of the regions of interest

a. Representation of a mouse brain section, modified from the mouse brain atlas in stereotaxic coordinates (Paxinos *et al.*, 2004). The corpus callosum was delimited by the extremities of the cingulum (cg) marked by blue lines and the cortical area was delimited by the beginning of the Piriform cortex (Pir) marked by black lines.

b. Nissl stained mouse brain section analysed with Fiji showing the three regions of interest: cortex area delimited in black, corpus callosum area delimited in red, ventricle area delimited in blue.
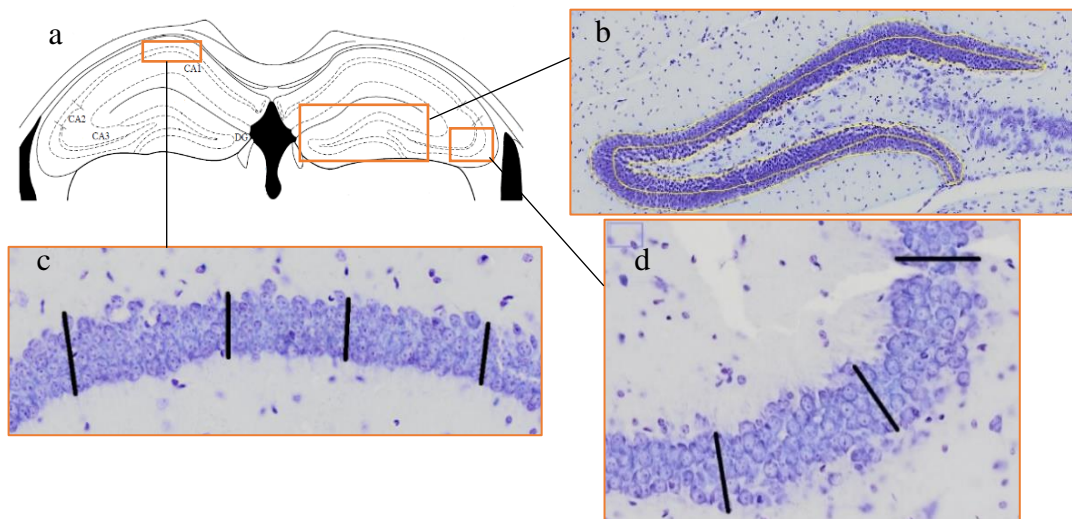
Figure 2.5: Structural analysis of the hippocampus

a. Representation of a mouse brain section of the hippocampus, modified from the mouse brain atlas in stereotaxic coordinates (Paxinos *et al.*, 2004). Orange squares to identify area of interest.

b. Zoom of the dentate gyrus, contour in yellow and yellow line to measure its length.

c. Zoom of the CA1, 4 black line to measure its thickness

d. Zoom of the CA3, 3 black lines to measure its thickness

2.4.4   Cell density

To automatically count the number of cells in a specific area on each section, a matrix which can be used with Fiji was created. This allowed me to calculate a density for each section of the prefrontal cortex using a box of width=1122 and height=930 micron (figure 2.6). The density of cells in the hippocampus was analysed in two regions of interest: the CA1 and the srlm zone. In the CA1, a box of 100 μm^2 was set at the same location for each section and the cells were counted manually using the cell counter plug in on Fiji (figure 2.7). In the srlm zone a box of 200 μm^2 was set at the same place on each section and the cells were counted manually as mentioned before using Fiji (figure 2.7). An average density of cell for the cortex prefrontal, the CA1 and the srlm was obtained for each animal.
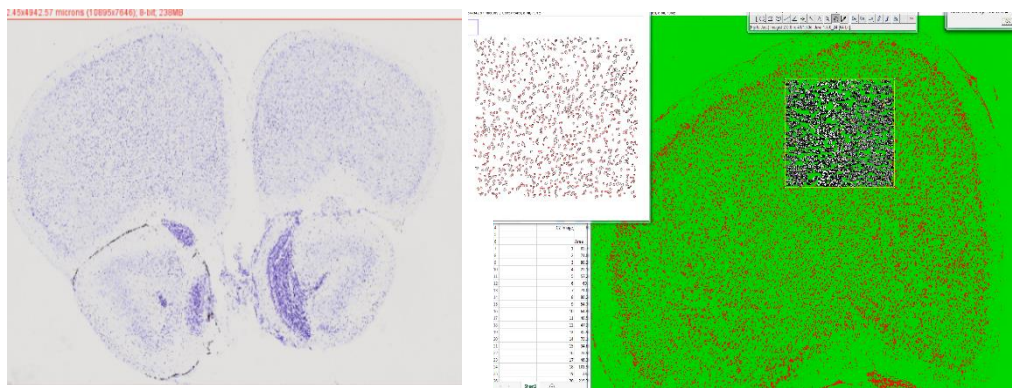
Figure 2.6: Cell count on a PFC section

a. Nissl sections of PFC. b. Automated cell count using Fiji.
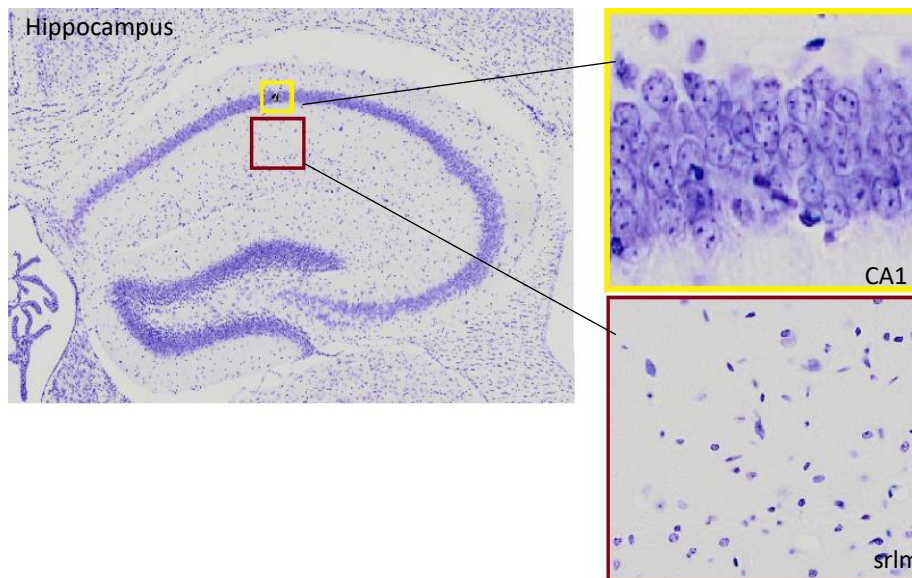
Figure 2.7: Cell count in the hippocampus

The yellow square indicates the box used to count cells in the CA1 and the red square indicates the box used to count cells in the srlm.

2.4.5   Count of Parvalbumin positive cells

To analyse the distribution of interneurons, the PFC was separated into its different regions of interest. Brain areas were distinguished using anatomical features and the Mouse brain atlas in stereotaxic coordinates (Paxinos *et al.*, 2004) (figure 2.8). The cells were counted manually using the cell counter plug in from Fiji. For each brain, data from the two hemispheres were collected and averaged for further analysis.
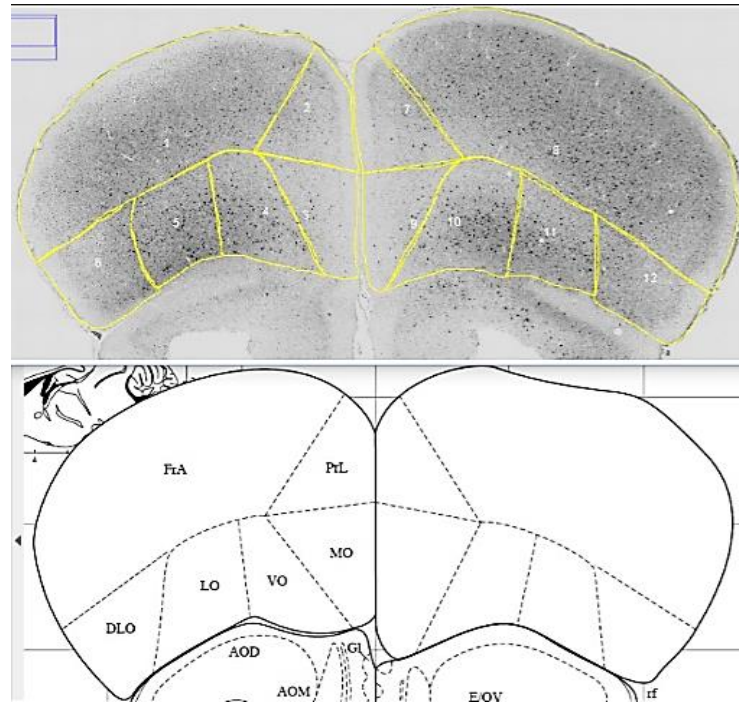
Figure 2.8: Areas of the prefrontal cortex used to count parvalbumin positive cells

a. Image of prefrontal cortex section stained for parvalbumin. Yellow lines have been manually traced using the mouse brain atlas in stereotaxic coordinates in order to define the different areas present.
b. Image from the mouse brain atlas in stereotaxic coordinates which correspond to the sections used in this analysis.

The interneuron count was also completed in the hippocampus. The area of the CA1 and the DG were drawn using anatomical cues and the cells were counted using Fiji, as previously mentioned (figure 2.9). Then the cells were counted in the whole hippocampus.
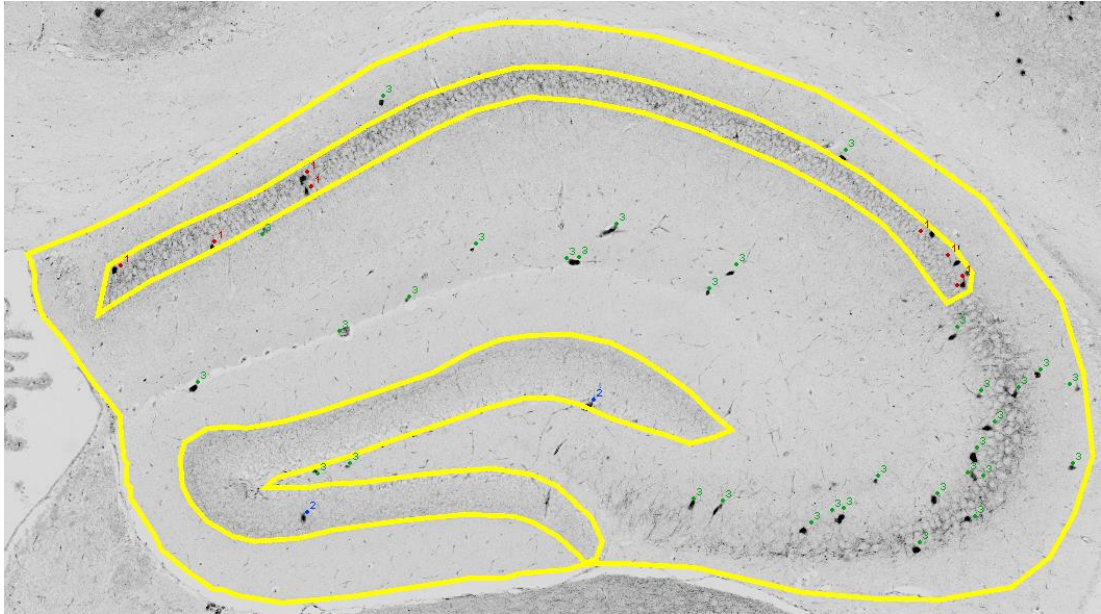
Figure 2.9: Image of hippocampus stained for parvalbumin

The different areas where the cells were counted are delimited in yellow. In each area, the cells have been counted using markers of different colours. Blue markers indicate parvalbumin positive cells in the dentate gyrus, turquoise markers are for the positive cells in the CA1 and green markers are for the rest of the hippocampus.

2.4.6   Count of caspase-3 positive cells

To study apoptosis, positive activated caspase-3 cells were counted in the PFC and in the hippocampus. Because of the low expression of activated caspase-3, the positive cells were counted manually in half of the PFC (figure 2.10). The cells were counted directly on OLIVIA software. Caspase-3 positive cells were marked by a green cross and their positions were also indicated on the pictures. In the hippocampus, the cells were counted in the CA1, the dentate gyrus and the whole hippocampus as presented for the count of the parvalbumin positive cells (figure 2.11). The counts were done using the cell counter plug in on Fiji, as previously.
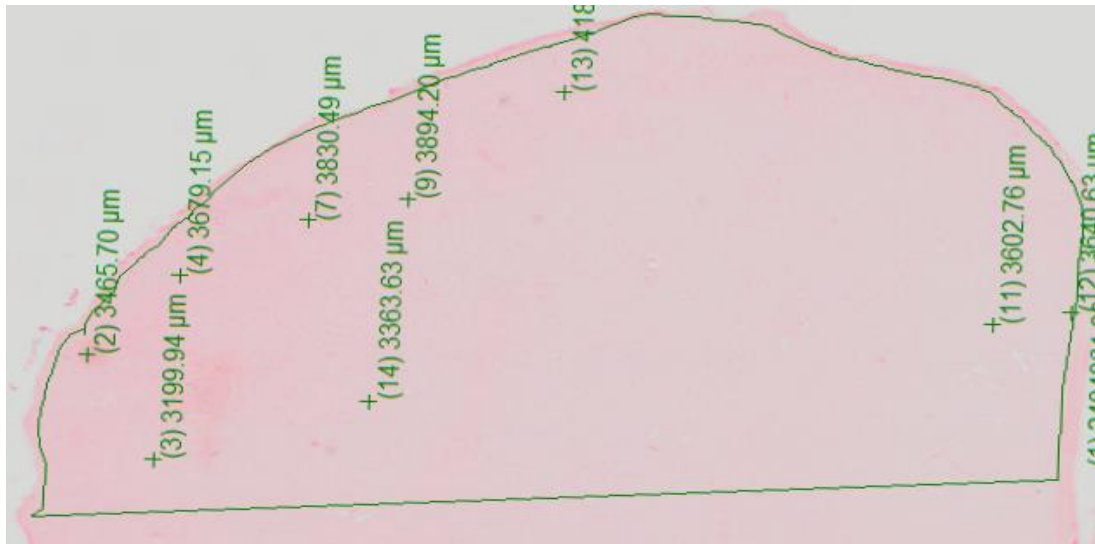
Figure 2.10: Image of a section of the prefrontal cortex stained with caspase-3 activated antibody

The green crosses indicate positive cells. The green line defines the area in which the cells were counted.

Figure 2.11: Image of a section of the hippocampus stained with caspase-3 activated antibody

The blue markers indicate positive cells. The yellow lines define the different areas in which the cells were counted.

2.4.7   DCX/BrdU count

Doublecortin and BrdU positive cells were counted exclusively in the dentate gyrus. The cell counter plug-in from Fiji was used to mark each counted cell. BrdU and DCX positive cells were first counted on single channel images. To count any double stained cells, the multiple channel images were used (figure 2.12). Number of positive cells per dentate gyrus were assessed and a mean per dentate gyrus was measured.
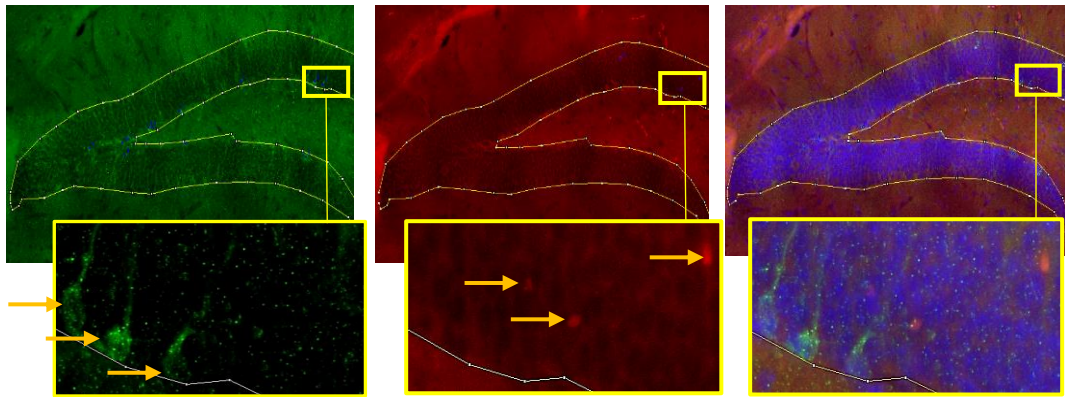
Figure 2.12: Images of the dentate gyrus stained for DCX/BrdU

In green, the cells are stained for DCX. The yellow box represents a zoom in, three positive cells can be detected (yellow arrows). In red, the cells are stained for BrdU. The yellow box represents a zoom in, three positive cells can be detected (yellow arrows). The multiple channel image shows cell nuclei stained with Dapi in blue, and red and green staining for BrdU and DCX respectively. The yellow box represents a zoom in, 3 BrdU positive cells and the 3 DCX positive cells can be seen, no DCX and BrdU co-labelled cells were visible here. BrdU incorporate the cell during phase S of the cell cycle and DCX is expressed in immature neuron (neuron progenitors) in post mitotic phase. It is possible that BrdU^{+}/DCX^{-} cells are not yet neuron progenitors or not meant to be neurons and BrdU^{-}/DCX^{+} immature neurons did not proliferate during BrdU incorporation.

2.4.8   Statistical analysis

All measurements were performed blind to genotype. All statistical analysis was performed using IBM SPSS statistics 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). A univariate general linear model 2-way ANOVA statistic test was performed to determine group-level differences, where $p \leq 0.05$ was considered significant. Genotype and littermate trios were considered as fixed factors in order

to take into account any littermate effect in our analysis. A Dunnett's Two-Tailed post hoc test was used to compare the wild-type group to the heterozygote and homozygote groups.

In order to take into account, the littermate effect, this effect was included as an independent categorical variable in the 2-way ANOVA and therefore labelled as a fixed factor. With fixed-effects factor, the effects of the independent variable are treated as fixed constants to be estimated (statistics.laerd.com, 2-way ANOVA with SPSS). The 'main effect' of this independent variable was analysed which allowed to assess the effect of the littermate on the dependant variable. The statistical result was obtained through the Wilk's lambda tests performed by SPSS software. This indicates how well the independent variable, here being the littermate effect, contributes to the model.

2.5 Magnetic resonance imaging (MRI)

2.5.1   Tissue collection

Brains were collected as previously described in the histology section 1.3.1. The brains were then left for 24h in 4% NFB after which they were transferred into 70% ethanol solution. They were then soaked for 3 weeks in contrast agent Gadolinium containing PBS in order to improve the quality of the MRI images.

2.5.2   Scanning protocol

Brains were scanned in pairs in a proton-free fluid Fomblin at the Queens Medical Research *Institute* of *Edinburgh* (QMRI). One male brain was placed at the bottom of the vial, hindbrain first, then a female brain of the same genotype (also hindbrain first) was placed on top. Foam soaked in Fomblin was added to secure the brains and to prevent them from moving. No air bubbles should be visible. The vials were placed in the centre of the 26 mm radiofrequency coil. The Acquisition parameters were:

matrix: 512   x   192   x   192   (reconstructed   to   512   x   256   x   256)
FOV: 40   x   10   x   10   mm
TR/TE: 30/10 ms

The 3D scans were run overnight.

2.5.3   Image analysis

MR images were processed using a combination of FSL (Jenkinson *et al.*, 2012), ANTs (Avants *et al.*, 2011) and in-house C++ software utilizing the ITK library, available from https://github.com/spinicist/QUIT (Richetto *et al.*, 2016; Wood *et al.*, 2016). In brief, multi-head scans were bias-field corrected (Tustison *et al.*, 2010) before being split into individual sample images. Registration was then performed between each subject and the Dorr atlas image (Dorr *et al.*, 2008) to ensure all samples were aligned. An average study template image was then constructed using MR images from all animals (Avants *et al.*, 2010). The resulting template was then non-linearly registered to the atlas image. All subject images were then non-linearly registered to the study template. The inverse transforms from the atlas to the study template and from the study template to each subject were applied to calculate the total brain volume and individual brain region of interest (ROI) volumes of each subject. ROIs match those found in the Dorr atlas (Dorr *et al.*, 2008).

2.5.4   Statistical analysis

A multivariate general linear model 2-way MANCOVA statistical test was performed using IBM SPSS statistics 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) to determine group-level differences in brain ROI volumes, where $p \leq 0.05$ was considered significant. Whole brain volume was considered as a co-variate. Genotype and littermate trios were considered as fixed factors in order to take into account any littermate effect in our analysis. All the brain regions were considered as dependent variables. A *Dunnett's Two-Tailed* post hoc test was used to compare the wild-type group to the heterozygote and homozygote groups. In order to take into account, the littermate effect, this effect was included as an independent categorical variable in the 2-way MANCOVA and therefore labelled as a fixed factor. With fixed-effects factor, the effects of the independent variable are treated as fixed constants to be estimated (statistics.laerd.com, 2-way MANCOVA with SPSS). The 'main effect' of this independent variable was analysed which allowed to assess the effect of the littermate on the dependant variables collectively. The statistical result was obtained through the Wilk's lambda tests performed by SPSS software. This indicates how well the independent variable, here being the littermate effect, contributes to the model.

2.6 RNA samples preparation and analysis

2.6.1   Preparation

a.   Collection of the tissue

The tissue was collected from 9 weeks old mice. Each group (wild type, heterozygotes, homozygotes) were composed of 4 males and 4 females. Mice were culled under the schedule 1 procedure by trained staff at the animal facility. The brains were then directly removed and washed in ice-cold PBS. The cortices and hippocampi from each hemisphere were dissected. The tissues from the right hemisphere were incubated overnight at 4ºC, in 5 volumes of RNA later (Ambion). After 24h, the RNA later was discarded to prevent the formation of salt crystals and the samples were stored at -80 ºC until they were processed.

b.   Processing of the samples

The samples were purified using QIAGEN RNA extraction kit, according to the manufacturer's instructions. To homogenise the tissues, the Tissueruptor was used. Then insoluble materials were removed, and nucleoprotein complex dissociated. The RNA was re-dissolved in 100 µl of RNA free water. At that point, the RNA concentration was assessed using the nanodrop for a first time to assess its quality. To obtain the purest RNA possible, RNA clean-up was performed, according to the manufacturer's instructions. A maximum of 100 µg RNA can be cleaned up in this protocol. RLT lysis buffer and RPE washing buffer were used in order to obtain optimum cleaned RNA. The RNA was then collected in in RNase-free water. Additional on-column DNase digestion was then done by treating the samples with DNase I, as indicated by the manufacturer's instructions. At the end, the RNA was eluted once for the hippocampal samples and twice for the cortical samples using 30 µl of RNase free water, therefore 60 µl of pure cortical RNA and 30 µl of pure hippocampal RNA were obtained.

2.6.2   Computational analysis

The RNA sequencing and analysis (mapping, read counts, fusion search) has been performed by the German company CeGAT, using Star for mapping and cuffdiff for differential expression. They used TruSeq Stranded mRNA Library Prep Kit (Illumina) in order to prepare the library. The sequencing parameters were the following: HiSeq4000, high output mode, 1x 100 base pair (bp). Demultiplexing of the sequencing reads was performed with Illumina CASAVA (1.8.2) and adapters were trimmed with Skewer (version 0.1.116) (Jiang et al.

2014). However, the expression analysis was re-done by Philippe Gautier, at the Institute of Genetic and Molecular Medicine (IGMM) in Edinburgh. Raw count of reads from the mapping data using htseq-count were produced and analyzed using the R package DESeq2 (Love et al, 2014) and for an analysis at exon level, the DEXSeq package was used (Anders et al, 2008). Therefore, differential gene expression was analysed using DESeq2 and differential exon expression was analysed using DEXSeq. To verify some of the results, the mapping files (. bam files) were also examined using IGV (Thorvaldsdóttir et al, 2013). Differential expression and statistical significance can only be obtained if the samples can be clustered into groups, each containing at least three replicates. Four biological replicate samples were used per group, in total 6 group for the hippocampal study and 6 group for the cortical study (wild-type female/male, heterozygotes female/male, homozygous female/male). The differential analysis is obtained through the DESeq function and using the Wald statistical test (Love, Huber and Anders, 2014; Love *et al.*, 2018). The adjusted p-value obtained as a result of significance correspond to the p-value corrected for multiple testing, in these packages the Benjamin-Hochberg correction is used, which allows to control the false discovery rate correspond to false positive results. Following this analysis, gene ontology analysis was done using the **G**ene **O**ntology en**RI**chment ana**L**ysis and visua**L**iz**A**tion tool GOrilla (http://cbl-gorilla.cs.technion.ac.il/) (Eden *et al.*, 2007, 2009). The full list of expressed genes was used as the background gene set while the significant gene list was used as the target. An expression base mean cut-off of half the base mean of disc1 expression was applied to all differentially expressed data (corrected p<0.05) to minimise quantitation errors from genes expressed at very low levels. The GOrilla database is updated regularly, here the database updated on February 2018 was used, the p-value threshold was set at 10^{-3}. Additionally, a protein annotation through evolutionary relationship Panther v.8 (http://pantherdb.org/) (Mi *et al.*, 2013), was also used to study gene ontology. Significant genes were compared to a reference gene list, and the software determined if there was a particular class-enrichment of GO terms categorised under Process, Function and Component. Heat maps of gene expression were generated using R version 3.4.2 and RStudio version 1.0.143.

In addition, pathway analysis of the heterozygous samples was done using Ingenuity pathway analysis (IPA) in order to complement the gene ontology analysis. Pathway analysis was not performed for the homozygous samples as the group revealed a sub division indicating the presence of 2 different groups within the homozygote group. It seemed that one subgroup had a gene expression pattern similar to the heterozygote group while the other subgroup had a gene expression pattern similar to the wild-type group. So far, we cannot explain the reason of this division and therefore we decided not to perform the pathway analysis yet.

CHAPTER 3

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EFFECT OF THE DER1 MUTATION ON BRAIN STRUCTURE

3.1 Introduction

There are few consistent pathologies associated with mental illness, but some changes have been found recurrently. Patients suffering from mental illness show several changes in their brain structure such as important loss of grey matter (De Peri *et al.*, 2012; Haijma *et al.*, 2013; Goodkind *et al.*, 2015; Schmaal *et al.*, 2017). This seems to impair their cognitive functions and their memory. However, some disorders have specific structural modifications. For instance, in schizophrenia patients the volume of the hippocampus and the corpus callosum has been often found to be reduced (Woodruff, McManus and David, 1995)(Smiley, Konnova and Bleiwas, 2012a) while lateral ventricles are enlarged. Major depressive disorders have been characterized by a decrease of the amygdala and the hippocampus (Lorenzetti *et al.*, 2009; Cole *et al.*, 2011).

In patients carrying the translocation t(1;11) reduced cortical thickness and gyrification was found as well as reduced white matter integrity (Whalley *et al.*, 2015; Thomson *et al.*, 2016). Moreover, patients carrying the translocation and unrelated schizophrenia patients without the translocation share the same cortical phenotype (Doyle *et al.*, 2015). Notably, translocation carriers do not exhibit lateral ventricular enlargement.

Rodent models of mental illness show critical changes in brain function and structure. Previous research on different DISC1 mouse models showed major changes in the brain structure such as reduction of the total brain volume and of the cerebral cortex, as well as enlargement of the ventricles, which correlate with characteristics of schizophrenia patients (Clapcote *et al.*, 2007; Hikida *et al.*, 2007; Shen *et al.*, 2008). However, none of those mutants exactly model mental disorders and animal models which reproduce these diseases with precision are needed.

In this chapter, the aim is to discover the effect of the Der1 mutation on brain structure and whether any of the structural changes previously mentioned are reproduced in the new Der1 mouse model so as to gain more understanding of how the translocation affects brain structure.

Histological analysis combined with three-dimensional imaging is the most frequent and thorough way to investigate brain structure. These two techniques allow us to obtain substantial information about the brain structure of this mouse model.

3.2 Histological analysis: Structural changes between wild-type, heterozygous and homozygous mutant mice

To analyse brain structure, histological techniques were used first. This enabled close examination of the corpus callosum, the cortical layer, the lateral ventricles and the hippocampus. Those areas are known to be particularly affected in patients with mental illnesses and in animal models of those diseases.

To study structural changes in young adult mice, the brains of five male nine-week-old littermate genotype trios were dissected. Each wild-type brain is therefore associated with one heterozygous and one homozygous brain from the same litter. To concentrate on modifications due to the mutation, it is necessary that only the genotypes in question are compared, without additional variables. Using littermate trios will ensure that the genetic background and the environment are comparable between controls and mutants (Holmdahl and Malissen, 2012). Mice were taken at nine weeks of age which is when mice transition from adolescence to adulthood (Brust, Schindler and Lewejohann, 2015). This time point corresponds to the first major onset time point in schizophrenia disorder. The sections were cut approximatively 0.74 mm after the Bregma point (figure 3.1) to see the ventricles and the corpus callosum, and 2.00 mm before the Bregma point (figure 3.2) to see the hippocampus.
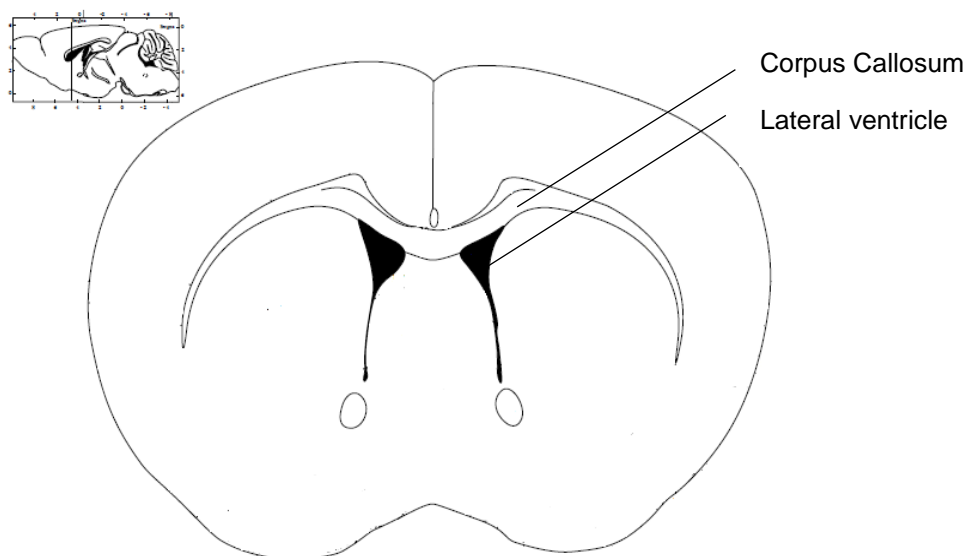
Figure 3.1: Representation of a mouse brain section localised 0.74 mm after the Bregma point

Image modified from 'The Mouse Brain in Stereotaxic Coordinates' by George Paxinos and Keith B. J. Franklin. The insert in the top left corner represents a mouse brain in sagittal position and shows where the section was cut.
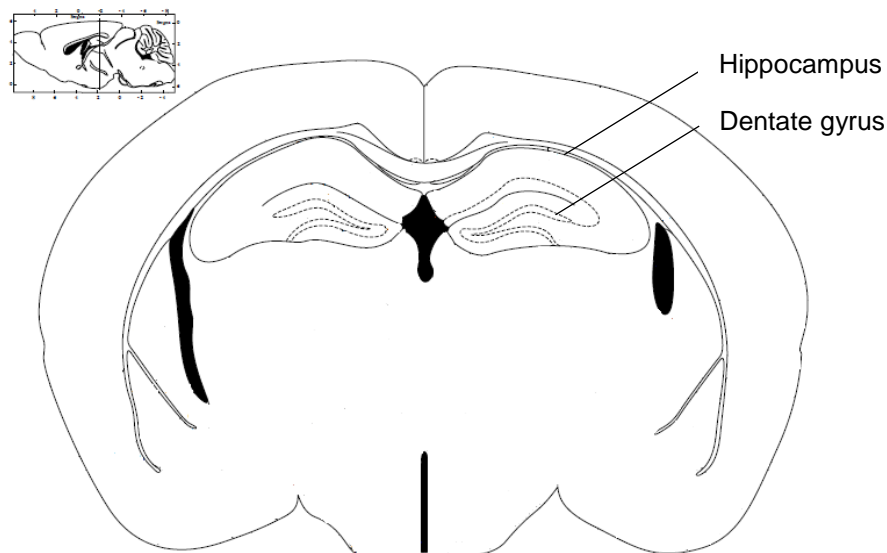
Figure 3.2: Representation of a mouse brain section localised 2.00 mm after the Bregma point

Image modified from 'The Mouse Brain in Stereotaxic Coordinates' by George Paxinos and Keith B. J. Franklin. The insert in the top left corner represents a mouse brain in sagittal position and shows where the section was cut.

For both coordinates and each animal, two consecutive sections of 10 μm were stained for Nissl bodies using cresyl violet acetate solution. This solution stains nucleic acid such as RNA and DNA, and is used to highlight important structural features of neurons and glial cells. The Nissl bodies are part of the endoplasmic reticulum and appear dark blue/purple due to the staining of ribosomal RNA, consequently the cytoplasm has a spotted appearance. DNA present in the nucleus stains a similar colour. Thus, stained Nissl bodies reveal the cell bodies of neurons and glial cells and can be used to define each structure of the brain in order to look at its gross and precise structure. The images were acquired using a slide scanner and their analysis was carried out using Fiji software. This software allows measurement of the area of interest for each image of each section. For each region of interest, the area was drawn manually on both hemispheres. Using structural landmarks, an equivalent area for each section was delimited (figure 3.3). The values of each area were collected from Fiji. The total area of the cortical layer, the lateral ventricles, the dentate gyrus, the CA1 and the CA3 were measured as well as the right and left areas separately except for the corpus callosum. First, the sections enclosing the cortical layer, the corpus callosum and the lateral ventricles were analysed then the sections encompassing the hippocampus, including the dentate gyrus and the Cornu Ammonis 1 and 3 (CA1 and CA3), were investigated.
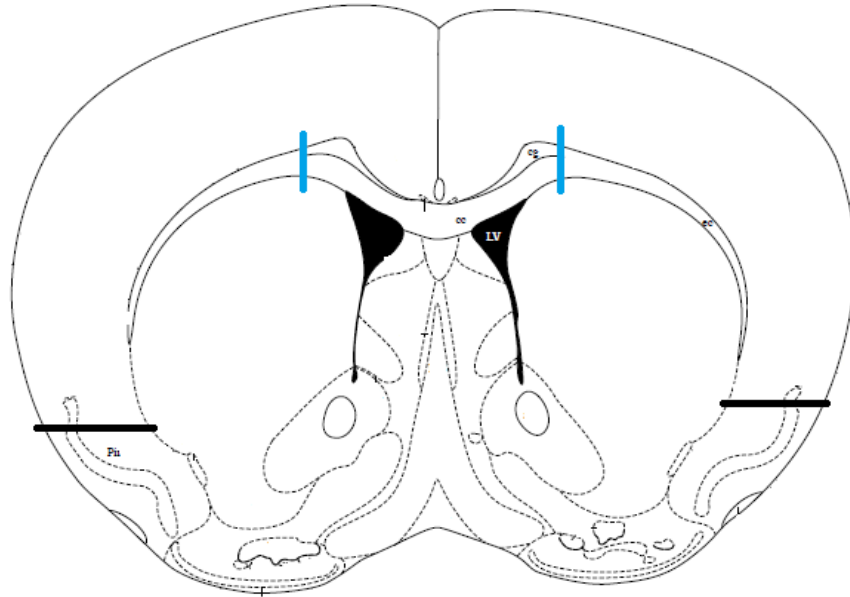
Figure 3.3: Delimitation of the area of the regions of interest

The corpus callosum was delimited by the extremities of the cingulum (cg) marked by blue lines and the cortical area was delimited by the beginning of the piriform cortex (Pir) marked by black lines. Image modified from 'The Mouse Brain in Stereotaxic Coordinates' by George Paxinos and Keith B. J. Franklin.

3.2.1 Changes in the cortical layer, corpus callosum and lateral ventricles

First, the lateral ventricles, the corpus callosum and the cortex were investigated to see possible changes in those areas in the homozygous and heterozygous mice when compared to their wild type littermates. Nissl stained sections were used to assess the area of the regions of interest (figure 3.4).
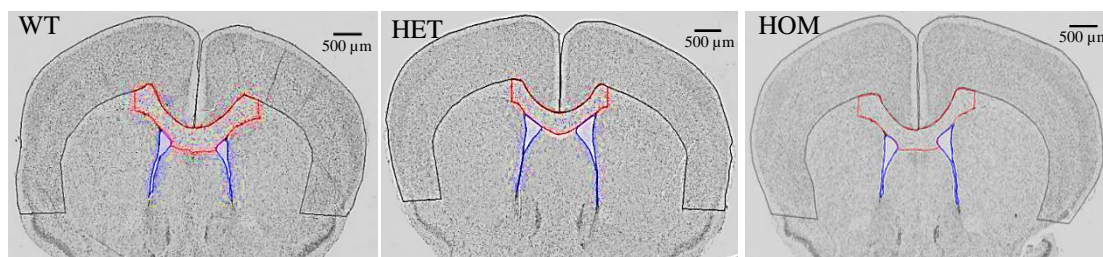
Figure 3.4: Analysis of Nissl brain sections

a. image of a brain section from a wild type mouse.

b. image of a brain section from a heterozygous mouse.

c. images of brain section from a homozygous mouse.

The black ROIs represent the cortical area measured, the red ROI represents the area of the corpus callosum measured and the blue ROIs represent the area of the ventricles measured.

The statistical analysis was done using SPSS and considered the fact that the mice were separated in trios to evaluate the possibility of a litter effect. A generalized linear model (GLM) was used. The genotypes and the trios were both set as fixed variables. The analysis of the total cortical area showed that there is a trend (GLM p=0.086) toward a smaller cortical area in the heterozygous group compare to the wild-type (figure 3.5, a). The observed power indicated by SPSS is of 0.45 which is quite low as the desired power level is typically 0.80, which would mean that there is 80%. Here, the low observed power indicates that the effect size does not allow to reach statistical significance. To reach a statistical significance and a power level of 0.8, an estimated sample size can be assessed using G*3 Power software (Faul and Erdfelder, 2007; Erdfelder *et al.*, 2009). Knowing the mean of variation of the area for each genotype and the global standard deviation, the analysis indicated that 48 samples (16 per group) would be required to see a significant difference in the total cortical area measurement (figure 3.6). The analysis of the right cortical layer showed no difference in its area between the genotypes (GLM p=0.224, figure 3.5, b). However, the results indicate a significant difference in the area of the left cortical layer between the genotypes (state test GLM p=0.044, figure 3.5, c). Yet, post-hoc analysis did not indicate a significant difference in the area of the left cortical layer between the heterozygotes and the wild type or between the homozygotes and the wild types, only between heterozygotes and homozygotes, therefore this difference might not be relevant to mental illness. This could indicate a hemisphere-specific effect.
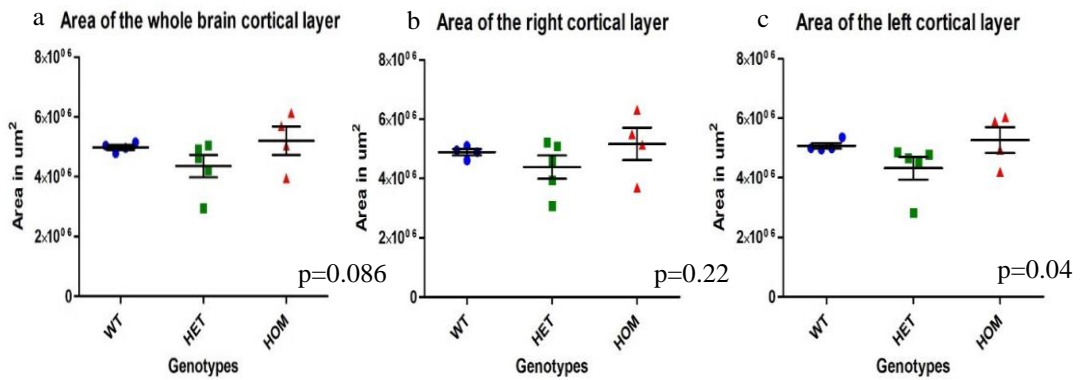
Figure 3.5: Cortical area measurements

a. Measure of the whole brain area shows a trend between the genotypes.

b. Measure of the right cortical layer shows no difference between the genotypes.

c. Measure of the left cortical layer shows a significant difference between the genotypes.

n = 5 animals per group; univariate generalized linear model with dunnetts' post hoc test. Means ± SEM are indicated.
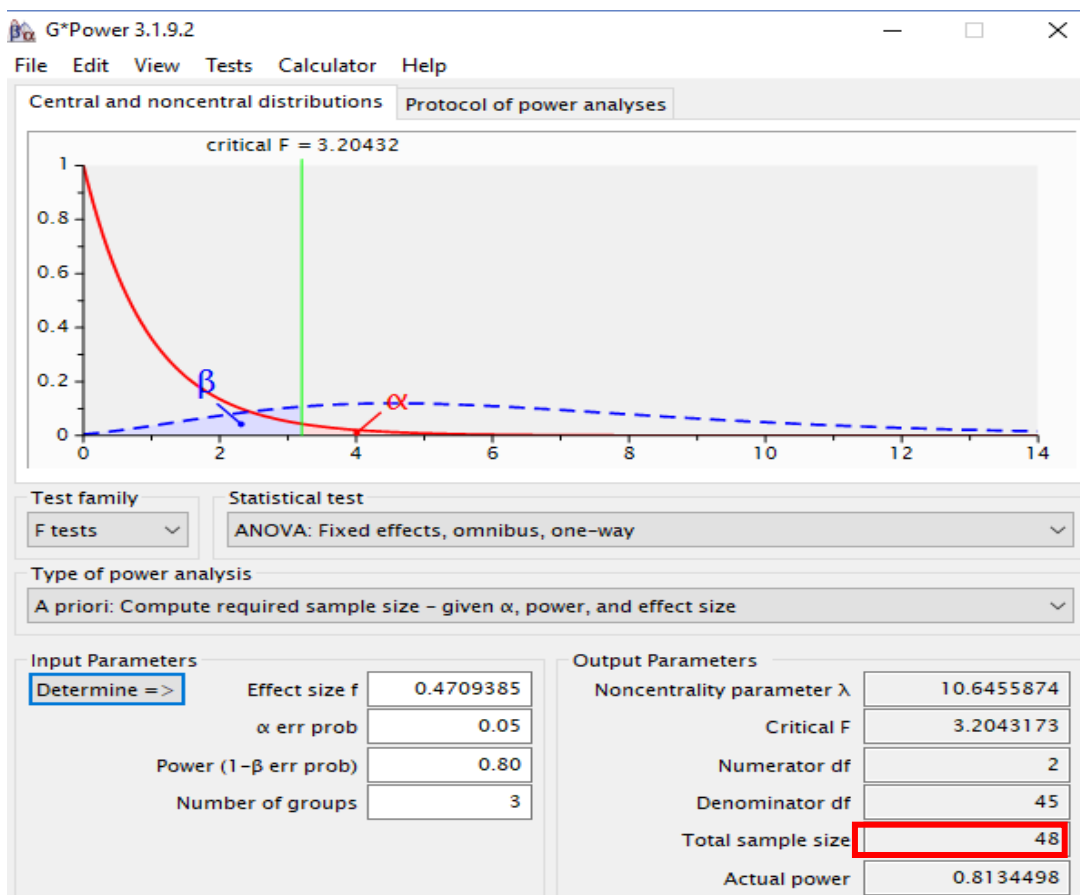
Figure 3.6: Screenshot of the power calculation done using G*Power 3 the red square indicates the sample size necessary to reach significance.

The analysis of the area of the corpus callosum shows a trend toward significance (p=0.072, figure 3.7). The observed power indicated by SPSS is of 0.42 which is quite low as the desired power level is typically 0.80, which would mean that there is 80%. Here, the low observed power indicates that the effect size does not allow to reach statistical significance. To reach a statistical significance and a power level of 0.8, an estimated sample size can be assessed using G*3 Power software (Faul and Erdfelder, 2007; Erdfelder *et al.*, 2009). Knowing the mean of variation of the area for each genotype and the global standard deviation, the analysis indicated that 36 samples (12 per group) would be required to see a significant difference in the total area of the corpus callosum.
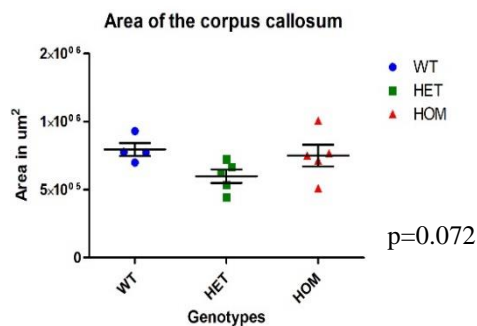
Figure 3.7: Corpus callosum measurements

The measure of the corpus callosum area shows a trend between the genotypes, toward a smaller corpus callosum in the heterozygous mice.

n = 5 animals per group; univariate generalized linear model with Dunnetts' post hoc test. Means ± SEM are indicated.

The analysis of the area of the ventricles indicates that there is a significant difference between the genotypes (GLM p=0.005, figure 3.8, a). Post hoc analysis indicates that the lateral ventricle area is greater in the heterozygotes compared to the wild types (p=0.028), however no difference between the homozygotes and the wild types was found (p=0.343). The analysis of the right ventricles also shows a significant difference in area between the genotypes (GLM p=0.007, figure 3.8, b), confirmed by post-hoc analysis which indicates that the area of the right ventricles of the heterozygotes is larger compared to the wild type (p=0.028) but that again the homozygotes don't significantly differ from the wild types (p=0.629). Likewise, the analysis of the left ventricles shows a significant difference of area between genotypes (GLM p=0.016, figure 3.8, c) but the post-hoc analysis only reveals a trend (p=0.093) between the heterozygotes and the wild type and no significant difference between the wild type and the homozygotes (p=0.352).
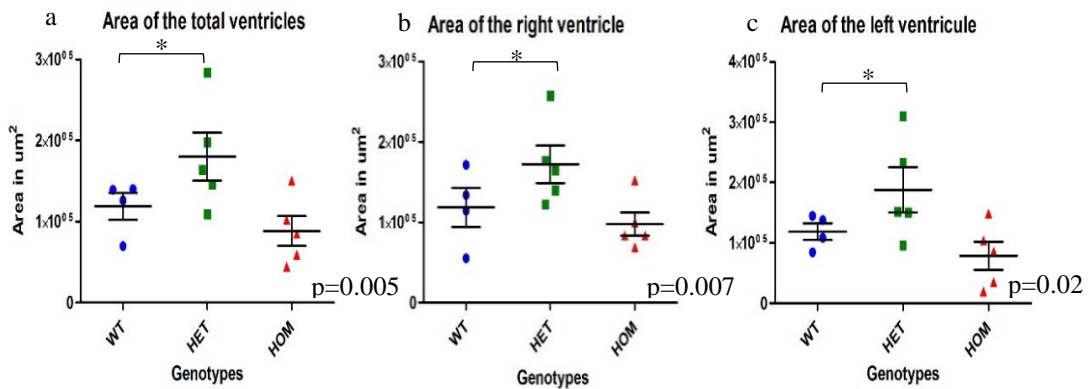
Figure 3.8: Ventricle area measurements

a. Measurement of the total ventricle area shows a significant difference between genotypes indicating larger ventricles in the heterozygous mice. b. Measurement of the right ventricle area shows a significant difference between genotypes indicating larger ventricles in the heterozygous mice. c. Measurement of the left ventricle area shows a significant difference between genotypes indicating larger ventricles in the heterozygous mice. n = 5 animals per group; univariate generalized linear model with dunnetts' post hoc test: * $p<0.05$. Means ± SEM are indicated.

3.2.2 Changes in the hippocampus

After studying those three areas of the prefrontal brain, the hippocampus was analysed. More specifically, the area of the dentate gyrus (DG) was measured and the thickness of the CA1 and the CA3 as well. The sections were stained for Nissl bodies (figure 3.9). The dentate gyrus average area was measured by dividing its area by its length while the thickness of the CA1 and CA3 were respectively measured by assessing three and four measurements of their width (figure 3.9).
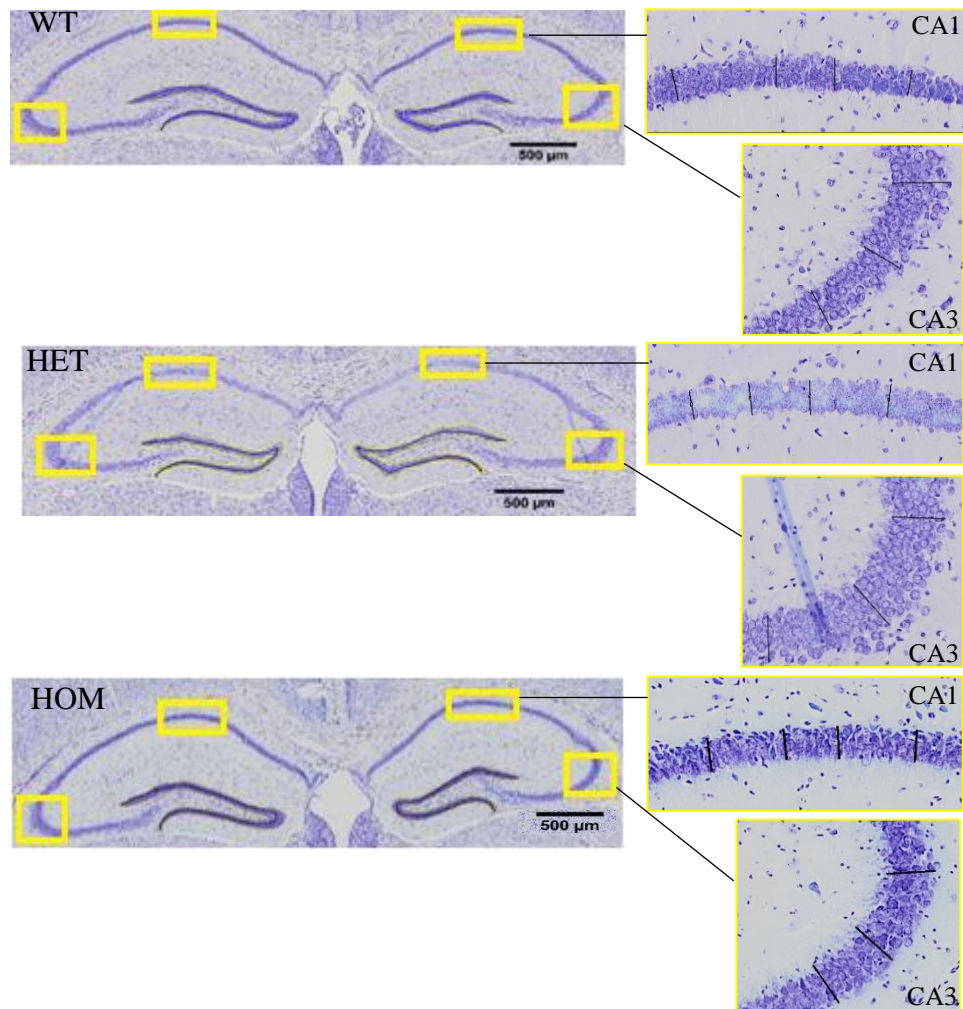
Figure 3.9: Analysis of Nissl brain section of the hippocampus

a. Brain section from a wild type mouse.

c. Brain section from a heterozygous mouse.

b. Brain section from a homozygous mouse.

The yellow ROIs represent the area of the CA1, CA3 and DG measured. Zoom on the CA1 and CA3 box showing how their thickness was measured using several black lines a homozygous mouse.

The statistical analysis was done as previously, using SPSS. Mouse trios were taken into account to evaluate the possibility of a litter effect. A generalized linear model with fixed effect was used. The genotypes and the trios were set as fixed values. The analysis of the dentate gyrus average area showed no significant difference between genotype (p=0.9, figure 3.10, a). Looking at the right and left dentate gyrus average area separately, no significant difference was found either (respectively p=0.7 and p=0.8; figure 3.10, b and c).
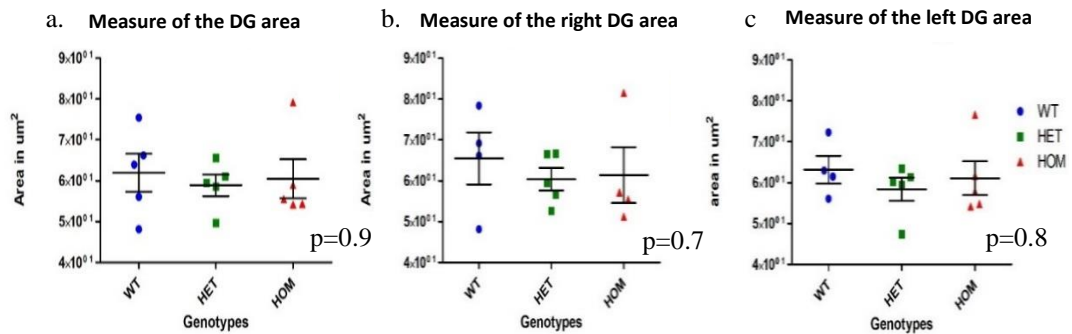
Figure 3.10: Measurement of the dentate gyrus average area

a. Measurement of the global dentate gyrus thickness shows no significant difference between genotypes.

b. Measurement of the right dentate gyrus thickness shows no significant difference between genotypes.

c. Measurement of the left dentate gyrus thickness shows no significant difference between genotypes.

n = 5 animals per group; generalized linear model with fixed effect followed by dunnetts' post hoc test when significant. Mean ± SEM are indicated.

The analysis of the CA1 thickness indicates that there is a significant difference between the genotypes (GLM p=0.027, figure 3.11 a). Indeed, post-hoc testing indicates that the CA1 is thinner in homozygous compared to wild type (p=0.04). No difference was found between the heterozygotes and the wild type. The analysis of the right and left CA1 thickness separately showed no statistical difference between the genotypes (figure 3.11, b and c). To finish, the analysis of the CA3 thickness revealed no difference between the genotypes (p=0.8; figure 3.12, a). Similarly, analysis of the right and left CA3 showed no difference (respectively p= 0.9 and p=0.5; figure 3.12, b and c).
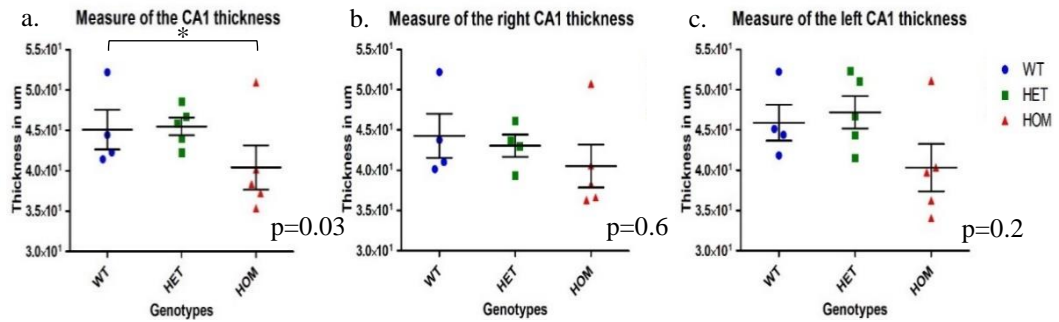
Figure 3.11: Measurement of the CA1 thickness

a. Measurement of the global CA1 thickness reveals a thinner CA1 in the homozygotes compared to the wild types, p=0.027.

b. Measurement of the right CA1 thickness shows no significant difference between genotypes.

c. Measurement of the left CA1 thickness shows no significant difference between genotypes.

n = 5 animals per group; generalized linear model with fixed effect followed by dunnetts' post hoc test when significant: * p<0.05. Values are shown with mean ± SEM.
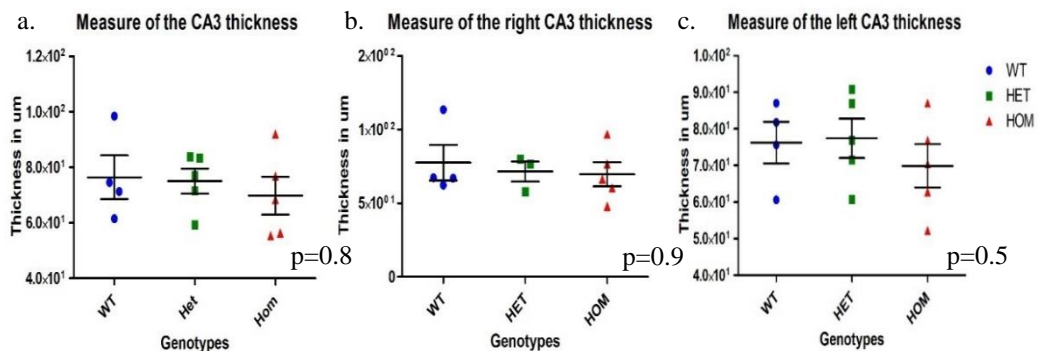
Figure 3.12: Measurement of the CA3 thickness

a. Measurement of the global CA3 thickness shows no significant difference between genotypes.

b. Measurement of the right CA3 thickness shows no significant difference between genotypes.

c. Measurement of the left CA3 thickness shows no significant difference between genotypes.

n = 5 animals per group; generalized linear model with fixed effect followed by dunnetts' post hoc test when significant. Values are shown with mean ± SEM.

3.3 MRI analysis reveals no major structural change

To investigate if those changes also take place in the whole brain of the Der1 mouse model, analysis of magnetic resonance imaging (MRI) was performed.

Six male trios and six female trios of nine weeks of age were used. The brains were enhanced with the contrast agent gadolinium to obtain a better-quality image and were then scanned in pairs in Fomblin, a proton-free medium which produces no magnetic signal and matches the magnetic susceptibility of tissue, reducing scan artefact. One male brain was placed at the bottom of the vial, cerebellum first, followed by one female brain of the same genotype, cerebellum first. The MR images were analysed using a combination of FSL 1, ANTs 2 and in-house C++ software utilizing the ITK library (Avants *et al.*, 2011; Jenkinson *et al.*, 2012; Richetto *et al.*, 2016; Wood *et al.*, 2016). This was done at King's College, London with the help of Robert Chester from Dr Anthony Vernon's laboratory. First, the image of the two brains together was split into two separate images, then these images were registered to the Dorr atlas (Dorr *et al.*, 2008) and a study template image was created. Finally, the study template was registered to the Dorr atlas and to the ROI labels. The olfactory bulbs and the cerebellum were excluded from our analysis as they are difficult to preserve during the dissection and most often damage. The individual images were registered to the study template. This allowed the extraction of ROI volume measurements for all brains (figure 3.13).
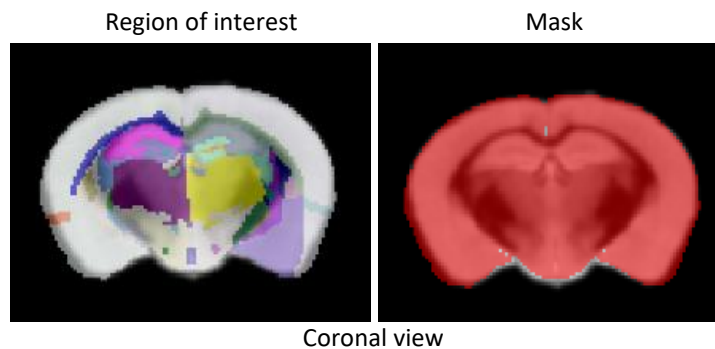
Figure 3.13: Regions of interest

The regions were indicated by the software (left) and a mask was created by

the software to assess whole brain volume (right).

Overall 93 regions of interest were measured across the two hemispheres, and 51 when the hemispheres were studied separately (table 3.1).

Region of interest	
corpus callosum	
medial septum	
lateral septum	
stria terminalis	
mammilo-thalamic tract	
fimbria	
internal capsule	
bed nucleus of stria terminalis	
cerebral peduncle	White matter
posterior commissure	
corticospinal tract or pyramids	
medial lemniscus or medial longitudinal fasciculus	
fornix	
pars posterior anterior commissure	
fasciculus retroflexus	
pars anterior anterior commissure	
optic tract	
ventral tegmental decussation	
periaqueductal grey	
superior olivary complex	
striatum	
stratum granulosum of hippocampus	
dentate gyrus of hippocampus	
globus pallidus	
pontine nucleus	Gray matter
fundus of striatum	
nucleus accumbens	
amygdala	
hippocampus	
pre-para subiculum	
interpedunclar nucleus	
cerebral cortex entorhinal cortex	
mammillary bodies	
thalamus	
pons	Brainstem
midbrain	
medulla	
basal forebrain	
cerebral cortex frontal lobe	
facial nerve (cranial nerve 7)	
stria medullaris	
habenular commissure	
cerebral cortex occipital lobe	
lateral ventricle	
third ventricle	
cerebral aqueduct	
cerebral cortex parieto-temporal lobe	
superior colliculus	
subependymale zone or rhinocele	
inferior colliculus	

Table 3.1: Table of all the brain regions analysed

The grey and white matter as well as the brainstem regions are indicated on the right.

The turquoise highlited regions are the ones constituting the limbic system.

Each ROI value was normalized to its corresponding whole brain volume. Within the 36 mice used for this analysis, two wild-types, one heterozygote and three homozygotes had to be excluded due to brain damage preventing the analysis. The statistical analysis was done applying a general linear model with genotype effect, sex effect and the littermate effect as fixed factors.

First, the overall brain volume was analysed to see if there was any change between the genotypes. Then all the brain regions detected by this method were analysed together, for whole brain or considering each hemisphere separately. Finally, the volumes of additional regions of interest were investigated.

3.3.1   Whole brain volume analysis

The volumes of the whole brains were compared between heterozygous, homozygous and wild-type mice. The sex effect was also studied. A univariate general linear model statistical test was performed in SPSS. The analysis was done considering the genotype and the sex as fixed effects. The results showed that there was no difference in the brain volumes between the three genotypes (GLM p=0.859, figure 3.14, a). Moreover, no sex effect on the brain volumes was discovered (p=0.523). To confirm that there was no difference between the genotypes regardless of the sex, males and females were investigated separately (figure 3.14, b).
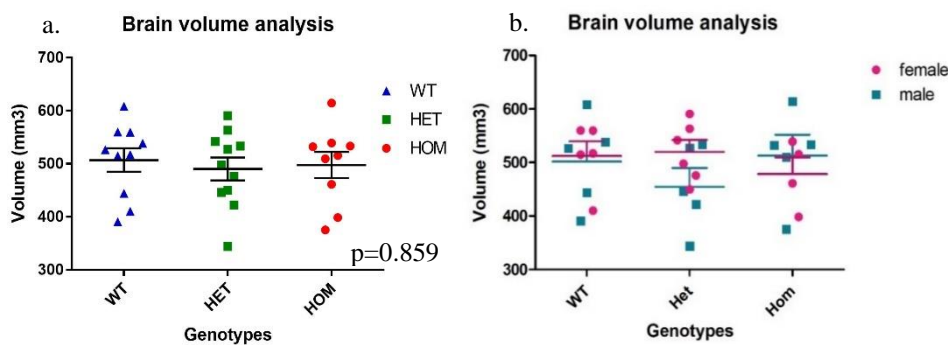
Figure 3.14: Whole brain volume measurement

a. Brain volume is not affected by genotype. The graph represents the distribution of the whole brain volume in each group. b. Brain volume is similar between male and females. Generalized mixed linear model, univariate analysis. Values shown are mean ± SEM

The genotype was defined as fixed effect and the trios as fixed effect. The results indicate that there is no genotype effect in the male's analysis (p=0.583) as well as in the female's (p=0.273). Therefore, the result of the analysis of the whole brain volume show no difference between the genotype and between the sex.

3.3.2   Volume analysis of multiple brain regions

a.   No changes in the 93 brain regions of both hemispheres

The volumes of the 93 regions normalized to their respective total brain volumes, were compared between wild-type, heterozygotes and homozygotes. A multivariate general linear model analysis was performed using SPSS. A Wilks' lambda statistical test was used to assess p values. The first statistical analysis was performed considering the genotype and trios as fixed factors. The results show that there is no significant difference in the volumes of the regions of interest between the genotypes (p=0.198). To investigate the effect of the sex on the volume of the 93 regions of interest between genotypes, the same statistical analysis was repeated considering the sex also as a fixed factor. The results show no overall difference of volume between the genotypes (p=0.103). This demonstrates that the sex doesn't have an influence over the volume of these regions, between the genotypes.

Overall, there were no changes in the volume of the 93 brain regions of interest between genotypes, when both hemispheres were considered separately.

b.   No changes in the overall 51 brain regions

When adding the two hemispheres together, 51 regions of interest were obtained as some areas cannot be separated in two, such as the corpus callosum. Similar to what was done for the 93 regions of interest, the volume of the 51 regions was compared between wild-type, heterozygotes and homozygotes. A multivariate general linear model analysis was performed using SPSS, and a Wilks' lambda statistical test was used to assess p values. First, the genotype and the trios were set as a fixed factor. The result shows that there is no significant difference in the volumes of the regions of interest between the genotype (p=0.376). The same statistical analysis was repeated with the sex set as fixed factor. The results show no significant differences in the volume of the 51 regions between genotypes (p=0.368).

Overall, there were no changes in the volume of the 51 brain regions of interest between genotypes, when both hemispheres were considered together.

3.3.3 Analysis of additional regions of interest

To finish, several areas were grouped together in order to look at related brain features. Several publications have shown that multiple brain areas are affected by mental illness, for example, brainstem morphometric alterations are reported to be associated with first-episode schizophrenia (Källstrand *et al.*, 2012; Hirjak *et al.*, 2013). Numerous studies have also indicated a reduction of grey matter volume in schizophrenic patients in the frontal cortex, temporal lobe, and insula with evidence of progression over time (Vita *et al.*, 2012; Yue *et al.*, 2016). A similar abnormality was found in the white matter which is believed to indicate a reduction of myelination (Kubicki, McCarley and Shenton, 2005; Lener *et al.*, 2015). The limbic system is composed principally of the hippocampus, hypothalamus, amygdala, anterior thalamic nuclei, fornix, mammillary body, septum and entorhinal cortex. It is very important for the process of emotions and memories and also affected in mental disorders.

Here, the brainstem, the limbic system, the grey and white matter were investigated. The same statistical test was used to analyse those regions and determine if their combined volume was different between the three genotypes. The genotype, sex and trio were considered as fixed variable. The statistical analysis indicated that there was no significant difference in the volume of the brainstem (table 3.1) between genotype (p=0.45, figure 3.15). The limbic system (table 3.1) analysis indicates no difference of volume between the genotype (p=0.26, figure 3.15). The white matter (table 3.1) analysis revealed no difference of volume between the genotypes (p=0.55, figure 3.15). The grey matter (table 3.1) analysis shows no difference of volume between the genotypes (p=0.66, figure 3.15).
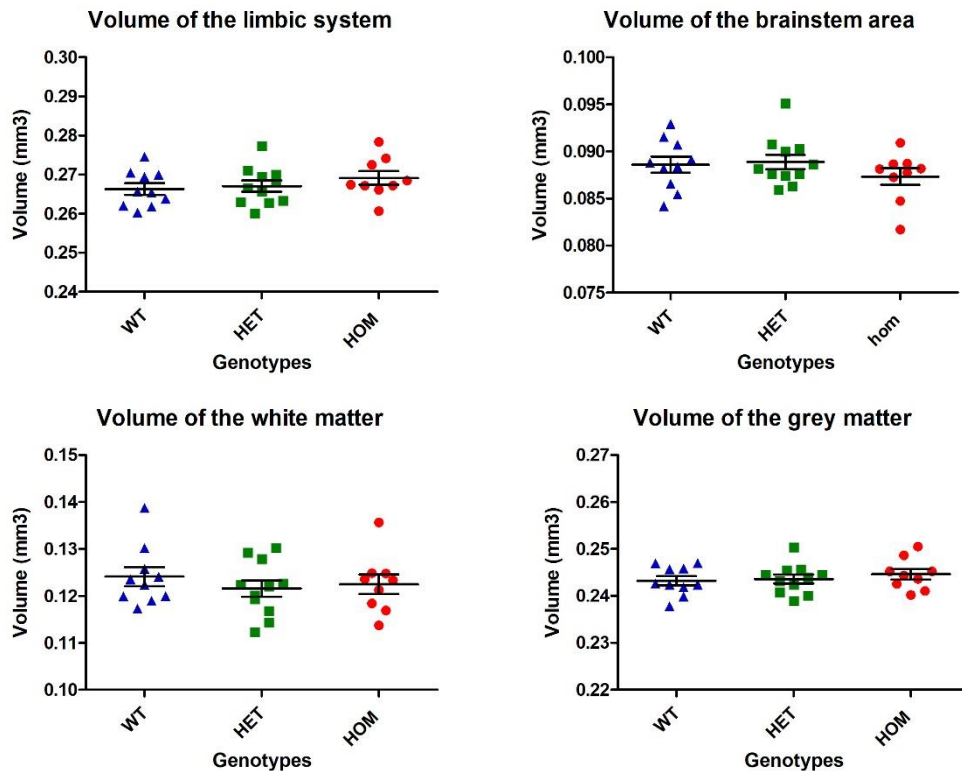
Figure 3.15: Analysis of four additional brain areas

a. No change in the volume of the limbic system between the genotype.

b. No change in the volume of the brainstem area between the genotype.

c. No change in the volume of the white matter between the genotype.

d. No change in the volume of the grey matter between the genotype.

Generalized linear model, univariate analysis. Values have been normalized to the corresponding brain volume. Values shown are mean ± SEM. WT n=10, HET n=11, HOM n=9

3.4 Littermate effect

Littermate effects could indicate that in-utero environment and/or sample handling influence the volumetric variation between animal and therefore should be analysed as a possible variable causing important changes (Holmdahl and Malissen, 2012). Indeed, studies have revealed that mice behaviours are variable depending on the litter they come from (Lazic and Essioux, 2013). Here the littermate "main effect" was assessed in the histological and MRI analysis using SPSS software. The p-value was obtained using the Wilk's lambda tests as mentioned in the material and methods.

Histological analysis of the cortical area indicates that in utero environment or sample handling does not have a significant effect, although there is a trend (p=0.065) toward a

possible effect (table 3.2). There is also an effect on the ventricle area indicating that depending of the litter the ventricle seems to have a different volume (p=0.02, table 3.2). There was no evidence for littermate influences upon other brain regions analysed by histology (table 3.2).

Overall then, the histological analysis revealed little evidence for a littermate effect. This suggests that in-utero environment and sample handling don't substantially influence the results of the volumetric analysis. The ventricles, however, may be particularly sensitive to sample handling effects because they are essentially fluid-filled spaces rather than solid tissue.

MRI analysis indicated strong litter effects on several measures (table 3.2), confirming the need to use littermates and to take individual litters into account to ensure that the results obtained reflect changes due to the different genotypes only. The discrepancy between the histological and MRI analyses, which used samples generated at different times, may indicate that the effects are mainly due to sample handling rather than differing in utero environments.

Analysis	ROI	Litter effect
Histology	Cortical layer	Trend (p=0.065)
	Corpus callosum	No (p=0.2)
	Ventricle	Yes (p=0.02)
	DG	No (p=0.3)
	CA1	No (p=0.5)
	CA3	No (p=0.3)
MRI	Whole brain	Yes (p=0.0001)
	93 ROI	Yes (p=0.001)
	51 ROI	Yes (p=0.01)
	Brainstem	No (p=0.23)
	Limbic system	Yes (p=0.03)
	White matter	Yes (p=0.001)
	Grey matter	No (p=0.59)

Table 3.2: Litter effect on the different analysis performed in this chapter

There is a strong litter effect observed in the MRI analysis.

3.5 Discussion

The analysis of brain structure using histology and MRI techniques has demonstrated very subtle changes, at most, in both the homozygous and the heterozygous mutant mice.

The histology analysis indicates that there are differences between the genotypes however the homozygotes and the heterozygotes do not exhibit the same differences when compared to the wild type. The results indicate that the heterozygotes have a larger ventricle area compared to the wild type (figure 3.7, a) while the homozygotes have a thinner CA1 compared to the wild type (figure 3.10, a.). Additionally, a trend toward a change in the cortical thickness has been found between the genotypes (figure 3.5, a) and a trend toward a change in the corpus callosum was found as well (figure 3.6). Ventricular enlargement is consistent with cytoarchitectural abnormalities reported in multiple *DISC1* transgenic mice (Pletnikov *et al.*, 2008; Shen *et al.*, 2008) (table 1.1) and in schizophrenic patients (Johnstone *et al.*, 1976; Nesvåg *et al.*, 2008). This feature could be the result of altered neuronal outgrowth or number of neurons in the heterozygous Der1 mice. This may also indicate neurodegenerative changes and neurodevelopmental impairments in the heterozygous mice. The modified ventricular shape/volume in heterozygous Der1 mice could be representative of altered brain development which could lead to impaired circulation of the cerebrospinal fluid (CSF) (Ming and Song, 2011). This could also affect the distribution of the cells around the ventricles, leading to defects of the white matter fibre tracts in the corpus callosum, which would affect the communication between the different regions of the brain. Moreover, ependymal cells of the sub-ventricular zone could be affected by the modification of the shape of the ventricles, which could lead to a dysregulation of the production of the CSF. These cells are also a source for neurogenesis (Ming and Song, 2011), therefore their modification could lead to the impairment of neurogenesis in the PFC. The modification of the lateral ventricles in the heterozygous mice could indicate an impairment of neurogenesis process in those mice. A thinner CA1 was also found in schizophrenic patients with stronger positive symptoms (Musso *et al.*, 2012). Moreover, a study showed a reduction of CA1 volume in persistently symptomatic ultra-high risk patients (Ho *et al.*, 2017). The CA1 is believed to be involved in the pathogenesis of hallucinations and delusions, core symptoms in schizophrenia (Zierhut *et al.*, 2013). Structural modification of the CA1 could reflect a pyramidal cell disarray which has been found in post mortem studies of schizophrenic patients and could indicate an impairment of the neuronal migration (Altshuler *et al.*, 1987; Casanova and Rothberg, 2002). Moreover, synaptic pathology in CA1 has also been associated with schizophrenia. Additionally, the pyramidal cell size was found reduced in bipolar disorder (Liu *et al.*, 2007). A DISC1 knock out model

study also showed that Disc1 suppression impairs migration of the CA1 pyramidal neurons (Tomita *et al.*, 2011) (table 1.1). Thinning of the CA1 could reveal a decrease in neuronal density in this area in the homozygous mice. Moreover, this could also be an indication about impairment of neuronal morphology which could affect the shape and the volume of these areas. Added to our results, this supports the importance of the CA1 in mental disorder and confirm that Disc1 is required for the formation of the CA1 during hippocampal development. A study investigating the cortical thickness of t(1;11) translocation carriers indicated a reduction in cortical thickness of these individuals (Doyle *et al.*, 2015). This result adds to our findings indicating that the disruption of *DISC1* might lead to a modification of the cortical thickness. Therefore, impairment in neuronal neurogenesis, migration and positioning could lead to a modification of the shape of these different regions. And lastly, increase of apoptotic activity could lead to cell loss or synaptic/dendritic loss which could also affect the volume and shape of these areas. Involvement of the apoptotic mechanism as well as reduced neuronal density, migration and positioning and change in neuronal morphology have been observed in in the pathophysiology of schizophrenia (Jarskog *et al.*, 2005a; Boksa, 2012; Muraki and Tanigaki, 2015; Iannitelli *et al.*, 2017) and DISC1 has also been found to be implicated in these mechanisms (Ishizuka *et al.*, 2006; Thomson *et al.*, 2013). This could therefore be plausible in the Der1 model and linked to the subtle structural changes observed. Overall, these results suggest that the mutation affects the homozygous and heterozygous Der1 mice in different ways. The differences observed between the heterozygotes and the homozygotes could be due to the possible presence of a chimeric protein which could be expressed on its own in the homozygous mice or could interact with the full length DISC1 in the heterozygous mice, causing heterozygote-specific dominant-negative effects. Otherwise, the imbalance of DISC1 concentration could lead to a different phenotype compared to not expressing the protein at all. So far, no Disc1 mouse model indicated that the heterozygous mice could be more severely affected than the homozygous, indeed they either similarly affected or the homozygous are slightly more affected (Koike *et al.*, 2006; Kvajo *et al.*, 2008, 2011; Lepagnol-Bestel *et al.*, 2013) (table 1.1). To understand this mechanism, the discovery of the presence of the chimeric proteins in the Der1 mice is necessary. For now, the antibodies used to confirm their presence did not lead to conclusive results, better antibodies might be necessary to conclude on the existence of these chimeric proteins. However, the MRI analysis does not indicate any significant differences in the volume of the regions of interest between the three genotypes. It is possible that the histology analysis reflects what is happening in the brain at a local level. The differences seen in the ventricles and CA1 using histology but not with the MRI could indicate a change in the shape of these regions in the mutant mice, or a

very localised volumetric change. Nevertheless, it is important to highlight that the MRI demonstrates clear litter effects (table 3.2). Indeed, results indicate that in utero environment or handling processes might affect whole brain volume as well as white matter and limbic system. The litter effect is mostly observed in the MRI analysis which suggests that this would rather be a sample handling issue. As a consequence, the significant effect found on the ventricles could be due to sample handling as ventricles might be the most susceptible to this effect. Additionally, analysis of the multiple brain regions acquired using MRI (tables 3.2) were also affected by this variable. While, the histology analysis revealed that there was a litter effect only in the ventricular investigation (table 3.2). The litter effect could be one of the reasons why histology and MRI leads to different results. Additionally, the lack of correlation between the two analyses could be due to the fact that a larger number of mice were used for the MRI analysis (10-12 per group), which is carried out at the whole brain level. In contrast, half that number were used for the histology (5 per group) which was carried out at a very localized level using two sections per brain region. Volumetric analyses carried out at the whole brain level using MRI are most likely more accurate than area quantification done using a couple of sections. Previous analysis of *DISC1* mouse models revealed numerous changes in their brain structure. Histology analysis of a transgenic mice model expressing two copies of truncated *DISC1 (*DISC1tr Hemi) encoding the first 8 exons indicated reduced thickness of the cortex and reduced corpus callosum development (Shen *et al.*, 2008; Dawson *et al.*, 2015) (table 1.1). MRI analysis of mouse models carrying *Disc1* missense mutations (L100P and Q31L) also revealed changes in brain structure of these mice. Indeed, reduction of brain volumes especially of the cerebellum, cortex, and thalamus was observed (Clapcote *et al.*, 2007) (table 1.1). Enlarged ventricles have also been shown using MRI, in transgenic mice in which human *DISC1* expression is induced in forebrain regions, although in at least one model this was reported to be developmental stage-specific (Hikida *et al.*, 2007; Pletnikov *et al.*, 2008) (table 1.1). It is possible that, the changes found in previous DISC1 mouse models are due to alterations, such as transgenic overexpression of truncated *DISC1*, that are more deleterious than the *Der1* mutation. Similarly to the findings from the DISC1 mouse models, brain structure analysis of schizophrenic patients more consistently shows deficits in whole brain volume, and ventricular enlargement (Harrison, 1999; Ross *et al.*, 2006). Moreover, reduction of the volume areas such as the hippocampus, corpus callosum, the prefrontal cortex and the thalamus have also been found in patients suffering from schizophrenia (Shenton *et al.*, 2001; Ellison-Wright *et al.*, 2010). Additionally, shape abnormality of the hippocampus has been revealed in schizophrenic patients which indicate a deformation of this region (Styner *et al.*, 2004; Kalmady *et al.*, 2017). Similar analysis of ventricular shape indicated a difference

in the shape of the lateral ventricle of schizophrenic patients while no volume change was found (Styner *et al.*, 2005). T(1;11) translocation carriers were found to have a decrease in cortical thickness, a reduction of white matter integrity underlying impairments of corpus callosum development, ,reduced gyrification in prefrontal cortex, but no ventricular enlargement (Doyle *et al.*, 2015; Whalley *et al.*, 2015; Thomson *et al.*, 2016). These features correlated with general psychopathology of mental illness. However, volumetric changes of the cortical thickness and the corpus callosum were not observed in the Der1 mouse model which indicates that this new model does not exactly reproduce the structural effects of the human translocation found in the Scottish family. But it is important to take into account the fact that the Der1 mouse model was studied at 9 weeks of age which correspond to early adulthood in humans; which is not equivalent to the age at which the translocation carriers were analysed as they were between 45 and 65 years old. Moreover, drug treatments taken by the carriers, or their exposure to environmental risk factors could lead to confounding effects. Indeed, gene expression and brain development is influenced by the environment (Tung and Gilad, 2013; Romero, Ruvinsky and Gilad, 2014; Ziats, Grosvenor and Rennert, 2015). Additionally, human and mouse brain are structurally quite different and human and mouse genomes are not exactly the same. *DISC2* and *DISC1FP1* which are disrupted by the translocation in humans, are not present in mice. Moreover, the transcript created by the human translocation on chromosome 11 is not present in our model, while this transcript is not translated its presence could have an impact on cellular process as it could act as a non-coding gene and perturb the regulation of other genes (Rinn and Chang, 2013; Romero-Barrios *et al.*, 2018). Mouse and human genomes share 85% of their protein coding regions (NIH, www.genome.gov, 2010). However, non-coding genes and regulatory regions are not as conserved. Those regions are potentially important in psychiatric disorders as they have been found to regulate the expression of genes involved in those disorders (Xiao, Chang and Li, 2017). Moreover, the surface of the mouse brain is known to be smooth, while the human brain is more complex with numerous sulci and gyri. Furthermore, environmental context can change gene expression patterns and brain structure in human and mouse. Finally, evidence has recently been found for the presence of genetic modifiers in the translocation family (Ryan et al, Molecular Psychiatry, under revision). It is conceivable that the effects of these modifiers contribute to the differences between the human translocation carriers and the Der1 mouse. Altogether these observations underline some of the limitations of using mouse models for understanding psychiatric disorders and indicate that future studies of the Der1 mouse should incorporate effects of stressors and drug treatments.

CHAPTER 4

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EFFECT OF THE DER1 MUTATION ON NEURONAL CELLS IN TWO DIFFERENT BRAIN REGIONS

4.1 Introduction

Patients suffering from mental disorders are characterised by brain impairments at a cellular level. In patients diagnosed with schizophrenia and major depressive disorder increased cell density in the cortex and hippocampus was found (Stockmeier *et al.*, 2010; Smiley, Konnova and Bleiwas, 2012b; Cotter *et al.*, 2014). While in bipolar disorder a reduced neuronal density was discovered (Rajkowska, Halaris and Selemon, 2001). Post mortem studies of bipolar patients show decreased cell number and density of GABA neurons in the cortex while a decrease of parvalbumin-positive neurons was found in the hippocampus of schizophrenic patients (Zhang and Reynolds, 2002; Pantazopoulos *et al.*, 2007), which indicates that specific groups of neurons are affected in mental illness. Additionally, schizophrenic patients showed reduced proliferation of hippocampal neural stem cells and impaired neurogenesis (Reif *et al.*, 2006; Allen, Fung and Shannon Weickert, 2016; Iannitelli *et al.*, 2017).

Similar impairments have been found in *DISC1* mouse models such as reduced cortical neurons, abnormal neuronal migration, decreased neurogenesis and reduced number of parvalbumin-positive interneurons (Hikida *et al.*, 2007; Shen *et al.*, 2008; Kim *et al.*, 2011; Lee, Fadel, *et al.*, 2011).

These findings suggest that similar impairments could be found in the Der1 mice. Here investigation of cellular deficits in these mice was performed so as to gain a more comprehensive understanding of how the translocation affects cellular organization in the brain. This will help us to characterise this mouse model and determine if it can be used as a model of mental illness. For this, immunohistochemistry and immunofluorescence techniques were used. For each analysis, the three groups (heterozygote, homozygote, wild-type) were composed of five males. For each animal, two 10μm paraffin embedded brain sections from the prefrontal cortex (PFC) and from the hippocampus were stained for Nissl, Parvalbumin, activated caspase-3 and DCX/BrdU. Statistical analysis was done using SPSS and considering the fact that the mice belong to littermate trios to evaluate the possibility of a litter effect. A generalized mixed linear model was used, both genotypes and trios were set as fixed variables.

4.2 Cellular density analysis in three different brain regions of the Der1 mice

A prominent feature of mental disorder is alteration of neuronal density which has also been found in mouse models of mental disorder (Brennand *et al.*, 2011; Lee, Fadel, *et al.*, 2011). To determine if there was any loss of cells in Der1 mouse PFC and hippocampus, sections were Nissl stained with cresyl fast violet. Nissl bodies are part of endoplasmic reticulum with ribosomes, where proteins are produced. Cresyl fast violet stains the RNA of these ribosomes. Cells were counted in a square of 1122 µm X930 µm in the PFC (figure 4.1), and in a 100 µm^2 square in the CA1 of the hippocampus and 200 µm^2 the srlm (stratum radiatum and lacunosum molecular layer) (figure 4.2). Cells were counted manually using the cell counter plug in on Fiji (figure 4.2).
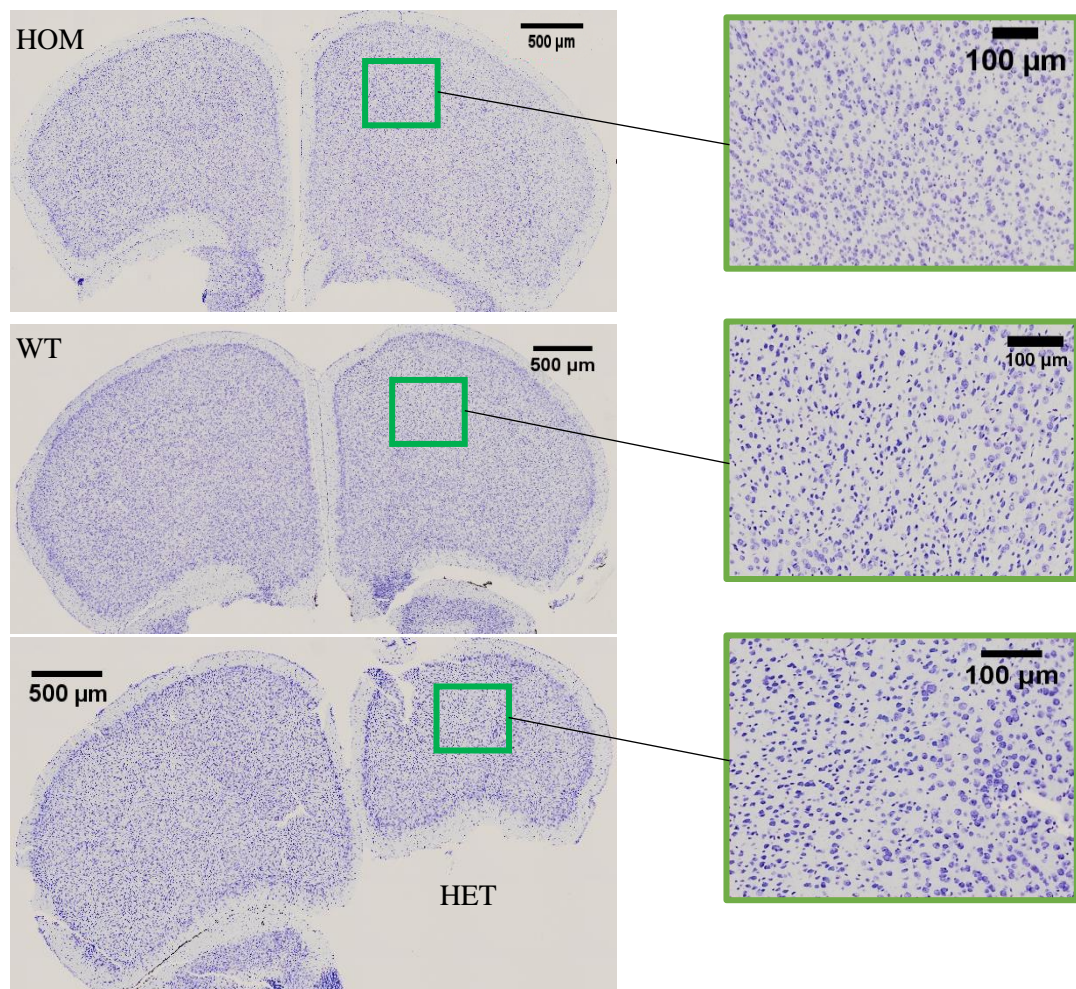
Figure 4.1: Image of the PFC stained with Nissl.

One section of each genotype is represented. The green square shows the area where cells were counted. The area is zoomed out.

a.
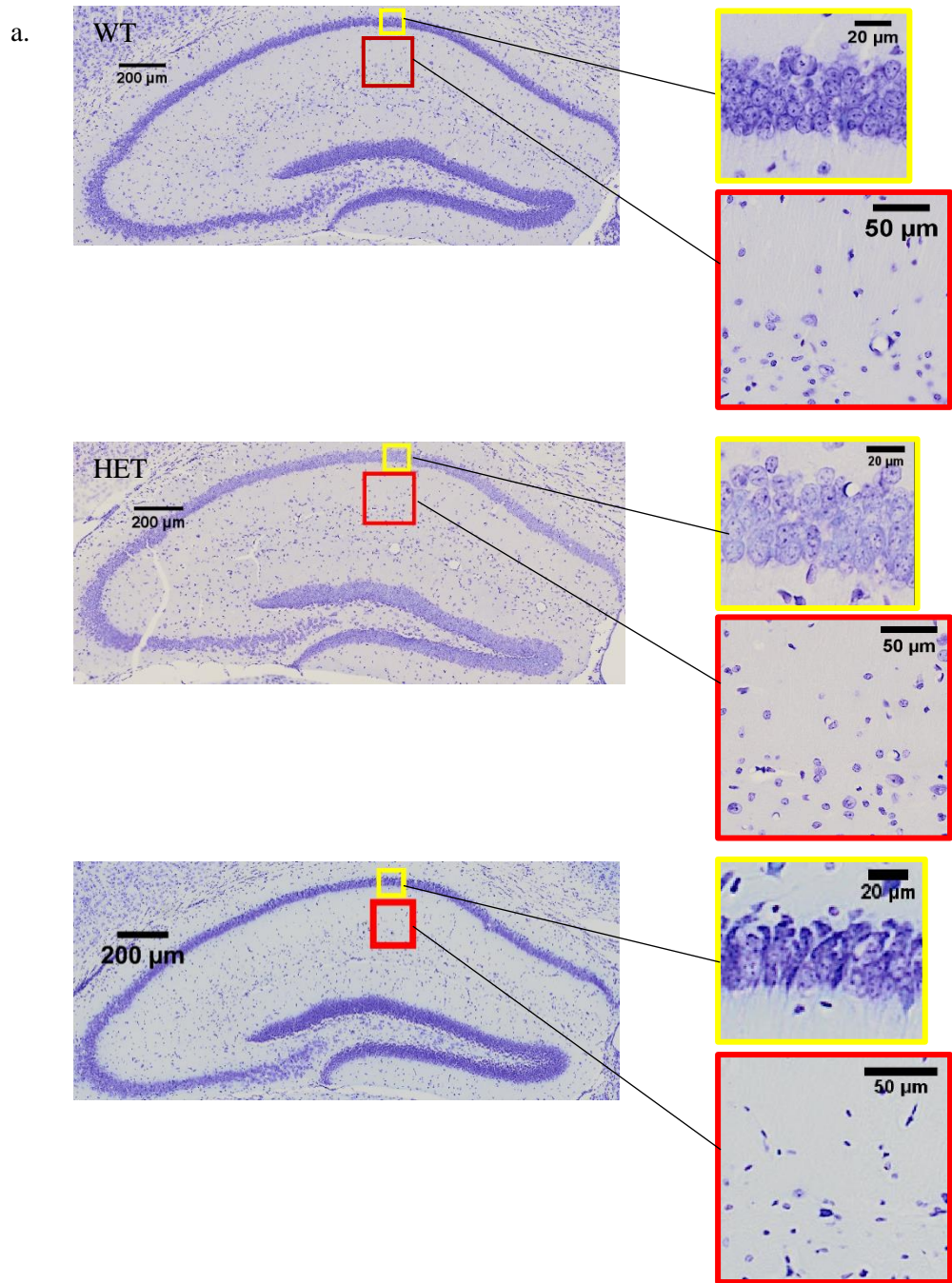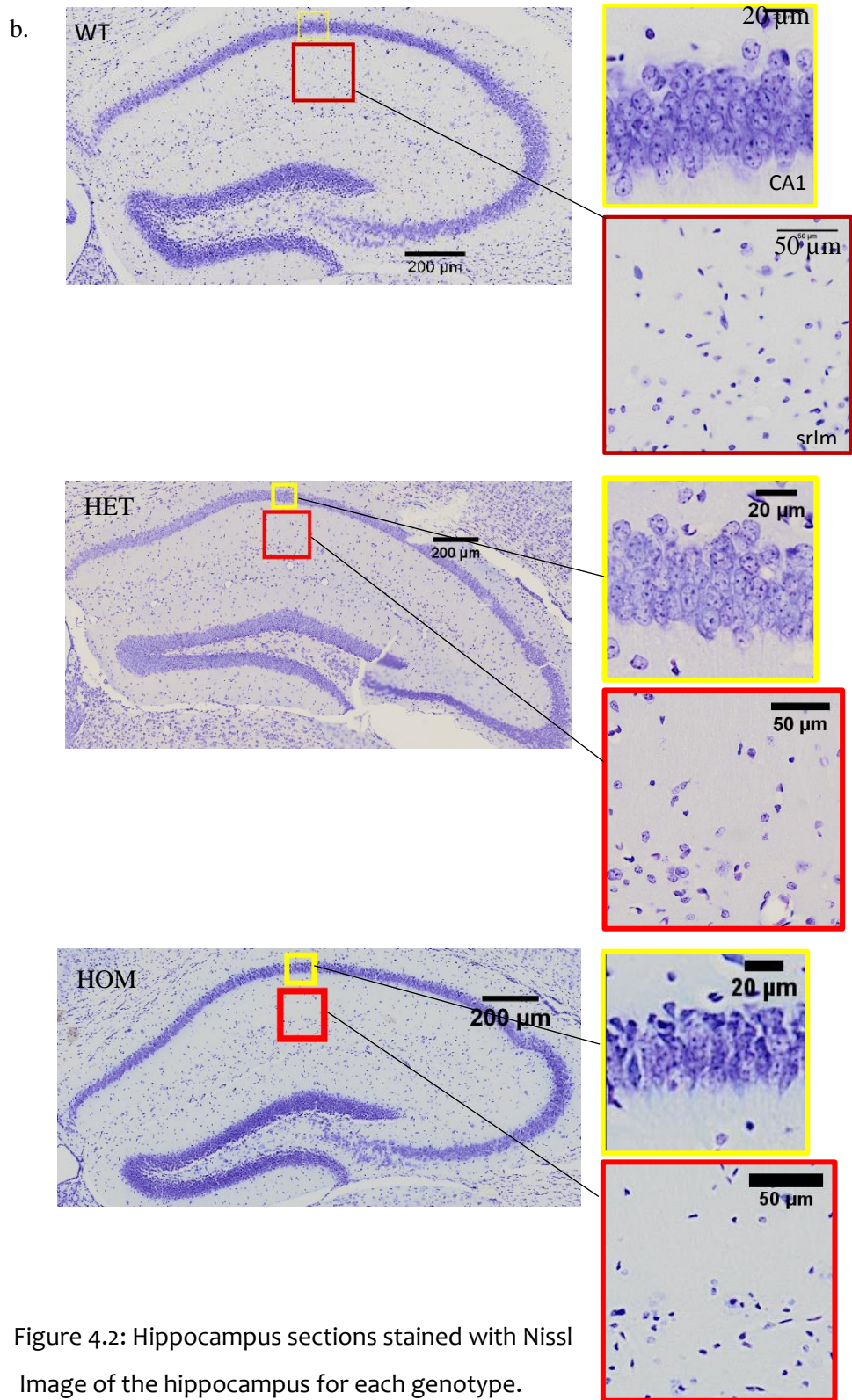
Figure 4.2: Hippocampus sections stained with Nissl
Image of the hippocampus for each genotype.

a. right hippocampus, b. left hippocampus

The yellow and red squares represent the areas where cells were counted.

Analysis of cellular density in the PFC showed no difference between genotype (p=0.4) (figure 4.3). Additionally, the left and right hemisphere were analysed separately, and no significant differences were found between the genotypes (figure 4.3).
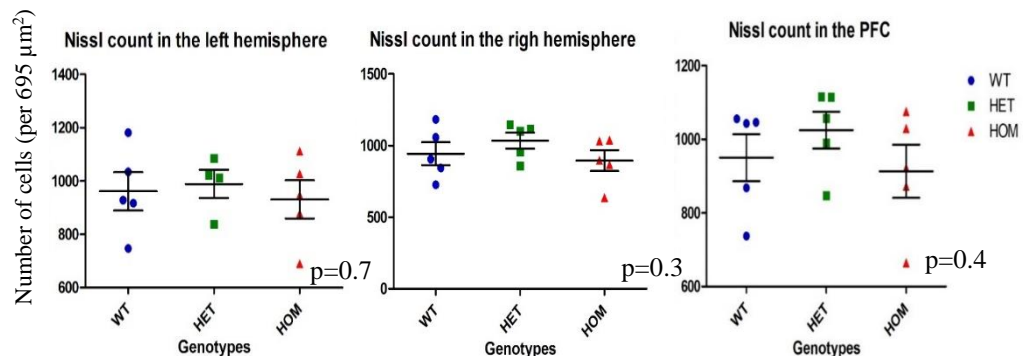
Figure 4.3: Cellular density analysis in the PFC

The analysis was done in an area of 695 μm², considering the left and right hemisphere as well as total brain. No difference was found between the genotypes.

n = 5 mice for each genotype; Generalized linear model with fixed effect. Values shown are mean ± SEM.

The analysis of the neuronal density in the stratum radiatum and lacunosum molecular (srlm) and CA1 showed no difference between genotypes (figure 4.4) (respectively p=0.8 and p=0.3). The analysis of the right and left hemisphere also indicated no difference of cellular density between genotypes (figure 4.4) in the hippocampus, except for the left srlm analysis (p=0.045). Indeed, the result indicates that the cellular density is increased in the homozygotes compared to the wild-type mice (p=0.007, figure 4.4).

The findings reveal that there is no significant change in cellular density in the PFC or in the CA1, while a subtle increase was discovered in the left srlm of the homozygous mice. This last discovery could indicate an asymmetrical neurodevelopment in the homozygous mice, possibly revealing an impaired neuronal migration in the left hemisphere. Although subtle, these results could be seen as similar to findings in schizophrenia and bipolar disorder patients and in animal models of schizophrenia as higher neuronal density has been previously reported in the hippocampus in a few studies, however other studies did not discover changes in overall cellular density.
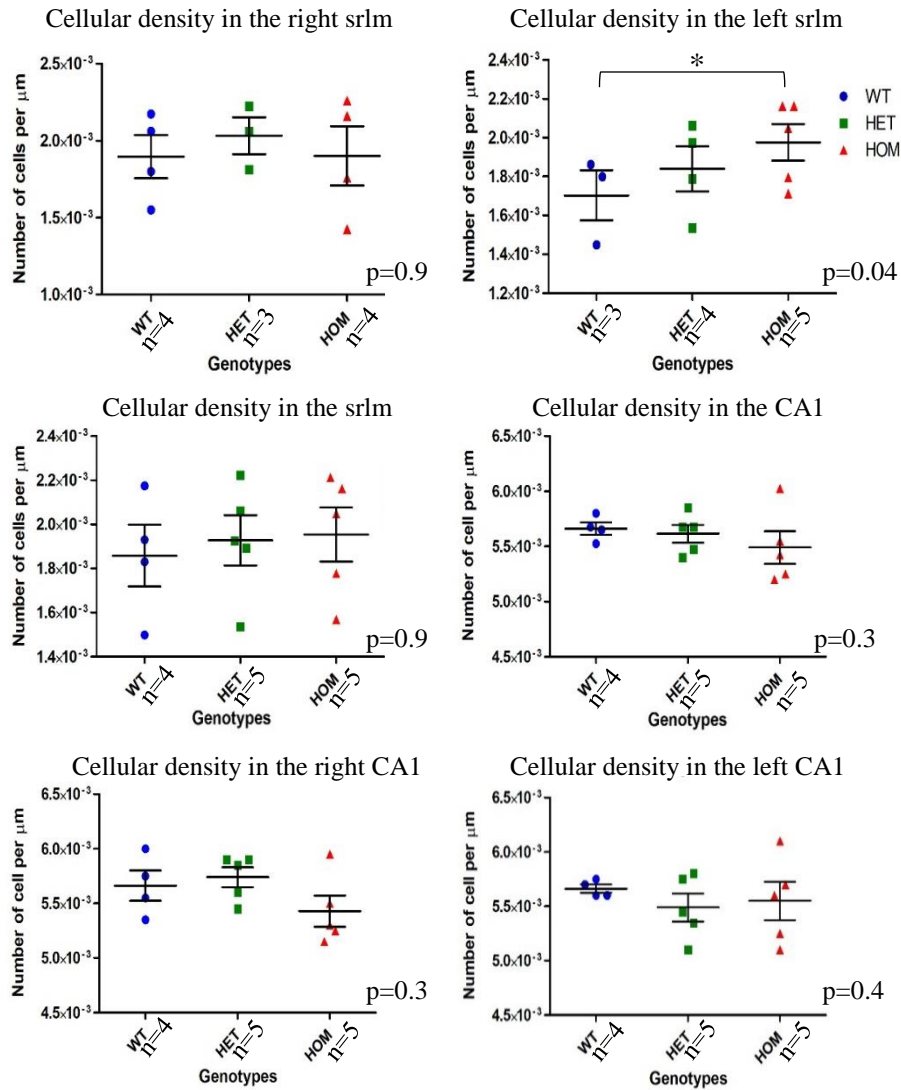
Figure 4.4: Cellular density analysis in the hippocampus

Analysis of the cellular density shows no difference between the genotypes except in the left srlm where the density in the homozygous mice is increased compared to the wild type.

n = 5; in some case the n number is reduced due to faulty sections.

generalized mixed linear model with fixed effect followed by dunnetts' post hoc test when significant: * p<0.05Values shown are mean ± SEM.

4.3 Interneurons density analysis in the Der1 mice

Numerous studies on schizophrenic patients have shown a decrease of interneurons especially in the prefrontal cortex and the hippocampus (Kalus, Senitz and Beckmann, 1997; Jiang, Cowell and Nakazawa, 2013). Moreover, other Disc1 model such as the L100-P model reported a decrease of parvalbumin staining, indicating a decrease of interneurons (Shen *et al.*, 2008; Lee *et al.*, 2013; Lipina and Roder, 2014). To find out if there is a similar impairment in the GABAergic pathway of the Der1 mice, the expression of parvalbumin neurons in the prefrontal cortex and the hippocampus was investigated. Parvalbumin is a calcium binding albumin protein present in GABAergic neurons. The PFC was separated into different regions of interest following anatomical features and the Mouse brain atlas in stereotaxic coordinates (Paxinos *et al.*, 2004): prelimbic cortex (prl), frontal association cortex (fra), ventral orbital cortex (vo), medial orbital cortex (mo), lateral orbital cortex (lo) dorsolateral orbital cortex (dlo) (figure 4.5). The PFC was also analysed as one entity. In the hippocampus, the area of the CA1 and the DG were drawn using anatomical cues to count PV positive cells separately, then the cells were counted in the whole hippocampus (figure 4.6). All the counts were made using the cell counter plug in of Fiji software.
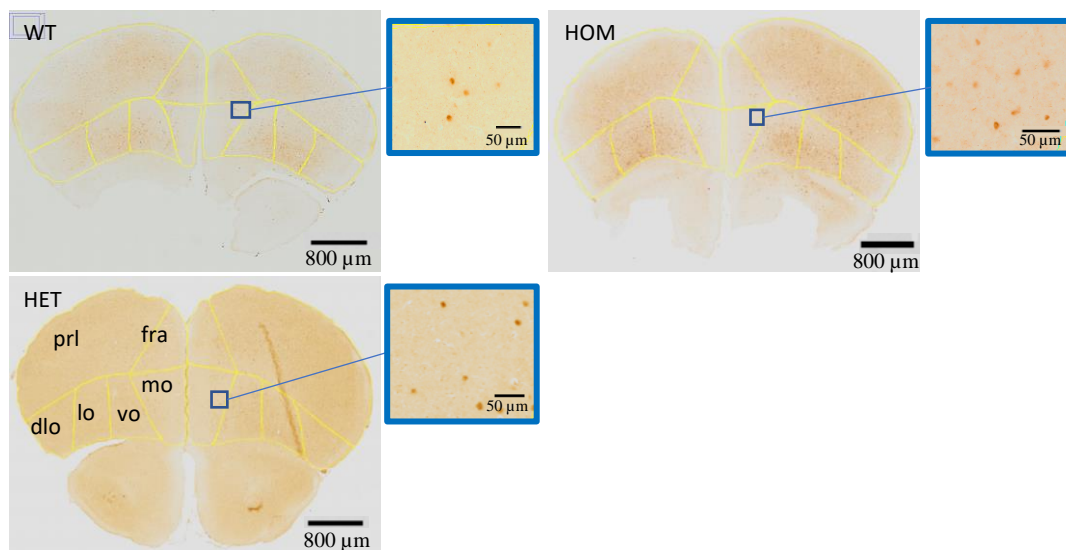
Figure 4.5: Representation of the PFC stained for parvalbumin

Images showing positive PV cells for each genotype: WT: wild type; HOM: homozygote; HET: heterozygote. Yellow lines outline the different area of the prefrontal cortex. The blue squares show zoomed areas containing PV stained cells.

a.
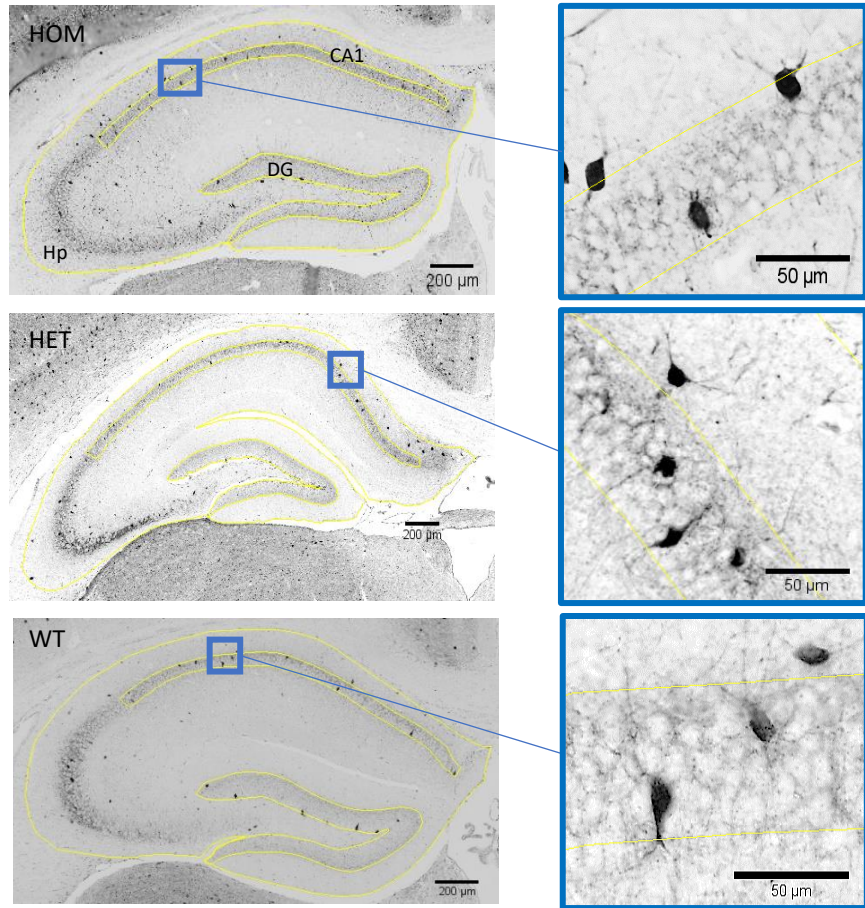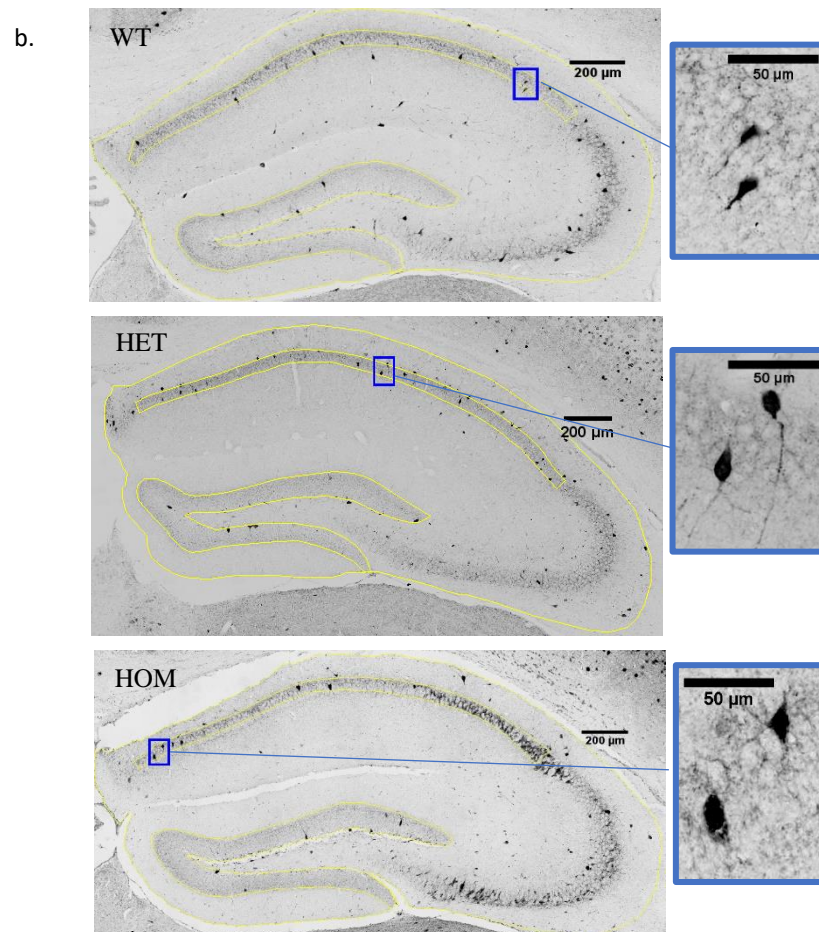
Figure 4.6: Representation of the hippocampus stained for parvalbumin

Images showing PV positive cells for each genotype: WT: wild type; HOM: homozygote;

HET: heterozygote. a. left hemisphere b. right hemisphere

Yellow lines outline the CA1, DG and the hippocampus (Hp).

The blue squares show zoomed areas containing PV stained cells.

The analysis of the density of the PV positive cells in the different area of the PFC showed no difference between genotype (p=0.9) (figure 4.7). When the left and right hemisphere were analysed separately no change was discover either (respectively p=0.9 and p=0.1). The analysis of the whole PFC didn't reveal any significant differences in the density of cells expressing PV between the genotypes (p= 0.2, figure 4.7.c), as well as the analysis of the left and right hemisphere separately (p=0.5, p=0.2; figure 4.7 a., b.).
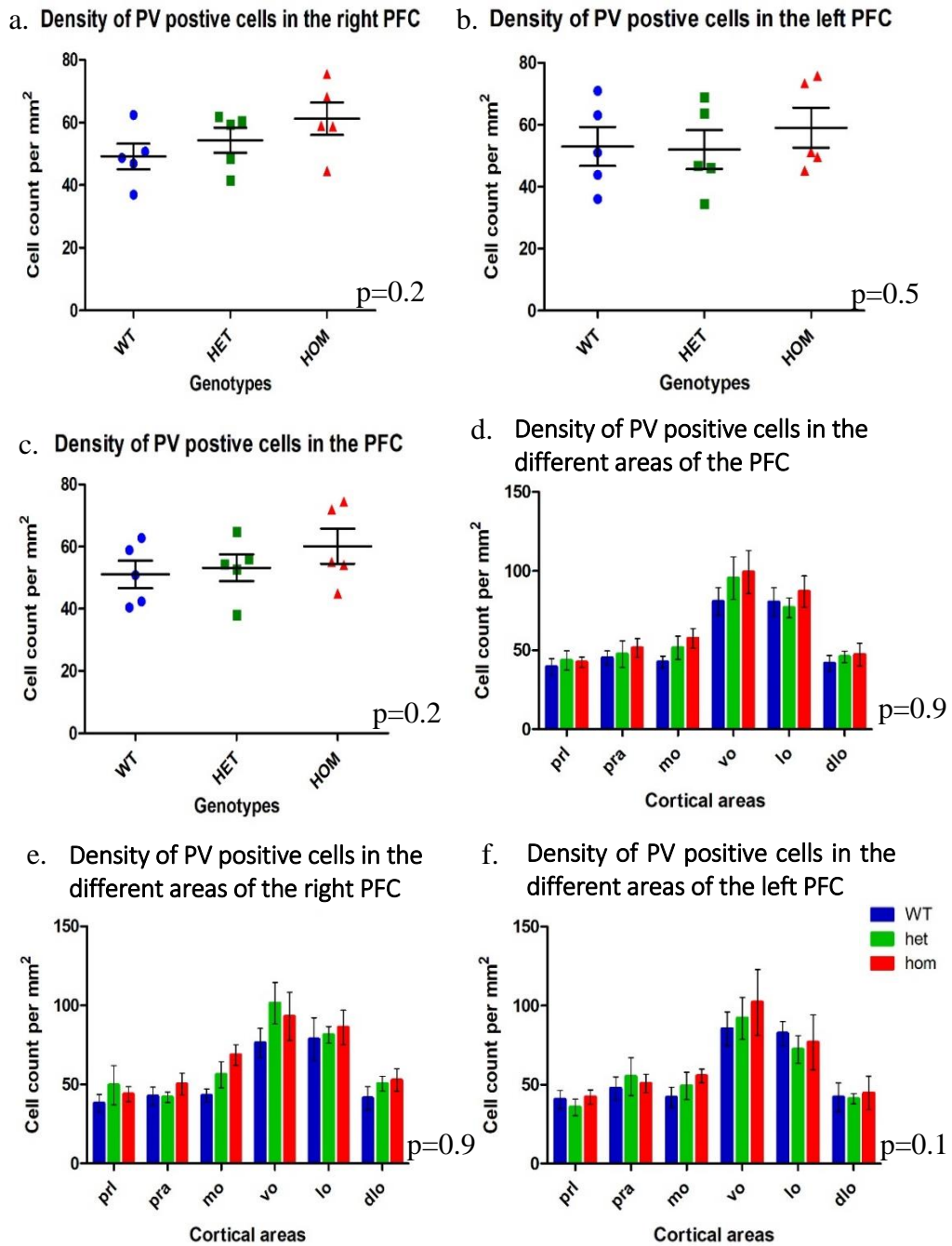
Figure 4.7: Analysis of PV positive cells density in the PFC

Analysis of the PV positive cell density shows no difference between the 3 genotypes. a.b.c. show results for the PFC as a whole; d.e.f. show results for the different area of the PFC. prl: prelimbic cortex, fra: frontal association cortex, vo: ventral orbital cortex, mo: medial orbital cortex, lo: lateral orbital cortex, dlo: dorsolateral orbital cortex. n = 5; generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

The analysis of the density of PV positive cells in the CA1 (p=0.1) and the DG (p=0.1) showed no difference between genotype (figure 4.8). The overall analysis of the hippocampus did not reveal any difference either between the three genotypes (p=0.2, figure 4.8). The analysis of the right and left hemisphere also indicated no difference of PV positive cell density between genotypes in the CA1 and the DG as well as when the whole hippocampus was considered (figure 4.8).

The results revealed that there was no significant change in the density of cells expressing parvalbumin in the PFC or in the hippocampus.
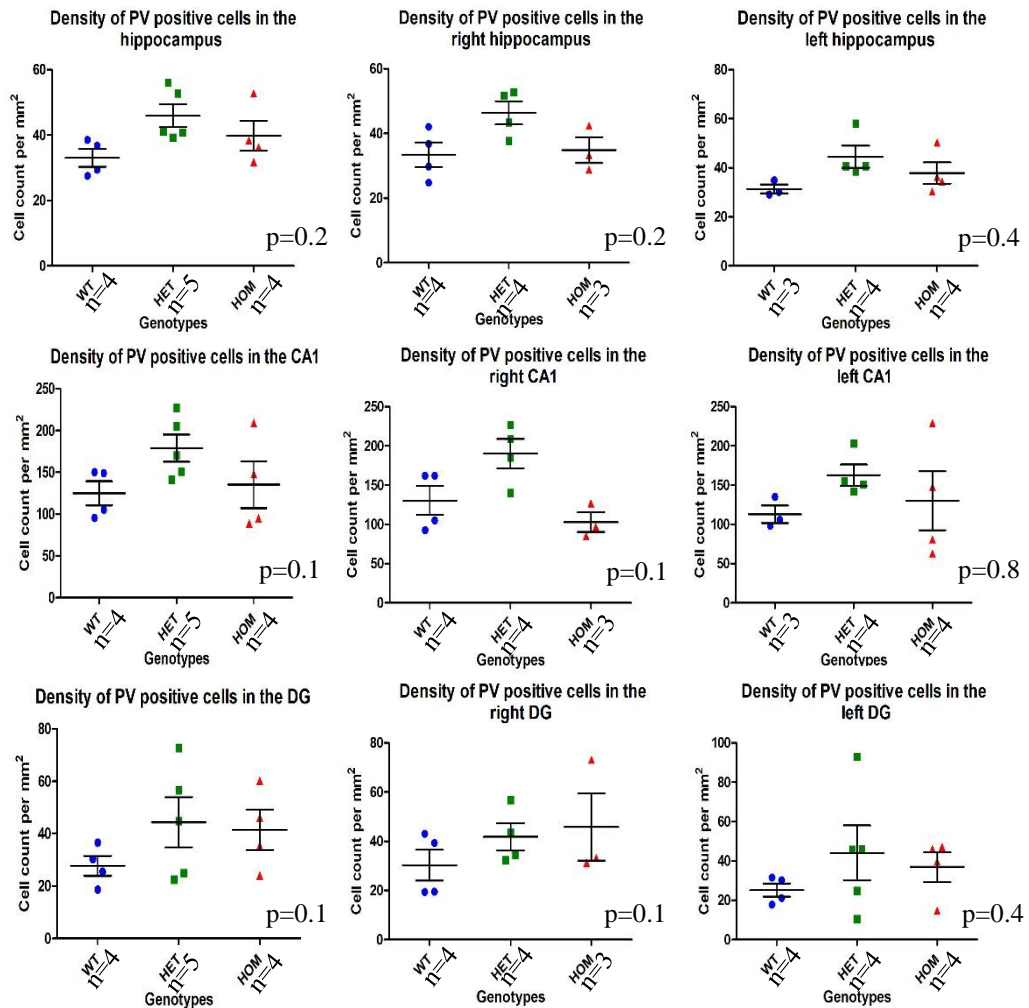
Figure 4.8: Analysis of PV positive cell density in the hippocampus

There was no difference of PV positive cell density between the 3 genotypes.

n = 5; in some case the n number is reduced due to faulty sections. generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

4.4 Cellular apoptosis analysis in the Der1 mice's brain

In post mortem studies on schizophrenic patients dysregulation of apoptosis has been found in several cortical regions (Catts and Catts, 2000; Glantz *et al.*, 2006). Moreover, results from our laboratory suggest that neurons might be susceptible to apoptosis in the Der1 mouse. Indeed, mitochondrial motility is impaired (Laura Murphy, unpublished) which could lead to neuronal damage or apoptosis. Moreover, NMDA receptor surface expression is increased (Elise Malavasi, unpublished) which could lead to excitotoxicity and therefore to apoptosis. To validate our hypothesis, the expression of activated caspase-3 in the PFC and the hippocampus was analysed. Caspase-3 is activated when cells undergo apoptosis therefore indicating that cells expressing activated caspase-3 are dying. As the number of stained cells was low, the PFC was analysed without taking into account its different region (figure 4.9 a.). Then stained cells were counted in the CA1, the DG and the whole hippocampus (figure 4.9 b.). All the counts were made using the cell counter plug in Fiji.
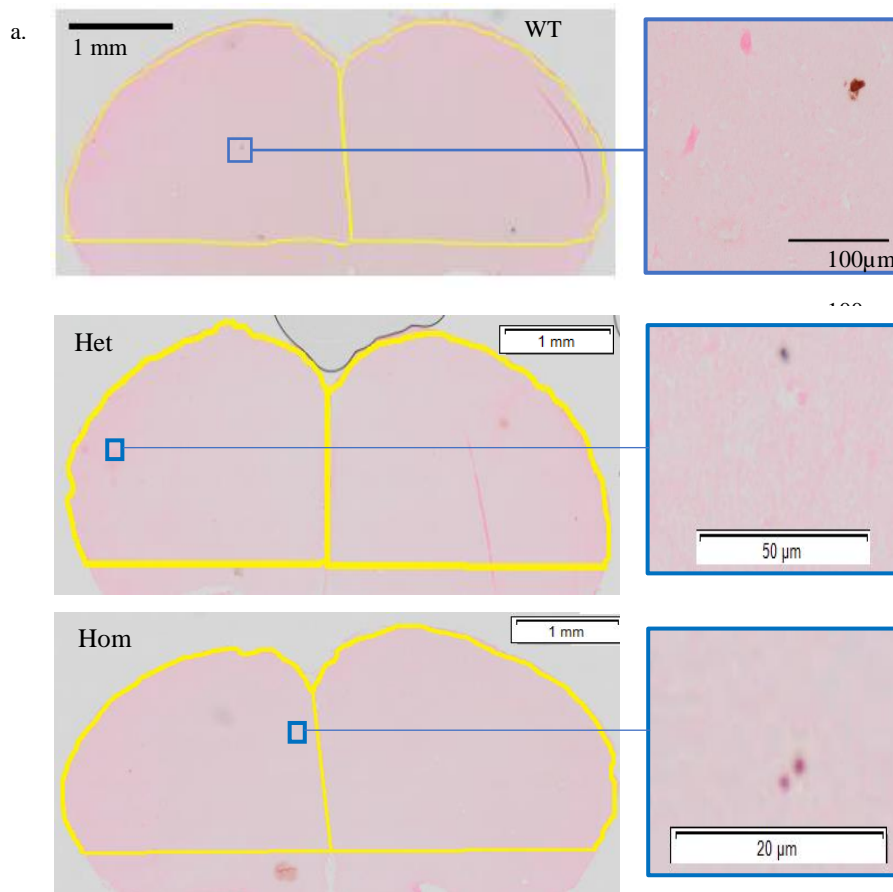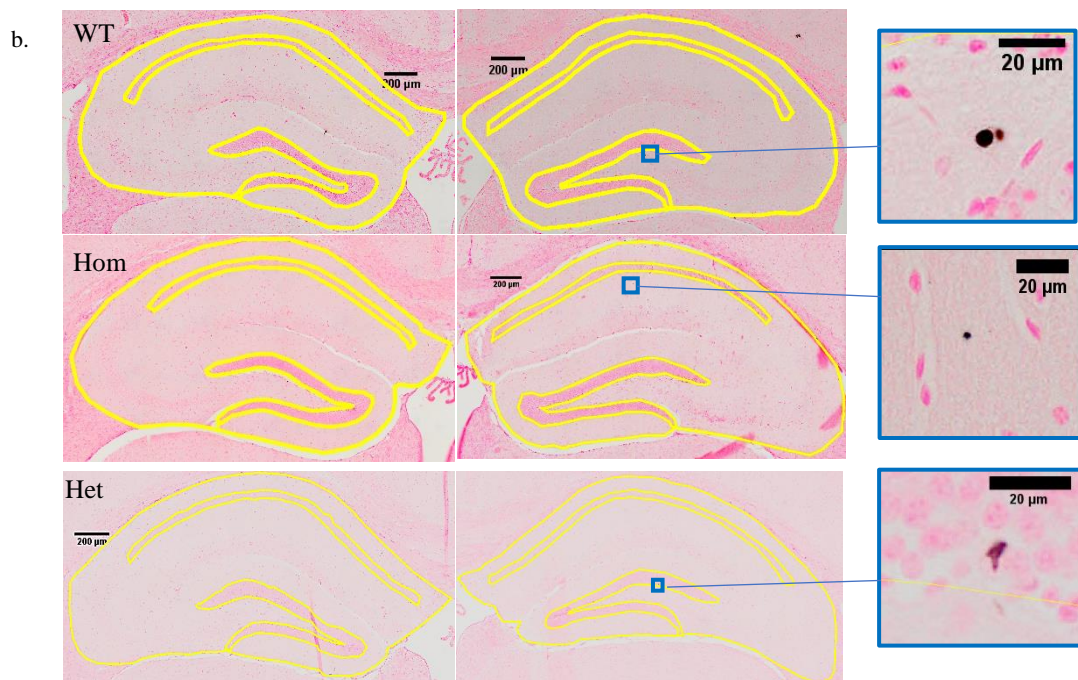
Figure 4.9: Staining of activated caspase-3 in the PFC and the hippocampus

a. Yellow line outline the area of the PFC where stained cells were counted.

b. Yellow lines outline the CA1, DG and the hippocampus (Hp).

The blue squares show zoomed areas containing activated caspase-3 stained cells.

The count of the cells expressing activated caspase-3 in the PFC showed a trend toward a change in the density of these cells between genotype (p=0.07) (figure 4.10). The observed power indicated by SPSS is of 0.52 which is quite low as the desired power level is typically 0.80. Here, the low observed power indicates that the effect size does not allow to reach statistical significance. To reach a statistical significance and a power level of 0.8, an estimated sample size can be assessed using G*3 Power software (Faul and Erdfelder, 2007; Erdfelder *et al.*, 2009). Knowing the mean of variation of the area for each genotype and the global standard deviation, the analysis indicated that 45 samples (15 per group) would be required to see a significant difference in this analysis. Similarly, the analysis of the left and right hemisphere shows a trend toward a change in the density of the activated caspase-3 positive cells (respectively p=0.06 and p=0.06; figure 4.10). The observed power indicated by SPSS is of 0.53 in the left hemisphere analysis and of 0.54 in the right hemisphere analysis. Power calculation indicates that for both analysis, 36 samples (12 per group) would be required to see a significant difference.
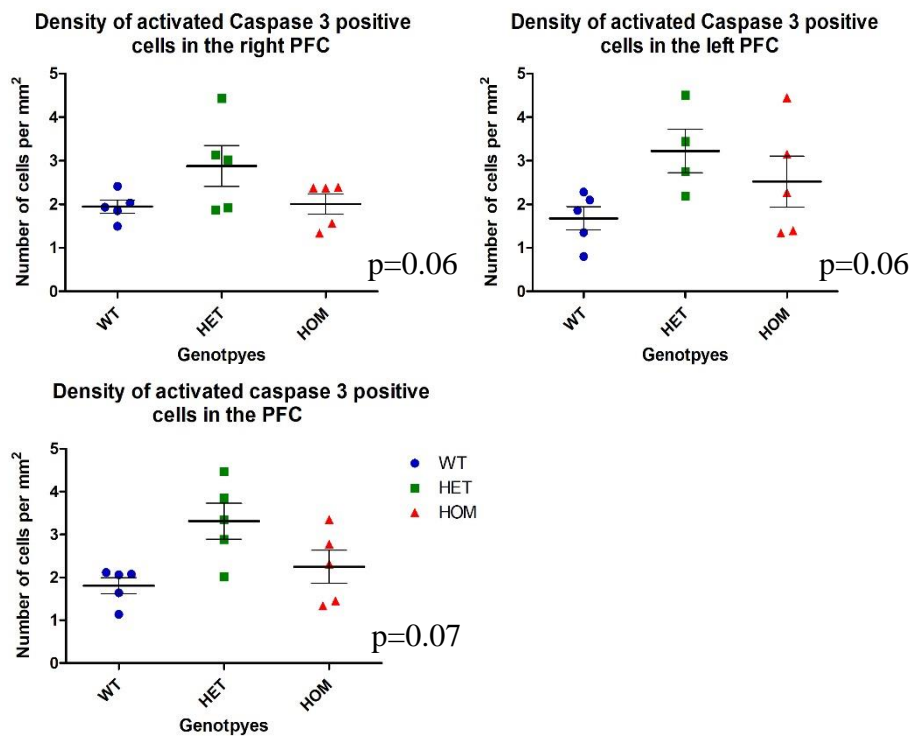
Figure 4.10: Activated caspase-3 positive cells density in the PFC

Quantification of activated caspase-3 positive cells indicates a trend toward an increased density of these cells in both the mutants Der1. n = 5; except in the left PFC n(HET)=4. generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

In the CA1 the analysis revealed a trend toward a change in the density of the activated caspase-3 positive cells between genotypes (p=0.06). The observed power indicated by SPSS is of 0.55. Power calculation indicates that for this analysis, 27 samples (9 per group) would be necessary to obtain a significant difference between genotype. No change was discovered in the DG (p=0.1) and in the hippocampus (p=0.6) (figure 4.11).
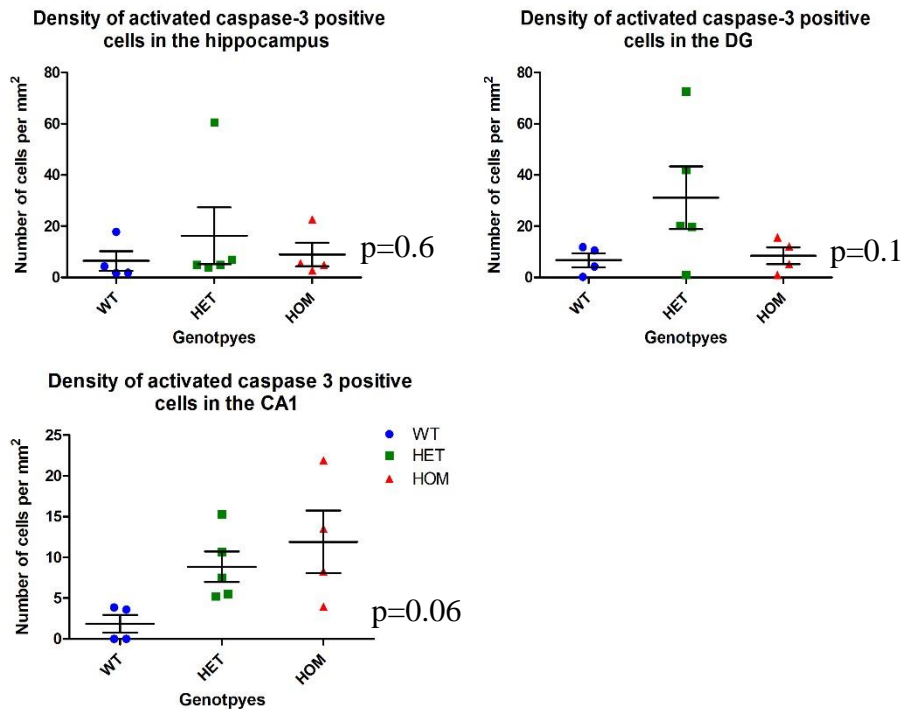
Figure 4.11: Activated caspase-3 positive cell density in the hippocampus

Count of the activated caspase-3 positive cells shows a trend towards an increased density of these cells in the CA1 in both heterozygous and homozygous groups.

n(HET) = 5; n(HOM)=4; n(WT)=4.

Generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

The analysis of each hemisphere revealed a trend towards a difference of density of activated caspase-3 cells between the genotypes in the left CA1 (p=0.07) (figure 4.12). The observed power indicated by SPSS is of 0.51. Power calculation indicates that for this analysis, 51 samples (17 per group) would be necessary to see a significant difference between genotype. Additionally, a significant difference in the right DG (p=0.02) between genotype indicating an increase of activated caspase-3 in the heterozygous mice compared to the wild type (p=0.02) (figure 4.12). However, the analysis of the right and left hippocampus as well as the right CA1 and the left DG did not show any difference (figure 4.12).

Overall the results indicate a trend towards increased density of caspase-3 positive cells in the PFC and the CA1 for both genotype and a significant increase of activated caspase-3 density in the right DG of the heterozygous mice. This might indicate an impairment in the apoptotic pathway of the heterozygous resulting in additional cell death in their prefrontal cortex and hippocampus, however in the homozygous mice a simple trend is not enough to suggest an impairment in this pathway.
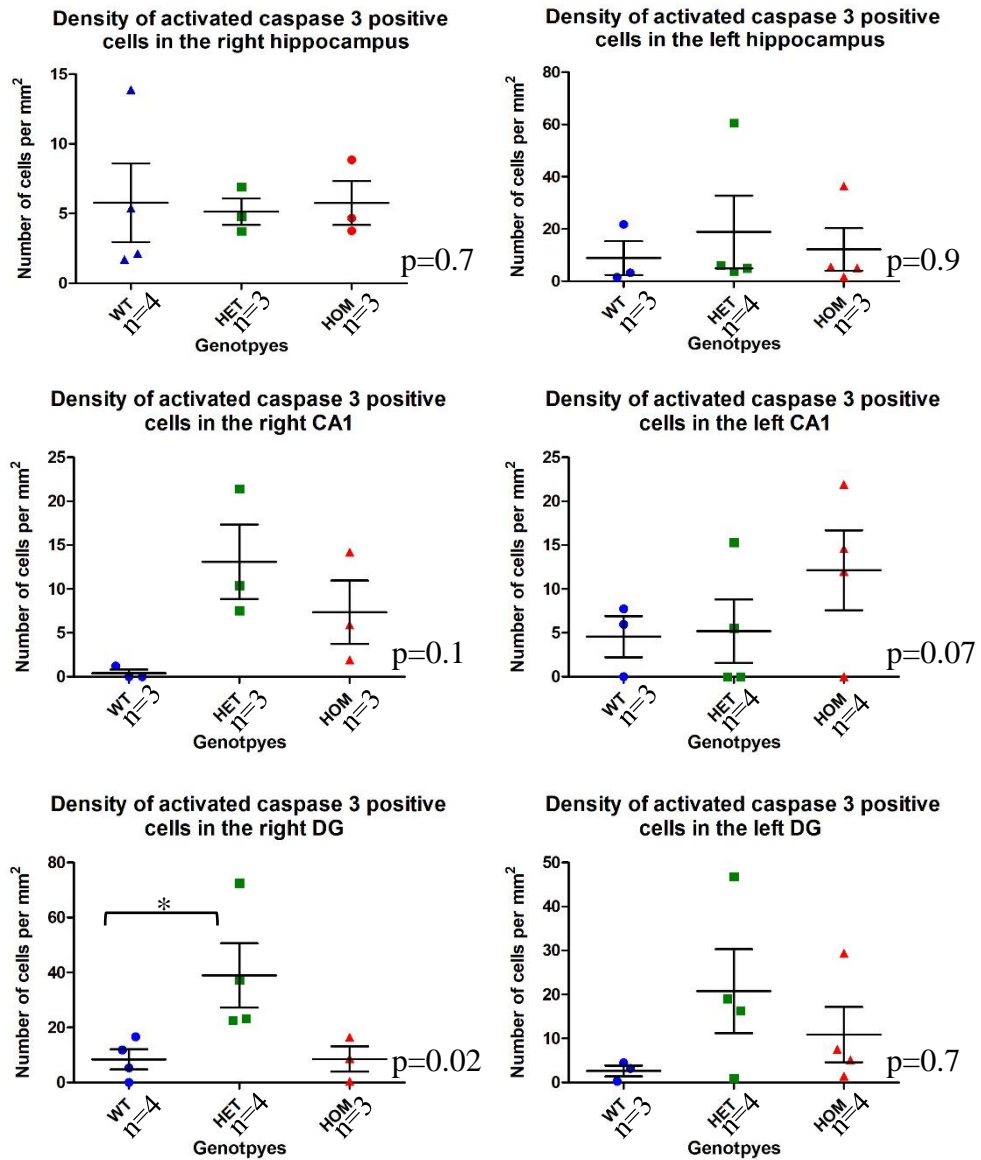
Figure 4.12: Activated caspase-3 positive cells density in the hippocampus in both hemisphere

Count of the activated caspase-3 positive cells shows a trend towards an increased density of these cells in the left CA1 in the homozygous groups and a significant difference of cell density in the right DG indicating an increase of density in the heterozygous group. The n number is variable due to faulty sections. Generalized mixed linear model with fixed effect followed by Dunnetts' post hoc test when significant: * p<0.05; ** p<0.01; *** p<0.001. Values shown are mean ± SEM.

4.5 Cell proliferation and migration analysis in the Der1 mice's brain

DISC1 is known to have an important role in adult neurogenesis (Duan *et al.*, 2007). According to post mortem studies in patients with mental disorders, neuronal proliferation is impaired, and the same has been found in animal models such as the DISC1-shRNA model and the Disc1[Tm1Kara] mouse model, where DCX positive cell expression was altered (Reif *et al.*, 2006; Kvajo *et al.*, 2008; Mao *et al.*, 2009).

In order to see if neurogenesis is impaired in our model proliferating cells were labelled with BrdU, a thymidine analogue that incorporates into the genomic DNA of dividing cells. Then BrdU labelled cells were counted in the dentate gyrus of the hippocampus only, as it is one of the major regions where neurogenesis occurs in adult mouse brain (figure 4.13 a) and as Disc1 is known to be highly expressed in this area (Austin *et al.*, 2004). Additionally, neuronal migration of new-born neurons was investigated using doublecortin (DCX), a microtubule-associated protein present in immature neurons (figure 4.13 b). Double stained cells are visible in the overlay image (figure 4.14 c).
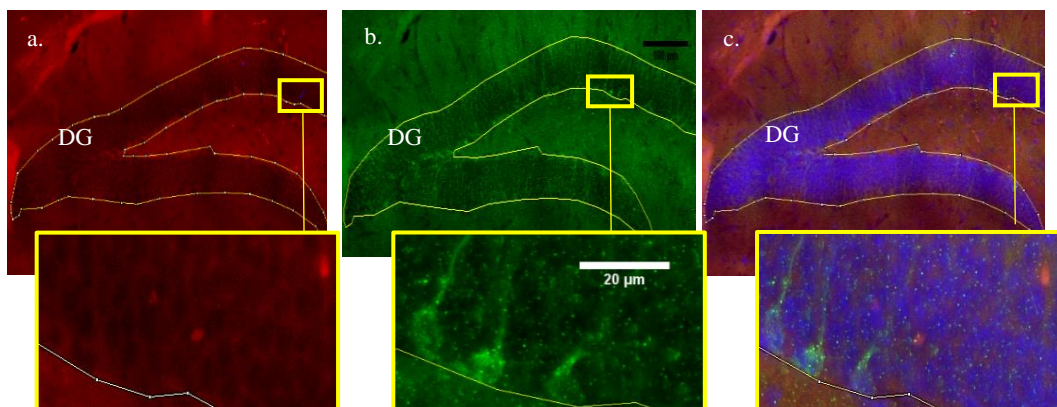
Figure 4.13: BrdU and DCX positive cells in the dentate gyrus

a. staining of BrdU positive cells in red

b. staining of DCX positive cells in green

c. Overlay of the different staining. Cells are stained with Dapi (blue) to visualise the nuclei. Yellow squares outline zoomed areas.

(blue) to visualise the nuclei. Yellow squares outline zoomed areas.
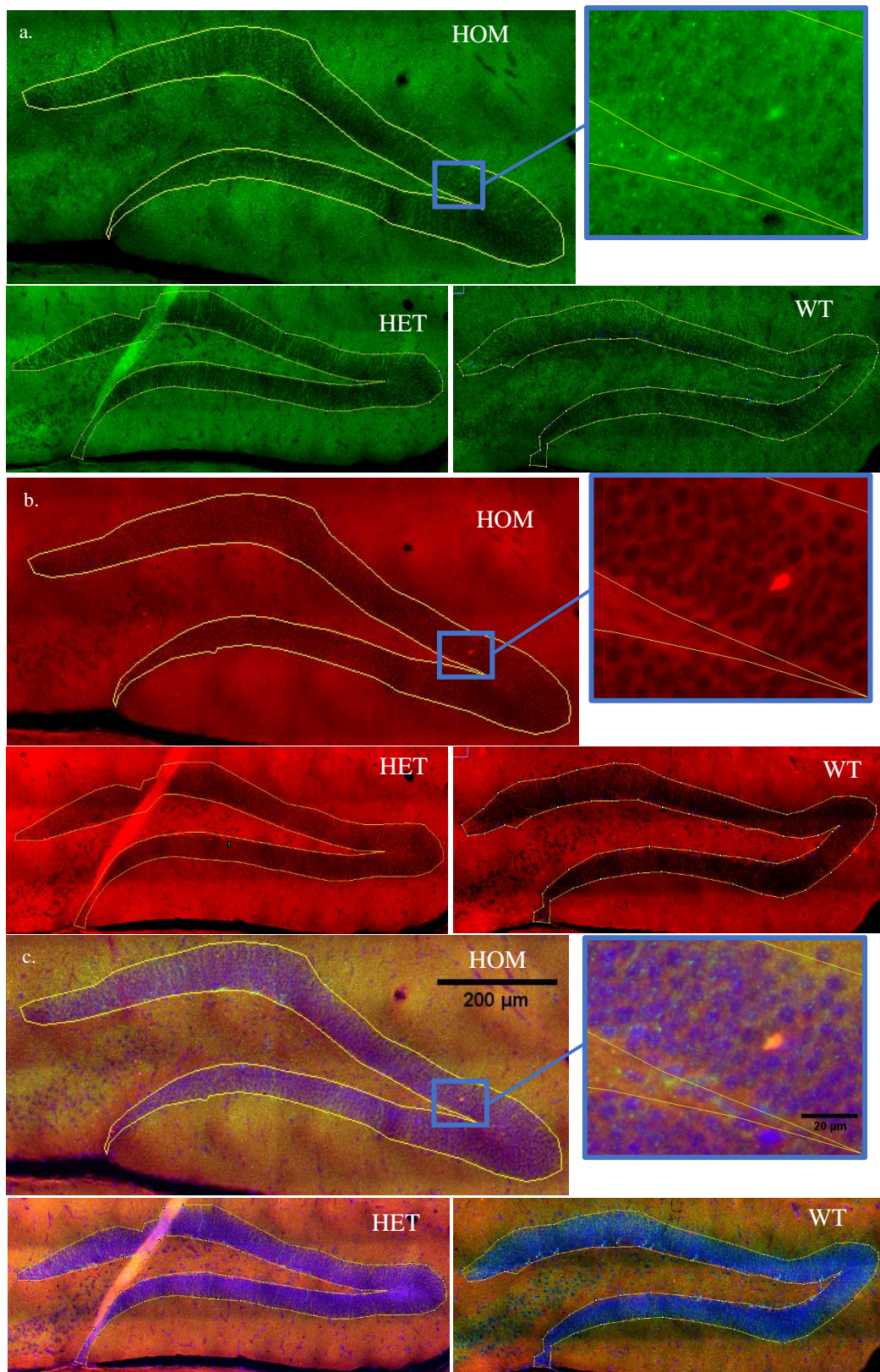
Figure 4.14: BrdU and DCX positive cells in the dentate gyrus

a. Staining of DCX positive cells in green

b. Staining of BrdU positive cells in red

c. Overlay of the different staining. Cells stained with Dapi (blue) to visualise the nuclei. A double stained cell is yellow in appearance.

Blue squares outline zoomed areas. All genotypes are represented

BrdU is incorporated during phase S of the cell cycle and DCX is expressed in immature neurons (neuron progenitors) in the post mitotic phase. It is possible that BrdU$^+$/DCX$^-$ cells are not yet neuron progenitors or not fated to become neurons. BrdU$^-$/DCX$^+$ immature neurons, however, may have been produced prior to BrdU treatment. since newly generated granule cells need at least 5 weeks to mature and become fully integrated into the existing network, in the adult brain (Zhao, 2006).

The count of the cells expressing both BrdU and DCX did not show any differences between genotype (p=0.1, figure 4.15). Additionally, the density of DCX positive cells were not changed between genotypes (p=0.4, figure 4.15). However, the density of BrdU positive cells showed a trend ($0.05 \leq p \leq 0.09$) toward a reduction of BrdU positive cells between the three groups (p=0.09, figure 4.15). The observed power indicated by SPSS is of 0.45 while the desired power level is typically 0.80. The low observed power indicates that the effect size does not allow to reach statistical significance. To reach a statistical significance and a power level of 0.80, an estimated sample size can be assessed using G*3 Power software (Faul and Erdfelder, 2007; Erdfelder *et al.*, 2009). Knowing the mean of variation of the area for each genotype and the global standard deviation, the analysis indicated that 90 samples (30 per group) would be required to see a significant difference in this analysis.
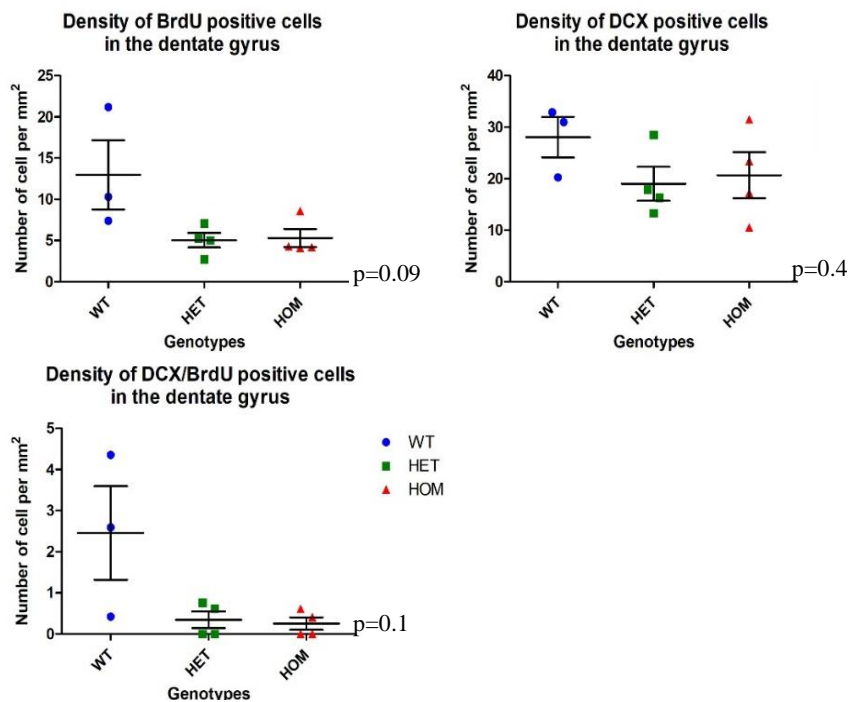
Figure 4.15: Density of DCX, BrdU and DCX/BrdU cells in the dentate gyrus

Count of DCX, BRDU and DCX/BrdU positive cells show a trend toward a reduction of BrdU positive cells in homozygous and heterozygous groups. No other differences were found. n(HET) = 4; n(HOM)=4; n(WT)=3. Generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

When the left and right hemisphere were analysed separately, there was no difference of density of BrdU/DCX positive cells as well as DCX positive cells between genotype (figure 4.16), however a trend toward a change in the density of BrdU positive cells between genotypes was found in the dentate gyrus of the right hemisphere (figure 4.16). For the BrdU analysis in the right hemisphere, the observed power was 0.49. Power calculation indicates that for this analysis, 150 samples (50 per group) would be necessary to see a significant difference between genotype.
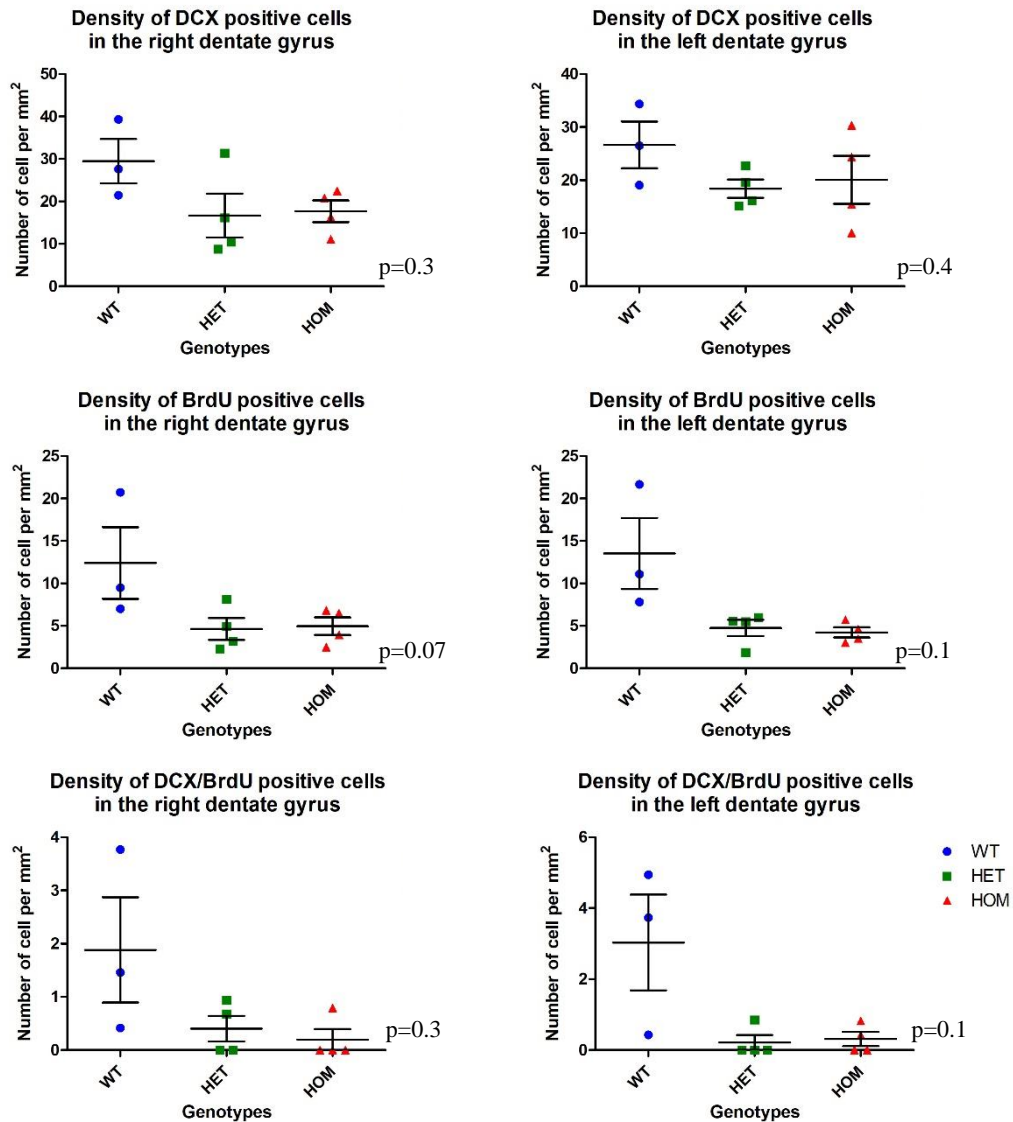
Figure 4.16: Density of DCX, BrdU and DCX/BrdU cells in the dentate gyrus of both hemisphere

Count of DCX, BRDU and DCX/BrdU positive cells in both hemisphere separately show a trend toward a reduction of BrdU positive cells in homozygous and heterozygous groups in the right hemisphere. No other differences were found.

n(HET) = 4; n(HOM)=4; n(WT)=3. Generalized mixed linear model with fixed effect. Values shown are mean ± SEM.

4.6   Littermate effect

As mentioned in chapter 3, littermate effects could indicate that in-utero environment and/or sample handling influence variation of cellular density between animal. Mild evidence for littermate effects was detected, affecting only srlm cell density and interneuron density in the dentate gyrus (table 4.1). Trends towards a littermate effect were observed in other analyses however. Overall though, the cellular expression and cellular density analyses revealed little evidence for a littermate effect.

Analysis	Brain regions	Litter effect
Cell density	PFC	No (p=0.2)
	CA1	No (p=0.3)
	Srlm	Yes (p=0.01)
PV positive cells	Different areas of PFC	No (p=0.5)
	Whole PFC	Trend (p=0.08
	CA1	Trend (p=0.05)
	DG	Yes (p=0.01)
	Whole hippocampus	No (p=0.2)
Caspase-3 positive cells	PFC	Trend (p=0.06)
	Hippocampus	No (p=0.6)
	CA1	No (p=0.3)
	DG	No (p=0.5)
BrdU/DCX positive cells	DG	No (p=0.8)
DCX positive cells	DG	No (p=0.8)
BrdU positive cells	DG	No (p=0.5)

Table 4.1: Litter effect on the different analyses performed through this chapter

4.7   Discussion

This analysis of the Der1 mouse demonstrated no profound changes in the prefrontal cortex or the hippocampus in the heterozygous and the homozygous mice. However, the homozygous mice have an increased cell density on the left srlm of the hippocampus compared to the wild-type mice. Additionally, the heterozygotes express more cells containing activated caspase-3 compare to the wild type mice, in the dentate gyrus. Furthermore, a trend towards increased activation of caspase-3 in the heterozygous and homozygous mice was found in the PFC and the CA1 of the hippocampus as well as a trend toward a reduction of BrdU positive cells in the dentate gyrus in both mutants.

Increased cellular density in the srlm of the hippocampus was not found in other DISC1 models. This could indicate that this phenotype is much more subtle in other models than in the homozygous Der1 mouse, and concerns a more specific area as well as being asymmetric. Asymmetrical changes have often been noticed in neuropsychiatric disorders, as mentioned in the introduction. Nevertheless, brain asymmetry is overall an indication of an impaired neurodevelopment therefore this could be an indication of an impaired neurodevelopment in the De1 mice model. However, a mouse model in which Disc1 expression was supressed in the CA1 region of the developing mouse hippocampus showed that a large number of migrating cells were distributed in the srlm (Tomita *et al.*, 2011). This indicates an impairment in cell migration in the srlm, (Tomita *et al.*, 2011). Therefore, in the homozygous Der1 mice, the higher concentration of neurons in the srlm could be due to cell migration defect leading to the cells staying in the srlm layer. However, the analysis of the homozygous Der1 didn't reveal a defect of cell proliferation in the dentate gyrus but cell proliferation defect has been found in other Disc1 mutants (Kim *et al.*, 2011; Wu *et al.*, 2017) and is known to happen in schizophrenic patients (Reif *et al.*, 2006). In schizophrenic patients, several post mortem studies of hippocampal cells have reported no significant change in cell density of its sub-structures (Heckers *et al.*, 1991; Heckers and Konradi, 2002; Walker *et al.*, 2002; Eastwood and Harrison, 2005).

Additionally, analysis in DISC1 KO mice revealed that there was no change in the total number of neurons in the cortex (Umeda *et al.*, 2016) which is similar to the result found in the Der1 mice model. However, analysis of other DISC1 models such as Q31L and L100P indicated a reduction in neuron density across the neocortex (Lee, Fadel, *et al.*, 2011) and in mental disorders, investigation of neuronal density revealed both decreased and increased density of cortical neurons (Chana *et al.*, 2003; Stockmeier *et al.*, 2010; Smiley, Konnova and

Bleiwas, 2012b; Bakhshi and Chance, 2015). This indicates that this feature is variable when studying mental disorders but change in the neuronal density is often discovered.

Regional cell density changes could result from altered rates of proliferation, neuronal migration and/or cell death. However, consistent with the lack of evidence for strong cell density changes in the Der1 mutants, the present data indicate only subtle effects upon neural precursor proliferation and cell death, with neuronal migration yet to be examined. Dentate gyrus cell proliferation defects are another feature that have been found in other Disc1 mutants (Kim *et al.*, 2011; Wu *et al.*, 2017) and this is known to happen in schizophrenia and depression patients (Reif *et al.*, 2006). However, in schizophrenic patients, several post mortem studies of hippocampal cells have reported no significant change in cell density of its sub-structures (Heckers *et al.*, 1991; Heckers and Konradi, 2002; Walker *et al.*, 2002; Eastwood and Harrison, 2005). Additionally, DISC1 is known to be involved in neurogenesis (Dranovsky and Hen, 2007; Duan *et al.*, 2007; Soares *et al.*, 2011). Reduced neurogenesis was observed in several mice carrying a *DISC1* mutation such as Q31L, L100P and DISC1$_{tr}$ (Shen *et al.*, 2008; Lee, Fadel, *et al.*, 2011) (table 1.1). Moreover, this has also been seen in psychiatric disorders (Eisch *et al.*, 2009; Schoenfeld and Cameron, 2015; Apple, Fonseca and Kokovay, 2017). This indicates that the *Der1* mutation might also affect neurogenesis. Only a statistical trend toward a reduction of neurogenesis in the dentate gyrus was observed in both the heterozygote and homozygote der1 mice. However, sample numbers were quite low for this experiment and it would therefore be worth examining more brains to find out if there is a consistent change. A strong hypothesis is that DISC1 regulates neurogenesis through one of its partners, GSK3β. DISC1 stabilizes β-catenin, a neurogenesis regulator, by preventing its phosphorylation by GSK3β to maintain neurogenesis (Mao *et al.*, 2009; Ming and Song, 2009). It is possible that in the Der1 mice, DISC1 is not able to bind to GSK3β which would be predicted to cause over activity of GSK3β and a consequent decrease of neurogenesis (Mao *et al.*, 2009). However, previous investigation of GSK3β in the Der1 mouse indicates that there is no change in the activity of hippocampal GSK3β (Laura Murphy, unpublished). Nevertheless, GSK3β is believed to bind to DISC1 at two sites: 1–220 and 356–595, both of which would be retained if the chimeric transcripts expressed in the Der1 mouse are translated (Mao *et al.*, 2009). GSK3β could therefore bind to the hypothetical chimeric protein which could cause, for example, changes in its localisation, or ability to regulate β-catenin due to altered protein complex composition. Such changes could influence neurogenesis. It will therefore be important to examine these possibilities in the future.

The increased density of activated caspase-3 indicates possible activation of the apoptotic pathway. This has also been seen in post mortem brain of patients (Glantz *et al.*, 2006).

Activated caspase 3 is present in both extrinsic and intrinsic apoptotic pathways. It is possible that a higher concentration of TNF (tumour necrosis factor) and Fas-Fas molecules in the Der1 model could lead to activation of the apoptosis pathway. However, no change in the gene expression of those molecules was found (chapter 6). Activation of apoptosis could also be due to mitochondrial effects. Indeed, mitochondria pause less during their traffic, in both heterozygous and homozygous hippocampal Der1 neurons (Laura Murphy, unpublished). It is therefore possible that their ability to supply functions such as calcium buffering, and the energy required to maintain neuronal ion gradients following depolarisation is impaired. In turn this could render neurons sensitive to excitotoxicity, damage and death. Additionally, increase of NMDA receptor surface expression has been previously found in the Der1 mice (Elise Malavasi, unpublished). This could lead to excitotoxicity, where activation of the higher concentration of glutamatergic receptors leads to increased intracellular calcium, which may activate apoptotic pathways.

Since the cell types expressing activated caspase-3 were not identified, it is possible that another type of cell is affected by the activation of apoptotic mechanisms. Indeed, loss of glial cells have been found in numerous psychiatric illness (Stark *et al.*, 2004; Elsayed and Magistretti, 2015). Glial cells are important for the formation and the survival of neurons and the formation of dendrites and synapses, therefore a reduction in the number of glial cells could also affect the neurons. As glial cells express glutamate transporters, apoptosis of glia through activation of caspase-3 could lead to a high concentration of glutamate in the synaptic cleft which would lead to excitotoxicity and further glial damage as well as neuronal death (Rajkowska *et al.*, 1999; De Biase *et al.*, 2011). However, this would most likely affect the cell density, and cluster of apoptotic cells should be seen. Therefore, this mechanism is a slightly less probable than the others. Additionally, activation of apoptosis could only affect the dendrite or spine of neurons affecting their morphology but allowing them to survive (Jarskog *et al.*, 2005b). Indeed, a study showed that applying glutamate to distal dendrites can lead to increase caspase-3 activity in synaptic terminals without affecting the whole cell (Mattson, Keller and Begley, 1998). However, the focal Caspase-3 staining that was quantified does not correlate with expression at synaptic terminals, and therefore does not provide evidence of this mechanism.

No difference in parvalbumin expression was found in the Der1 mice. This indicates that the Der1 mutation does not affect the density of interneurons. This is quite unexpected as several DISC1 mutant mice feature a defect in GABAergic neurons (Lee *et al.*, 2013; Borkowska, Millar and Price, 2016; Delevich *et al.*, 2016). Moreover, mental disorders also display a reduction of GABAergic interneurons (Marín, 2012; Fung *et al.*, 2014). However it is believed

that the fast-spiking of interneurons makes them especially sensitive to overload and excitotoxicity, which would lead to their death in schizophrenia (Nakazawa, 2011). It is therefore possible that neuronal stimulation would be required to see a decrease in interneuron density as well as an intensified activation of apoptosis.

Overall, these data indicate that heterozygous and homozygous mice do not always express the same phenotypes. It seems that cellular density of the srlm is specifically affected in the homozygous mice while in the heterozygous mouse there is a specific activation of apoptotic mechanisms in the dentate gyrus. These genotype-specific effects might be due to the fact that the homozygous mice carry two copies of the *Der1* mutation, therefore expressing no full-length DISC1 protein, but possibly maximum amounts of the deleterious chimeric proteins. On the other hand, he heterozygous mice carry only one copy of the Der1 mutation and therefore express full length DISC1 and possibly also chimeric proteins, with consequent potential for dominant-negative effects.

Moreover, on the measures examined to date, it appears that the Der1 mutation has more subtle effects than what have been observed in other DISC1 mutant mice.

CHAPTER 5

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EFFECT OF THE DER1 MUTATION UPON NEURONAL MORPHOLOGY

5.1 Introduction

In order for the brain to function properly, intricate connections between neurons must be formed during brain development. During this time dendrites and axons grow out from the cell body of each neuron. Impairment of neuronal growth can lead to a wide range of disorders. Sholl analysis is often used to study the morphology of a neuron, more specifically to study the formation and organization of their dendrites (Sholl, 1953). It is achieved by counting the number of intersections between dendrites and concentric circles centered around the cell body. Sholl analysis is used to investigate the mechanism and the evolution of some brain disorders. It is known that in mental disorders neuronal function is affected, their communication is deficient, and their shape and outgrowth is also affected. These studies highlight the importance of dendrite outgrowth in neuronal function (Hyman, 2000; NIH, 2007; Brennand *et al.*, 2011; Uhlhaas, 2015). Indeed, it is through the dendrites and the axons that the information is transferred from one neuron to another and in turn from one brain area to another. In schizophrenic patients, it has been shown that neuronal outgrowth is impaired. Using Sholl analysis, 3-D image analysis of the dendritic arborization of Golgi-impregnated prefrontal pyramidal neurons in post mortem tissue from patients showed that the basilar dendritic structures were reduced. The distal dendrites were the ones affected (Kalus *et al.*, 2000). Another study showed that apical dendritic trees of internal pyramidal neurons were also reduced in schizophrenic patients (Rosoklija *et al.*, 2000). Additionally, a decrease in the number of primary and secondary dendrites in the pyramidal neurons of layers III and V in medial prefrontal cortex was found in schizophrenic patients (Broadbelt, Byne and Jones, 2002). Two other studies also indicated a reduction of the soma size of pyramidal neurons from schizophrenic patients (Rajkowska G, Selemon LD and Goldman-Rakic PS, 1998; Pierri *et al.*, 2001).

Mutation of the *DISC1* gene in animals leads to similar phenotypes to those found in schizophrenic patients. The importance of DISC1 in neuronal outgrowth has been investigated in several studies mentioned in the introduction and showed that DISC1 plays an important role in morphological development of adult-born neurons (Duan *et al.*, 2007; Kim *et al.*, 2009) (table 1.1). DISC1 is also known to influence neurite outgrowth with the help of its interactors (Miyoshi *et al.*, 2003).

In this study, neuronal outgrowth in homozygous Der1 mice was investigated to see if the mutation would lead to similar effects to those previously found in other models of mental illness. Sholl analysis was performed on primary neuronal cultures from E18 mice, as neurons are easier to visualise in culture than in tissue, and astrocytes are present in a small quantity.

The number and length of the dendrites was studied at five days in vitro (DIV) in order to have young neurons and easily see their dendrites. Moreover, studying neurons at this age allows us to look at individual neurons as their processes are not yet very long and neurons have not yet formed an interconnected network, but cells still display robust neurite extension. This choice had no relevance to mental illness or Disc1 and was purely practical.

Primary cortical neuronal cultures, set up by E. Malavasi, were used for this analysis. These neurons were grown at low density (10^5 neurons per well) in six well plates with astrocyte feeders in order to allow trophic factors to diffuse into the shared media and support the growth of the neurons. Low density co-culture enabled neuronal morphology to be easily examined. Map2 antibody was used to stain the dendrites in order to perform Sholl analysis on 5 DIV (days *in vitro*) neurons. However, MAP2 is sometimes present in axons as immature neurons can express MAP2 in all their processes. There is a loss of MAP2 immunoreactivity in axons with time following an initial period when its distribution is uniform throughout the cell (Caceres, Banker and Binder, 1986). Compared to dendrites, axons are longer processes which develop from a conical prolongation and then tend to have a constant radius. Therefore, when axons were spotted due to their characteristic length and width, they were excluded from the analysis. Wild-type and homozygous cultures were analysed blind to genotype. The study was carried out on four independent cultures for each genotype. From the cultures, neurons which were distinct from each other were selected in order to be able to assess the dendrites extending from the cell bodies. The images were taken with a fluorescent microscope (figure 5.1). The analysis was done using Fiji software.
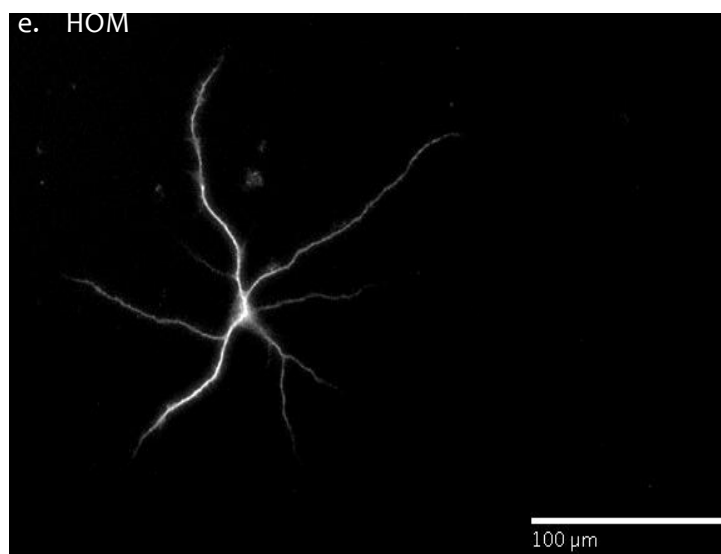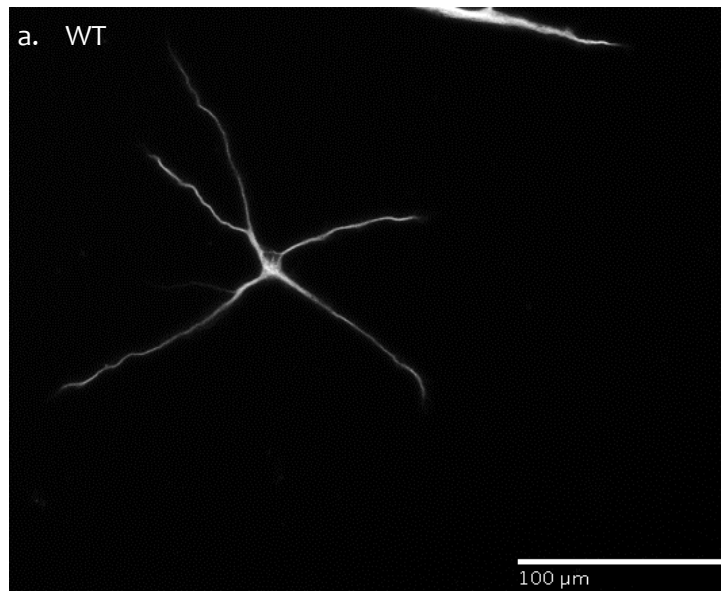
Figure 5.1: Wild type and homozygous Der1 neurons
Representative fluorescent images at 20x magnification of
wild type (a) and homozygous Der1 neurons (b) in primary
neuronal culture at DIV 5.

5.2 Sholl analysis and dendrite length analysis

In order to perform Sholl analysis, the Fiji plug in "Neurite tracer" was first used to reconstruct each dendrite from the chosen neurons. The "Sholl analysis" plug-in was applied, and circles of increasing radius centred on the cell body were created every 5 µm. The number of circle crossings by dendrites was then quantified.

The results of the Sholl analysis indicate a significant difference interaction between genotype and distance with respect to the number of crossings between the wild-type and the Der1 homozygotes (2way ANOVA with repeated measure p=0.0005, figure 5.2).
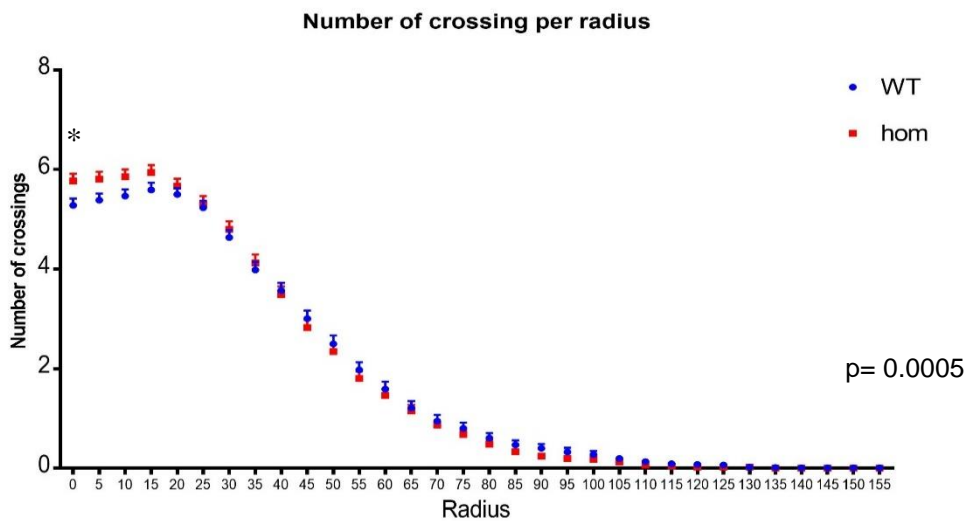
Figure 5.2: Neuronal outgrowth alteration in homozygous neuronal cell cultures

The mean number of dendrite crossings is shown for every 5µm for each group. There is an overall significant difference between the wild-type and the homozygote group.

n=132 neurons for each group, for each group 4 plate of 3 wells were analysed, each plate was cultures from one litter from one pregnant damn composed of between 3 to 8 embryos, depending on the size of the litter. Error bars represent standard error of the mean (SEM). 2-way ANOVA with repeated measures indicated a significant interaction between the number of crossings and the genotype p=0.0005; genotype p>0.05; distance from soma p<0.0001. Sidak's multiple comparison post-hoc test revealed a significant difference at radius 0, p=0.02, indicating that the number of crossings is increased in the homozygotes compared to the wild types at radius 0 and there was a trend at radius 5, p=0.09.

These neurons show a significant increase in the number of crossings in the homozygous mice compared to the wild-type mice in the first micron, at radius 0 (p=0.02, figure 5.2) as well as a trend (p=0.09) at radius 5 toward a similar change (p=0.09, figure 5.2). At larger radii, the number of crossings is similar between the two groups. The Der1 homozygous neurons form more crossings at radius 0 and 5, indicating that the outgrowth of the homozygous neurons is increased compared to that of the wild-type neurons.

To investigate further, the number of dendrites and branches and their length were investigated using imageJ software. No significant differences were found between the homozygous group and the wild-type group in the number of branches or dendrites (figure 5.3.b and c). However, a trend (p=0.09) was found indicating less branches per neuron in the homozygotes compare to the wild-type (figure 5.3.a). Using the information obtained from "neurite tracer", the length of the dendrites and branches for each neuron selected was analysed. Looking at the length of dendrites and branches separately, no significant overall difference was found between the homozygous and the wild-type groups (figure 5.3.d e.).

However, when the number of neurite extensions at 0 µm and 5 µm were analysed a significant difference was discovered at 0 µm, indicating that the homozygous neurons have more extensions compared to the wild type neurons (p=0.03) and the same was discovered at 5 µm (p=0.03) (figure 5.4). This indicates an early outgrowth with a higher number of neurite extension in the homozygous mice, indicating a possible neurodevelopment impairment.

a. Number of branches per neuron

p=0.09

b. Number of primary dendrites per neuron

p=0.18

c. Number of dendrites and branches per neuron

p=0.89

d. Length of primary dendrites

p=0.34

e. Length of branches

p=0.42
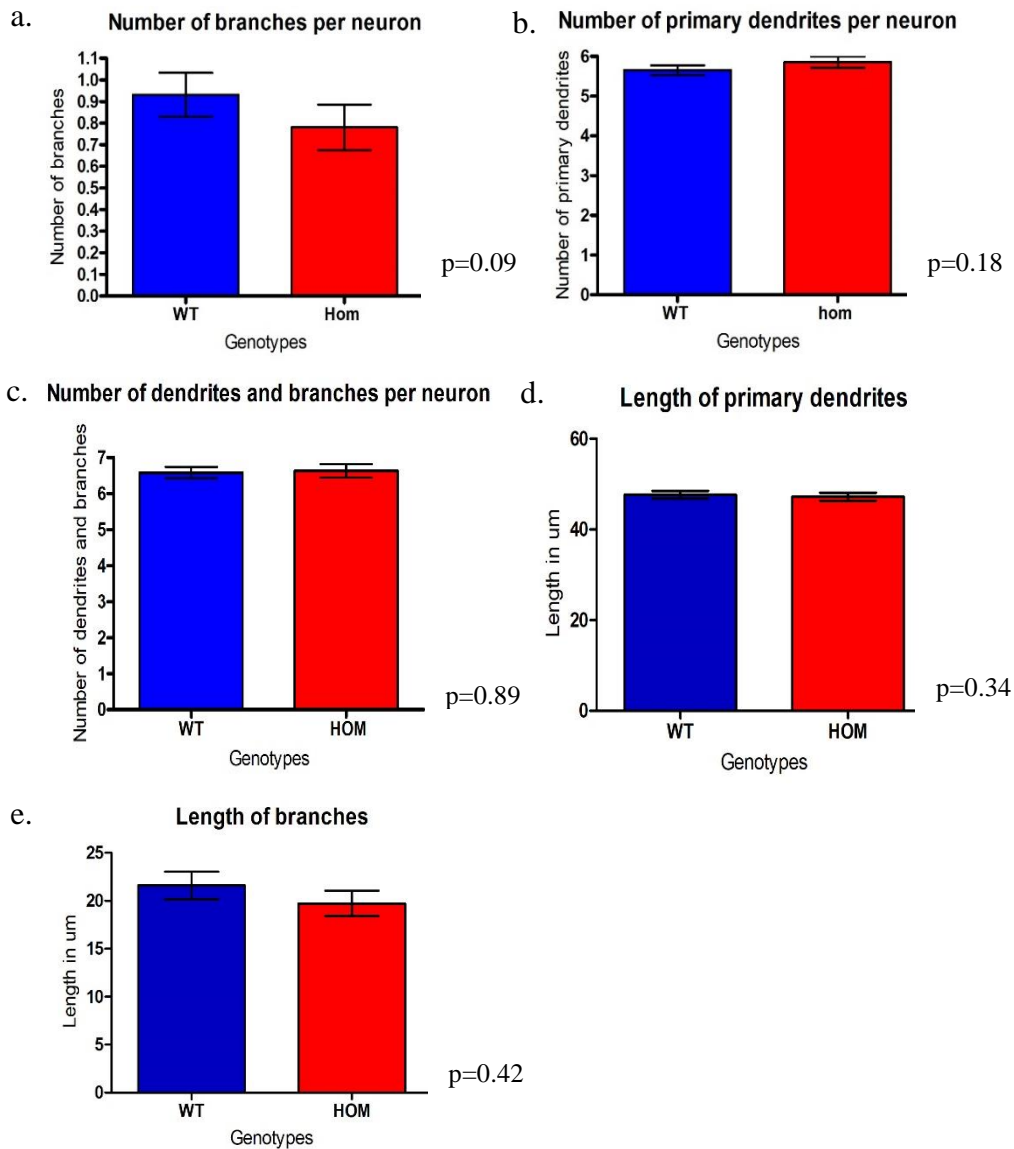
Figure 5.3: No change in the complexity of the dendritic arbour between wild-type and homozygous primary neuronal cultures

a. Number of branches per neuron. b. Number of primary dendrites per neuron. c. Number of primary dendrites and branches per neuron. d. Analysis of the length of primary dendrites from each neuron. e. Analysis of the length of branches from each selected neuron. n=132 neurons per group. For each group 4 plate of 3 wells were analysed, each plate was cultures from one litter from one pregnant damn composed of between 3 to 8 embryos, depending on the size of the litter. Weather each neuron is looked at separately or their mean is assessed for each plate no significance is found. The error bars represent SEM. D'Agostino & Pearson omnibus normality test was performed to assess normality of the data set. The distributions were not normal therefore a Mann-Whitney statistical test was used.
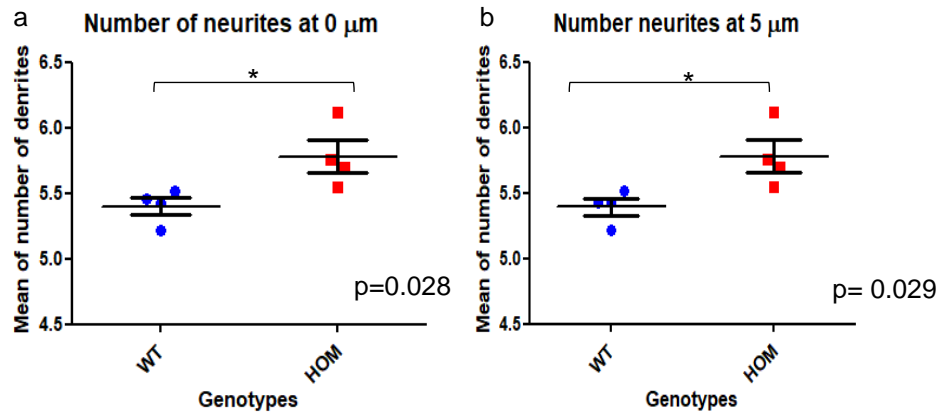
Figure 5.4: Change in the number of neurites

a. Number of neurites at radius 0. b. Number of neurite at radius 5.

A significant change was discovered indicating more neurites at radius 0 in the Der1 homozygote neurons compare to the WT (p=0.028). A trend towards the same indication was found at radius 5 (p=0.029). n=132 neurons per genotype, 33 neurons per plate/litter, 4 litters per genotype. For each genotype 4 plates of 3 wells were analysed, each plate was cultures from one litter from one pregnant damn composed of between 3 to 8 embryos, depending on the size of the litter. Each dot represents one litter. The black error bars represent SEM. D'Agostino & Pearson omnibus normality test was performed to assess normality of the data set. The distributions were not normal therefore a Mann-Whitney statistical test was used.

5.3 Cell soma size analysis

To assess cell soma size, the outline of the cell body was drawn manually for each neuron using Fiji software and the value of the defined area was obtained as an area measurement. The investigation of cell soma size revealed a significant increase in soma size in the homozygotes compared to the wild types (p=0.008, figure 5.5). This indicates that the Der1 mutation affects neuronal soma size and therefore neuronal outgrowth. Moreover, this could be an indication that neuronal communication is impaired as a change in the cell body could affect its postsynaptic role and neuronal integrative properties.
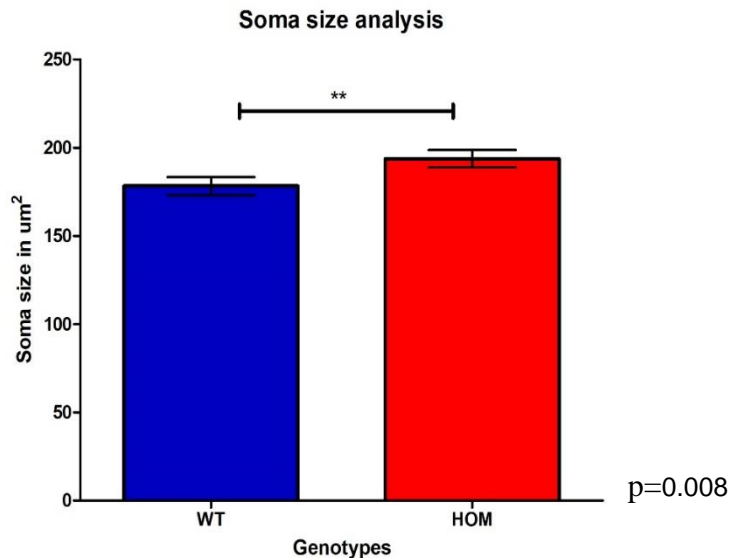
Figure 5.5: Variation of the soma size of the homozygous Der1 neuron

Homozygous neurons have a larger soma compared to the wild-type neurons. n=132 neurons per group. For each group 4 plate of 3 wells were analysed, each plate was cultures from one litter from one pregnant damn composed of between 3 to 8 embryos, depending on the size of the litter Error bars represent SEM. D'Agostino & Pearson omnibus normality test was performed to assess normality of the data set. The distributions were not normal, so a Mann Whitney statistical test was used to compare the two groups. **, p<0.01.

5.4 Discussion

In this study neuronal outgrowth in homozygous *Der1* cortical neurons was analysed. Homozygous mice carry the *Der1* mutation on both Disc1 alleles, therefore, there is no full length Disc1 plus a possible truncated Disc1 protein in these mutants. The investigation indicates that the *Der1* mutation subtly increases cortical neuronal outgrowth in this mouse model of mental disorders. It seems that the mutation increases the number of neurites, however the additional neurites remain very short therefore a difference in neurite extension is only visible close to the soma. Furthermore, cell soma hypertrophy was also discovered in the cultured neurons.

Impaired neuronal outgrowth is a frequent finding in schizophrenic patients and in animal models of mental illness (Arnold *et al.*, 1995; Harrison, 1999; Kalus *et al.*, 2000; Ozeki *et al.*, 2003; Li *et al.*, 2007). However, in schizophrenic patient, dendritic growth is reduced compared to control, with less extensive neurons (Kalus *et al.*, 2000; Rosoklija *et al.*, 2000).

Consistent with the data presented here, DISC1 knockdown is known to increase neurite outgrowth and neuronal soma size (Duan *et al.*, 2007). Therefore, it could be hypothesised that increased neurite outgrowth and soma hypertrophy found in the homozygous mice could be induced by hyper-activation of the AKT-mTOR pathway which has been shown to participate in Disc1-knockdown induced increased neurite extension and soma size (Duan *et al.*, 2007). Indeed, DISC1 regulates AKT signalling via KIAA1212. The AKT-mTOR pathway is known to be involved in the development of new-born neurons in the adult brain. The phosphorylation of various substrates by AKT regulates diverse processes of neuronal development, including morphogenesis, dendritic development, synapse formation and synaptic plasticity. Phosphoinositide-3′ kinase (PI3K)–Akt–mTOR signalling leads to the growth and branching of dendrites (Kwon *et al.*, 2003; Jaworski *et al.*, 2005). When DISC1 is knocked out, the association between DISC1 and KIAA1212 is abolished and AKT-mTOR signalling is over activated in neurons. This leads to increased neurite extension. Moreover rapamycin, which is an inhibitor of the mTOR pathway, rescues the soma size defect of new neurons affected by DISC1 knockdown or KIAA1212 overexpression (Kim *et al.*, 2009; Wu, Li and Xiao, 2013; Zhou *et al.*, 2013). This suggests that the mTOR pathway, a regulator of cell growth and metabolism, could be affected in the homozygous Der1 mice.

DISC1 might also be involved in other pathways affecting neuronal outgrowth. For example, NDEL1 is a major partner of DISC1 and, similarly to what has been found when DISC1 is downregulated, NDEL1 knockdown in new-born neurons also leads to outgrowth disruption. Moreover, when expression of both DISC1 and NDEL1 is knocked down, an increase of the number of dendrites was found as well as a drastic modification of neuronal outgrowth. Therefore, it seems that their association regulates the outgrowth of new-born neurons (Duan *et al.*, 2007). In our model Disc1 protein is absent and a truncated protein might be present but, if so, it lacks the binding site for NDEL1. It is therefore possible that the abolition of NDEL1 binding to DISC1 leads to abnormal neuronal outgrowth. Another partner of DISC1, FEZ1, also regulates neuronal outgrowth. Together they have been found to regulate dendritic development of new born dentate granule cells in the adult brain. Blocking the interaction between DISC1 and FEZ1 leads to an increase of the total dendritic length and complexity as well as soma hypertrophy (Kang *et al.*, 2011). FEZ1 and NDEL1 work together through the formation of a complex with DISC1 to ensure normal neuronal outgrowth (Kang *et al.*, 2011). In the *Der1* mice, the mutation abolishes full-length Disc1 expression, but may also produce aberrant Disc1 species lacking the full binding sites for FEZ1 and NDEL1. Transcripts of these species were found in the Der1 mice but not the chimeric proteins. Therefore, the role of the complex might be impaired which could be why there is an abnormal outgrowth of the neurons

represented by an increased neurite extension as well as soma hypertrophy. Overall then, DISC1 may interact with different partners to regulate distinct aspects of neuronal outgrowth. It could be postulate that soma hypertrophy could indicate an increase in the density of organelle such as mitochondria, lysosome, vesicles or endosome as this would lead to extension of the cell matrix. Moreover, it could possibly indicate a reorganization of the cytoskeleton and of the vesicular trafficking, as the area covered is greater, additional association between microtubule and actin could be occurring. Moreover, it is known that DISC1 plays a role in the organisation of the cytoskeleton through its association with microtubules (Morris *et al.*, 2003; Ishizuka *et al.*, 2006). Moreover, hypertrophy of cortical neuron could ultimately lead to a decrease of neuronal density in the cortex. Indeed, other mice models expressing disrupted DISC1 have indeed found a reduction in neuron number (Lee, Fadel, *et al.*, 2011). Moreover, post mortem study using samples from patient suffering from psychiatric illness also reported a reduced total number of neuron (Rajkowska, Halaris and Selemon, 2001; Manji *et al.*, 2003; Kreczmanski *et al.*, 2007).

Neuronal outgrowth impairment could lead to a deficit in neuronal connection and communication in the brain of this mouse model. Indeed, the growth of neurites determines neuronal connectivity, and disruption of this process could lead to defects in cortical development and cognition. Additional neurite extension could lead to impairment of synaptic signal and connections therefore impairing the brain circuitry. Consequently, integration and positioning of neurons in the brain could be affected by the change in neuronal morphology. Moreover, this could alter neuronal excitability. This would mean that communication between neurons and brain areas could be altered, and brain function could be impaired as a consequence. These findings indicate that the *Der1* mutation could lead to abnormal neuronal circuitry which is a typical phenotype of schizophrenia.

Chapter 6

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CONSEQUENCES OF THE DER1 MUTATION FOR RNA EXPRESSION

6.1 Introduction

Data in the previous chapters indicates that there are very subtle brain changes in the Der1 mouse model at the anatomical and cellular level. However, molecular changes have not been studied so far.

DISC1 is known to have an important role in transcription. DISC1 involvement in gene transcription was first suggested by its interaction with activating transcription factors 4 and 5 (*ATF4* and *ATF5*) as well as the transcriptional repressor Nuclear receptor Co-Repressor (*N-CoR*) (Millar, Christie and Porteous, 2003; Morris *et al.*, 2003; Sawamura *et al.*, 2008). DISC1 is also believed to bind to mRNA such as *ITPR1* mRNA and to regulate synaptic plasticity (Tsuboi *et al.*, 2015) . DISC1 inhibits the transcriptional activity of ATF4, therefore it is possible that through its interaction with ATF4, DISC1 contributes to the regulation of the cellular transcriptome (Malavasi *et al.*, 2012; Soda *et al.*, 2013). Additionally, DISC1 was found to colocalize with promyelocytic leukaemia (PML) bodies which are nuclear compartments for gene transcription, also suggesting that DISC1 might be involved in transcriptional regulation (Sawamura *et al.*, 2008). Transcriptional dysregulation has been frequently found in psychiatric disorders (Nestler *et al.*, 2016). It seems that genes differentially expressed in those disorders are involved in neurodevelopment and neuronal function, and particularly in synaptic activity and cell communication (Lin *et al.*, 2011; Wen *et al.*, 2014; Sanders *et al.*, 2017). Additionally, altered transcript levels may be due to other effects of the *DISC1* mutation. DISC1 is known to affect neuronal properties such as neurogenesis, proliferation, neuronal morphology and brain development (Brandon, 2007). Such altered cellular properties would likely affect the RNA expression profile.

The aim of this chapter is to investigate RNA expression in the Der1 mice in order to discover if genes and pathways known to be affected in mental disorders are affected in this mouse model, as well as to discover possible new genes and pathways which could be affected by the t(1;11) translocation. After a global analysis of the data, the genes involved in apoptosis, neurogenesis, neurite outgrowth and GABAergic pathways corresponding to the proteins previously studied in this thesis such as caspase-3, PV and DCX were investigated as well to compare and confirm our previous findings but at a molecular level this time.

RNA sequencing (RNAseq) was carried out using RNA extracted from nine weeks old hippocampus and the cortex   of 4 males and 4 females for each genotype (wild type, heterozygous, homozygous), making a total of 48 samples.

6.2 Analysis of the effect of the heterozygous Der1 mutation on RNA expression

6.2.1 Quality control of the samples

Principal-component analysis (PCA) is used to inspect data for unwanted biological or technical effects. PCA analysis was performed using regularized log (rlog) of the counts from all samples. This transforms the counts to the log2 scale minimizing the differences between samples for rows with small counts and normalizing with respect to library size. This allows checking for outliers and clustering of samples.

For the hippocampus, the PCA plot revealed a wild-type male outlier which was removed for further analysis. To balance the sex ratio one female wild-type was removed as well. Following outlier removal, the PCA plot showed wild-type and heterozygous samples clustering by genotype and sex (figure 6.1, a). In addition, a heatmap was created to look at the samples from the hippocampus, with outliers excluded (figure 6.1, b). This confirmed that the heterozygous samples group together, as do the wild-type samples. The cortex PCA plot also revealed a wild-type male outlier, and a wild-type female sample was removed as well for balance. As in hippocampus, following outlier removal the PCA plot indicates that the samples cluster by genotype and sex (figure 6.2, a). The heatmap issued from the RNAseq results of the cortical samples revealed that the heterozygous samples cluster together, as do the wild type samples (figure 6.2, b). These initial analyses indicate that the heterozygous samples are clearly different from the wild-type samples in both regions of interest. Moreover, heterozygous mutant mice correspond to the human t(1;11) carriers and are therefore of most relevance to mental illness.
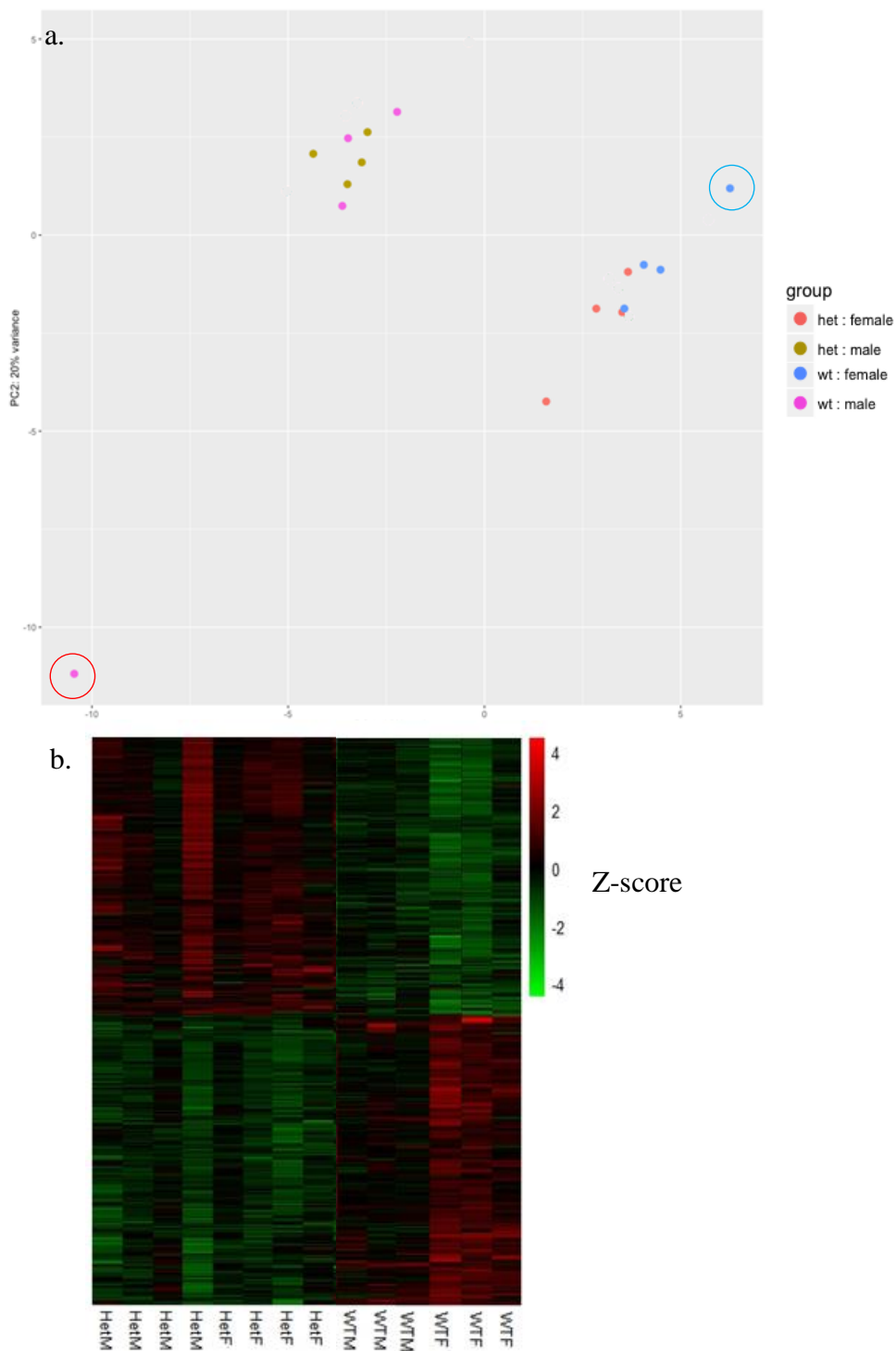
Figure 6.1: Principal component analysis of the hippocampus samples

a. PCA plot two WT outliers circled.

b. Heatmap: each row represents the expression of one gene and each column represents one sample. Z-score demonstrates the standard deviation of the log2 fold change for genes with a significant differential expression between genotype (log2 fold change <0.05), in each sample. Differential gene expression is expressed in red and green respectively representing high and low expression.
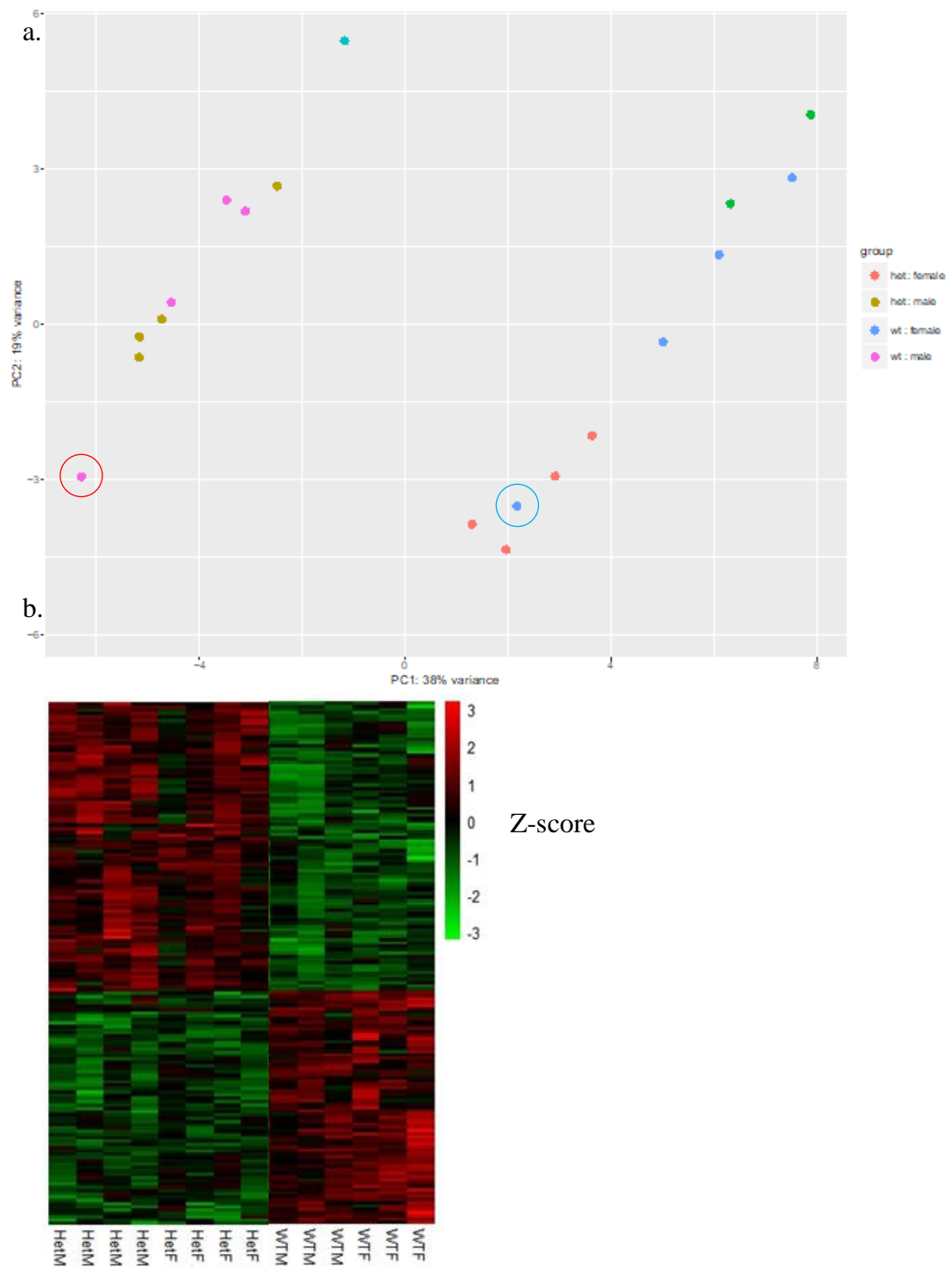
Figure 6.2: Principal components analysis of the cortex samples

a. PCA plot with the two WT outliers circled.

b. Heatmap: each row represents the expression of one gene and each column represents one sample. Z-score demonstrates the standard deviation of the log2 fold change for genes with a significant differential expression between genotype (log2 fold change<0.05), in each sample. Differential gene expression is expressed in red and green respectively representing high and low expression.

6.2.2   Differential gene and exon expression analysis

For the full analysis, RNAseq was analysed at the whole gene level using DESeq2 (Love, Huber and Anders, 2014), and at the single exon level using DEXSeq (Anders, Reyes and Huber, 2012). The latter analysis was performed because a single gene can give rise to numerous different transcripts due to alternative splice sites, transcription start sites, and polyadenylation sites. Therefore, investigation at the exon level can lead to additional and more precise information. For each exon and each sample, reads mapped to this exon and reads mapped to any of the other exons of the same gene were counted. Then, the ratio of these two counts was assessed. Changes across genotypes were observed in order to deduce variations specific to the exon usage. Change in internal exon usage is believed to be due to a change in the rate with which this exon is spliced into transcripts, while change in first and last exons reveals altered usage of start sites and polyadenylation sites. The same comparisons performed using the gene level analysis were done at the exon level.

a.   *Disc1* expression in Der1 brain

Extraction of the *Disc1* expression data indicates that it is downregulated in heterozygous samples from both brain regions, which confirms the heterozygosity of the samples used (Figure 6.3). Moreover, it was confirmed by its expression value which indicates a negative log2fc and a p value less than 0.05 in the hippocampus and the cortex of the heterozygous Der1 samples (table 6.1). In addition to this reduction, *Disc1* expression was analysed exon by exon. As the *Der1* mutation produces transcripts containing *Disc1* exons 1-8 (Malavasi et al), reduced expression of *Disc1* exons 9-13 was predicted, with little effect on exons 1-8 in heterozygotes. This was confirmed in both the cortex (figure 6.4, a) and the hippocampus (figure 6.4, b). A study has reported the expression of over 50 *DISC1* splice variants in human brain (Nakata *et al*, 2009), with a higher expression in the hippocampus. Similarly, in adult mouse brain *Disc1* expression in the hippocampus was higher compared to other regions. Here, *Disc1* expression is higher in the hippocampus (table 6.1) and some exons seem to be more or less expressed depending on the region. For example, in the hippocampus exon 2 (expression>15) compared to in the cortex (expression<10) (figure 6.4).

	Base Mean	log2FoldChange (log2fc)	padj
hippocampus	114.614663	-0.3239909	0.00523277
cortex	87.5821453	-0.4633902	0.0007188

Table 6.1: Disc1 RNAseq data from the hippocampus and the cortex of the heterozygous Der1 mice

The negative log2fc indicates that the expression of DISC1 is reduced in both areas. Moreover, the adjusted p value (padj) indicates that DISC1 expression in the heterozygous mice is significantly different compared to the WT.
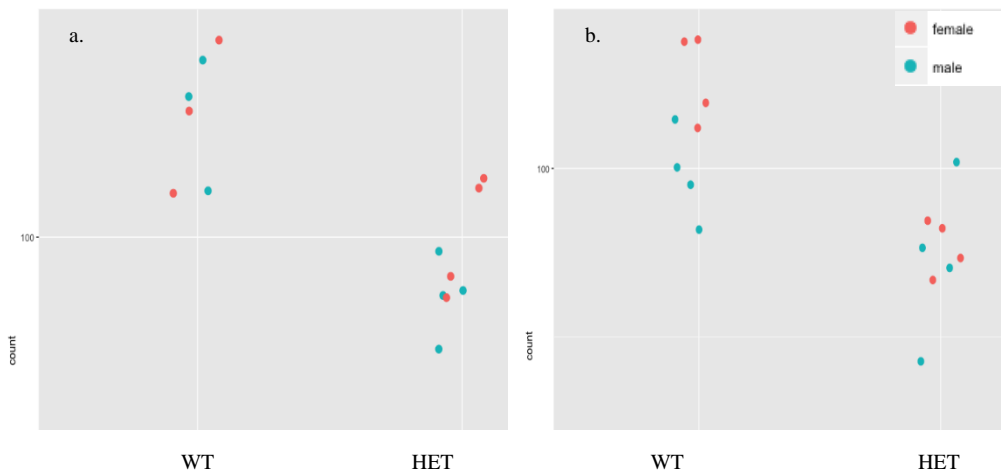
Figure 6.3: Normalised counts for *DISC1* in WT and Hets

DISC1 counts from the heterozygotes are lower than the counts from the WT, in both the hippocampus (a) and the cortex (b).
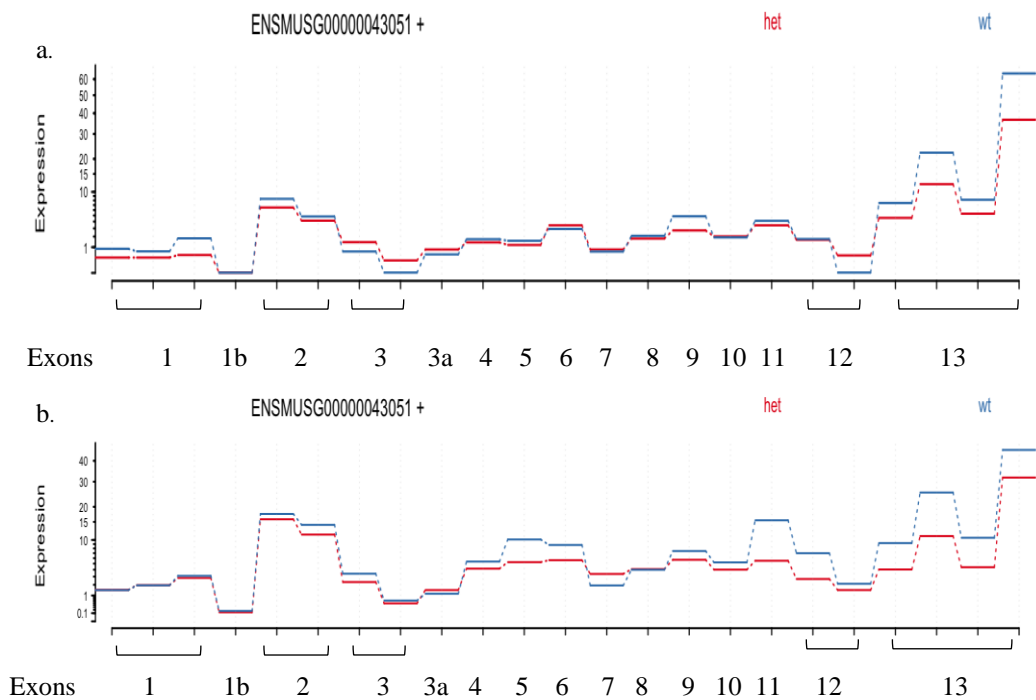
Figure 6.4: Result of the DEXSeq analysis for DISC1 in the heterozygotes versus wild-type
Both shows cortex (a.) and hippocampus (b.) show a drop mainly after exon 8.

b. Overview of the gene-level and exon-level expression analysis of heterozygous mice compared to WT

The whole gene-level analysis using DESeq2 revealed that in the samples from the hippocampus of the heterozygous Der1 mice there were 175 differentially expressed genes compared to wild type (padj<0.05). These genes are represented in the volcano plot below (figure 6.5. a). From those genes 76 had a reduced expression (log2 fold change<0) and 99 had a higher expression (log2 fold change>0), compared to the wild-type. From the cortical samples of the heterozygous mice, 2124 genes were found differentially expressed compared to the wild-type (padj<0.05). These genes are represented in the volcano plot below (figure 6.5. b). From those genes 1108 are down regulated and 1016 are up regulated compared to the genes expression from the wild-type cortical samples. A cut off at base mean (DISC1)/2 was applied, three and seven genes were respectively excluded from the down and up regulated gene list obtained with the hippocampus samples and five and four genes were excluded from the down and up regulated genes from the cortical sample analysis. The exon level analysis using DEXSeq also revealed a number of significant changes. In the cortex, the expression of 3304 genes was found to be significantly different compared to the wild-type, while 50 genes with differentially expressed exons were discovered in the hippocampus compared to the WT. For further analysis, both gene lists obtained with exon and gene level analysis were studied

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combined together, for the cortex and the hippocampus global investigation. In order to assess the potential biological significance of the set of genes found to be differentially expressed in the cortex and the hippocampus, gene ontology (GO) was performed using Gorilla (http://cbl-gorilla.cs.technion.ac.il/).
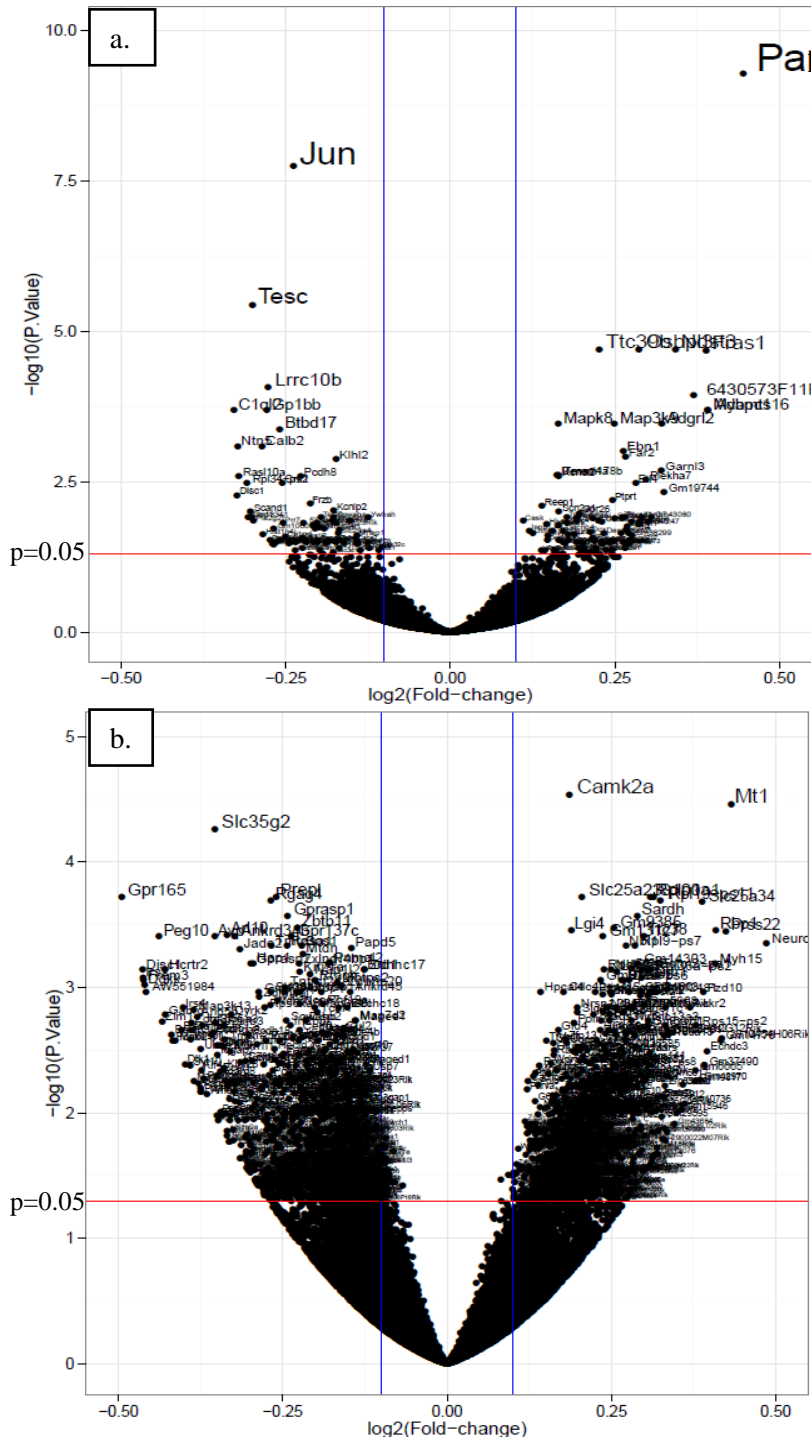
Figure 6.5: Volcano plot of the gene expression in the heterozygous mice.

a. Representation of the gene expression in the hippocampus

b. Representation of the gene expression in the cortex

All the genes above the red line are significantly differently expressed in the heterozygous mice compared to the wild-type mice (p<0.05). The blue lines represent -0.1<log2FC<0.1.

6.2.3 Analysis of the cortical samples

a. Gene ontology of the cortical samples from the heterozygous mice

GO analysis of the differentially expressed genes from the cortical samples revealed that multiples processes are affected, with a total of 293 GO component, 279 GO function, and 1220 GO process terms significantly affected by the *Der1* mutation (table 6.2 to 6.4). GO Processes mainly affected by the *Der1* mutation in the cortex of the heterozygous mice include localization and transport of entities, neurodevelopment, neurogenesis, dendrite development and organization of various cell parts such as synapses, vesicles and the cytoskeleton (table 6.2). Furthermore, it seems that binding activity is the major GO Function affected as well as channel, transporter and receptor activity (table 6.3). The major GO Components affected are organelles such as mitochondria and vesicles, transporter and receptor complexes, the cytoskeleton and synapses (table 6.4).

Overall, the heterozygous *Der1* mutation is predicted to affect neuronal development, transport of molecules and organelles, synapse function and therefore excitatory and inhibitory neurotransmission through the dysregulation of the expression of neurotransmitter receptors and possibly the dysregulation of neurotransmitter circulation.

Description	FDR q-value (padj)	Enrichment	Number of genes
localization	2.56E-90	1.62	1277
transport	3.90E-72	1.62	1072
protein localization	1.28E-52	1.76	618
intracellular transport	1.49E-43	1.87	437
organelle organization	1.26E-34	1.55	651
vesicle-mediated transport	5.99E-34	1.82	372
regulation of neurogenesis	8.61E-33	1.88	328
regulation of neuron differentiation	1.98E-31	1.96	676
protein transport	9.81E-31	1.73	398
regulation of cell communication	1.42E-24	1.36	880
regulation of signalling	2.36E-24	1.35	883
developmental process	2.89E-24	1.27	1292
intracellular protein transport	1.20E-22	1.84	242
regulation of cell death	2.80E-18	1.44	505
neuron projection development	9.39E-18	2.19	120
anatomical structure development	4.94E-17	1.28	903
regulation of apoptotic process	5.30E-15	1.42	451
nervous system development	9.25E-15	1.83	163
regulation of programmed cell death	1.42E-14	1.41	453
cytoskeleton organization	1.65E-14	1.59	260
neuron projection morphogenesis	2.94E-14	2.02	118
anatomical structure morphogenesis	2.21E-12	1.38	431
cation transport	2.24E-10	1.52	222
regulation of neurotransmitter levels	2.18E-09	1.85	100
endocytosis	2.65E-09	1.69	132
microtubule-based transport	1.11E-08	2.17	60
cell communication	1.43E-08	1.47	217
dendrite morphogenesis	4.21E-08	2.88	31
synapse organization	1.17E-07	2	65
brain development	2.28E-07	1.98	64
vesicle organization	2.43E-07	1.77	89
RNA transport	1.06E-06	1.97	59
dendrite development	2.40E-06	2.82	25
synaptic signalling	3.26E-06	1.61	105
mitochondrial transport	6.91E-06	1.94	53
dendritic spine organization	1.84E-04	2.75	18
microtubule-based movement	2.32E-04	1.58	77
glutamate receptor signalling pathway	2.46E-04	2.56	20
signalling	2.72E-04	1.36	158
synaptic transmission, GABAergic	3.54E-04	3.34	12

Table 6.2: <u>Processes affected by the *Der1* mutation in the cortex of the heterozygous mice</u>

'FDR q-value', or padj, is the correction of the p-value for multiple testing using the Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value * number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

Description	FDR q-value (p-value)	Enrichment	Number of genes
protein binding	6.24E-136	1.41	2581
binding	3.35E-135	1.28	3420
catalytic activity	1.29E-68	1.41	1678
ion binding	5.40E-62	1.41	1565
transferase activity	2.20E-35	1.52	724
RNA binding	3.05E-27	1.7	377
kinase activity	3.24E-22	1.75	279
transporter activity	1.04E-15	1.49	368
cation transmembrane transporter activity	2.43E-10	1.55	631
mRNA binding	4.24E-08	1.86	219
enzyme regulator activity	5.02E-07	1.36	278
tubulin binding	4.46E-06	1.61	104
beta-catenin binding	9.12E-06	2.2	39
glutamate receptor binding	2.68E-05	2.41	29
SNARE binding	3.02E-05	1.89	52
ion channel activity	5.63E-05	1.47	124
voltage-gated sodium channel activity	7.01E-05	3.15	16
channel regulator activity	1.71E-04	1.83	49
ribosome binding	1.88E-04	2.28	27
actin binding	3.01E-04	1.43	122
microtubule binding	4.22E-04	1.58	73
syntaxin binding	4.83E-04	1.91	38
channel activity	7.92E-04	1.39	127
GABA receptor activity	2.68E-03	2.8	13
p53 binding	3.78E-03	1.91	29
transmembrane receptor protein tyrosine kinase activity	5.28E-03	1.97	25
GABA-A receptor activity	9.24E-03	2.74	11
growth factor binding	9.47E-03	1.57	48
L-glutamate transmembrane transporter activity	1.03E-02	3.04	9
GABA-gated chloride ion channel activity	1.44E-02	3.15	8

Table 6.3: <u>Functions affected by the *Der1* mutation in the cortex of the heterozygous mice</u>

'FDR q-value', or padj, is the correction of the p-value for multiple testing using the Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value * number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

Description	FDR q-value (padj)	Enrichment	Number of genes
intracellular part	1.74E-287	1.37	3857
organelle	2.31E-235	1.43	3308
neuron part	7.79E-97	2.03	731
membrane	1.39E-92	1.38	2189
neuron projection	1.39E-74	2.11	520
synapse	5.35E-45	2.24	278
dendrite	6.18E-41	2.29	242
neuronal cell body	7.35E-39	2.16	263
vesicle	7.28E-35	1.59	590
axon	1.93E-34	2.35	192
mitochondrion	3.24E-33	1.59	557
postsynaptic density	4.11E-28	2.57	130
endosome	2.02E-26	1.83	281
cytoskeleton	2.20E-24	1.56	452
post-synapse	1.49E-21	2.56	101
neuron spine	2.84E-18	2.51	89
dendritic spine	1.01E-17	2.5	87
pre-synapse	3.11E-17	2.31	100
transporter complex	4.59E-14	1.95	124
lysosome	4.60E-14	1.84	146
microtubule	2.85E-12	1.76	148
transport vesicle	1.32E-11	2.03	91
exocytic vesicle	3.03E-10	2.12	72
synaptic vesicle	8.22E-10	2.17	65
excitatory synapse	7.94E-09	3.24	26
mitochondrial respiratory chain complex I	2.52E-06	2.77	24
dendrite cytoplasm	2.79E-06	3.05	20
receptor complex	5.63E-05	1.46	115
AMPA glutamate receptor complex	1.21E-04	2.77	17
ionotropic glutamate receptor complex	1.37E-04	2.28	25
microtubule cytoskeleton	1.74E-04	1.69	56
neurotransmitter receptor complex	2.86E-04	2.19	25
inhibitory synapse	3.55E-04	2.88	14
GABA receptor complex	5.09E-04	2.93	13
actin cytoskeleton	7.88E-04	1.55	65
GABA-A receptor complex	3.34E-03	2.74	11
SNARE complex	5.99E-03	1.95	21

Table 6.4: <u>Components affected by the Der1 mutation in the cortex of the heterozygous</u>

<u>mice</u>

'FDR q-value', or padj, is the correction of the p-value for multiple testing using the

Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value *

number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

In addition, Panther online (http://pantherdb.org/) was used in order to get a representation of the molecular function, biological process, cellular component, and pathways in which the differentially expressed genes are involved. Panther does not use the entire GO catalogue and allow additional functional classification of the different genes by combining gene function, families, pathways and biological process. Similar molecular functions were affected by the change in gene expression in the cortex (figure 6.6, a). Additionally, when looking more particularly at transporter and receptor activity (figure 6.6, b and c), GABA, glutamate and acetylcholine receptor activity is predicted to be affected, together with transmembrane transporter activity, including neurotransmitter transporter activity.

The processes found to be affected when using Panther online are quite broad but again similar to what was previously found using Gene Ontology analysis. Indeed, processes involved in cellular component organization, development, growth, localization and cellular biology are predicted to be affected by the *Der1* mutation (figure 6.7, a). Moreover, it seems that protein and RNA localization may be particularly affected as well as overall major transport of entities which account for most of the localization processes affected (figure 6.7, b). The main cellular processes (figure 6.7, c) affected are involved in cell communication which would involve synaptic transmission, signal transduction and receptor signalling.

Similar cellular components were also found using Panther. Indeed, genes found to be differentially expressed are involved in cellular part and junction, macromolecule complex, membrane and above all in organelles and synapses (figure 6.8, a). The organelles predicted to be most affected are the nucleus, Golgi, endoplasmic reticulum and mitochondria (figure 6.8, b and c).

The analysis also revealed pathways in which those genes are involved. The cortical set of genes affected is involved in numerous pathways (figure 6.9). Most of these pathways are involved in signalling and cellular communication, cell growth and death, and molecular synthesis. The most important pathway in which the differentially expressed genes are involved seems to be the Wnt pathway (figure 6.10). This pathway is important for brain development.

a.

■	antioxidant activity (GO:0016209),8 genes
■	binding (GO:0005488), 1232 genes
■	catalytic activity (GO:0003824), 1239 genes
■	channel regulator activity (GO:0016247), 2 genes
■	receptor activity (GO:0004872), 165 genes
■	signal transducer activity (GO:0004871), 153 genes
■	structural molecule activity (GO:0005198), 135 genes
■	translation regulator activity (GO:0045182), 39 genes
■	transporter activity (GO:0005215), 314 genes

b.

c.

■ G-protein coupled receptor activity (GO:0004930), 39 genes
■ GABA receptor activity (GO:0016917), 11 genes
■ acetylcholine receptor activity (GO:0015464), 2 genes
■ cytokine receptor activity (GO:0004896), 6 genes
■ glutamate receptor activity (GO:0008066), 16 genes
■ ligand-activated sequence-specific DNA binding
RNA polymerase II transcription factor activity (GO:0004879), 10 genes
■ receptor activator activity (GO:0030546), 1 genes
■ receptor inhibitor activity (GO:0030547), 3 genes
■ receptor regulator activity (GO:0030545), 4 genes
■ transmembrane receptor protein serine/threonine
kinase activity (GO:0004675), 3 genes
■ transmembrane receptor protein tyrosine kinase activity (GO:0004714), 22 genes

■ amino acid transmembrane transporter activity (GO:0015171), 30 genes
■ carbohydrate transmembrane transporter activity (GO:0015144), 5 genes
■ lipid transporter activity (GO:0005319), 15 genes
■ neurotransmitter transporter activity (GO:0005326), 8 genes
■ transmembrane transporter activity (GO:0022857), 262 genes

Figure 6.6: Molecular function in which the genes differentially expressed in the heterozygous Der1 mice are involved.

a. Overall representation of the molecular functions in which the genes are involved
b. Molecular functions involved in receptor activity
c. Molecular functions involved in transporter activity

The number of significant genes included in each function is indicated at each line.

a.

- biological adhesion (GO:0022610), 87 genes
- biological regulation (GO:0065007), 699 genes
- cellular component organization or biogenesis (GO:0071840), 598 genes
- cellular process (GO:0009987), 2231 genes
- developmental process (GO:0032502), 366 genes
- growth (GO:0040007) 3 genes
- immune system process (GO:0002376), 74 genes
- localization (GO:0051179) 620 genes
- locomotion (GO:0040011), 77 genes
- metabolic process (GO:0008152) 1668 genes
- multicellular organismal process (GO:0032501), 392 genes
- reproduction (GO:0000003) 51 genes
- response to stimulus (GO:0050896), 539 genes
- rhythmic process (GO:0048511), 5 genes

b

- RNA localization (GO:0006403) 22 genes
- protein localization (GO:0008104), 175 genes
- transport (GO:0006810), 544 genes

c.

- cell communication (GO:0007154), 690 genes
- cell cycle (GO:0007049), 193 genes
- cell growth (GO:0016049), 30 genes
- cell proliferation (GO:0008283), 6 genes
- cell recognition (GO:0008037) 2 genes
- cellular component movement (GO:0006928), 126 genes
- chromosome segregation (GO:0007059), 17 genes
- cytokinesis (GO:0000910) 52 genes

Figure 6.7: Processes in which the genes differentially expressed in the heterozygous Der1 mice are involved

a. Overall representation of the processes in which the genes are involved

b. Processes involved in localization

c. Processes involved in cellular process

The number of significant genes included in each function is indicated at each line.

a.

- cell junction (GO:0030054), 32 genes
- cell part (GO:0044464), 1662 genes
- extracellular matrix (GO:0031012), 30 genes
- extracellular region (GO:0005576), 96 genes
- macromolecular complex (GO:0032991) 698 genes
- membrane (GO:0016020), 596 genes
- organelle (GO:0043226), 1113 genes
- synapse (GO:0045202), 53 genes

b.

- Golgi apparatus (GO:0005794), 103 genes
- chromosome (GO:0005694) 47 genes
- cytoplasmic membrane-bounded vesicle (GO:0016023) 58 genes
- cytoskeleton (GO:0005856) 146 genes
- endoplasmic reticulum (GO:0005783), 120 genes
- endosome (GO:0005768) 55 genes
- lysosome (GO:0005764), 21 genes
- mitochondrion (GO:0005739), 104 genes
- nucleus (GO:0005634), 541 genes
- peroxisome (GO:0005777) 10 genes
- plastid (GO:0009536) 2 genes
- vacuole (GO:0005773), 61 genes

c.

- apical part of cell (GO:0045177) 4 genes
- cell projection (GO:0042995), 130 genes
- external encapsulating structure (GO:0030312), 2 genes
- intracellular (GO:0005622), 1589 genes
- neuronal cell body (GO:0043025), 14 genes
- nuclear outer membrane-endoplasmic reticulum membrane network (GO:0042175) 69 genes
- plasma membrane (GO:0005886), 356 genes

Figure 6.8: Cellular components in which the genes differentially expressed in the heterozygous Der1 mice are involved

a. Overall representation of the cellular components in which the genes are involved.

b. Cellular components organelles.

c. Cellular components of cell part.

The number of significant genes included in each function is indicated at each line.

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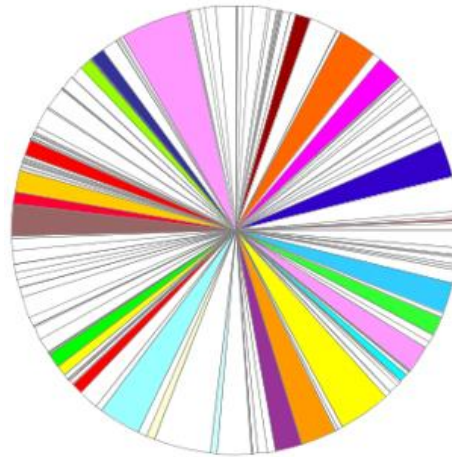
PANTHER Pathways	Number of genes
Circadian clock system	8
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	20
GABA-B receptor II signaling	20
Hypoxia response via HIF activation	16
Metabotropic glutamate receptor group III pathway	34
Alzheimer disease-amyloid secretase pathway	35
Ionotropic glutamate receptor pathway	24
Ras Pathway	33
FGF signaling pathway	55
Endothelin signaling pathway	37
Metabotropic glutamate receptor group II pathway	20
PI3 kinase pathway	23
Transcription regulation by bZIP transcription factor	24
CCKR signaling map	66
Ubiquitin proteasome pathway	27
Apoptosis signaling pathway	46
EGF receptor signaling pathway	53
Gonadotropin-releasing hormone receptor pathway	90
Parkinson disease	37
Huntington disease	57
Integrin signalling pathway	71
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	45
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	57
Angiogenesis	61
PDGF signaling pathway	49
Wnt signaling pathway	94

Figure 6.9: Panther most enriched pathways in which the genes differentially expressed in the heterozygous Der1 mice are involved.

The number of significant genes included in each pathway is indicated at each line.
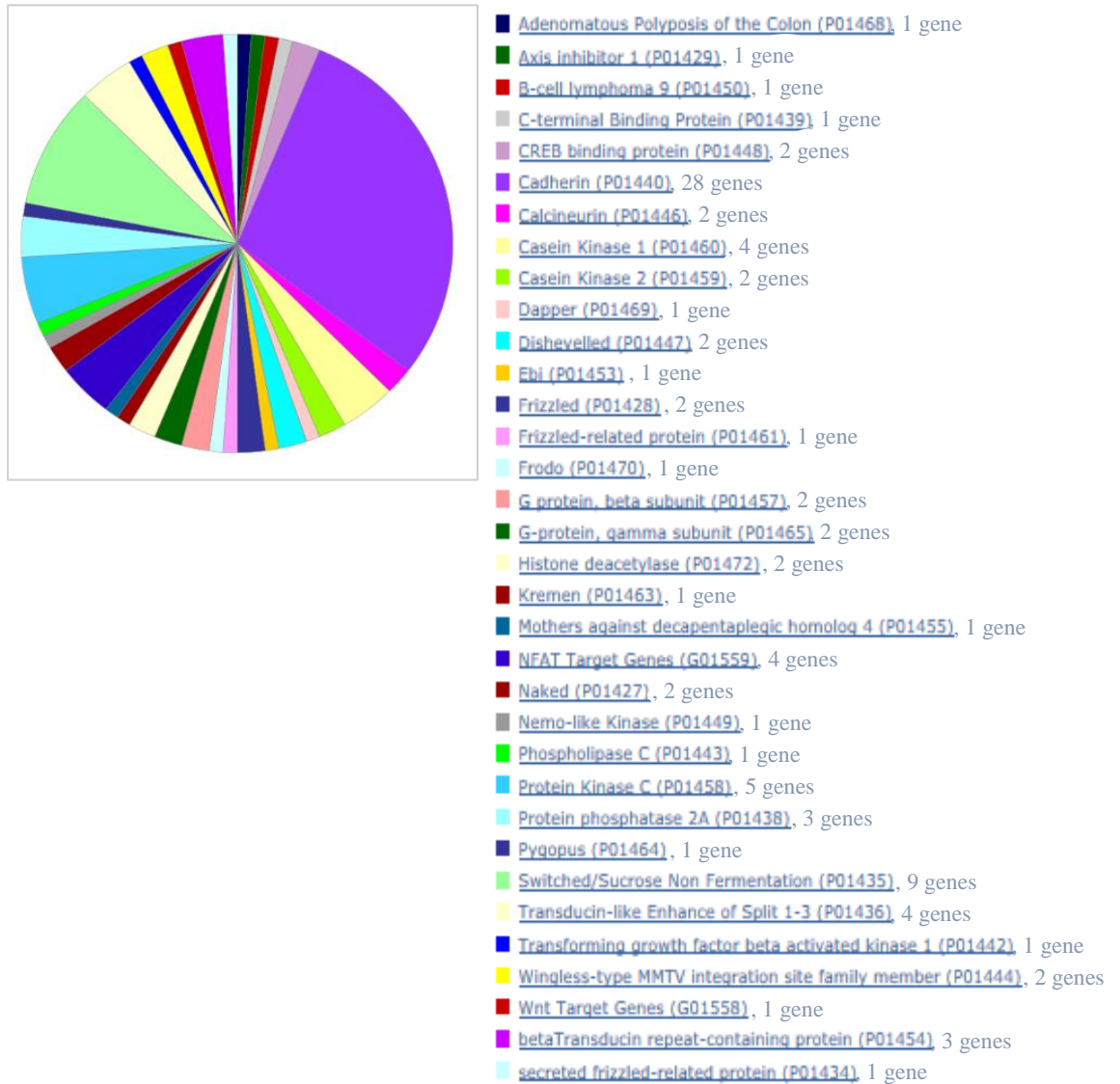
Figure 6.10: Representation of the Wnt pathway in which the differentially expressed genes are involved.

The number of significant genes included in each pathway is indicated at each line.

b. Specific genes and pathways

b.i Ion channels

RNAseq analysis revealed that multiple genes corresponding to ion channels were significantly differentially expressed at the whole gene and at the exon level (table 6.5 and table 6.6), in particular voltage gated channels which are transmembrane proteins activated by the membrane potential. They are localized along the axon and at the synapse and play a role in the regulation of neuronal excitation. Their activity is crucial for neuronal signalling. Moreover, numerous studies have implicated them in mental disorders (Judy and Zandi, 2013; Askland, 2015; Heyes *et al.*, 2015).

Genes coding for potassium channel proteins are the most affected in the heterozygous Der1 mice. Potassium channels set the resting membrane potential and repolarize neurons, modulating neuronal excitability in neurons which is believed to be altered in psychiatric disorders. Previous genomic expression studies have also provided evidence that gene expression of potassium channels is dysregulated in schizophrenia, autism disorders and bipolar disorders, which indicate that regulation of expression of the potassium channel might be impaired in mental disorders (Judy and Zandi, 2013). This indicates that neuronal plasticity and signalling might be altered in the Der1 heterozygous mice.

Calcium regulates multiple functions such as neurotransmitter release, cell growth and differentiation, neuronal excitability or gene transcription. Alteration of the concentration of calcium lead to various disorders, therefore altered expression of calcium channels could lead to major disruptions of neuronal signalling (Catterall, 2011). *CACNA1C* is a consistent finding from GWAS analysis of mental disorders (Bhat *et al.*, 2012). This gene is implicated in cell membrane depolarization which leads to changes in signalling (Bhat *et al.*, 2012), which is known to be altered in psychiatric disorders.

Sodium channels transmit action potentials through cells and are essential for the regulation of neuronal excitability, thereby for neuronal signalling. As mentioned for the other channels, modification of their expression would alter neurotransmission and signalling (Marban, Yamagishi and Tomaselli, 1998; Catterall, 2014).

In the axon initial segment, the voltage-gated sodium and potassium channels activation leads to action potential generation. Action potentials propagate along the axon and reach the nerve terminals, where activation of voltage-gated calcium channels causes calcium influx and neurotransmitter release. Potassium channels on dendrites further control action potential propagation to the synapse. This affects synaptic plasticity and leads to long-term potentiation or long-term depression. Additionally, voltage-gated sodium, calcium and potassium channels

in dendrites control the propagation of action potentials into dendrites, and help spread synaptic potentials, contributing to neuronal signalling processing in dendrites.

Those three type of voltage gated channel are spread along the neurons to relay the information between neurons as well as intracellularly, therefore the regulation of their expression and their localization is essential for neuronal signalling. Dysregulation of their expression would lead to alteration of long-term potentiation/depression, neurotransmitter flux and signalling.

	Gene	log2FC	padj
Sodium channels	*Scn1b*	0.158	0.042
	Scn3a	-0.197	0.029
	Scn3b	-0.153	0.013
	Scn9a	-0.325	0.014
	Scn5a	-0.554	5.29E-06
Calcium channels	*Cacna2d2*	-0.202	0.049
	Cacnb3	0.160	0.010
	Cacng3	0.123	0.006
Potassium channels	*Kcna1*	-0.112	0.016
	Kcnab2	0.117	0.046
	Kcnc2	-0.151	0.045
	Kcnd3	-0.262	0.006
	Kcnj10	0.182	0.032
	Kcnj6	-0.262	0.030
	Kcnt2	-0.187	0.005

Table 6.5: <u>Genes coding for voltage-gated channels found to be differentially expressed, at gene level, in the heterozygous mice</u>

Calcium channel			Potassium channel			Sodium channel		
gene	padj	log2fc	gene	padj	log2fc	gene	padj	log2fc
Cacna1a	9.81E-03	1.55E-01	*Kcna6*	8.33E-03	-6.65E-02	*Scn1a*	3.68E-02	1.35E-01
Cacna1a	1.36E-02	1.26E-01	*Kcna6*	1.75E-02	2.29E-01	*Scn1a*	4.93E-02	1.29E-01
Cacna1a	1.48E-02	1.53E-01	*Kcnab1*	4.01E-02	-7.14E-02	*Scn2a1*	2.18E-02	6.30E-03
Cacna1a	1.50E-02	7.51E-02	*Kcnab3*	3.34E-03	-6.71E-02	*Scn2a1*	4.53E-02	2.17E-02
Cacna1a	2.13E-02	1.06E-01	*Kcnab3*	3.41E-02	8.80E-02	*Scn2b*	9.08E-03	2.54E-02
Cacna1a	2.30E-02	1.25E-01	*Kcnc1*	4.35E-03	1.02E-01	*Scn2b*	3.33E-02	-4.79E-02
Cacna1a	2.79E-02	1.47E-01	*Kcnc1*	2.78E-02	-6.45E-02	*Scn3b*	4.43E-03	1.43E-01
Cacna1a	3.14E-02	-9.30E-02	*Kcnc1*	2.91E-02	7.55E-02	*Scn3b*	9.67E-03	1.12E-01
Cacna1a	4.19E-02	1.44E-01	*Kcnc2*	9.99E-03	-6.90E-02	*Scn3b*	1.83E-02	-7.24E-02
Cacna1a	4.81E-02	1.19E-01	*Kcnc2*	1.71E-02	9.82E-02	*Scn3b*	2.00E-02	1.01E-01
Cacna1b	3.29E-02	1.41E-01	*Kcnc2*	1.90E-02	4.55E-02	*Scn3b*	3.67E-02	5.30E-02
Cacna1b	3.38E-02	1.85E-01	*Kcnc2*	4.55E-02	1.27E-02	*Scn3b*	3.81E-02	2.10E-03
Cacna1b	3.52E-02	1.87E-01	*Kcnc3*	2.65E-02	-4.13E-02	*Scn3b*	4.00E-02	9.20E-02
Cacna1c	3.11E-02	1.47E-01	*Kcnc3*	3.59E-02	3.09E-02	*Scn4b*	1.83E-02	4.36E-02
Cacna1e	1.91E-02	-4.72E-03	*Kcnc4*	8.36E-03	1.78E-02	*Scn8a*	4.42E-02	8.04E-02
Cacna1g	4.00E-02	1.58E-01	*Kcnc4*	2.58E-02	-4.21E-02	*Scn8a*	4.58E-02	1.74E-02
Cacna1h	6.99E-03	1.02E-01	*Kcnd1*	1.00E-02	7.67E-02			
Cacna1h	3.76E-02	1.25E-01	*Kcnd1*	2.32E-02	-9.59E-02			
Cacna1i	6.85E-03	8.00E-02	*Kcng1*	7.15E-03	2.56E-01			
Cacna1i	3.09E-02	4.72E-02	*Kcng1*	1.43E-02	-5.79E-02			
Cacna1i	3.09E-02	-8.68E-02	*Kcng1*	2.11E-02	1.00E-01			
Cacna1i	4.91E-02	1.97E-01	*Kcnh1*	2.59E-02	4.85E-02			
Cacna2d1	1.46E-02	NA	*Kcnh2*	2.25E-02	-8.84E-02			
Cacna2d1	3.73E-02	NA	*Kcnh3*	4.78E-02	5.48E-02			
Cacna2d1	4.10E-02	NA	*Kcnj3*	2.43E-02	2.00E-04			
Cacna2d3	1.24E-02	1.10E-01	*Kcnj3*	4.79E-02	-6.23E-02			
Cacna2d3	2.42E-02	-1.11E-01	*Kcnn2*	3.49E-02	1.21E-01			
Cacna2d3	4.87E-02	6.88E-02	*Kcnv1*	1.52E-02	2.60E-02			
Cacna2d3	4.97E-02	1.32E-01	*Kcnv1*	2.91E-02	-4.11E-02			
Cacnb1	2.29E-02	-5.82E-02						
Cacnb1	4.71E-02	4.25E-02						
Cacnb2	2.32E-02	-1.06E-01						
Cacnb2	2.62E-02	1.23E-01						
Cacnb2	3.61E-02	-7.95E-02						
Cacng3	1.60E-02	2.86E-02						
Cacng3	2.08E-02	1.29E-01						
Cacng3	4.40E-02	1.03E-01						

Table 6.6: <u>Genes coding for voltage-gated channels found to be differentially expressed, at exon level, in the heterozygous mice</u>

b.ii   Neurotransmitter receptors

Additionally, the results of the RNAseq analysis showed significant changes in the expression of genes coding for neurotransmitter receptor subunits, as well as exon-specific expression changes (table 6.7 and 6.8). In the cortex, genes encoding cholinergic and GABAergic

receptors are affected in these mice. Both these systems have been showed to be impaired in mental illness and selected as potential drug targets for schizophrenia. The glutamatergic system was also found affected as several genes involved in the glutamate pathway were found differentially expressed. In the cortex, multiple genes encoding serotonin receptors have been found down regulated which could lead to deficits in memory and learning. Overall, the receptors for neurotransmitters seem to be down regulated which would indicate an important alteration of neuronal signalling and an imbalance between excitatory and inhibitory signals.

	Gene	log2FC	padj	Receptors name
Cholinergic receptors	*Chrm2*	-0.347	0.003	Cholinergic Receptor Muscarinic
	Chrna7	-0.236	0.033	Cholinergic Receptor Nicotinic
	Chrnb2	-0.126	0.044	
GABAergic receptors	*Gabrb2*	-0.239	0.030	
	Gabre	-0.3296	0.010	GABA A
	Gabrq	-0.429	0.001	
Glutamatergic receptors	*Gria2*	-0.177	0.010	Glutamate Ionotropic Receptor AMPA
	Grik3	-0.199	0.029	Glutamate Ionotropic Receptor Kainate
	Grm1	-0.198	0.009	Glutamate Metabotropic Receptor 1
Serotoninergic receptors	*Htr1b*	-0.273	0.028	
	Htr1f	-0.257	0.047	
	Htr2c	-0.222	0.039	5-Hydroxytryptamine
	Htr4	-0.255	0.047	
	Htr5a	-0.168	0.018	

Table 6.7: Genes coding for neurotransmitter receptors and found to be differentially expressed at gene level, in the heterozygous mice

GABAergic receptors			Glutamatergic receptors			Cholinergic receptors		
gene	padj	log2fc	gene	padj	log2fc	gene	padj	log2fc
Gabarapl1	8.64E-03	6.10E-03	Gria1	1.58E-02	6.62E-03	Chrm1	3.85E-02	-2.08E-02
Gabarapl1	1.58E-02	-1.91E-02	Gria1	2.50E-02	2.35E-02	Chrm2	4.13E-02	-1.06E-01
Gabbr1	3.12E-02	4.36E-02	Gria1	3.17E-02	5.73E-02	Chrm3	1.34E-02	-7.43E-02
Gabbr1	3.77E-02	-3.32E-02	Gria1	3.64E-02	6.27E-02	Chrm3	2.44E-02	2.66E-03
Gabbr1	4.71E-02	1.62E-02	Gria1	4.38E-02	4.54E-02	Chrm3	3.55E-02	8.91E-02
Gabbr1	4.90E-02	5.04E-02	Gria1	4.60E-02	-4.88E-02	Chrm3	4.39E-02	1.02E-01
Gabbr2	4.89E-02	1.92E-02	Grik2	3.76E-02	-8.70E-02	Chrnb2	3.02E-02	1.80E-01
Gabpa	1.78E-02	-7.52E-02	Grik3	7.70E-03	1.46E-01	Serotoninergic receptors		
Gabra1	1.02E-02	2.77E-02	Grik3	4.79E-02	1.28E-01	gene	padj	log2fc
Gabra1	2.35E-02	1.77E-02	Grik5	2.51E-02	7.95E-02	Htr2c	1.75E-02	8.13E-02
Gabra1	3.09E-02	7.99E-04	Grin1	1.21E-02	2.02E-01	Htr2c	3.77E-02	7.74E-02
Gabra1	3.47E-02	-6.11E-02	Grin1	1.40E-02	8.72E-02			
Gabra2	1.53E-02	5.80E-02	Grin1	1.41E-02	7.75E-02			
Gabra2	2.03E-02	1.43E-01	Grin1	1.70E-02	9.15E-02			
Gabra2	4.55E-02	1.06E-01	Grin1	1.72E-02	9.30E-02			
Gabra3	1.60E-02	4.74E-02	Grin1	1.78E-02	-8.00E-02			
Gabra3	2.21E-02	4.55E-02	Grin1	2.01E-02	6.64E-02			
Gabra3	2.34E-02	1.26E-02	Grin1	2.15E-02	-5.72E-02			
Gabra3	2.89E-02	-5.55E-02	Grin1	2.82E-02	7.72E-02			
Gabra3	3.80E-02	3.33E-02	Grin1	2.89E-02	6.35E-02			
Gabra4	1.95E-02	-7.92E-02	Grin1	3.37E-02	7.91E-02			
Gabra4	2.36E-02	5.56E-02	Grin1	3.47E-02	5.08E-02			
Gabra4	4.93E-02	4.74E-02	Grin1	4.87E-02	-5.70E-02			
Gabra5	2.54E-02	1.14E-01	Grin3a	1.28E-02	4.28E-02			
Gabra5	3.00E-02	-6.52E-02	Grin3a	2.89E-02	-9.18E-02			
Gabrb3	2.55E-02	-8.87E-02	Grina	4.16E-02	1.33E-02			
Gabrb3	2.82E-02	6.12E-02	Grina	4.55E-02	3.31E-02			
Gabrb3	3.02E-02	-1.93E-02	Gripap1	5.98E-03	-6.65E-02			
Gabrb3	4.95E-02	5.81E-02	Grk6	1.46E-02	1.15E-01			
Gabrd	1.68E-02	-7.06E-02	Grk6	2.68E-02	8.07E-02			
Gabrd	4.28E-02	3.88E-02	Grm2	2.54E-02	1.25E-01			
Gabrd	4.80E-02	3.10E-02	Grm4	5.80E-03	1.22E-01			
Gabrg2	6.98E-04	8.85E-02	Grm5	1.66E-02	8.62E-03			
Gabrg2	3.04E-03	7.08E-02	Grm7	9.09E-03	1.66E-02			
Gabrg2	1.12E-02	1.11E-01	Grm7	4.14E-02	-1.04E-01			
Gabrg2	1.59E-02	-3.69E-02						
Gabrg2	3.72E-02	-5.97E-02						

Table 6.8: <u>Genes coding for neurotransmitter receptors and found to be differentially expressed, at exon level, in the heterozygous mice</u>

Additionally, genes necessary for vesicle trafficking have been found abnormally expressed at the gene and exon level (table 6.9 and 6.10) which would indicate a possible impairment of neurotransmitter release and overall cell communication and signalling. Overall, the expression of implicated genes is mainly downregulated (table 6.9) which would suggest that there is a diminution of the vesicular traffic in the cortex of the heterozygous mice.

Gene	log2FC	padj	Protein family
Nsf	-0.10607	0.034844	Vesicle Fusing ATPase
Stx16	-0.08754	0.03149	Syntaxin
Stx1a	0.159658	0.021553	
Synj1	-0.17764	0.004892	Synaptojanin
Synj2	-0.27338	0.011166	
Syt10	-0.39057	0.004215	Synaptotagmin
Syt4	-0.17802	0.021778	

Table 6.9: Genes coding for proteins involved in vesicular trafficking and found to be differentially expressed at gene level in the heterozygous mice

Genes involved in vesicle trafficking					
gene	padj	log2fold	gene	padj	log2fold
Nsf	7.26E-03	2.42E-02	*Syn1*	4.84E-02	4.51E-02
Nsf	1.62E-02	1.33E-02	*Syn2*	1.42E-03	-7.51E-02
Nsf	2.17E-02	2.18E-02	*Syn2*	1.62E-02	3.12E-02
Nsf	2.17E-02	4.19E-02	*Syn2*	1.75E-02	5.48E-02
Nsf	2.28E-02	-5.66E-02	*Syngr1*	1.79E-02	2.82E-02
Nsf	2.36E-02	6.46E-02	*Syngr1*	3.87E-02	-2.77E-02
Nsf	2.72E-02	5.29E-02	*Synj1*	9.16E-05	2.22E-01
Nsf	3.24E-02	6.92E-02	*Synj1*	4.35E-03	1.27E-01
Nsf	4.78E-02	2.43E-02	*Synj1*	1.41E-02	1.19E-01
Nsf	4.80E-02	2.41E-02	*Synj1*	2.72E-02	7.97E-02
Nsf	4.84E-02	4.78E-02	*Synj2*	1.60E-02	1.41E-01
Stx1b	4.24E-02	1.36E-02	*Synpr*	5.00E-02	7.59E-03
Stx4a	4.08E-02	-1.19E-01	*Synrg*	1.78E-02	1.22E-01
Stx6	2.71E-02	-2.77E-02	*Synrg*	1.80E-02	1.84E-01
Stxbp1	4.56E-03	2.58E-02	*Synrg*	1.92E-02	1.62E-01
Stxbp1	6.83E-03	4.59E-02	*Synrg*	4.66E-02	1.27E-01
Stxbp1	7.03E-03	4.33E-02	*Synrg*	4.85E-02	1.26E-01
Stxbp1	7.19E-03	-4.35E-02	*Syp*	2.15E-02	-1.38E-03
Stxbp1	7.46E-03	-1.59E-01	*Syp*	2.91E-02	-3.95E-02
Stxbp1	1.60E-02	4.23E-02	*Syp*	3.00E-02	1.87E-02
Stxbp1	2.47E-02	6.03E-03	*Syp*	3.62E-02	1.96E-03
Stxbp1	2.69E-02	2.59E-02	*Syt1*	2.07E-02	1.04E-02
Stxbp1	3.10E-02	3.05E-02	*Syt1*	2.70E-02	1.18E-02

Gene			Gene		
Stxbp1	3.76E-02	3.88E-02	*Syt1*	2.71E-02	-1.79E-03
Stxbp1	4.67E-02	4.30E-03	*Syt1*	2.89E-02	-4.64E-02
Stxbp1	4.79E-02	2.77E-03	*Syt1*	3.54E-02	-8.87E-03
Stxbp1	4.79E-02	2.09E-02	*Syt1*	3.82E-02	3.31E-02
Stxbp5	1.41E-02	1.50E-01	*Syt1*	4.40E-02	3.57E-02
Stxbp5	3.98E-02	1.43E-01	*Syt1*	4.41E-02	2.41E-02
Stxbp6	1.44E-02	2.96E-02	*Syt1*	4.94E-02	2.66E-02
Stxbp6	3.50E-02	-4.16E-02	*Syt1*	4.96E-02	2.93E-02
			Syt11	1.86E-02	6.25E-02
			Syt11	2.01E-02	2.34E-02
			Syt11	3.46E-02	-2.47E-02
			Syt13	1.36E-03	2.85E-02
			Syt13	2.76E-02	-3.07E-02
			Syt2	1.44E-02	-9.79E-02
			Syt4	1.53E-02	2.69E-02
			Syt4	1.59E-02	-5.35E-02
			Syt4	3.34E-02	2.37E-02
			Syt9	1.34E-02	-6.83E-02

Table 6.10: <u>Genes coding for proteins involved in vesicular trafficking and found to be differentially expressed at exon level, in the heterozygous mice</u>

b.iv   Synapses

Genes necessary for the formation and function of synapses were also found to be differentially expressed at gene and exon level compared to the wild-type (table 6.11 and 6.12). Those genes code for membrane proteins which are essential for the propagation of synaptic signals between neurons. This indicate that synaptic transmission is altered and therefore the formation of memory could also be affected in the heterozygous mice.

Post-synaptic membrane			Pre-synaptic membrane		
Gene	log2FC	padj	Gene	log2FC	padj
Clstn3	-1.19E-01	3.73E-02	*Stx16*	-8.75E-02	3.15E-02
Dnm3	-1.72E-01	6.67E-03	*Stx1a*	1.60E-01	2.16E-02
Dnmt1	-1.50E-01	3.54E-02			
Shisa4	2.06E-01	4.62E-03			
Shisa6	-2.71E-01	2.93E-02			

Table 6.11: <u>Expression of genes coding for protein involved in synaptic membrane</u>

Post-synaptic membrane			Pre-synaptic membrane		
Gene	padj	log2FC	Gene	padj	log2FC
Clstn1	2.34E-03	3.50E-02	*Rims3*	3.73E-02	-3.09E-02
Clstn1	4.65E-03	5.63E-02	*Rims3*	4.05E-02	1.42E-01
Clstn1	8.76E-03	4.16E-02	*Stx1b*	4.24E-02	1.36E-02
Clstn1	9.99E-03	4.94E-02	*Stx4a*	4.08E-02	-1.19E-01
Clstn1	1.34E-02	5.29E-02	*Unc13a*	2.93E-02	4.14E-02
Clstn1	1.45E-02	-4.43E-02	*Unc13a*	3.01E-02	8.43E-02
Clstn1	1.86E-02	2.33E-02	*Unc13a*	3.26E-02	2.13E-02
Clstn1	2.01E-02	6.91E-02	*Unc13a*	4.00E-02	6.57E-02
Clstn1	3.57E-02	8.74E-03	*Nrxn1*	4.68E-02	2.86E-02
Clstn2	2.06E-03	1.27E-01	*Nrxn2*	8.38E-03	1.14E-01
Clstn2	4.97E-02	3.66E-02	*Nrxn2*	1.27E-02	1.41E-01
Clstn3	5.65E-05	6.01E-02	*Nrxn2*	3.50E-02	-4.87E-02
Clstn3	6.98E-04	3.91E-02	*Nrxn3*	1.74E-02	4.95E-02
Clstn3	1.89E-02	-7.88E-02	*Nrxn3*	2.31E-02	1.75E-01
Clstn3	2.07E-02	7.98E-02	*Nrxn3*	2.88E-02	1.00E-01
Clstn3	2.18E-02	-5.46E-02	*Nrxn3*	2.94E-02	1.79E-01
Clstn3	2.46E-02	-6.68E-02	*Nrxn3*	3.79E-02	-7.52E-02
Clstn3	2.62E-02	2.01E-02	*Nrxn3*	4.44E-02	1.82E-01
Clstn3	3.00E-02	8.36E-02	*Pclo*	9.64E-03	1.14E-01
Clstn3	3.23E-02	5.36E-02	*Pclo*	2.54E-02	1.51E-01
Clstn3	3.27E-02	6.56E-02			
Clstn3	4.50E-02	1.34E-01			
Clstn3	4.63E-02	8.09E-02			
Dnm1	2.27E-03	-1.81E-01			
Dnm1	2.41E-03	-1.28E-01			
Dnm1	2.62E-03	-6.41E-02			
Dnm1	1.37E-02	8.31E-02			
Dnm1	1.85E-02	7.69E-02			
Dnm1	3.09E-02	-4.55E-02			
Dnm1	3.87E-02	6.59E-02			
Dnm1	3.93E-02	5.74E-02			
Dnm1	4.15E-02	-4.45E-02			
Dnm1	4.39E-02	8.36E-02			
Dnm3	3.26E-03	1.51E-01			
Dnm3	1.36E-02	1.35E-01			
Dnm3	2.71E-02	9.01E-02			
Dnm3	4.71E-02	8.68E-02			
Dnm3	4.83E-02	-8.39E-02			
Shank1	6.12E-03	2.31E-01			
Shisa5	3.47E-02	-6.41E-02			
Shisa6	2.02E-02	-1.01E-01			
Shisa9	9.59E-03	2.94E-02			
Nlgn2	3.69E-03	1.16E-04			
Nlgn2	1.42E-02	-5.33E-02			
Nlgn2	2.68E-02	2.69E-02			
Nlgn2	3.63E-02	4.28E-02			
Nlgn2	3.95E-02	6.36E-02			
Nlgn2	4.41E-02	1.45E-02			

Table 6.12: Exon expression of genes coding for proteins involved in synaptic membrane

Overall, several systems believed to be affected in mental disorders are predicted to be affected in the heterozygote Der1 mice. Multiple genes affected are involved in cell communication and signalling. Indeed, it seems that the vesicles trafficking is affected which in turn could affect the release of neurotransmitters, which would then affect the receptor stimulation. A decrease in neurotransmitter release could lead to a lack of stimulation therefore leading to an increase expression of the post-synaptic receptors such as AMPA, NMDA, GABA receptors, leading to hypersensitivity of the neuron (Barnes, 1996; Tallent, 2007; Condon and Ehlers, 2010). Indeed, a very small release of neurotransmitter would activate the receptors and lead to neuronal activity. However, the RNAseq does not indicate any dysregulation regarding neurotransmitter release but a majority of the receptors are up-regulated in the Der1 mice which could support this hypothesis.

The global effect would lead to a dysfunction of the synapses which would not be able to function properly therefore leading to a miscommunication between neurons and possibly between the different cortical regions and between the hippocampus and the cortex.

c. Ingenuity pathway analysis of the differentially expressed genes in the cortex of the heterozygous mice

In order to gain more insight on which pathways are affected, and how they are affected, Ingenuity Pathway Analysis (IPA) was used. The network of canonical pathways shows the relations between the 25 most significant pathways and indicates that most of the genes are involved in several pathways (figure 6.11). The representation of the canonical pathways reveals that there are 71 significant pathways in which the genes are involved in (figure 6.12, table 6.13). In the cortex, 10 pathways seem to be activated and 15 inhibited. The pathway that is most significantly affected is EIF2 (Eukaryotic initiation factor-2), and it has the highest number of genes differentially expressed. This pathway is involved in the regulation of mRNA translation and so of protein synthesis and seems to be highly affected in the heterozygous mouse cortex. The second most significantly affected canonical pathway is the oxidative phosphorylation. This process is localised in the mitochondria and is essential to produce the necessary energy for the cell function. Exchange of electron allows the production of ATP in the cell which form a PH gradient and membrane potential (Smeitink *et al.*, 2006). Defect of this process can lead to bioenergy deficiency, imbalance of electrons and metabolites, disturbed production of ROS, production of nitric oxide and altered apoptosis pattern (Smeitink *et al.*, 2006). Similarly, mitochondrial dysfunction is also a significant canonical pathway affected by the genes differentially expressed. Mitochondria are the powerhouse of

the cell and mainly regulate apoptotic pathways. Dysfunction of mitochondria could lead to the same consequences as the ones of oxidative phosphorylation defect (Pieczenik and Neustadt, 2007). Those three most affected pathways indicate that protein synthesis might be altered in the Der1 mice, therefore affecting multiple functions. Additionally, mitochondria activity is mainly affected indicating that basic cellular functions are likely to be affected due to the possible change in ATP production.
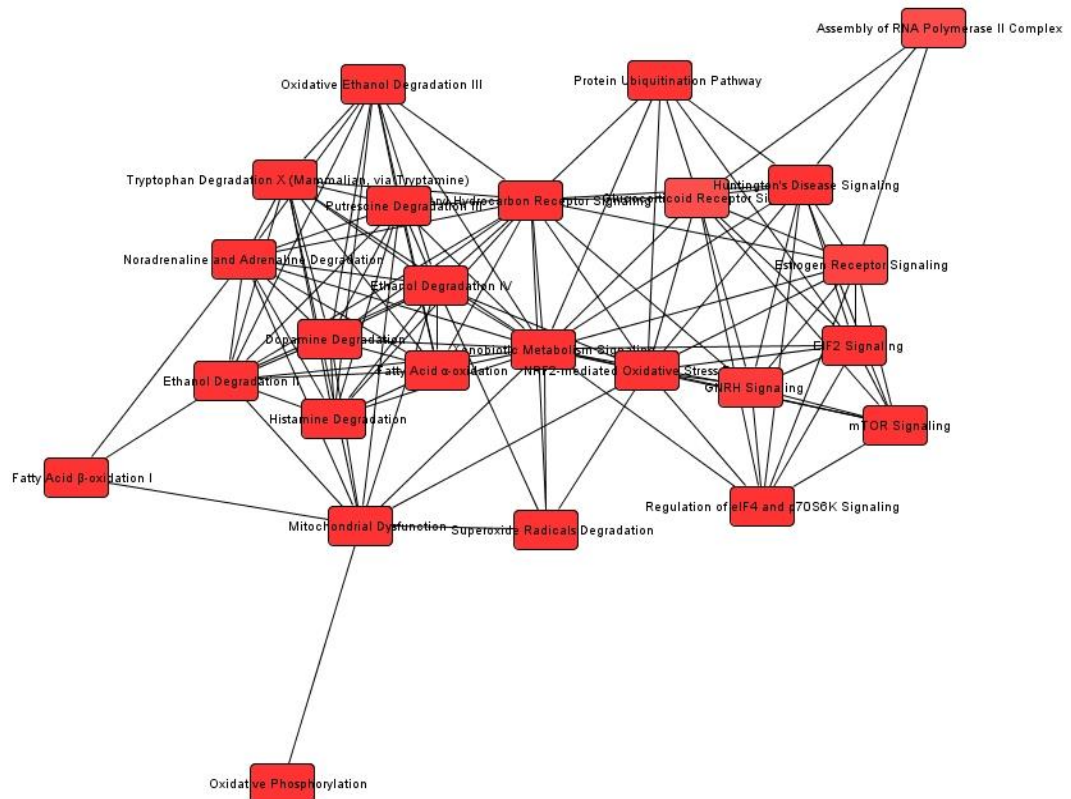
Figure 6.11: Map of the overlapping canonical pathways in the cortex

This represents shared biology among the identified candidate genes. Canonical pathways linked with black lines share one or more genes in common. The brighter the red of the node, the more significant the canonical pathway.
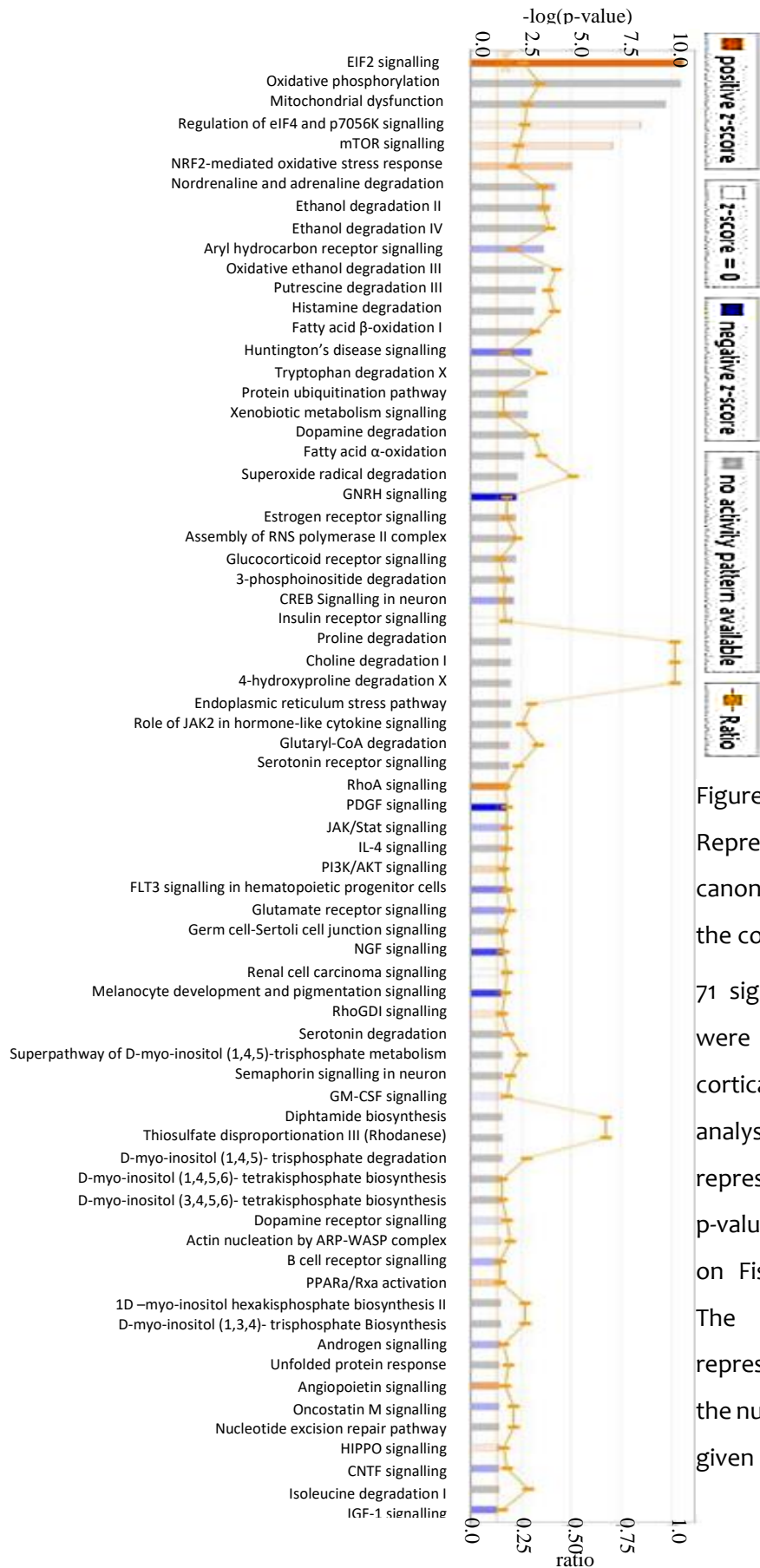
Figure 6.12: Representation of the canonical pathways of the cortex

71 significant pathways were found from the cortical RNAseq analysis. The columns represent the -log of the p-value calculated based on Fisher's exact test. The dot points represent the ratio of the number of genes in a given pathway.

significant Ingenuity Canonical Pathways	-log(p-value)	number of genes
EIF2 Signaling	1.06E+01	54
Oxidative Phosphorylation	1.04E+01	34
Mitochondrial Dysfunction	9.66E+00	44
Regulation of eIF4 and p70S6K Signaling	8.43E+00	40
mTOR Signaling	7.03E+00	44
NRF2-mediated Oxidative Stress Response	5.01E+00	38
Noradrenaline and Adrenaline Degradation	4.19E+00	12
Ethanol Degradation II	3.91E+00	11
Ethanol Degradation IV	3.67E+00	9
Aryl Hydrocarbon Receptor Signaling	3.58E+00	27
Oxidative Ethanol Degradation III	3.57E+00	8
Putrescine Degradation III	3.22E+00	8
Histamine Degradation	3.11E+00	7
Fatty Acid Î²-oxidation I	3.10E+00	10
Huntington's Disease Signaling	3.01E+00	39
Tryptophan Degradation X	2.92E+00	8
Dopamine Degradation	2.82E+00	9
Xenobiotic Metabolism Signaling	2.82E+00	42
Protein Ubiquitination Pathway	2.82E+00	41
Fatty Acid Î±-oxidation	2.63E+00	7
Superoxide Radicals Degradation	2.30E+00	4
GNRH Signaling	2.26E+00	22
Estrogen Receptor Signaling	2.22E+00	22
Assembly of RNA Polymerase II Complex	2.18E+00	11
Glucocorticoid Receptor Signaling	2.17E+00	41
3-phosphoinositide Degradation	2.14E+00	25
CREB Signaling in Neurons	2.11E+00	29
Insulin Receptor Signaling	2.01E+00	23
4-hydroxyproline Degradation I	2.00E+00	2
Choline Degradation I	2.00E+00	2
Proline Degradation	2.00E+00	2
Endoplasmic Reticulum Stress Pathway	1.95E+00	6
Role of JAK2 in Hormone-like Cytokine Signaling	1.94E+00	8
Glutaryl-CoA Degradation	1.90E+00	5
Serotonin Receptor Signaling	1.89E+00	9
RhoA Signaling	1.85E+00	20
PDGF Signaling	1.80E+00	16
IL-4 Signaling	1.78E+00	15
JAK/Stat Signaling	1.78E+00	15
PI3K/AKT Signaling	1.74E+00	20
FLT3 Signaling in Hematopoietic Progenitor Cells	1.69E+00	15
Glutamate Receptor Signaling	1.68E+00	11
NGF Signaling	1.64E+00	19
Germ Cell-Sertoli Cell Junction Signaling	1.64E+00	25

Renal Cell Carcinoma Signaling	1.62E+00	14
Melanocyte Development and Pigmentation Signaling	1.59E+00	16
RhoGDI Signaling	1.58E+00	25
Serotonin Degradation	1.57E+00	11
GM-CSF Signaling	1.56E+00	13
Semaphorin Signaling in Neurons	1.56E+00	10
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	1.56E+00	6
D-myo-inositol (1,4,5)-trisphosphate Degradation	1.55E+00	5
Diphthamide Biosynthesis	1.55E+00	2
Thiosulfate Disproportionation III (Rhodanese)	1.55E+00	2
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	1.54E+00	21
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	1.54E+00	21
Dopamine Receptor Signaling	1.52E+00	13
Actin Nucleation by ARP-WASP Complex	1.51E+00	10
B Cell Receptor Signaling	1.48E+00	26
PPARÎ±/RXRÎ± Activation	1.47E+00	25
1D-myo-inositol Hexakisphosphate Biosynthesis II	1.46E+00	5
D-myo-inositol (1,3,4)-trisphosphate Biosynthesis	1.46E+00	5
Androgen Signaling	1.44E+00	17
Unfolded protein response	1.41E+00	10
Angiopoietin Signaling	1.39E+00	13
CNTF Signaling	1.38E+00	11
HIPPO signaling	1.38E+00	14
Nucleotide Excision Repair Pathway	1.38E+00	7
Oncostatin M Signaling	1.38E+00	7
Isoleucine Degradation I	1.36E+00	4
IGF-1 Signaling	1.31E+00	16

Table 6.13: <u>Canonical pathway significantly affected by the differentially expressed genes in the heterozygote's cortex</u>

71 significant pathways were found. -log (p-value)>1,3 = p-value<0.05. Number of genes differentially expressed for each pathway are indicated.

Additionally, several neurotransmitters signalling pathways are affected. The dopaminergic receptor signalling pathway (figure 6.13) reveals that the adenylyl cyclase (*AC*) and protein kinase A (*PKA*) is predicted to be upregulated while *PP1* would be downregulated in the cortices of the heterozygote mice. This results also indicates that modification of the expression of these genes affect the synaptic compartment. Overall, the results indicate that dopamine synthesis, firing of dopaminergic neurons and CA2+ influx are down regulated however dopaminergic receptors would be up-regulated which would lead to the upregulation of the modulation of phosphoinositide metabolism in pre-synaptique neuron and up-regulation of phenotypes such as eye blinking and emesis process which are two mechanism regulated

by dopaminergic receptors function (O'Brien, 2003; Jongkees and Colzato, 2016). Disturbed dopaminergic neurotransmission has been known to be involved in mental disorder and to affect the mood. It seems that in the cortex of these heterozygote mice glutamate receptor signalling is also affected (figure 6.14). If this glutamatergic pathway is affected due to multiple genes differentially expressed involved in this pathway, then IPA software predict that the gene *DLG4* and *GRIA3* and *GRIK3* (glutamate receptors) would be upregulated while glutamate-ammonia ligase (*GLNS*) would be downregulated. Overall, glutamate would be predicted to be upregulated as well as several receptors and PSD95 even though they were not found to be significantly different in the data obtained with RNAseq analysis. It seems that this would lead to upregulation of neuronal depolarization, synaptic plasticity, neurotoxicity and excitatory post-synaptic potential (EPSPs) and activation of the clustering of receptors. Impairment of glutamate pathway is known to be link to mental disorder as well. The serotonin pathway (figure 6.15) also seems to be affected by the differential expression found in the cortical samples of the heterozygous mice. Serotonin receptors *5HT1B*, *5HT2*, *5HT4* are downregulated as well as adenylate cyclase (*ADCY*) while mono-amine oxidase (*MAO*) and 6-pyruvoyltetrahydropterin (*6-PTPS*) synthase are upregulated. This is predicted to lead to activation of CA2+ influx and inhibition of phosphoinositide hydrolysis as well as to inhibit the activation of adenylate cyclase. This would mean that serotonin would accumulate in the synapse. The mTOR pathway involved in cell growth, proliferation and survival is also affected in the cortices of the heterozygous mice (figure 6.16). Indeed, multiple genes from the pathway are dysregulated and the results predict that this could lead to a downregulation of the autophagy process as well as an inhibition of the translation via *4EBP* and an activation of the translation via activation of the ribosome. mTOR pathway is also known to be disturbed in mental disorder, moreover it is a target of *DISC1*, indeed, *DISC1* binds to KIAA1212 to regulate AKT-mTOR signaling pathway and therefore regulates neuronal development through this function (Kim *et al.*, 2009).

In addition to impairments of several pathways involved in cellular functions, the cortical analysis predicts impairment of neurotransmitter function and therefore could indicate that activation and inhibition of neurons following the receipt of an information is disturbed in the cortices of the heterozygous mice. This could indicate impairment of synaptic plasticity which is regulated in part via neurotransmitter release and receptor expression.
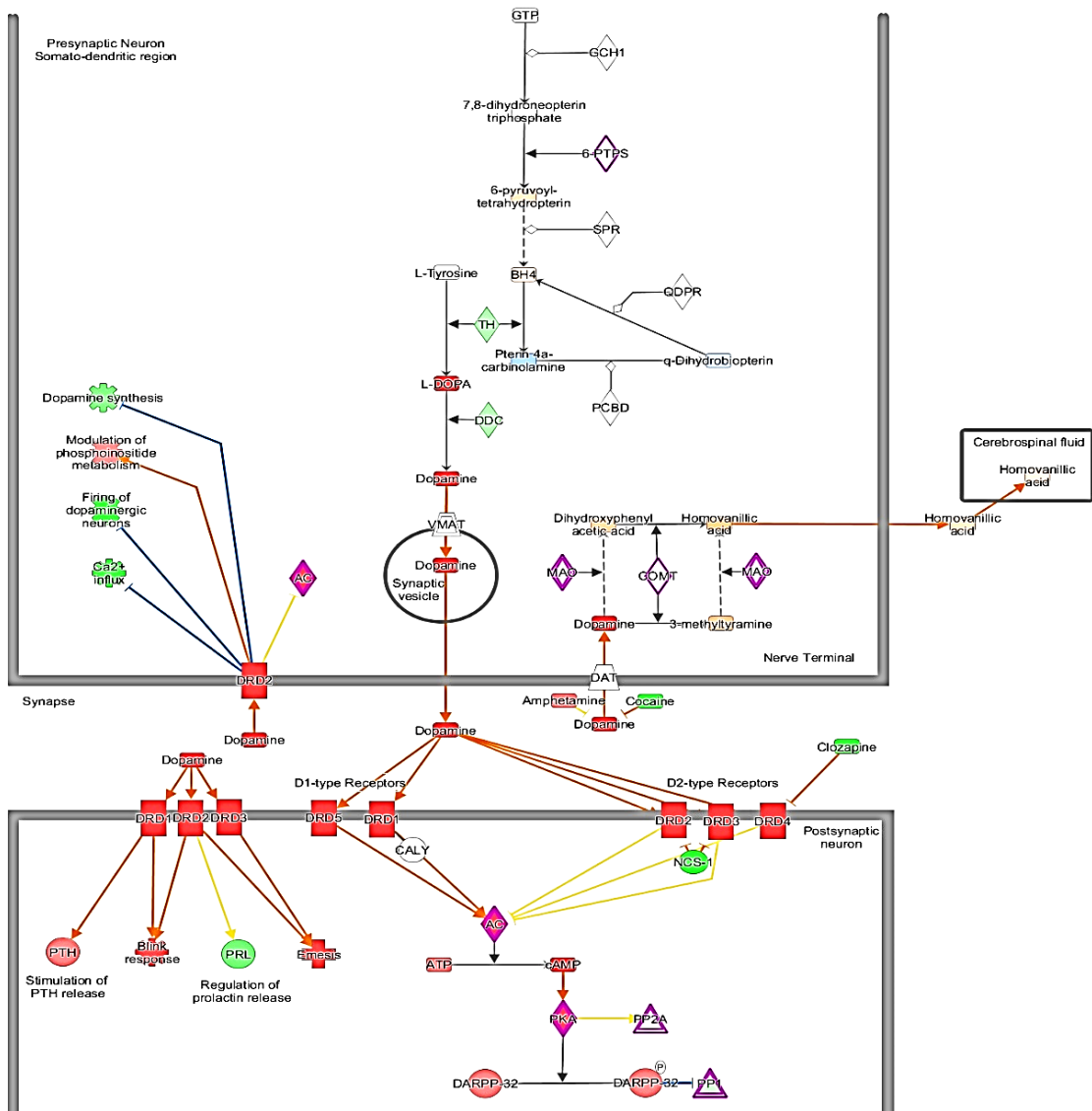
Figure 6.13: Representation of the expected activation state of the dopamine receptors signalling pathway obtain from the cortical RNAseq analysis using IPA

Several genes involved in this pathway have been found to be differentially expressed and the IPA analysis predict which molecules and process would be activated/inhibited and upregulated/down regulated in the cortex.

The genes in magenta/purple have been found in our analysis to be differentially expressed in the cortex of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.
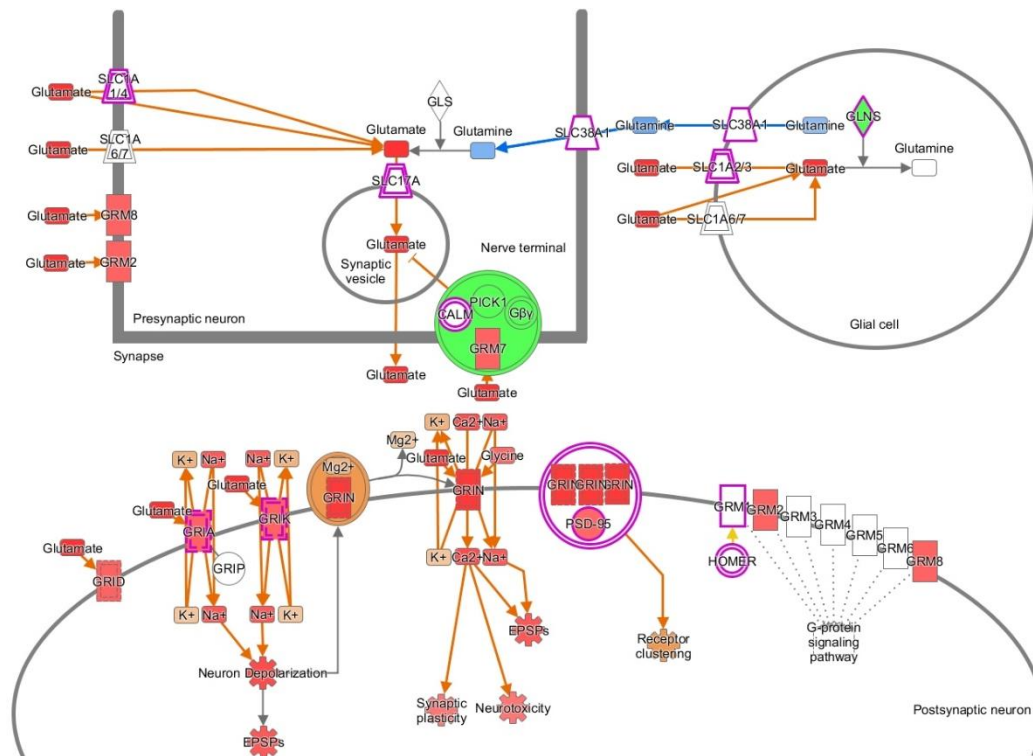
Figure 6.14: Representation of the expected activation state of the glutamate signalling pathway obtain from the cortical RNAseq analysis using IPA.

Several genes involved in this pathway have been found to be differentially expressed and the IPA analysis predict which molecules and process would be activated/inhibited and upregulated/down regulated in the cortex.

The genes circled in magenta have been found in our analysis to be differentially expressed in the cortex of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.

Figure 6.15: Representation of the expected activation state of the serotonin receptors signalling pathway obtain from the cortical RNAseq analysis using IPA

Several genes involved in this pathway have been found to be differentially expressed and the IPA analysis predict which molecules and process would be activated/inhibited and upregulated/down regulated in the cortex.

The genes circled in magenta have been found in our analysis to be differentially expressed in the cortex of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.
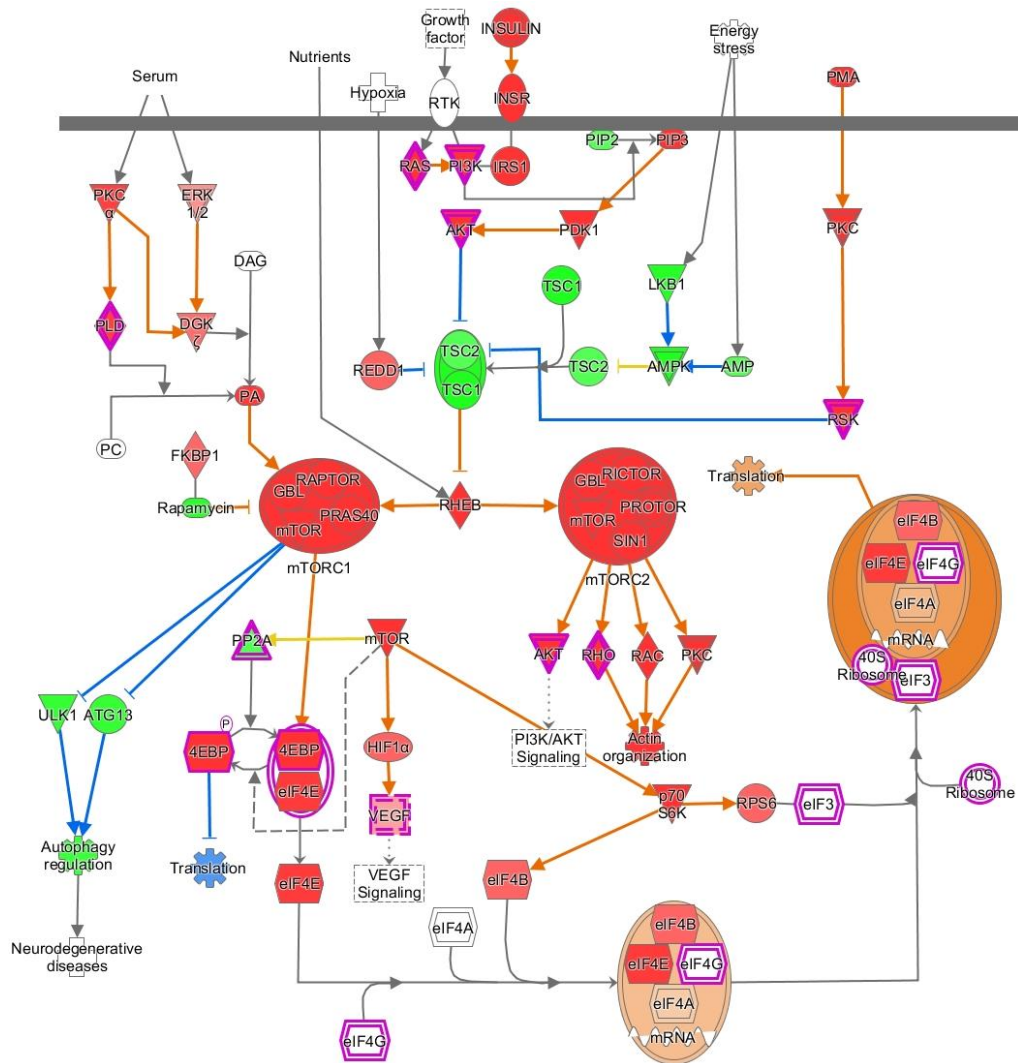
Figure 6.16: Representation of the expected activation state of the mTOR signalling pathway obtain from the cortical RNAseq analysis using IPA

Several genes involved in this pathway have been found to be differentially expressed and the IPA analysis predict which molecules and process would be activated/inhibited and upregulated/down regulated in the cortex.

The genes circled in magenta have been found in our analysis to be differentially expressed in the cortex of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.

6.2.4 Hippocampus samples

a. Gene ontology of the cortical samples from the heterozygous mice

GO analysis of the differentially expressed genes from the hippocampal samples revealed that several processes are likely to be affected by the mutation, with a total of 27 GO component, 20 GO function, 63 GO process terms which are significantly affected by the genes differentially expressed. Overall, there were less changes in the hippocampus compared to the cortex. These results were obtained from the analysis of the differentially expressed genes and exons found to be significantly different in the hippocampus of the heterozygous Der1 mice compared to the wild-type.

The main processes predicted to be affected by the *Der1* mutation in the hippocampus of the heterozygous mice are involved in synaptic organization and function, electrical activity, development, transport, localization, neurogenesis and neurotransmitter secretion (table 6.14). Furthermore, some of the genes affected are involved in channel activity with voltage-gated cation channel activity as the major function enriched. Transporter activity and binding activity are also enriched function (table 6.15). The major components affected are the synapses as well as channel and transporter complex. Genes affecting neuron parts such as projections and axons are also enriched in the dysregulated gene set from the hippocampus of the heterozygous mice (table 6.16).

Overall, the genes that are differentially expressed in the hippocampus are involved mainly in synaptic function. This indicates that cell communication and signalling could be impaired in the hippocampus of the heterozygous Der1 mice due to a dysfunction of the channels and transporters, therefore leading to an impaired action potential and membrane depolarization.

Description	FDR q-value (padj)	Enrichment	Number of genes
regulation of membrane potential	1.51E-03	4.31	19
synapse organization	4.83E-03	6.86	11
signalling	1.56E-02	3.41	18
regulation of ion transport	1.89E-02	3	20
developmental process	2.14E-02	1.56	74
action potential	2.20E-02	8.57	7
membrane depolarization	2.68E-02	10.41	6
regulation of neuron differentiation	2.56E-02	2.87	24
regulation of transmembrane transport	3.05E-02	3.16	17
regulation of ion transmembrane transport	3.65E-02	3.38	15
regulation of neurogenesis	3.74E-02	2.57	26
synaptic signalling	3.67E-02	4.02	13
trans-synaptic signalling	3.50E-02	4.02	13
anterograde trans-synaptic signalling	8.26E-02	3.83	12
chemical synaptic transmission	7.97E-02	3.83	12
signal release from synapse	7.60E-02	7.44	6
cell communication	7.68E-02	2.65	19
positive regulation of cell differentiation	7.58E-02	2.3	25
cell-cell signalling	7.86E-02	3.11	15
localization	8.16E-02	1.55	60
regulation of neuron projection development	1.01E-01	2.86	18
regulation of localization	1.13E-01	1.67	45
transport	1.25E-01	1.59	52
ion transmembrane transport	1.45E-01	2.63	16
nervous system development	1.57E-01	2.98	12
neurotransmitter secretion	1.52E-01	10.91	4
regulation of dendrite development	1.61E-01	4.36	8
membrane depolarization during action potential	1.56E-01	10.61	4
regulation of ion transmembrane transporter activity	1.67E-01	3.85	9
cation transport	1.81E-01	2.44	18
cell proliferation in forebrain	1.83E-01	16.84	3
regulation of dendrite morphogenesis	1.89E-01	5.56	6
protein localization to membrane	2.41E-01	3.07	11
regulation of cation channel activity	2.38E-01	4.48	7

Table 6.14: <u>Processes affected by the Der1 mutation in the hippocampus of the heterozygous mice</u>

'FDR q-value' or padj is the correction of the p-value for multiple testing using the Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value * number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

Description	FDR q-value (padj)	Enrichment	Number of genes
voltage-gated cation channel activity	8.76E-03	6.92	10
cation channel activity	6.50E-03	4.53	14
ion channel activity	1.95E-02	3.74	15
ion gated channel activity	1.47E-02	4.25	13
gated channel activity	1.31E-02	4.21	13
substrate-specific channel activity	1.24E-02	3.66	15
voltage-gated channel activity	1.39E-02	5.27	10
voltage-gated ion channel activity	1.21E-02	5.27	10
passive transmembrane transporter activity	1.56E-02	3.47	15
channel activity	1.41E-02	3.47	15
calcium ion binding	4.07E-02	2.87	17
metal ion transmembrane transporter activity	5.50E-02	3.17	14
inorganic cation transmembrane transporter activity	6.88E-02	2.81	16
voltage-gated potassium channel activity	1.04E-01	6.51	6
protein dimerization activity	1.20E-01	2.01	27
syntaxin binding	1.30E-01	6.09	6
protein binding	1.29E-01	1.27	109
cation transmembrane transporter activity	1.41E-01	2.56	16
outward rectifier potassium channel activity	1.36E-01	17.9	3
binding	1.85E-01	1.17	142

Table 6.15: <u>Functions affected by the Der1 mutation in the hippocampus of the heterozygous mice</u>

'FDR q-value' or padj is the correction of the p-value for multiple testing using the Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value * number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

Description	FDR q-value (Padj)	Enrichment	Number of genes
synapse part	1.58E-07	3.88	31
synaptic membrane	9.50E-07	5.72	19
synapse	5.39E-06	3.98	24
cation channel complex	1.43E-05	6.58	14
ion channel complex	1.15E-05	5.59	16
transmembrane transporter complex	1.66E-05	5.38	16
transporter complex	2.00E-05	5.25	16
plasma membrane region	6.14E-05	2.88	29
postsynaptic density	2.90E-04	5.21	13
postsynaptic specialization	2.73E-04	5.19	13
neuron part	5.63E-04	2.18	38
postsynaptic membrane	1.11E-03	4.87	12
axon part	1.42E-03	3.62	16
plasma membrane bounded cell projection part	1.43E-03	2.23	33
cell projection part	1.33E-03	2.23	33
cell junction	1.48E-03	2.47	27
plasma membrane part	1.43E-03	1.87	47
cell projection	5.66E-03	1.93	38
presynaptic membrane	5.89E-03	8.81	6
plasma membrane protein complex	9.63E-03	2.98	16
plasma membrane	9.96E-03	1.58	59
neuron projection	1.25E-02	2.18	26
terminal bouton	1.88E-02	5.71	7
pre-synapse	2.37E-02	4.21	9
plasma membrane bounded cell projection	2.69E-02	1.88	32
membrane part	2.97E-02	1.38	80
axon	4.85E-02	3.01	12

Table 6.16: <u>Components affected by the Der1 mutation in the hippocampus of the heterozygous mice</u>

'FDR q-value' or padj is the correction of the p-value for multiple testing using the Benjamini and Hochberg (1995) method: for the ith term the FDR q-value is (p-value * number of GO terms) / i. Number of genes correspond to the number of significant differentially expressed genes belonging to the descriptive term.

Analysis with Panther software (http://pantherdb.org/) was also used. Similar molecular functions were affected by the change in gene expression in the hippocampus such as binding and transporter activity (figure 6.17, a). Additionally, the genes are also involved in catalytic activity, signal transducer activity and structural molecule activity. The receptor activity (figure 6.17, b) indicates that genes affected are involved in GABAergic and glutamatergic receptors activity as well as G-protein coupled receptor activity, ligand activated transcription factor and transmembrane receptor activity. The transporter activity (figure 6.17, c) indicates that the genes affected are mainly involved in ion channel activity and also in hydrogen ion trans-membrane transporter activity and cation trans-membrane transporter activity.

a.
- binding (GO:0005488), 56 genes
- catalytic activity (GO:0003824) 42 genes
- receptor activity (GO:0004872), 15 genes
- signal transducer activity (GO:0004871), 10 genes
- structural molecule activity (GO:0005198), 7 genes
- transporter activity (GO:0005215), 18 genes

b.

c.

- G-protein coupled receptor activity (GO:0004930), 3 genes
- GABA receptor activity (GO:0016917) 1 gene
- glutamate receptor activity (GO:0008066), 1 gene
- ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity (GO:0004879), 2 genes
- transmembrane receptor protein tyrosine kinase activity (GO:0004714), 1 gene
- transmembrane transporter activity (GO:0022857), 17 genes

Figure 6.17: Molecular function in which the genes differentially expressed in the hippocampus of the heterozygous Der1 mice are involved.

a. Overall representation of the molecular functions in which the genes are involved.

b. Molecular functions involved in receptor activity.

c. Molecular function involved in transporter activity.

The number of significant genes included in each function is indicated at each line.

The processes found to be affected are quite broad when using Panther online, but the results are similar to what was previously found with Gene Ontology analysis. Indeed, processes involved in development, growth, localization and cellular biology could be affected by the modification of the expression of these genes (figure 6.18, a). Moreover, the cellular processes are particularly affected, indicating that the differentially expressed genes are involved in functions linked to cellular communication, proliferation, cycle as well as cellular component movement and cytokinesis (figure 6.18, c). Additionally, genes affected are involved in functions associated with localization, mainly with transport (figure 6.18, b).
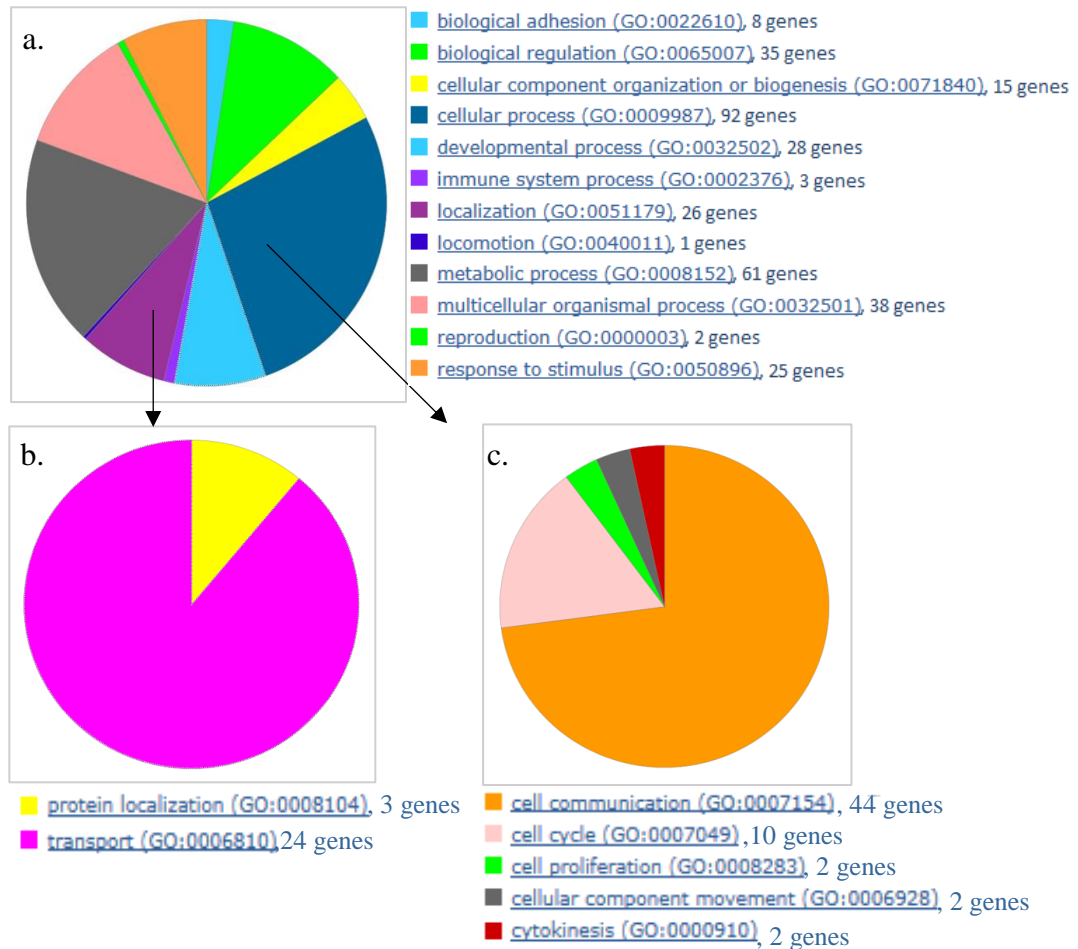
Figure 6.18: Process in which the genes differentially expressed in the hippocampus of the heterozygous Der1 mice are involved.

a. Overall representation of the process in which the genes are involved

b. Process involved in localization

c. Cellular process

The number of significant genes included in each function is indicated at each line.

The genes affected seem to also be important for the cellular components. Indeed, they seem to be involved in cellular part and junction, macromolecule complex, membrane, extracellular matrix and region, synapse and organelles (figure 6.19, a). The genes are particularly important for the nucleus, the vesicle membrane, the cytoskeleton, the Golgi apparatus, the chromosomes, the endoplasmic reticulum, the peroxisome, the endosome and the mitochondrion (figure 19, b). Most of the genes involved in the cell parts are mostly involved in intracellular part then plasma membrane, cell projection and basal part (figure 6.19, c).

a.

- cell junction (GO:0030054) 3 genes
- cell part (GO:0044464) 49 genes
- extracellular matrix (GO:0031012) 1 gene
- extracellular region (GO:0005576) 5 genes
- macromolecular complex (GO:0032991) 25 genes
- membrane (GO:0016020) 27 genes
- organelle (GO:0043226) 39 genes
- synapse (GO:0045202) 5 genes

b.

c.

- Golgi apparatus (GO:0005794), 2 genes
- chromosome (GO:0005694), 2 genes
- cytoplasmic membrane-bounded vesicle (GO:0016023) 5 genes
- cytoskeleton (GO:0005856), 5 genes
- endoplasmic reticulum (GO:0005783), 1 genes
- endosome (GO:0005768), 1 genes
- mitochondrion (GO:0005739), 1 genes
- nucleus (GO:0005634), 26 genes
- peroxisome (GO:0005777), 1 gene

- basal part of cell (GO:0045178), 1 genes
- cell projection (GO:0042995), 8 genes
- intracellular (GO:0005622), 46 genes
- plasma membrane (GO:0005886), 24 genes

Figure 6.19: Cellular component in which the genes differentially expressed in the hippocampus of the heterozygous Der1 mice are involved.

a. Overall representation of the cellular component in which the genes are involved.

b. Cellular component of the organelles.

c. Component of cellular part.

The number of significant genes included in each function is indicated at each line.

The analysis also revealed pathways in which those genes are involved. The cortical set of genes affected is involved in numerous pathways (figure 6.20). It seems that most of these pathways are involved in signalling, molecular synthesis, glutamatergic pathway. The most important pathway in which the differentially expressed genes are involved seems to be the Wnt pathway (figure 6.21), similarly to what we found with the cortical samples.

5-Hydroxytryptamine degredation (P04372), 1 gene
5HT2 type receptor mediated signaling pathway (P04374), 3 genes
ATP synthesis (P02721), 1 gene
Adrenaline and noradrenaline biosynthesis (P00001), 1 gene
Alpha adrenergic receptor signaling pathway (P00002), 1 gene
Alzheimer disease-amyloid secretase pathway (P00003), 3 genes
Alzheimer disease-presenilin pathway (P00004), 3 genes
Angiogenesis (P00005), 6 genes
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911), 1 genes
Apoptosis signaling pathway (P00006), 3 genes
B cell activation (P00010), 3 genes
Beta1 adrenergic receptor signaling pathway (P04377), 2 genes
Beta2 adrenergic receptor signaling pathway (P04378), 2 genes
Blood coagulation (P00011), 2 genes
CCKR signaling map (P06959), 4 genes
Cadherin signaling pathway (P00012), 6 genes
Cholesterol biosynthesis (P00014), 1 gene
Dopamine receptor mediated signaling pathway (P05912), 1 gene
EGF receptor signaling pathway (P00018), 4 genes
Endogenous cannabinoid signaling (P05730), 1 gene
Endothelin signaling pathway (P00019), 1 gene
FAS signaling pathway (P00020), 2 genes
FGF signaling pathway (P00021), 4 genes
Gonadotropin-releasing hormone receptor pathway (P06664), 6 genes
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027), 2 genes
Histamine H1 receptor mediated signaling pathway (P04385), 1 gene
Huntington disease (P00029), 4 genes
Inflammation mediated by chemokine and cytokine signaling pathway (P00031), 4 genes
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032), 1 gene

Integrin signalling pathway (P00034), 2 genes
Interferon-gamma signaling pathway (P00035), 2 genes
Interleukin signaling pathway (P00036), 2 genes
Ionotropic glutamate receptor pathway (P00037), 1 gene
JAK/STAT signaling pathway (P00038), 1 gene
Metabotropic glutamate receptor group I pathway (P00041), 2 genes
Metabotropic glutamate receptor group III pathway (P00039), 1 gene
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042), 2 genes
Nicotine pharmacodynamics pathway (P06587), 2 genes
Nicotinic acetylcholine receptor signaling pathway (P00044), 3 genes
Oxidative stress response (P00046), 4 genes
Oxytocin receptor mediated signaling pathway (P04391), 3 genes
PDGF signaling pathway (P00047), 3 genes
Parkinson disease (P00049), 6 genes
Ras Pathway (P04393), 4 genes
Salvage pyrimidine ribonucleotides (P02775), 1 gene
Synaptic vesicle trafficking (P05734), 2 genes
T cell activation (P00053), 2 genes
TGF-beta signaling pathway (P00052), 3 genes
Thyrotropin-releasing hormone receptor signaling pathway (P04394), 3 genes
Toll receptor signaling pathway (P00054), 4 genes
VEGF signaling pathway (P00056), 2 genes
Wnt signaling pathway (P00057), 7 genes
p38 MAPK pathway (P05918), 2 genes
p53 pathway feedback loops 2 (P04398), 1 gene

Figure 6.20: Pathways in which the genes differentially expressed in the hippocampus of the heterozygous Der1 mice are involved
The number of significant genes included in each function is indicated at each line.

- Cadherin (P01440), 4 genes
- Frizzled-related protein (P01461) 1 gene
- Phospholipase C (P01443), 1 gene
- Wingless-type MMTV integration site family member (P01444), 1 gene

Figure 6.21: Representation of Wnt pathway in which some differentially expressed genes are involved.

The number of significant genes included in each function is indicated at each line.

The GO analysis shows some similarity between the genes expression in the hippocampus and the cortex of the heterozygous Der1 mice. However, the cortex is a much bigger, and less homogeneous area, and more genes are affected, leading to more affected characteristics.

b. Genes and pathways analysis

b.i Voltage-gated cation channel

Voltage-gated cation channel activity is the most enriched function obtained from the RNAseq analysis of the hippocampal samples. Genes coding for potassium channels are the most affected in the hippocampus of the heterozygous Der1 mice (table 6.17). Moreover, expression of these genes is up regulated (log2fc>0) apart from one. A few calcium and sodium channels are also affected and are also mainly up regulated (table 6.17). This is similar to what was found in the cortex, however less genes are affected. This indicates that neuronal plasticity and signalling might be altered in the Der1 heterozygous mice. Voltage-gated channels are necessary for the polarization and depolarization of the membrane, which might therefore be altered in the heterozygous mice. Changes in their expression triggers changes in neuronal excitability.

	genes	log2fc	padj	Analysis
Potassium channels	*Kcna2*	0.164	2.54E-03	DESeq
	Kcnab3	0.215	4.56E-02	DESeq
	Kcnh7	0.290	1.23E-02	DESeq
	Kcnip2	-0.177	9.39E-03	DESeq
Calcium channel	*Cacna2d2*	0.230	2.24E-02	DESeq
	Cacnb4	0.171	1.49E-02	DESeq
	Cacnb2	-0.478	2.02E-02	DEXSeq
Sodium channel	*Scn2a1*	0.165	-4.78E-01	DESeq

Table 6.17: Genes coding for voltage-gated channels found to be differentially expressed at gene or exon level, in the hippocampus of the heterozygous mice

Log2fc indicates if the gene is up (log2fc>0) or down (log2fc<0) regulated. Padj indicates the significant difference compare to the gene expression in the WT, after correcting for multiple testing. The analysis indicates weather the gene is a hit at the gene (DESeq) or exon level (DEXseq). Only one gene (Cacnb2) was found to have a differentially expressed exon.

b.ii Synapse

Multiple genes involved in synaptic composition and organization have been found differentially expressed in the hippocampus of the heterozygous mice and significantly different compared to the wild-type (table 6.18). This indicates that synaptic function might be altered in those mice. Several genes coding for neurotransmitter receptors such as *Gabrg3* and *Grin2a* are essential for synaptic signalling and have been found to be differentially expressed compared to wild-type. Receptors at the synapse might not be normally distributed due to their altered expression leading to disturbed synaptic function. Additionally, expression of multiple calcium channels involved in synaptic function are also altered which would lead to altered synaptic signalling as well.

Synapse organization process				Synapse components			
genes	log2fc	padj	Analysis	genes	log2fc	padj	Analysis
Myo5a	0.148	2.51E-02	DESeq	*Cask*	0.111	1.40E-02	DESeq
Plxnd1	0.265	1.21E-02	DESeq	*Chrna4*	0.261	1.29E-02	DESeq
Unc13c	0.268	3.44E-02	DESeq	*Cnih2*	-0.17	2.87E-02	DESeq
Utrn	0.232	3.40E-02	DESeq	*Cryab*	-0.201	3.24E-02	DESeq
Erbb4	0.225	1.41E-02	DESeq	*Disc1*	-0.324	5.23E-03	DESeq
Kalrn	-0.712	4.32E-02	DEXSeq	*Gabrg3*	0.193	1.21E-02	DESeq
Nrxn1	-0.084	4.82E-02	DEXSeq	*Olfm1*	-0.179	1.41E-02	DESeq
Wnt7a	0.286	3.92E-02	DEXSeq	*Pcdh10*	0.144	4.29E-02	DESeq
				Pcdh8	-0.227	2.59E-03	DESeq
				Prr7	-0.254	1.51E-02	DESeq
				Prss12	0.259	2.24E-02	DESeq
				Syt2	0.242	3.10E-02	DESeq
				Vwc2l	0.229	3.40E-02	DESeq
				Kcna2	0.165	2.54E-03	DESeq
				Lzts1	0.189	2.38E-02	DESeq
				Calb2	-0.286	8.05E-04	DESeq
				Gabrg3	0.193	1.21E-02	DESeq
				Homer3	-0.184	3.12E-02	DESeq
				Ank2	NA	3.01E-02	DEXSeq
				Atp2b1	-0.035	1.77E-02	DEXSeq
				Nrxn1	-0.084	4.82E-02	DEXSeq
				Anks1b	0.239	3.01E-02	DEXSeq
				Grin2a	-0.208	3.01E-02	DEXSeq

Table 6.18: <u>Genes involved in synaptic function found to be differentially expressed at gene or exon level, in the hippocampus of the heterozygous mice</u>

Log2fc indicates if the gene is up (log2fc>0) or down (log2fc<0) regulated. Padj indicates the significant difference compare to the gene expression in the WT, after correcting for multiple testing. The analysis indicates weather the gene is a hit at the gene (DESeq) or exon level (DEXseq).

b.iii Signalling

Signalling is an important affected process in the hippocampus of the heterozygous mice. 18 genes have been found differentially expressed compared to the wild-type (table 6.19). This adds to the possible alteration of the organization of the synapse which was also found and confirms that signalling might be the main affected process in the hippocampus. This would lead to a disrupted neurodevelopment and would be linked to the alteration of membrane potential which were found to be enriched.

Signalling process			
gene	log2fc	padj	Analysis
Apln	-0.2429	0.030991	DESeq
Cacnb4	0.171492	0.020223	DESeq
Cnih2	-0.17041	0.028676	DESeq
Gabrg3	0.193485	0.012098	DESeq
Hcn4	0.265428	0.040402	DESeq
Myo5a	0.147957	0.02505	DESeq
Chrna4	0.261261	0.012902	DESeq
Pcdh8	-0.22739	0.002591	DESeq
Myo5a	0.147957	0.02505	DESeq
Pcdh10	0.143996	0.042912	DESeq
Unc13c	0.268396	0.034412	DESeq
Snap91	-0.11396	0.025119	DEXSeq
Cnr1	-0.52954	0.048201	DEXSeq
Nrxn1	-0.08367	0.048201	DEXSeq
Jam3	0.229917	0.006761	DEXSeq
Wnt7a	0.28617	0.039217	DEXSeq
Cacnb2	-0.47813	0.000135	DEXSeq
Grin2a	-0.20791	0.030134	DEXSeq

Table 6.19: <u>Genes involved in signalling found to be differentially expressed in the hippocampus of the heterozygous mice</u>

Log2fc indicates if the gene is up (log2fc>0) or down (log2fc<0) regulated. Padj indicates the significant difference compare to the gene expression in the WT, after correcting for multiple testing. The analysis indicates weather the gene is a hit at the gene (DESeq) or exon level (DEXseq).

b.iv   Neurotransmitter secretion

Neurotransmitters are stored in synaptic vesicles. Their release occurs via exocytosis and it is a principal mode of communication in the nervous system. Neurotransmission processes were found to be enriched in the hippocampus, and four genes involved in this process are differentially expressed compared to the wild-type (table 6.20). Fusion of the vesicles with the presynaptic membrane is necessary for the release of neurotransmitters and could be altered as expression of genes involved in this dysregulated (table 6.20). Fusion happens when intracellular calcium concentrations increase during an action potential and therefore could also be altered due to dysregulated expression of the voltage-gated channels mentioned previously. Indeed, Genes coding for proteins involved in calcium transport have also been found differentially expressed in the hippocampus.

Neurotransmitter secretion			
gene	log2fc	padj	Analysis
Syt2	0.241895	0.030991	DESeq
Nrxn1	-0.08367	0.048201	DEXSeq
Snap91	-0.11396	0.025119	DEXSeq
Wnt7a	0.039217	0.28617	DEXSeq

Table 6.20: Genes involved in neurotransmitter secretion found to be differentially expressed in the hippocampus of the heterozygous mice

Log2fc indicates if the gene is up (log2fc>0) or down (log2fc<0) regulated. Padj indicates the significant difference compare to the gene expression in the WT, after correcting for multiple testing. The analysis indicates weather the gene is a hit at the gene (DESeq) or exon level (DEXseq).

c. Ingenuity pathway analysis of the differentially expressed genes in the hippocampus of the heterozygous mice

Ingenuity Pathway Analysis (IPA) was used to determine which pathways were affected in the hippocampus of the heterozygous mice and if they were activated or inhibited. The network of canonical pathways shows the relations between the 25 most significant pathways and indicates that most of the genes are involved in several pathways (figure 6.22). The representation of the canonical pathways obtained from the differentially expressed genes from the hippocampus reveals that there are 48 significant pathways in which the differentially expressed genes are involved (figure 6.23, table 6.21).

This reveals that in the hippocampus of the heterozygous mice there are three pathways that are likely to be activated. Those pathways are: 1) gonadotrophin releasing hormone (GNRH) signalling which regulates the production and release of the gonadotropins, luteinizing hormone and follicle stimulating hormones; 2) signalling by Rho family GTPases which regulates actin dynamics and is particularly involved in cell morphology and migration, and spine formations; 3) cardiac hypertrophy signalling.

There are also three pathways that are likely to be inhibited, which are 14-3-3 mediated signalling which regulates the cell cycle, cell survival, intracellular trafficking, and signal transduction, GO12/13 signalling involved in signal transduction, and Wnt/β-catenin signalling which regulates cell pluripotency and fate during development.

The most significantly affected pathway is Agrin Interactions at the Neuromuscular Junction which is involved in synaptogenesis, which did not come up when investigating the cortex. This pathway is also necessary for the post-synaptic localization of many proteins, aggregation of cholinergic receptors and reorganization of the position of other neurotransmitter receptors

and numerous protein involved in the formation of those junction are present in the brain Therefore, this pathway is necessary for synaptic transmission and the fact that it is affected in the Der1 mice suggest that synaptogenesis and neuronal transmission might be affected as well (Zong and Jin, 2013).

Figure 6.22: Map of the overlapping canonical pathways in the hippocampus. This figure represents shared biology among the identified candidate genes. Canonical pathways linked with black lines share one or more genes. The brighter the red of the node, the more significant the canonical pathway.

Figure 6.23: Canonical pathways of the hippocampus 48 significant pathways were found from the hippocampus RNAseq analysis. The columns represent the -log of the p-value calculated based on Fisher's exact test. The dot points represent the ratio of the number of genes in a given pathway.

Canonical Pathways	-log(p-value)	number of genes
Agrin Interactions at Neuromuscular Junction	2.71	4
GNRH Signalling	2.49	5
Signalling by Rho Family GTPases	2.48	7
April Mediated Signalling	2.43	3
14-3-3-mediated Signalling	2.42	5
MIF Regulation of Innate Immunity	2.37	3
B Cell Activating Factor Signalling	2.37	3
Xenobiotic Metabolism Signalling	2.27	7
ErbB Signalling	2.14	4
Cholecystokinin/Gastrin-mediated Signalling	2.11	4
Lanosterol Biosynthesis	2.1	1
CD27 Signalling in Lymphocytes	2.08	3
RANK Signalling in Osteoclasts	2.07	4
SAPK/JNK Signalling	2.07	4
IGF-1 Signalling	2.02	4
HGF Signalling	1.9	4
IL-17A Signalling in Gastric Cells	1.88	2
IL-2 Signalling	1.83	3
IL-10 Signalling	1.8	3
Chemokine Signalling	1.8	3
EGF Signalling	1.76	3
Gα12/13 Signalling	1.71	4
Toll-like Receptor Signalling	1.7	3
STAT3 Pathway	1.67	3
Aryl Hydrocarbon Receptor Signalling	1.65	4
GDNF Family Ligand-Receptor Interactions	1.65	3
Neurotrophin/TRK Signalling	1.65	3
LPS/IL-1 Mediated Inhibition of RXR Function	1.64	5
TNFR2 Signalling	1.62	2
4-1BB Signalling in T Lymphocytes	1.59	2
IL-17 Signalling	1.59	3
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.59	3
IL-17A Signalling in Fibroblasts	1.57	2
Inhibition of Angiogenesis by TSP1	1.54	2
LPS-stimulated MAPK Signalling	1.51	3
Glycerol-3-phosphate Shuttle	1.5	1
Salvage Pathways of Pyrimidine Ribonucleotides	1.45	3
PDGF Signalling	1.45	3
Cardiac Hypertrophy Signalling	1.43	5
Reelin Signalling in Neurons	1.42	3
Dermatan Sulfate Biosynthesis (Late Stages)	1.41	2
CXCR4 Signalling	1.38	4
Chondroitin Sulfate Biosynthesis (Late Stages)	1.35	2
Wnt/β-catenin Signalling	1.33	4
UVC-Induced MAPK Signalling	1.33	2
Glycerol Degradation I	1.33	1
Antioxidant Action of Vitamin C	1.31	3
PPARα/RXRα Activation	1.31	4

Table 6.21: Canonical pathways significantly affected by the differentially expressed genes in the heterozygote's hippocampus

48 significant pathways were found. -log (p-value)>1,3 = p-value<0.05. Number of genes differentially expressed in each pathway is indicated.

Additionally, the analysis of heterozygous hippocampus revealed that GNRH signalling is predicted to be the second most significantly affected pathway. The GNRH pathway is involved in regulation of the reproductive system, synthesis and release of hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) and mood regulation (Bliss *et al.*, 2011). In this pathway (figure 6.24), genes differentially expressed in the heterozygote mice compared to the wild-type mice are *PLCβ, MEKK, JNK, c-Jun, c-fos* and *Elk-1* and they are all upregulated. Here, this pathway is predicted to be activated, moreover the outcome of this pathway in the heterozygous mice is predicted to activate and up regulate cytoskeletal rearrangement processes as well as cell motility and migration process (figure 6.24). Indeed, through the activation of G protein, protein involved in those process such as *FAK*, and *Src* would be up-regulated. Another interesting pathway which is the third most significant and is downregulated is the Rho GTPase pathway (figure 6.25). This pathway plays a role in the organization of actin and microtubule cytoskeletons and links with surface receptors. It is also involved in neuronal morphology, survival and death (Schwartz, 2004). Here we can see that differentially expressed genes from our data set are involved in this pathway: *Cadherin, Citron, MLK, JNK, c-Fos, c-Jun* and *Elk-1*; and are all upregulated. The expression of the genes involved in this pathway indicate upregulation of actin polymerization, cell trafficking, cytokinesis, actin membrane linkage, membrane ruffling, cell-cell adhesion, cell contraction and proliferation, as well as cytoskeleton regulation and reorganization, while microtubule growth is down regulated. Moreover, it predicts that microtubule-organizing centre (MTOC) orientation processes would be activated and actin nucleation process inhibited. However, as mentioned previously, the disruption of this pathway suggests primarily a modification of the cytoskeleton. Therefore, similar to the GNRH pathway, the modification of this pathway would also indicate a change in cytoskeleton organization and therfore molecular trafficking and more particularly receptor trafficking would also be altered which could, overall, lead to a modification of synaptic plasticity. This analysis also revealed that the genes differentially expressed are involved in pathways such as signal transducer and activator of transcription 3 (*Stat3*) and Wnt/β-catenin. The Stat3 pathway plays an important role in cellular outgrowth and apoptosis. Here, expression of genes from the MAP kinase family such as *MKS* and *JNK* would be upregulated (figure 6.26). Moreover, the results indicate that in the heterozygote mice anti-apoptosis processes could be upregulated and transcription could be activated. Overall, in these pathways, several genes have their expression altered, weather up or down regulated, due to the Der1 mutation (figure 6.26). In the Wnt/β-catenin pathway, *SFRP* and *SOX* genes are down regulated while *GBP* and *c-Jun* are upregulated, in the hippocampi from the heterozygous mice. The changes in this pathway are predicted to lead to activation of cell

pluripotency (figure 6.27). Those affected pathways in the heterozygous mice are mainly involved in cellular growth and migration as well as regulation of the cytoskeleton. Which indicate that these processes could be impaired in the hippocampus of the heterozygous mice.
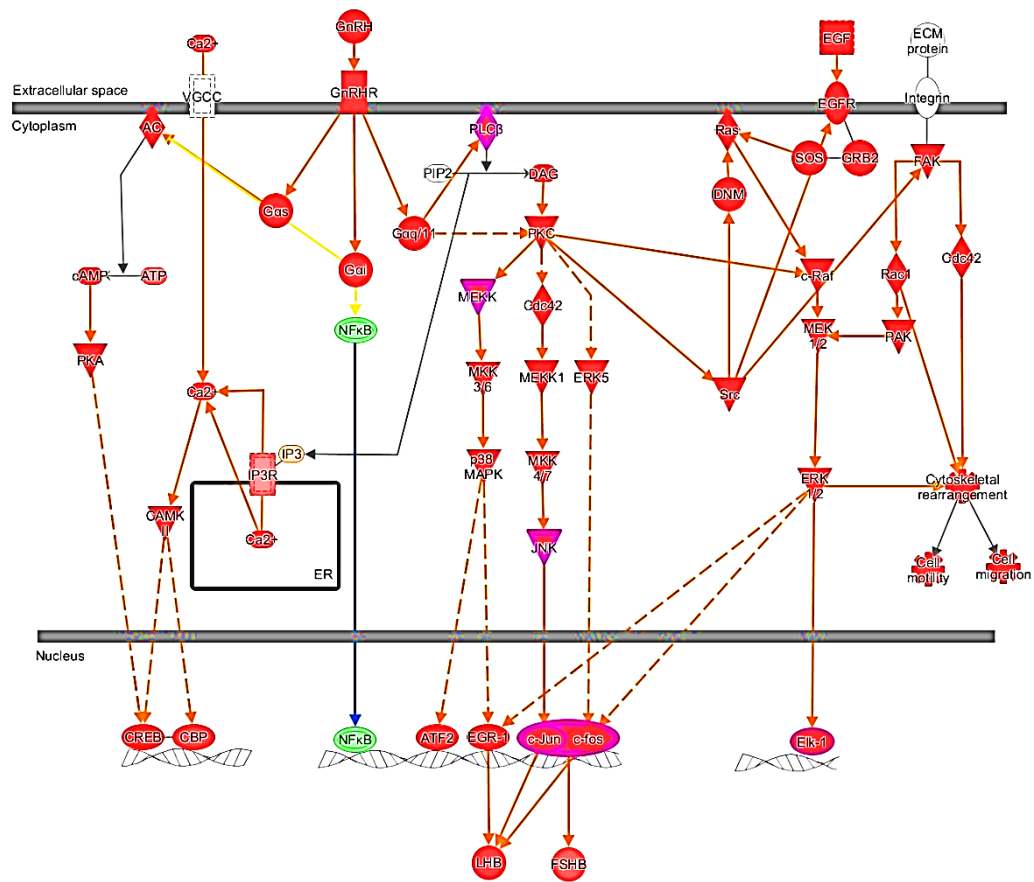
Figure 6.24: Representation of the expected activation state of the GNRH pathways

The hippocampus RNAseq analysis using IPA revealed that genes necessary for the GNRH signalling pathway were affected. Significant genes differentially expressed in the heterozygote mice compared to the WT mice are *PLCβ, MEKK, JNK, c-Jun, c-fos* and *Elk-1* and they all seem to be upregulated. The genes circled in magenta have been found in our analysis to be differentially expressed in the hippocampus of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.
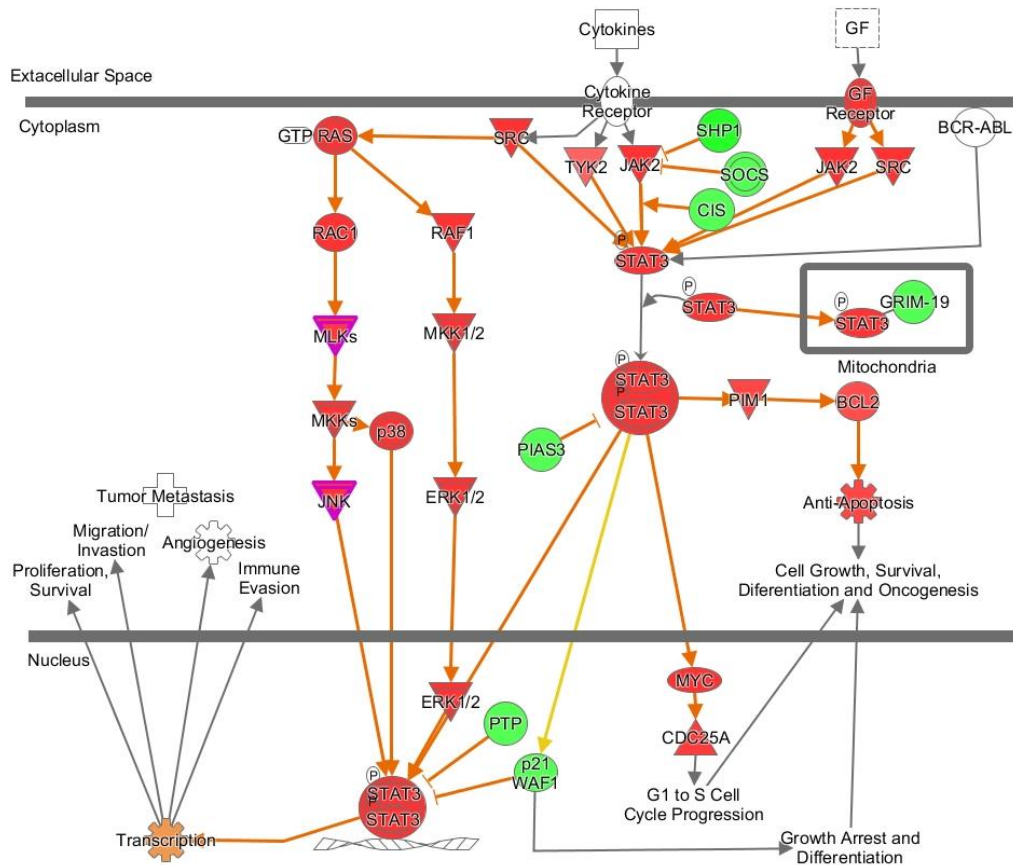
Figure 6.25: Representation of the expected activation state of the STAT3 pathways.

In the hippocampus, the Stat3 signalling pathway seemed to be affected. Significant genes differentially expressed in the heterozygote mice compared to the WT mice are JNK and MLKs, which both seem to be upregulated. The genes circled in magenta have been found in our analysis to be differentially expressed in the hippocampus of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.
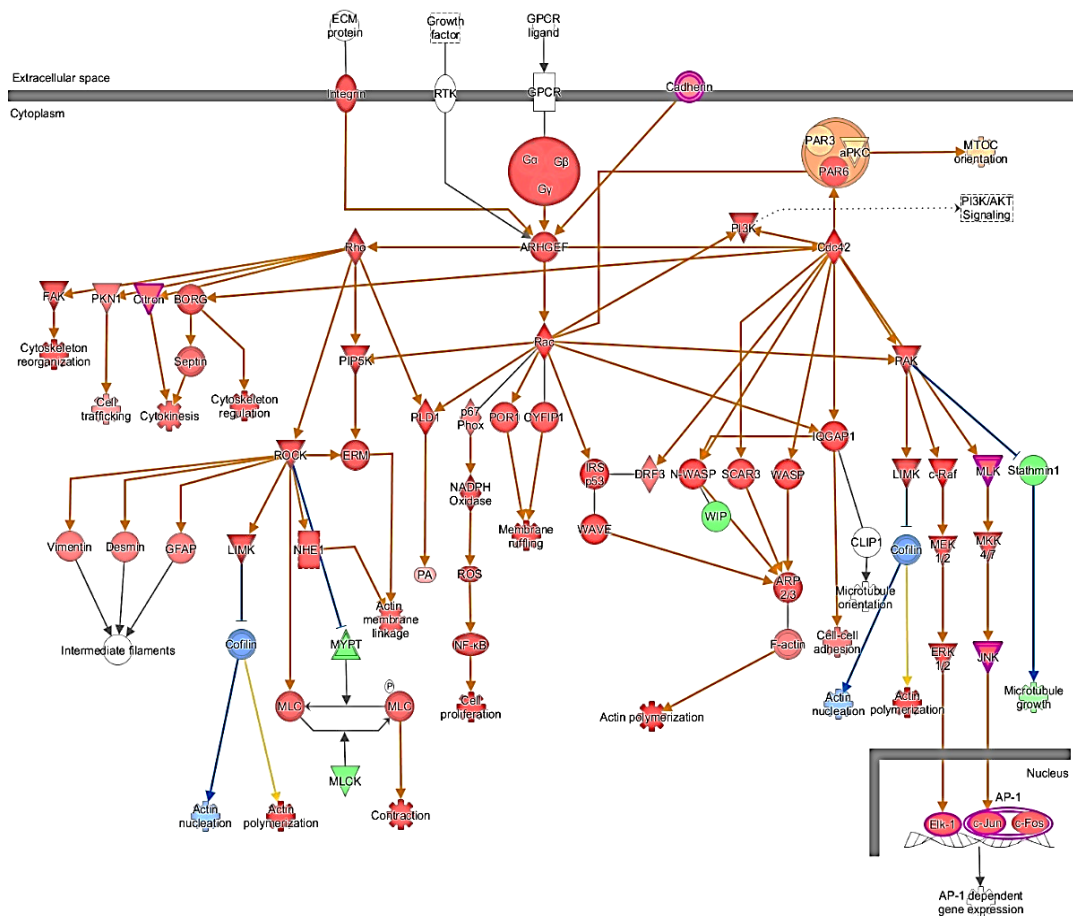
Figure 6.26: Representation of the expected activation state of the Rho GTPase pathways.

In the hippocampus of the heterozygous Der1 mice, Rho GTPase pathway might be affected by the Der1 translocation. The significant genes of this pathway differentially expressed in the heterozygote mice compared to the WT mice are *cadherin, citron, MLK, JNK, c-Fos, c-Jun* and *ElK1,* all are upregulated. The genes circled in magenta have been found in our analysis to be differentially expressed in the hippocampus of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.
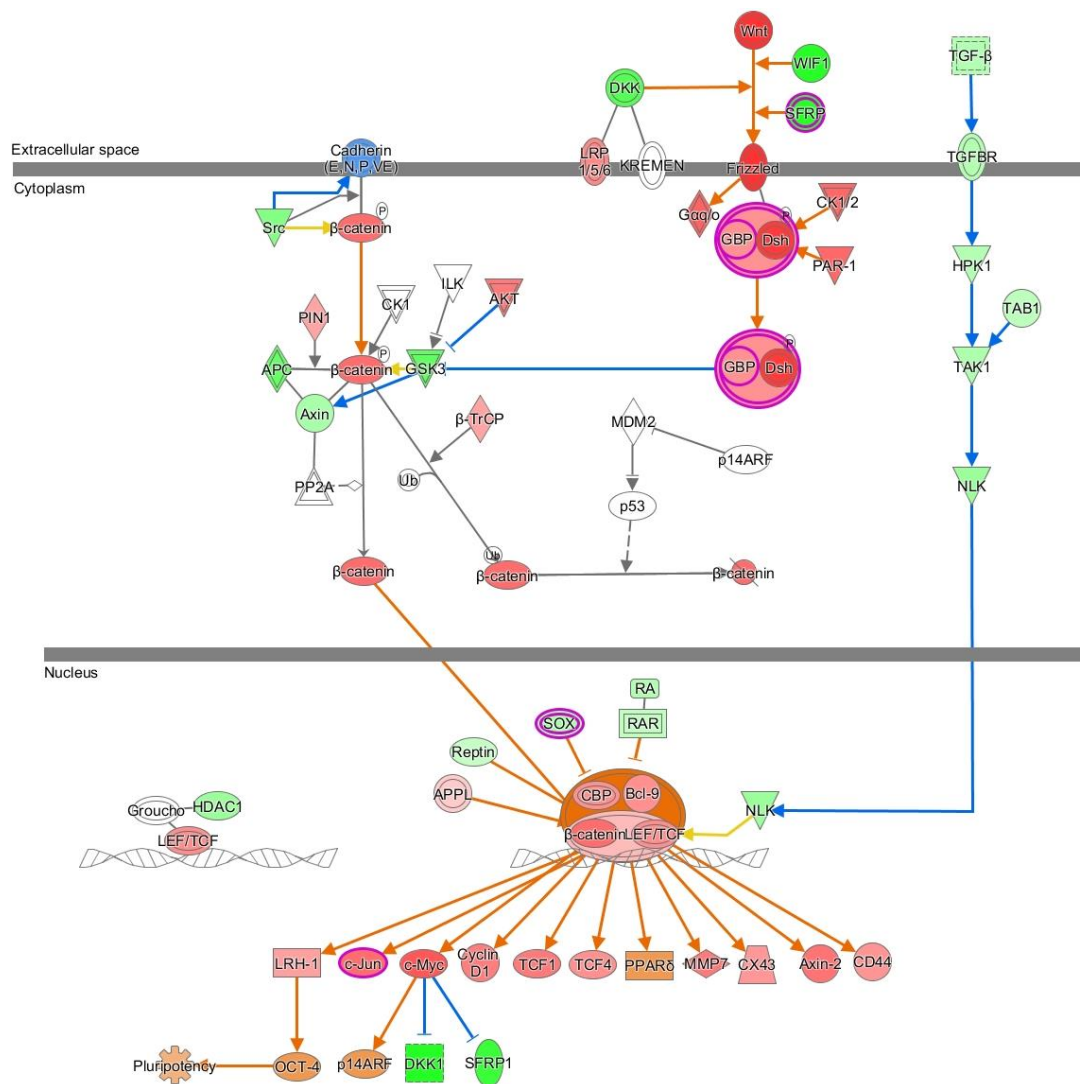
Figure 6.27: Representation of the expected activation state of the Wnt/β-catenin pathways.

In the hippocampus of the heterozygous Der1 mice, the Wnt/β-catenin pathway seem to be affected. Significant genes differentially expressed in the heterozygote mice compared to the WT mice are c-Jun and GBP, both upregulated, and SFRP and SOX both downregulated. The genes circled in magenta have been found in our analysis to be differentially expressed in the hippocampus of the heterozygous mice compared to the wild type mice. The genes in red or pink are expected to be up regulated and those in green are expected to be down regulated. Solid lines and broken lines respectively show direct or indirect interactions. Orange lines leads to activation, blue lines lead to inhibition, yellow lines represent inconsistent findings and grey line represent unpredicted results. Orange and blue fillings respectively indicate predicted activated or inhibited genes.

Overall RNAseq analysis revealed enrichments in a multitude of processes, function, and component in the cortex and hippocampus of the heterozygous mice. The cortical analysis indicates that the genes are mainly involved in transport and binding activity, vesicular function, synaptic signalling, neurogenesis, neuronal migration, GABAergic and glutamatergic pathway as well as Wnt pathway. While the hippocampal analysis revealed similar enrichment such as transport, signalling, Wnt pathway, synaptic activity, cell communication and neuronal development as well as more specific affected process such as the secretion of neurotransmitters. However, in the hippocampus, genes affected by the *Der1* mutation are not involved in specific neurotransmitter function as seen for the cortex analysis but in the global neurotransmitter secretion.

This shows some similarity between the two compartments; however, the cortex is a much bigger area, and has more complex functions therefore additional affected characteristics have been found in this area.

6.3 Analysis of the effect of the homozygous *Der1* mutation on RNA expression

6.3.1 Quality control of the homozygous samples

As described above, in the hippocampus, the PCA plot revealed a wild-type male outlier which was removed for further analysis. To balance the sex ratio one female wild-type was removed as well. Following outlier removal, the PCA plot showed wild-type and homozygous samples clustering by sex but not by genotype (figure 6.28, a). In addition, the heatmap also reveals that the homozygous samples do not cluster together (figure 6.28, b).

The cortex PCA plot also revealed a wild-type male outlier, and a wild-type female sample was removed as well for balance. Similarly, to what was found in the hippocampus, the cortical PCA plot indicates that the samples cluster by sex but not by genotype (figure 6.29, a). The heatmap issued from the RNAseq results of the cortical samples revealed that the homozygous samples do not cluster together (figure 6.29, b).

These initial analyses indicate that the cortical and hippocampal homozygous samples are heterogenous, therefore, homozygous RNASeq data from the homozygous samples was not studied using pathway analysis because no explanation for the extreme variability could be identified.
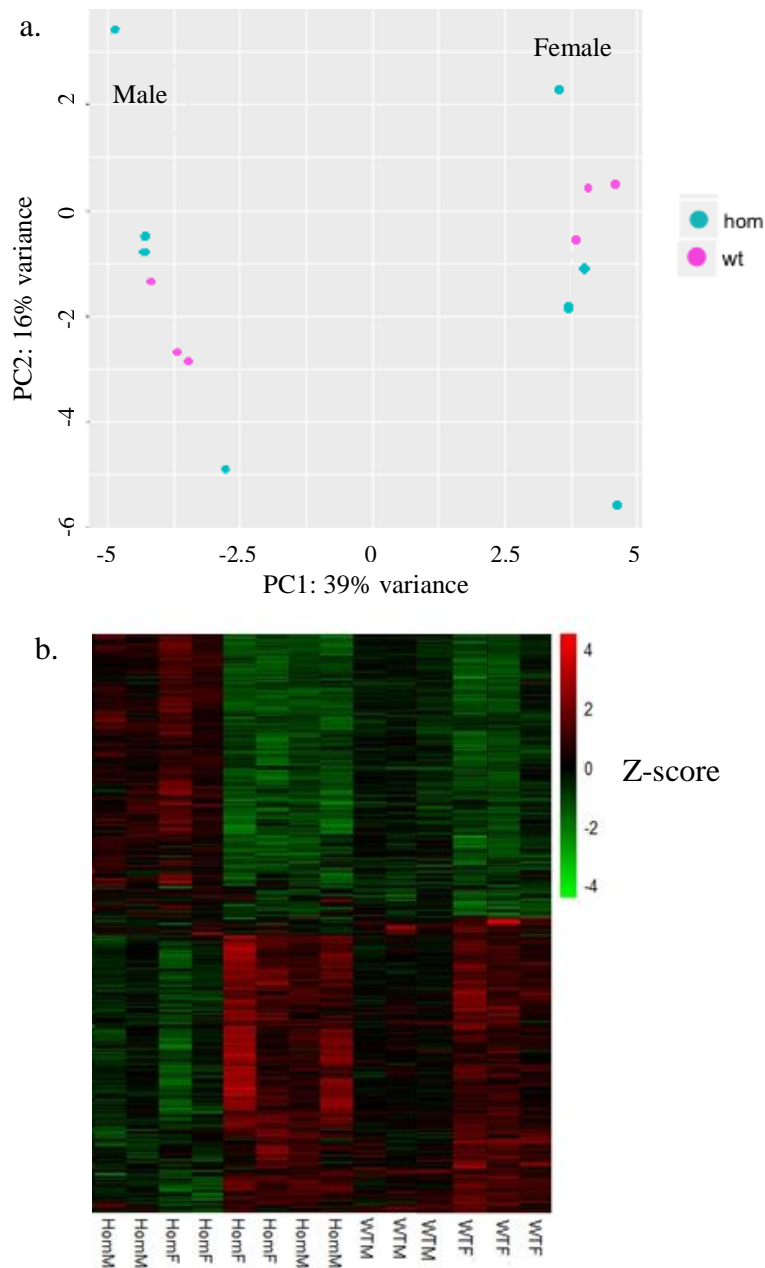
Figure 6.28: Quality control of the homozygote's hippocampus samples

a. PCA plot after removal of the 2 WT samples. Male samples cluster on the left of the graph while female samples cluster on the right

b. Heatmap: each row represents the expression of one gene and each column represents one sample. Z-score demonstrates the standard deviation of the log2 fold change for genes with a significant differential expression between genotype (log2 fold change <0.05), in each sample. Differential gene expression is expressed in red and green respectively representing high and low expression.
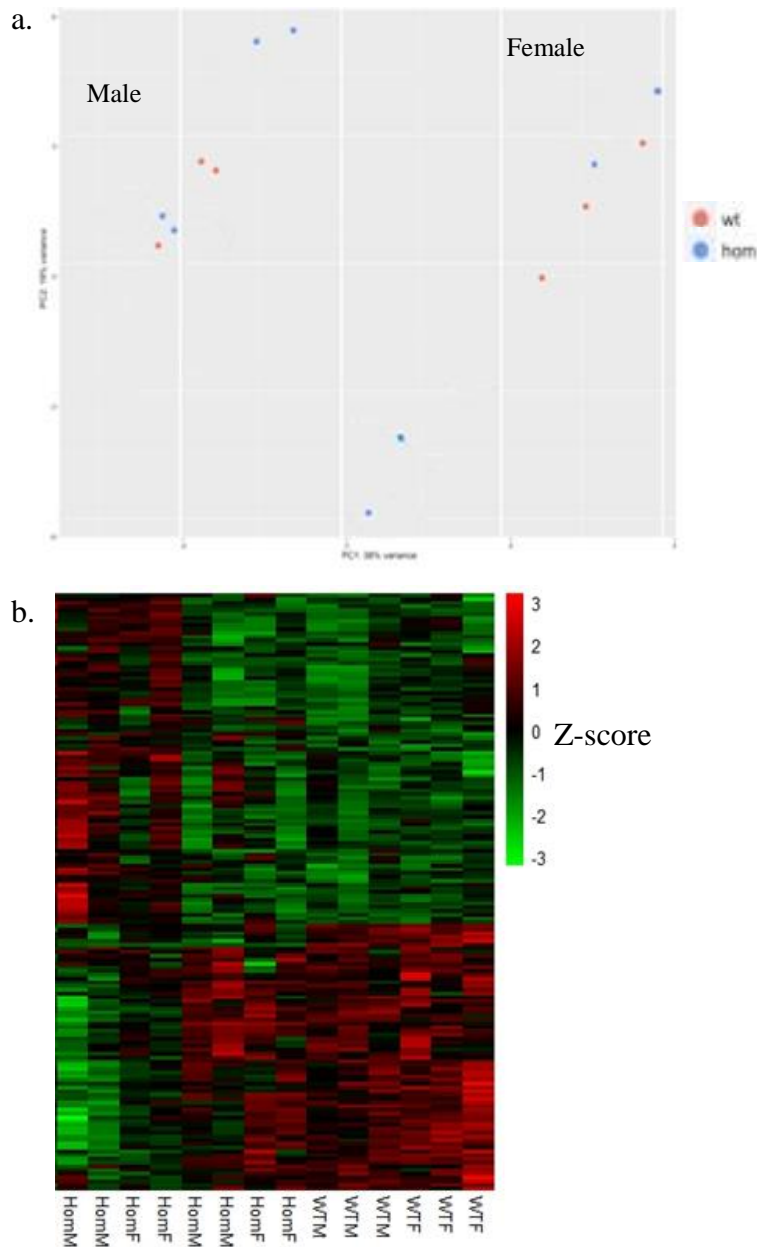
Figure 6.29: Principal components analysis of the cortex samples

a. PCA plot after removal of the 2 WT samples. Male samples cluster on the left of the graph while female samples cluster on the right.

b. Heatmap: each row represents the expression of one gene and each column represents one sample. Z-score demonstrates the standard deviation of the log2 fold change for genes with a significant differential expression between genotype (log2 fold change<0.05), in each sample. Differential gene expression is expressed in red and green respectively representing high and low expression.

6.3.2   DISC1 expression in the homozygous samples

Extraction of the *Disc1* expression data indicates that it is downregulated and almost non-existent in homozygous samples, which confirms the homozygosity of the samples used (figure 6.30). Moreover, it was confirmed by its expression value which indicates a negative log2fc and a p value less than 0.05 in the hippocampus and the cortex of the homozygous Der1 mice (table 6.22). In addition to this reduction, *Disc1* expression was analysed exon by exon as it was analysed in the heterozygous samples. In the homozygous samples, expression of exons 9-13 should be abolished, with expression of exons 1-8 still detectable. This was confirmed (figure 6.31).

Here, *Disc1* expression is higher in the hippocampus (table 6.22).

DISC1 expression	Base Mean	log2FoldChange (log2fc)	padj
Cortex	65.17	-2.455	8.91E-39
Hippocampus	80.88	-1.748	1.68E-30

Table 6.22: DISC1 differential expression in the homozygous Der1
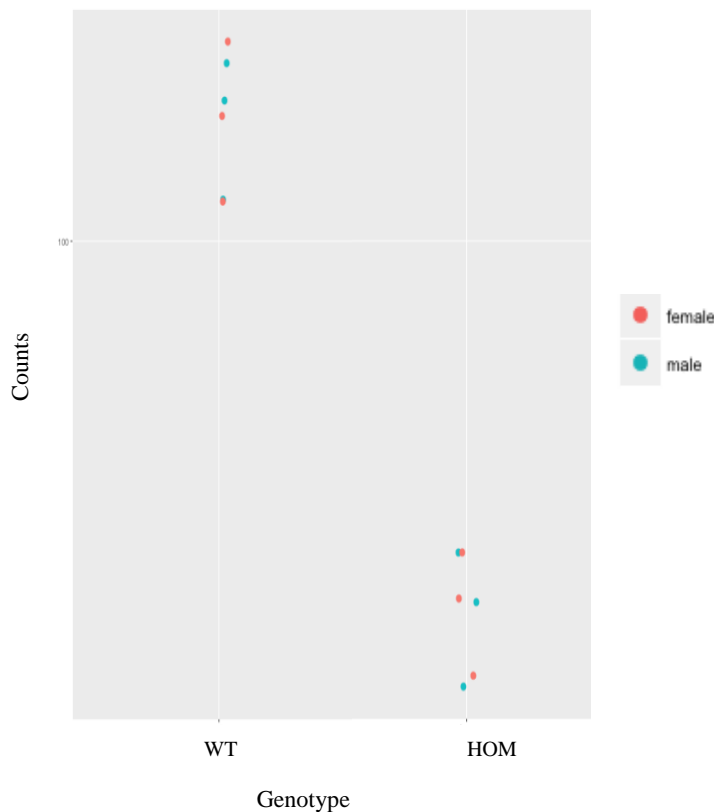
Figure 6.30: Normalised counts for *DISC1* in WT and Homs

DISC1 counts from the homozygotes are lower compared to the WT, as expected.

The results show differential expression in the cortex and in the hippocampus. In both regions *Disc1* expression is significantly reduced compared to the WT. log2fc<0 indicates that *Disc1* expression is reduced and the p adjusted value (padj) indicates that it is significantly different compared to the WT.
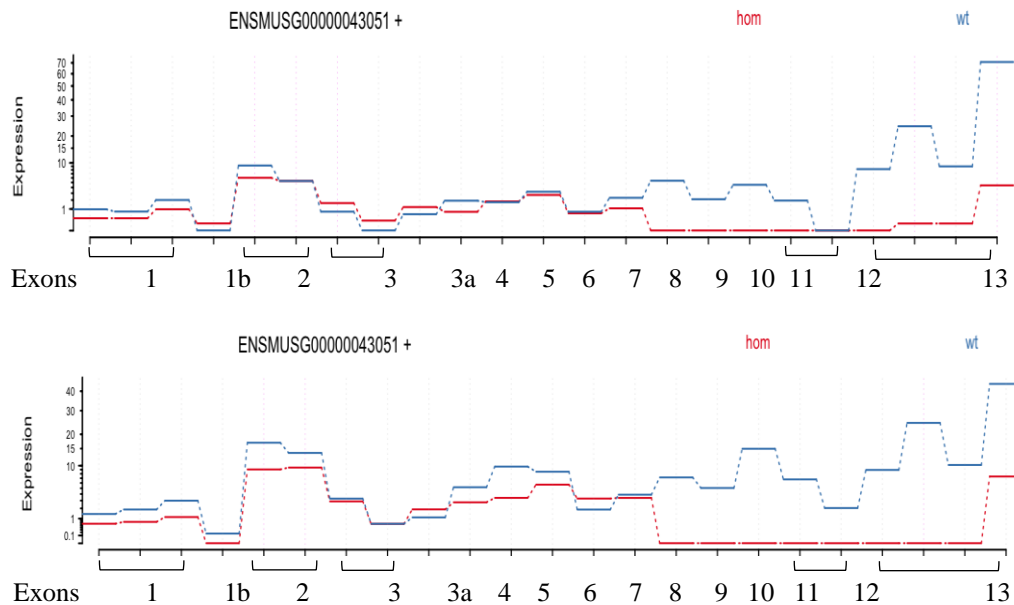
Figure 6.31: Result of the DEXSeq analysis for DISC1 in the homozygous
Both cortex (a.) and hippocampus (b.) show a drop mainly after exon 8.

6.4 Comparison with previous findings

6.4.1 Expression of DISC1 interactors is affected by the *Der1* mutation.

The RNAseq analysis revealed that several DISC1 interactors are differentially expressed in the cortex of the heterozygous mice, with some affected at gene level and others at exon level (table 6.23). Kif5a which is a microtubule motor involved in intracellular organelle transport; Atf5 which is involved in cell survival, proliferation, and differentiation; and Dtnbp1 which is necessary for neurite outgrowth, synaptic vesicle trafficking and neurotransmitter release, have been found up regulated (table 6.23) while Pcm1 which is necessary for the localization of proteins, and for anchoring microtubules to the centrosome have been found down regulated (table 6.23).

Additionally, DISC1 interactors involved in the assembly and organization of the cytoskeleton were found to be differentially expressed in the heterozygote's cortices such as TNIK which

plays a role in cytoskeletal rearrangements, Pafah1b1 (LIS1) which is involved in dynein regulation and in the Rho GTPase pathway linking this gene to microtubule and cytoskeleton assembly and organization (table 6.23). Cep63, MAP1a and PCNT (table 6.23) are also involved in microtubule assembly and network formation. Syne1 makes the link between organelles and cytoskeleton therefore influencing the cytoskeleton organization. Moreover, interactors involved in organelle trafficking such as LIS1, FEZ1, KLC1, KLC2 and DYNC1H1 (table 6.23) were also found differentially expressed. The interactors IMMT involved in mitochondria function and PDE4B involved in signal transduction were also found differentially expressed in the cortices of the heterozygous mice (table 6.23). This indicates that dysregulation of DISC1 affects the expression of its interactors by direct or indirect means.

No DISC1 interactors were found to be dysregulated in the hippocampus of the heterozygous Der1 mice.

Interactor found in DESeq			Interactor found in DEXSeq		
Name	log2FoldChange	padj	Name	log2FoldChange	padj
Kif5a	0.222701	0.001656	*Cep63*	-0.08363	0.019924
Atf5	0.232043	0.018415	*Map1a*	0.029333	0.043555
Dtnbp1	0.138686	0.045666	*Map1a*	-0.03224	0.046381
Ankhd1	-0.20359	0.031577	*Map1a*	0.249955	0.047062
Pcm1	-0.19108	0.017355	*Pafah1b1*	0.042378	0.017401
Trak2	0.14953	0.03537	*Pafah1b1*	0.048128	0.030475
			Pafah1b1	0.000955	0.035181
			Pafah1b1	-0.04427	0.039341
			Pafah1b1	0.033259	0.040981
			Syne1	0.146481	0.006835
			Syne1	0.178507	0.024372
			Syne1	0.144414	0.030863
			Syne1	0.172714	0.045021
			Pcnt	-0.12921	0.014708
			Pcnt	0.094812	0.045785
			Fez1	-0.02985	0.040702
			Immt	-0.08294	0.002124
			Immt	0.067315	0.031207
			Immt	0.062765	0.036451
			Pde4b	0.066066	0.029707
			Klc1	0.060091	0.010036
			Klc1	0.049129	0.031679
			Klc2	-0.05032	0.030418
			Dync1h1	0.051235	0.001421
			Dync1h1	0.045299	0.003061
			Dync1h1	0.051156	0.00537
			Dync1h1	0.060294	0.009221
			Dync1h1	0.05183	0.012286
			Dync1h1	0.048217	0.013774
			Dync1h1	0.06086	0.01854
			Tnik	0.076955	0.040087
			Tnik	0.078099	0.044203
			Tnik	0.086166	0.049313

Table 6.23: Genes encoding DISC1 interactors found to be differentially expressed in the cortex of the heterozygous mice at gene and exon level

6.4.2 Dysregulated expression of putative schizophrenia risk genes

a. Cortical samples analysis

The latest largest single cohort GWAS of schizophrenia using 40,675 cases and 64,643 controls identified 535 genome-wide significant genes from which 145 genetic loci have an altered expression (Pardiñas *et al.*, 2016). Overall, 535 genes were found to be enriched and associated with schizophrenia. New enriched genes were involved in metabotropic GABA-B signalling and acetyl cholinesterase, other genes confirmed that calcium channel function is altered in schizophrenia. Additionally, rare variants such as *NRXN1* and GABAergic signalling were also discovered to be enriched in this study as well as genes involved with autism such as *RBFOX1*, *FOXP1*, *FOXG1*.

Previously, another older schizophrenia GWAS using 36,989 cases and 113,075 controls discovered 108 loci associated to schizophrenia (Ripke *et al.*, 2014). Involvement of dopaminergic and glutamatergic signalling in schizophrenia were highlighted in this study, as well as genes involved in the immune system.

Additionally, a Copy number variations (CNVs) study on a cohort of 21,094 schizophrenic patients and 20,227 controls also identified numerous genes as putative schizophrenia risk factors (Marshall *et al.*, 2016). This study highlighted genes associated with schizophrenia which are involved in synaptic networks, glutamatergic ionotropic receptor function and diverse protein complexes.

Results of the RNAseq analysis using the samples of heterozygous cortices were compared with these different gene lists and multiple putative schizophrenia risk genes were found dysregulated in the Der1 cortical samples (table 6.24). For further functional analysis the lists were combined and analysed using GO term analysis (table 6.25).

These genes were found to be involved in neuron differentiation, neuronal death, organelle and synapse organization and regulation of action potential as well as in binding function, transporter and voltage-gated channel; cytoskeleton, organelle and synapse organization activity; glutamate pathway and vesicular trafficking. Interestingly, it was also indicating enrichment in the activation of astrocyte in response of immune activation which has also been linked with psychiatric disorder, in particular in schizophrenia. Glutamatergic signalling was also highlighted in the two GWA studies, which indicate that the Der1 heterozygous mice could have a similar RNA dysregulation compared to schizophrenic patients.

Additionally, acetyl cholinesterase gene (*ACHE*) were found in the analysis of the cortical samples from the heterozygote mice as well as in the GWAS (table 6.24) (Pardiñas *et al.*,

2016) indicating that the cholinergic signalling is affected in the Der1 heterozygote model as well as in schizophrenia. This gene is a potential therapeutic target in schizophrenia and has been found to be implicated in the pathology of autism. The activity-regulated cytoskeleton-associated protein complexes gene (*ARC*) is also known to be implicated in schizophrenia through GWAS analysis (Pardiñas *et al.*, 2016), and was found to be differentially expressed in the heterozygote Der1 cortices (table 6.24). This gene is involved in the regulation of long-term potentiation and depression therefore this indicates that synaptic plasticity is dysregulated in the heterozygous Der1 mice. Interestingly, Neurexine (NRXN1) was previously also found to be differentially expressed in the Der1 and to be a susceptibility gene for schizophrenia (table 6.24) (Marshall *et al.*, 2016; Pardiñas *et al.*, 2016). *NRXN*1 is involved in synaptic activity and formation and is crucial for neurotransmission (Viñas-Jornet *et al.*, 2014).

Overall, this indicate multiple similarities between the Der1 RNAseq analysis and these association studies, which confirm that DISC1 is involved in multiple process and might be a susceptibly gene for mental disorder. Genes found to be affected in the RNAseq analysis as well as these studies indicate that similar functions such as signalling, cytoskeleton organisation, cell communication, and synaptic signalling seem to be affected in schizophrenia patients and in the Der1 mice.

Exon level					Gene level		
GWAS 1		**GWAS 2**		**CNV**	**GWAS 1**	**GWAS 2**	**CNV**
ABCD2	KCNG1	ABCB9	NAGA	MPDZ	Ache	Apopt1	CHRNA7
ACHE	KCTD10	ALDOA	NGEF	RAPGEF2	Ankrd45	As3mt	L1CAM
ADAM15	KDM4C	AMBRA1	NISCH	DVL1	Arc	Atxn7	MPDZ
ADGRB3	KIF21A	ATG13	OSBPL3	NRXN1	B4galt2	Cenpm	MYO6
ANAPC7	LRRC4	ATP2A2	PCGF6	SEMA4C	Bach2	Clu	RAPGEF2
ANK3	MAP1A	CACNA1C	PITPNM2	DLG1	Bcl11a	Egr1	SEMA3E
ANKS1B	MARK3	CACNA1I	PJA1	DLGAP1	Brd1	Ep300	SNTB2
AP3B2	ME1	CACNB2	PLCH2	SNAP29	Cep170	Ephx2	STX1A
ARC	MOB4	CKB	PODXL	NRXN3	Chd2	Furin	
ATG2A	MSRA	CLU	PPP1R13B	SHANK1	Clip1	Fxr1	
ATP13A1	NCAPD3	CNKSR2	PRR12	GRIN1	Dgcr8	Gid4	
BAG4	NCSTN	CNOT1	PTPRF	SYT1	Emb	Gigyf2	
BRD1	NDFIP2	CNTN4	R3HDM2	NEDD4	Emx1	Gramd1b	
BRINP1	NIP7	DFNA5	RANGAP1	APP	Gmip	Hcn1	
CDIP1	NPTX1	DGKZ	RERE	WASF1	Hcn4	Ireb2	
CDK5R1	NTRK3	DOC2A	RGS6	DTNB	Inpp4b	Itih3	
CEP170	OLA1	DRG2	SATB2		Kcnd3	Mdk	
CLIP1	PCDH7	EGR1	SERPING1		Kif21a	Naga	
CNPPD1	PCLO	EPC2	SF3B1		Lamtor2	Ndufa13	
CNTN2	PDE4B	ERCC4	SLC32A1		Msra	Ndufa2	
COPA	PEPD	ETF1	SLC38A7		Nip7	Ndufa6	
CPEB1	PHF2	GIGYF2	SLC45A1		Nos1	Ngef	
CPT1C	PHF3	GLT8D1	SLC4A10		Nptx1	Nrgn	
CUL9	PRUNE	GRAMD1B	SLC7A6		Pcdha11	Pak6	
DDHD2	PSMD11	GRIA1	SMG6		Pcdha12	Pcdha3	
DMTF1	PSME4	HARS	SNAP91		Pcdhac2	Pcdha5	
DNAJC11	PTBP2	HARS2	SNX19		Pcnx	Pcdha7	
DPYSL5	PTK2B	HSPA9	SREBF2		Phf2	Pja1	
EIF3B	RBFOX1	HSPD1	SRPK2		Phf3	Ppp1r13b	
EIF5	RNF38	IK	STAB1		Rprd2	Ptn	
ELAVL4	RPTOR	IREB2	TAOK2		Rps19bp1	Rcn3	
EMB	SGCE	ITIH3	TCF4		Scn5a	Reep2	
FAM114A2	SIPA1	KCNV1	THOC7		Sfxn5	Rere	
FAM120A	SIPA1L3	KDM3B	TLE1		Trpc4	Rgs6	
FAM216A	SIRPA	KDM4A	TLE3		Whsc1l1	Rras	
FAM49B	SORCS3	KLC1	TOM1L2		Zc3h7b	Slc35g2	
FGFR1	ST3GAL3	L3MBTL2	TRANK1			Slc4a10	
GABBR2	TCTN1	LRP1	TSR1			Smdt1	
GLG1	THRB	MAN2A2	VPS45			Smim4	
HMOX2	VPS26B	MAU2	YPEL3			Stat6	
IFT81	YWHAE	MED19	YPEL4			Zdhhc5	
IPO13	ZDHHC8	MEF2C	ZDHHC5			Zfyve21	
			ZSWIM6				

Table 6.24: <u>Genes found to be enriched in the heterozygous Der1 mouse cortex and found to be putative schizophrenia risk genes</u>

Genes highlighted in orange have been found in both GWAS while genes highlighted in blue have been found in GWAS 1 and CNV analysis.

GO Component	FDR q-value (padj)	Enrichment	Number of genes
neuron projection	4.61E-22	4.74	61
synapse	2.67E-19	6.39	42
dendrite	2.26E-14	5.98	33
postsynaptic density	3.09E-14	9.13	24
organelle	5.92E-14	1.51	181
transporter complex	1.06E-09	6.45	21
vesicle	1.28E-08	2.61	50
presynaptic membrane	1.16E-07	13.83	10
excitatory synapse	1.11E-05	15.58	7
ionotropic glutamate receptor complex	7.37E-04	10.53	6
neurotransmitter receptor complex	8.56E-04	10.14	6
voltage-gated potassium channel complex	9.43E-04	7.89	7
potassium channel complex	1.75E-03	7.1	7
NMDA selective glutamate receptor complex	6.70E-03	22.82	3
trans-Golgi network transport vesicle membrane	8.63E-03	60.85	2
GO function	FDR q-value (padj)	Enrichment	Number of genes
protein binding	1.20E-12	1.66	157
voltage-gated channel activity	2.98E-03	5.37	11
ionotropic glutamate receptor binding	8.13E-03	12.68	5
transporter activity	8.73E-03	2.26	28
signalling receptor binding	9.06E-03	1.98	37
glutamate receptor binding	9.11E-03	8.98	6
channel activity	2.81E-02	3.01	14
GO Process	FDR q-value (padj)	Enrichment	Number of genes
regulation of neuron differentiation	3.15E-09	4.38	35
regulation of nervous system development	3.04E-09	3.77	40
regulation of neurogenesis	2.30E-09	3.94	38
cellular component organization	3.02E-08	1.92	96
synapse organization	4.25E-08	10.29	15
transport	5.19E-07	2.08	71
signalling	1.19E-06	4.41	24
cell communication	5.53E-06	3.73	26
modulation of excitatory postsynaptic potential	1.74E-05	15.54	8
protein localization to synapse	1.85E-05	19.97	7
synaptic signalling	9.64E-04	4.63	13
ionotropic glutamate receptor signalling pathway	2.91E-03	22.82	4
neuronal action potential	3.00E-03	14.26	5
neurotransmitter secretion	4.55E-03	13.04	5
regulation of glutamate receptor signalling pathway	1.12E-02	10.37	5
regulation of neurotransmitter receptor activity	1.19E-02	10.14	5
regulation of AMPA receptor activity	1.31E-02	14.6	4
dendrite morphogenesis	1.72E-02	9.13	5
positive regulation of neuron death	1.75E-02	5.76	7
regulation of actin cytoskeleton organization	2.23E-02	3.32	12
astrocyte activation involved in immune response	2.47E-02	60.85	2
glutamate receptor signalling pathway	4.13E-02	9.87	4

Table 6.25: <u>Gene ontology analysis of schizophrenia risk genes found in the homozygous cortical samples.</u>

Example of some interesting GO terms found to be enriched. FDR q-value correspond to

the p-value corrected for multiple testing. Number of genes differentially expressed in each pathway is indicated.

b. Hippocampus analysis

The genes found to be differentially expressed in the RNAseq analysis of the samples of the heterozygotes hippocampi were also compared with the results of these three analysis (Ripke *et al.*, 2014; Marshall *et al.*, 2016; Pardiñas *et al.*, 2016)(table 6.26). The GO term analysis (table 6.26) revealed that some of these genes were involved in synapse and vesicle composition, channel and transporter activity and composition, binding activity, synaptic signalling, neurogenesis, neurotransmitter secretion and transport, and localization of the receptors at the synapse. As mentioned previously, localization of the receptor at the synapse is dependant of the secretion of the neurotransmitter and modification of these process highlight an impairment in synaptic plasticity. A high enrichment in mitochondrial fragmentation involved in apoptotic process was found suggesting an impairment of the intrinsic apoptotic pathway.

NRXN1 is again highlighted and dysregulated in the hippocampus of the heterozygous mice. Moreover, the *ERBB4* gene was also found differentially expressed in the hippocampi of the heterozygous Der1 mice and was a hit in the GWAS (table 6.27) (Pardiñas *et al.*, 2016) implicating neuregulin signalling as a risk factor for schizophrenia and indicating that disruption of *DISC1* lead to dysregulation of this signalling pathway in the heterozygous Der1 mouse model. Moreover, *ERBB4* is also involved in the glutamatergic synapse function so is *NPTX* which is also a putative schizophrenia risk gene and differentially expressed in the hippocampus of the heterozygous mice (table 6.26). NPTX is particularly involved in clustering AMPARs at the cell surface. This indicates that the glutamatergic pathway is also altered in the hippocampus of the Der1 mice and that synaptic plasticity is dysregulated. Additionally, *osbpl3*, which codes for an intracellular lipid receptor and is necessary for cell adhesion and actin cytoskeleton organisation and is has been found to be involved in schizophrenia was also differentially expressed in the Der1 mice hippocampus. This indicates that cytoskeleton organization is also altered. *cntn4*, which is an axon-associated cell adhesion molecule and is necessary for neuronal network formation and plasticity and is believed to be a susceptibility gene for schizophrenia was found differentially expressed as well. This reinforces the fact that neuronal network, plasticity and signalization is affected in the Der1 mice hippocampus.

GO Component	FDR q-value (pasdj)	Enrichment	Number of genes
synapse part	9.58E-03	7.94	7
presynaptic membrane	2.56E-02	40	3
cell projection	3.00E-02	4.11	9
ion channel complex	4.72E-02	12.89	4
transporter complex	4.53E-02	12.08	4
synaptic vesicle	5.82E-02	18.98	3
exocytic vesicle	7.56E-02	17	3
GO Function	**FDR q-value (pasdj)**	**Enrichment**	**Number of genes**
cell adhesion molecule binding	4.49E-02	11.28	6
calcium channel activity	2.30E-01	23.86	3
cation channel activity	2.33E-01	11.87	4
calcium ion transmembrane transporter activity	2.69E-01	20.16	3
calcium channel regulator activity	2.45E-01	64.2	2
voltage-gated cation channel activity	2.30E-01	18.84	3
actin filament binding	3.41E-01	15.03	3
GO Process	**FDR q-value (pasdj)**	**Enrichment**	**Number of genes**
synapse organization	5.19E-03	32.83	5
synaptic signalling	1.67E-02	18.68	5
mitochondrial fragmentation involved in apoptotic process	7.76E-02	173.35	2
secretion	1.50E-01	9.14	5
localization	1.44E-01	2.89	12
transport	1.45E-01	3.15	11
developmental process	1.51E-01	2.58	13
regulation of neurogenesis	1.70E-01	5.99	6
neurotransmitter transport	1.69E-01	20.64	3
receptor localization to synapse	1.95E-01	59.78	2
neurotransmitter secretion	2.18E-01	49.53	2

Table 6.26: Gene ontology analysis of schizophrenia risk genes found in the heterozygous hippocampal samples

Example of some interesting GO terms found to be enriched. FDR q-value correspond to the p-value corrected for multiple testing

Exon level			Gene level		
GWAS1	GWAS2	CNV	GWAS 1	GWAS 2	CNV
ANKS1B	CACNB2	NRXN1	EHD1	OSBPL3	MYO5A
JAM3	GRIN2A		ERBB4	CNTN4	UTRN
TTBK1	R3HDM2		FAM120AOS		
	SNAP91		HCN4		
			NPTX1		
			NTN5		
			SOX5		
			THRB		
			TRPC4		

Table 6.27: Genes found to be enriched in the heterozygote Der1 mice' hippocampal samples and found to be putative schizophrenia risk genes

Genes highlighted in orange have been found in both GWAS while genes highlighted in blue have been found in GWAS 1 and CNV analysis

To conclude, multiple putative schizophrenia risk genes were found differentially expressed in the heterozygous Der1 mice which indicates that disruption of Disc1 affect the expression of multiple genes believed to be involved in schizophrenia. In the Der1 mouse model, the risk genes affected are mainly involved in cytoskeleton organisation, cell communication, synaptic signalling and neuronal plasticity. Moreover, the glutamatergic pathway is also dysregulated in this model. The glutamatergic theory is still the most prominent theory in schizophrenia, which could indicate that the Der1 mouse model could be a good model to study schizophrenia molecular mechanisms.

6.5 Discussion

Overall, the RNAseq analysis reveals that heterozygous Der1 mice have extensively altered transcript levels. Indeed, numerous genes were found differentially expressed. These genes are involved in multiple biological pathways and mechanisms. The differential expressed genes in the Der1 mice mainly affect synaptic activity and function, and therefore neuronal plasticity and signalling.

The heterozygous Der1 mouse analysis revealed numerous changes compared to the WT. Differentially expressed genes indicate an alteration of the dopaminergic, GABAergic, serotoninergic and glutamatergic system as well as impairment of vesicle trafficking. Additionally, GO term analysis indicated that neuronal development, transport of molecules and organelles, cell communication, signalling and death as well as synapse activity could be impaired in the heterozygous Der1 mice. Specific pathways such mTOR, Wnt, GNRH and stat3 might also be impaired in this mouse. Overall, the results are subtle but indicate that action potential and neuronal signalling may be altered.

Dopaminergic, serotoninergic, GABAergic and glutamatergic pathways are known to be affected in psychiatric illness (Belsham, 2001; Brambilla *et al.*, 2003; Tost, Alam and Meyer-Lindenberg, 2010; Dayer, 2014). The heterozygous Der1 mouse may exhibit impairments in similar pathways, therefore this mouse may indeed model mental illness. Moreover, multiple genes found enriched in our analysis were also found enriched in recent GWAS analysis (Ripke *et al.*, 2014; Pardiñas *et al.*, 2016) and CNVs analysis (Marshall *et al.*, 2016). This indicates that the change in gene expression in the Der1 mouse model might be similar to changes seen in the RNA expression of patients suffering from psychiatric disorders, especially schizophrenic patients. Moreover, similar processes and pathways seem to be

affected. The Der1 heterozygous mice can be compared to the patients carrying the t(1;11) as they also have only one *DISC1* allele affected. Studies on patients carrying the t(1;11) translocation showed a reduction of glutamate levels in the dorsolateral prefrontal cortex (Thomson *et al.*, 2016) which could synergise with our findings of a reduction of expression of glutamatergic receptors in the cortex of the Der1 heterozygote mice.

Several *Disc1* mouse models revealed a reduction of glutamate activity (Maher and LoTurco, 2012; Dawson *et al.*, 2015) (table 1.1). Moreover, the Disc1tr Hemi mice previously mentioned in the introduction, also revealed decreased expression of NMDA receptor subunits (Shen *et al.*, 2008; Dawson *et al.*, 2015) (table 1.1). Similarly, in the present study, the quantity of several isoform-specific transcripts of glutamatergic receptors was found reduced in the Der1 heterozygote mice. These data indicate that the Der1 translocation may induce subtle dysregulation of the glutamate system and altered brain connectivity. Multiple *Disc1* mouse models indicate dysregulation of the dopaminergic system (Dahoun *et al.*, 2017) which was also found in the Der1 mice model. However, studies indicate a decrease in cortical tyrosine hydroxylase levels (Niwa *et al.*, 2011, 2013) while the Der1 heterozygote mouse seem to reveal an increased expression of its transcript (table 1.1). A knock down model of *disc1* revealed that the regulation of dendritic development by DISC1 requires GABAergic pathway activity through AKT-mTOR pathway (Kim *et al.*, 2013). Similarly, in the Der1 model the quantity of transcripts of GABA receptors is reduced and the mTOR pathway is impaired, this could explain the neurite outgrowth impairment found in the Der1 mice (chapter 5). Our study reveals a link between DISC1 and GABA signalling, in controlling neuronal development in the Der1 mouse model.

In the Der1 heterozygote models Wnt/βcatenin signalling seems to be down regulated in the hippocampus. *Disc1* mouse models also revealed a linked between DISC1 and Wnt/βcatenin signalling (De Rienzo *et al.*, 2011; Singh *et al.*, 2011). Moreover, DISC1 has been found to affect neural progenitor proliferation through this pathway. Which indicates that proliferation could be altered in the Der1 mice model (Mao *et al.*, 2009). However, we did not find any change in DCX density (chapter 4) in our model. Further analysis is necessary.

Interestingly the GRNH pathway was also strongly affected and has been found affected in schizophrenia patients (Guilloux, Gaiteri and Sibille, 2010; Chen *et al.*, 2016; Kumar *et al.*, 2017). This adds weight to the idea that hormonal dysregulation is also a characteristic of psychiatric illness. Moreover, this finding links DISC1 to hormonal pathways. DISC1 has been previously found to be necessary for the normal function of the hypothalamic-pituitary-internal axis (Eachus *et al.*, 2017). It is possible that the Der1 heterozygote mice have an

impaired hypothalamus they should therefore express an impaired behaviour and a dysregulated neuroendocrine stress response.

Numerous voltage-gated channels were also found to be differentially expressed in the heterozygous Der1 mice. Those channels are crucial for the initiation and propagation of depolarisation and for membrane potential restoration and maintenance. These channels are therefore critical for synaptic and neuronal signalling. Numerous studies have highlighted those channels in the pathogenesis of psychiatric diseases (Imbrici, Camerino and Tricarico, 2013). This indicates that trafficking of ions such as calcium, potassium and sodium may be altered in the Der1 mutant, similarly to what has been found in psychiatric patients. Moreover, this also emphasizes the strong possibility that synaptic signaling is the main process affected in the Der1 mouse model.

Those various changes in gene expression could also indicate a modification in ratio of the different cell types of the brain. It has been shown that a mutation on exon 8 of *DISC1* leads to loss of function of the protein and therefore lead to an increase of WNT signalling in progenitor cells which then results in the reduction of the expression of Foxg1 and Tbr2, two fate markers (Srikanth *et al.*, 2015). Moreover, a study on human brain organoids also revealed that loss of DISC1 leads to alteration of the Wnt pathway, indicating that DISC1 was involved in neuronal progenitor migration and cell fate (Srikanth *et al.*, 2018). DISC1 might therefore be involved in cellular fate and the difference in gene expression discovered in the present work could be due to a change in cell fate and therefore in cell type. However, in the Der1 mice, no strong evidence that cell fate is affected has been discovered so far. Although, the modification of gene expression in both the cortex and the hippocampus revealed that the Wnt pathway was highly affected which could be an indication of a change in cell fate in the Der1 mice. To obtain evidence that cell fate could be affected by the *Der1* mutation cell-type specific and single cell RNAseq analysis could be performed.

The results of this RNAseq analysis corroborate our previous finding indicating an outgrowth impairment. Indeed, pathways involved in cellular growth and morphology such as AKT, mTOR and Wnt pathway were found to be enriched. Moreover, the cell death pathway seems to be dysregulated and this was also discovered when looking at the activation of caspase-3 (chapter 4). However, the RNAseq analysis indicates that DCX expression should be impaired while the histology analysis did not indicate any change. It is possible that the histology results don't indicate the same effect due to the small number of mice used. Further analysis is required.

Heterozygous mutant *Der1* have a reduced quantity of wild-type Disc1 protein and, furthermore, the expression of many genes related to synapses and psychiatric disorders are dysregulated in these mutants. Our results reveal that a psychiatric disorder relevant mutation causes extensive changes and potentially provide new insight into the molecular mechanisms underlying psychiatric disorders. Overall, our observations support previous findings from *Disc1* mouse models, and from patients affected by mental disorders.

Additionally, RNASeq has already been carried out using IPSC-derived neurons generated from the translocation family. Comparing these sets of results could then confirm whether the Der1 mouse is an accurate representation of the t(1,11) translocation and therefore a model of psychiatric disorders. This is currently being done by another PhD student.

Taken together, these results indicate that DISC1 is necessary for the regulation of the transcription. Disruption of *DISC1* lead directly or indirectly to the dysregulation of numerous genes. These preliminary results open future directions for the studies of multiple pathways involved in mental disorder and in which DISC1 plays a role.

CHAPTER 7

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CONCLUSION

DISC1 has been widely studied as a result of its potential involvement in mental disorders, (Tomoda *et al.*, 2016). It has been shown to play an important role in various processes such as development, neurogenesis, cell signalling, and neuronal plasticity which are believed to be involved in those disorders (Brandon, 2016). However, the functions of DISC1 have not yet been completely elucidated and its link with mental disorders is still being discussed. Therefore, further investigation into its biological roles and mechanism of action in those disorders is required.Animal models of disc1 have been frequently created in order to gain some insight on its function and to discover pathways which might be involved in mental illness (Tomoda *et al.*, 2016). In particular, mouse models relating to the t(1;11) translocation discovered in a Scottish family (K. Millar *et al.*, 2000) have been created in the hope of discovering the underlying mechanisms leading to the spread of mental disorders found in this family (table 1.1). However, most of these models do not reproduce the translocation found in the human carrier accurately. Nevertheless, these models have led to the discovery of novel functions of DISC1 and the possible mechanisms affected in mental disorders. Therefore, mouse models that more accurately recapitulate the t(1;11) translocation would allow for an easier path to translating such biological findings to patients suffering of mental illness. This would allow us to discover possible biological pathways affected in the patients carrying the translocation and therefore involved in psychiatric disorders.

The research presented in this thesis represents the first characterization of the Der1 mouse model. This model carries part of the human chromosome 11 genomic DNA from exons 4 to 8 of *DISC1FP1,* replacing part of the mouse *Disc1* gene from intron 8 to its 3' end, in order to mimic the translocation found in the Scottish family. The aim of the present work was to characterise this new Der1 mouse model in the hope to find a better mouse model for mental disorder and through the investigation of the effect of this *Der1* mutation at a structural, cellular and molecular level in order to have a comprehensive understanding of this new model. Moreover, this would give us new information on DISC1 function and its involvement in psychiatric disorders and on the mechanisms and consequences of the t(1;11) translocation in carriers as well. Investigation of local brain structure indicated a thinning of the CA1 area of the hippocampus in the Der1 homozygous mice and a ventricular enlargement in the Der1 heterozygous mice. However, MRI analysis did not corroborate these changes in either of the Der1 mutants (chapter 3). It is therefore possible that brain structure is only locally altered due to the Der1 mutation and this could reflect a change in the shape of these brain regions. Analysis of specific cell populations in fixed brain tissue revealed no difference in GABAergic neuron numbers in the PFC and the hippocampus (chapter 4). However, there was increased neuronal density in the left srlm of the hippocampus in the Der1 homozygous mice as well as

increase activation of caspase-3 in the dentate gyrus of the heterozygous mice (chapter 4). A trend toward a similar increase of caspase-3 was found in the PFC and the CA1 of both homozygous and heterozygous Der1 mice as well as a trend toward a reduction of BrdU density in the dentate gyrus in both Der1 mice (chapter 4). These results indicate that the Der1 mutation activates the apoptotic pathway and leads to minor decrease of neurogenesis. Analysis of primary neuronal cultures of homozygous Der1 mice showed that neurite outgrowth was increased and that neurons exhibited somal hypertrophy as compared to their wildtype counterparts (chapter 5). This demonstrated that despite no drastic changes to cell populations in the brain, the Der1 mutation appears to affect intracellular mechanisms in neurons. To better understand what the intracellular effects of the Der1 mutation are, RNA sequencing was performed. This analysis indicated important changes in the gene expression in the heterozygous mice (chapter 6). The changes in gene expression appear to affect processes such as cell communication, cell proliferation, cell death, molecular transport, trafficking of mitochondria and vesicles as well as synaptic plasticity and organization and neurotransmission (chapter 6). A summary of those results can be found in table 7.1, previous similar findings from other disc1 mice models were also indicated in the table.

Name	Description	Results	Comparison to previous mice models
Der1	carries part of the human chromosome 11 genomic DNA from exons 4 to 8 of *DISC1FP1*, replacing part of the mouse *Disc1* gene from intron 8 to its 3' end	thinning of the CA1 in the Hom	Not previously seen
		ventricular enlargement Het	Seen in Hikida et al, 2007 and Shen et al, 2008
		↑ neuronal density in the left srlm Hom	Not previously seen in Disc1 mice model
		↑ apoptosis trend Het and Hom	Not previously seen in Disc1 mice
		↓ neurogenesis trend Het and Hom	Shen et al, 2008 Lee et al, 2011
		↑ neurite outgrowth somal hypertrophy in Hom	Pletnikov *et al.*, 2008
		altered global gene expression through RNAseq	Not previously seen at this scale in Disc1 mice model

Table 7.1: summary of the results of Der1 analysis

Disturbances in these structural, cellular and molecular processes are believed to happen in psychiatric disorders. In the present study, those alterations are due to the disruption of DISC1 and therefore could confirm the link between DISC1 and psychiatric illness. Moreover, this indicates that the Der1 mouse model is a model of psychiatric illness which better reflect the human t(1;11) translocation. The consequences of these results will be further discussed in this chapter, as well as how they complement known functions of DISC1; then possible future directions will be presented.

7.1   The Der1 mutation has a subtle effect on brain structure

Enlargement of the lateral ventricles and thinning of the CA1 were observed respectively in the brains of heterozygous and homozygous Der1 mice (Chapter 3). However, MRI analysis of these mice did not support these results. This indicates that the observed histological changes are either very subtle and localised and would therefore not be enough to be detected by MRI or perhaps be due to a change of shape of these areas. A modification in the shape rather than volume of these two areas would affect volumes locally rather than in the whole brain and this would explain the fact that changes are seen using histological analysis and not using MRI. It is also possible that the difference of results between the two techniques are due to litter effects, sample number differences and to the fact that histology analysis reflects what happens locally on the two sections used while MRI analysis is a volumetric analysis done at the whole brain level.

As discussed in chapter 3, changes in the shape of several brain regions have been found in schizophrenic patients. However, the changes seen in the Der1 mice are not as extensive as structural modifications found in other mouse models of DISC1 (Tomoda *et al.*, 2016). Further, heterozygous and homozygous Der1 mice do not display the same structural alterations. This implies that there is a differential effect of DISC1 expression, with the Der1 mutation expressed on one or two alleles leading to different phenotypes. This could indicate that the Der1 mutation exerts different effects to those observed in other DISC1 mutant mice.

A change in the shape of brain regions could indicate a possible modification in cellular migration, distribution or possible modification of cellular morphology. In particular a change in the ventricular shape/ volume could indicate an impaired CFS circulation and impaired neurogenesis. However, when investigating neurogenesis and proliferation (chapter 4), only a trend toward reduced neurogenesis in the heterozygotes was discovered in the Der1 mice but the sample number was quite low. On the other hand, the RNAseq analysis indicates that proliferation could be affected as DCX expression was altered in the heterozygous mice. This indicates that neurogenesis might be affected in the heterozygous Der1 mice.

In the homozygous mice, the alteration of the CA1 region of the hippocampus could indicate a modification in neuronal communication in the hippocampus and the entorhinal cortex and therefore memory would be impaired (Ji and Maren, 2008; Wozny *et al.*, 2008). This could also indicate impairment of neurogenesis in the DG, migration and distribution of neurons in the hippocampus and may indicate a defect in the morphology of the cells. A trend toward a reduction in neurogenesis has been found (chapter 4) in the homozygotes which could confirm this and changes in neuronal outgrowth were discovered in the homozygous Der1 cortical

neurons (chapter 5) which could suggest that similarly, changes in neuronal outgrowth and therefore in neuronal morphology could be happening in the hippocampus. Moreover, this could also indicate a local increase of apoptotic activity. Indeed, as mentioned in chapter 4, a trend toward an increase of apoptosis was found in the CA1 of the heterozygous mice.

Overall, the Der1 mutation seems to affect the brain structure in very subtle ways. This may indicate that DISC1 involvement in the establishment of normal brain structure is rather subtle but very necessary. However, whether the Der1 mutation is expressed on one or two allele gives different results and seems to indicate that different mechanisms are affected depending on gene expression.

7.2 The Der1 mutation leads to cellular changes

Analysis of different cell population in Der1 heterozygous and homozygous mice was performed (Chapter 4). The heterozygous mice showed an increased activity of caspase-3 in the dentate gyrus and a trend in the same direction in the PFC and CA1. A trend towards a reduction of neurogenesis in the dentate gyrus was also observed. In the homozygous mice, an increase in the neuronal density of the left *srlm* of the hippocampus, as well as a trend towards an increase in caspase-3 activity and a trend toward a reduction of neurogenesis in the dentate gyrus were observed. Additionally, analysis of the morphology of cultured primary cortical neurons from homozygous Der1 mice revealed an increase in the dendritic growth near the soma as well as a somal hypertrophy (Chapter 5).

Overall, this indicates that homozygous Der1 mice might exhibit an impairment of neuronal migration from the dentate gyrus to the CA1. Additionally, both heterozygous and homozygous mice seem to express an increase of apoptotic activity. However, no decrease of cell density was found in those areas as well as no change in their shape and volume (Chapter 3). This suggest that rather than representing an increase of neuronal death, increase caspase-9 activation indicates an increase in apoptosis of neural extension or more likely that the subtle change in apoptosis activity is not enough to lead to a significant change in cell density. Additionally, RNAseq revealed that apoptosis mechanisms may be impaired in the heterozygous mice. This strongly suggests that apoptosis mechanisms are affected by the *Der1* mutation.

Moreover, in the homozygous Der1 mice, a thinned CA1 was also found (Chapter 3) which could be due to increase of cell death. Activation of apoptotic mechanisms could be due to impairment of mitochondrial function (Wang and Youle, 2016). It is possible that nitric oxide (NO) could make the mitochondria membrane more permeable which would lead to mitochondrial defect (Brown and Borutaite, 2001). NO is known to be release when the

immune system is activated which is known to happen in mental disorder (Nasyrova *et al.*, 2015). Activation of the apoptotic pathways could affect synaptic communication and neuronal communication. From the data presented in chapter 4, neurogenesis could be decreased in these mice, which could lead to an enhanced vulnerability to stress, decreased activity, and cognitive deficits.

Homozygous Der1 cortical neurons exhibit a defect in neuronal outgrowth in this mouse. This outgrowth defect could reflect an impairment of intracellular trafficking which in turn could affect mitochondrial function therefore activating apoptotic pathways. The different processes affected in the homozygous Der1 mouse would all lead to a defect in the organisation of the brain circuitry and the impairment of neuronal communication.

Therefore, it indicates that the *Der1* mutation leads to increased apoptosis, and possibly impaired neurogenesis. This confirms the role of DISC1 in these biological processes indicating that DISC1 could be a risk factor for mental illness and that this mouse model could be a model of mental illness. However, these analyses indicate that the homozygous and the heterozygous Der1 mice are again not presenting the same phenotype.

7.3 Gene expression is extensively altered by the Der1 mutation

RNAseq analysis revealed that numerous genes were affected by the Der1 mutation (chapter 6). Similar to previous analyses, the results between the homozygous and heterozygous mice were not exactly the same. In the homozygous mice there was no consistent change of gene expression, apart from for Disc1 itself. In the heterozygous Der1 mice, numerous genes were found differentially expressed compare to the WT. These changes indicate impaired neurodevelopment, neurogenesis, cellular growth, intracellular transport and cellular outgrowth in the heterozygous mice. Moreover, the results indicate impairment of the intrinsic apoptotic pathway. This is another indication that apoptotic mechanisms may be flawed in the Der1 mice. These changes support the hypothesis of defective mitochondrial activity that could lead to the activation of apoptotic mechanisms seen in this Der1 mouse. Other affected genes indicate modified neurotransmitter signalling therefore suggesting that the balance between inhibitory and excitatory signal is dysregulated. Abnormalities in the regulation of neurotransmitter release and an imbalance in excitatory and inhibitory activity have been associated with neuropsychiatric disorders (Gao and Penzes, 2015; Selten, van Bokhoven and Nadif Kasri, 2018).

7.4 Overall effect of the Der1 mutation

To conclude, both homozygous and heterozygous mice express different structural and cellular phenotypes which are rather subtle. The results indicate that neurogenesis is probably impaired in the Der1 mice model as well as neuronal outgrowth and possibly neuronal migration. Moreover, strong indications at the structural, cellular and molecular level indicate that apoptosis mechanisms are activated possibly via a mitochondrial defect. These changes also support impaired neurodevelopment and neuronal transmission in the Der1 model. Additionally, in the heterozygous mice RNASeq analysis strongly predicts that synaptic plasticity and neurotransmission is likely to be impaired. Overall, it is possible that the Der1 mutation leads to impairment of molecular transport which affects intracellular trafficking, leading to a mitochondrial defect and then to apoptosis. Impaired molecular transport could also affect neurotransmitter trafficking and release, leading to synapse dysregulation. However, there are important changes in the RNA expression of the heterozygous Der1 mice compared to WT while the changes in the homozygous mice were too heterogeneous to draw any conclusions. The heterozygous mutation mimics the t(1;11) translocation, the homozygous mutation does not. which could explain why more changes relevant to psychiatric disorder were found in the heterozygous Der1 mouse model. The difference between the heterozygous and homozygous mice could be explained by a haplo-insufficiency effect of the *Der1* mutation. Indeed, expressing only 50% of the full length DISC1 could lead to a more drastic phenotype than not expressing the protein at all. The presence of the full length DISC1 in a reduced quantity might lead to reduced interaction with other proteins and itself and therefore disrupt cellular function as there would not be enough protein to fulfil all of its function. Moreover, no DISC1 at all might allow the organisms to find a compensatory mechanism during the development reducing the impairment caused by the absence of DISC1. Another explanation could be that the Der1 mutation has a dominant negative effect. It is possible that chimeric proteins could be synthesized in both homozygous and heterozygous mice, however in the heterozygous mice the chimeric protein could form dimers with the full length *DISC1* which would lead to an adverse effect compared to the homozygous mice. The dominant negative effect could be tested by inducing the expression of the possible chimeric protein in cell culture first before moving to creating a whole new line, using a plasmid with a bacterial promoter. This would lead to overexpression of the chimeric transcript and indicate its effect. Nonetheless, chimeric proteins have not yet been found in the patient carrying the t(1;11) translocation or in the Der1 mice due to the lack of suitable antibodies but investigation is ongoing.

The consequences of the Der1 mutation seem to correlate with several phenotypes found in psychiatric illness. This suggests that the Der1 mutation could increase the susceptibly to psychiatric illness and therefore adds to the evidence that *DISC1* is a risk factor for mental illness. These results underline the importance of the investigation of rare variants in mental illness. Additionally, it shows the importance of mimicking known human mutations rather than knocking down or knocking out the gene involved, as results will not always be similar between the two. The fact that subtle and heterogenous phenotypes were discovered in the Der1 mouse model emphasizes the facts that psychiatric disorders are apparently specific to each patient affected. Indeed, schizophrenia patient subgroups could be explained by the heterogeneity of schizophrenia which could be due to the different gene-environment interactions involved in this disorder (Arango, Kirkpatrick and Buchanan, 2000; Takahashi, 2013).

7.5 Future directions

The experiments described in this thesis present preliminary characterization of the new Der1 mice. The results indicate that the Der1 mutation leads to subtle structural and cellular changes in the brains of these mice as well as very extensive transcriptional modifications. This confirms the role of DISC1 in neurodevelopment through multiple mechanisms and supports the idea that *DISC1* could be a susceptibility gene for psychiatric illness.

However, further characterization of this mice is required to uncover the exact impact of this Der1 mutation. Additionally, in order to study the role of DISC1 in psychiatric illness, a particularly relevant point would be to consider the environmental effects as well as other mutations believed to be risk factors. Thus, more work will be required to fully characterize the Der1 mice model and establish that this is a good model of mental illness. Potential future work which would help validate this new mouse model further is discussed below.

7.5.1   Further characterisation of the Der1 model

- The structural analysis of the Der1 mouse brains revealed subtle changes in the shape of some brain regions. In order to confirm this, additional histological analysis would be informative. The brains used for the MRI analysis could be processed in order to obtain brain sections all along the brain. Each section could be compared to an atlas of an average mouse brain built from control mice. By matching the shape of tissue sections, shape variation could then be assessed (Carson *et al.*, 2005).

Additionally, serial brain sections could be used to investigate inflammation and quantify glial cells as well as assess their activity. Microglial activation is a marker for inflammation and can be observed using immunohistochemistry technics. Microglial overactivation is believed to lead to exaggerated synaptic pruning and loss of grey matter as well as cognitive and negative symptoms, and structural brain modification associated with the psychiatric disorders (Müller *et al.*, 2015; Howes and McCutcheon, 2017). Autoimmune diseases and neuronal inflammation have been previously linked to neuropsychiatric disorders, however it is still being discussed weather it could be a cause or a result of mental disorders (Najjar *et al.*, 2013; Müller *et al.*, 2015). As well as being involved in the immune response, glial cells are also necessary for neuronal function and activity as well as involved in the underlying pathobiology of psychiatric disorders (Elsayed and Magistretti, 2015; Yamamuro *et al.*, 2015). This study would therefore reveal more information about inflammatory processes associated cell death. To go further, localization of cytochrome C could be studied using brain sections. Indeed, it has been shown that the release of the cytochrome C from the mitochondria to the cytosol lead to activation of apoptotic pathways (Cai, Yang and Jones, 1998). This would therefore help confirm whether intrinsic apoptotic pathways are activated in the Der1 mice and link DISC1 function to apoptosis.

- Outgrowth analysis of the homozygous der (1) cortical neurons revealed that this mutation leads to somal hypertrophy and excess dendritic formation. However, it would be necessary to investigate the neuronal outgrowth of heterozygous cortical neurons as well. This experience is currently being done in a collaborating lab that is part of the Marie Curie consortium. As previously mentioned, the structural and transcriptional changes observed in heterozygous Der1 mice more closely resemble the t(1,11) translocation found in the Scottish family, therefore this investigation could lead to additional information about the mechanisms affected by the translocation. Moreover, outgrowth analysis of iPSC-derived neurons from patients carrying the translocation could be performed as well and compared to the Der1 analysis. As previously discussed, neuronal outgrowth impairment has been found in psychiatric disorders and it is yet to be investigated in the patients carrying the t(1;11) translocation. This analysis could confirm the Der1 mouse model as a cellular model for the t(1;11) translocation found in humans.

- To confirm the results obtained from the RNAseq analysis, quantitative PCR could be carried out to quantify transcripts in each sample, as well as investigating the expression and activity of affected proteins in the Der1 mice. Experimental verification of the

RNAseq results by qRT-PCR was not performed by the time the present work was finished as qRT-PCR would also have to be done regarding the RNAseq analysis from the IPSCs from the patients carrying the t(1;11) translocation. This is currently being investigated. Performing qRT-PCR on the same set of RNA samples used for RNA-Seq would allow to control for technical errors while performing qRT-PCR on a new set of samples would add power to the study. Without this validation, the results obtained are only at a broad level and do not take into account possible errors happening during the RNAseq analysis. qRT-PCR would validate the results of the RNAseq and confirm the significance of the results.

- The results presented in chapter 6 underline several mechanisms which seem to be affected by the Der1 mutation. Investigation of the formation and the quantity of synapses and spine using live super resolution microscopy could lead to the discovery of additional impairments in the Der1 neurons. A first analysis investigating NMDA receptors and post synaptic density was already done (submitted, E. Malavasi et al). A follow up such as an in-depth study of the various receptors involved in synapses formation such as GABAergic, serotoninergic and dopaminergic receptors would be interesting to do. Indeed, their quantification and movement in the synapse could reveal clues about synaptic impairment in the Der1 mice. These experiments could also be done using iPSC-derived neurons. Indeed, it seem that synapses malfunction is a major cause of psychiatric disorders as well as many neurological disorders (Van Spronsen and Hoogenraad, 2010; Lepeta *et al.*, 2016). Therefore, investigation of this phenomenon could lead to discoveries which would have an impact on the understanding of multiple neuropathology. This could also offer the possibility to identify new targets for therapeutic intervention in these diseases. If the Der1 and the iPSC-derived neurons share similar phenotypes further work on the Der1 mice could then be performed to understand the development of psychiatric disorders. Investigation over time should be done to understand how and when neurodevelopment is affected. This would mean that multiple embryonic and post-embryonic time point should be studied to fully understand the extend of the disorder due to the Der1 mutation and the results could be translated to human pathology to a certain extent. The investigation of the synaptic formation would also provide information on the balance between excitatory and inhibitory signalling which is believed to be impaired in mental disorders and it would allow us to understand when this dysregulation occurs.

Overall, this could confirm that the Der1 mice can be used as a model of psychiatric disorders and help understanding the mechanisms of formation and the consequences of synapthopathies.

7.5.2   Improving the Der1 mouse model

- It is important to take into account that the Der1 mouse model only mimics the presence of the chimeric transcript existing on the chromosome 1. Therefore, the transcript created on the chromosome 11 when the t(1;11) translocation occurs is not present in the Der1 mice. Additionally, as *DISC2* and *DISC1FP* are not present in the mice genome so their disturbance is not reflected by the Der1 mutation. Because *DISC2* and *DISC1FP1* are non-coding RNAs, little investigation on their role has been done. However, their disruption could exert effects and therefore could be linked to the development of mental disorders occurring in the Scottish family carrying this translocation. Indeed, *DISC2* has been hypothesized to regulate DISC1 function through its RNA (K. Millar *et al.*, 2000; Porteous *et al.*, 2006) and therefore it is possible that *DISC2* could also be a risk factor for psychiatric disorders as well. Moreover, as discussed in the introduction, it is possible that *DISC1FP1* encodes a small protein which inhibits oxidoreductase activity, RNA expression, and protein synthesis. Further study indicated that its RNA expression was increased in post-mortem brain of patient suffering from psychiatric disorders (Thomson *et al.*, 2013; Ji *et al.*, 2015). This indicates that *DISC1FP1* could also be a susceptibility gene for psychiatric disorders. Disruption of these three genes could therefore contribute to the development of psychiatric illnesses. Therefore, creating a model which carries the exact same transcripts than those found in the patient carrying the t(1;11) translocation could be more accurate. Using gene editing strategies such as Cre/lox system (McLellan, 2017), those transcripts could be inserted into the mouse genome. This could be used to control the expression of these transcripts which would give new information on the importance of *DISC2* and *DISCFP1* in the development of psychiatric disorders. Additionally, the CRISPR-Cas9 technology (Patrick, Eric and Zhang, 2014) could be used on primary neuronal cells from control mice as well as proliferating cells derived from control human NPCs in order to reproduce the translocation by expression chimeric transcripts from both chromosome 1 and 11, and to reveal whether the dysregulation of these three genes would lead to the same phenotype than those found in NPCs from patient carrying the translocation or than those from a mice model carrying the same transcripts.

- It is well known that psychiatric illness is the result of gene-environment interactions. Therefore, it would be interesting to investigate environmental effects on the der (1) mice. Numerous epidemiological studies suggest that maternal immune activation can lead to development of mental disorders (Mednick *et al.*, 1988; Brown *et al.*, 2000; Brown, 2006; Canetta and Brown, 2012). Moreover, rodent models of maternal immune activation have been created and seem to successfully reproduce the pathology observed in humans (Boksa, 2010; Connor *et al.*, 2012). Polyinosinic–polycytidilic acid (Poly(I:C)) acts as a virus-like molecule and has been used on gestating mice to mimic viral action seen in humans (Reisinger *et al.*, 2015; Patrich *et al.*, 2016). Using this compound on the Der1 mice would therefore allow us to investigate the interaction between gene and environment in the very specific context of the t(1;11) translocation. Then, similar experiments to those mentioned in this thesis could be performed. This would provide new information about the extent of the effect of the environment on mental disorders. Possibly, a more critical phenotype could be uncovered indicating that the Der1 mutation could be activated by environmental change.

In conclusion, this thesis provided an initial characterisation of a novel Der1 mouse model, confirming of the role of DISC1 in various biological processes associated with the development of mental illness. The results indicate that this mouse model differs from previous animal models used to study mental disorders and expressed more subtle phenotypes. However, a few similar cellular phenotypes were found when compared to these previous animal model. Additionally, RNA expression was discovered to be significantly altered to an extend that was not observed previously on other animal models. Overall, this presents a new mouse model of mental illness and indicates that altered expression of *DISC1* might increase the risk of developing psychiatric disorders. The Der1 mice is a better model to study the human translocation t(1;11) but additional investigation of the molecular mechanisms affected by the Der1 mutation would be necessary to use this model for further understanding of mental disorders in its globality and possibly to help finding a better treatment for those suffering of such debilitating illness.

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