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SPECIFIC READING DISORDER: CELLULAR AND NEURODEVELOPMENTAL FUNCTIONS OF SUSCEPTIBILITY GENES

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ACADEMIC DISSERTATION

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ABSTRACT

Specific reading disorder (SRD), or developmental dyslexia, is defined as an unexpected difficulty in learning to read and write when intelligence and senses are normal. Hereditary factors are estimated to play a substantial role in the etiology of SRD, although the exact neurobiological mechanisms involved are rather poorly understood. In this thesis we have investigated the function of three SRD susceptibility candidate genes, *DYX1C1*, *DCDC2* and *ROBO1*, with the aim of finding neurodevelopmental and molecular pathways that might shed light on the etiology of SRD.

When research for this thesis began, knockdown of the rodent orthologs of *DYX1C1* and *DCDC2* had been shown to disturb radial neuronal migration in the developing cerebral cortex, but the function of human DYX1C1 and DCDC2 at the cellular level was still unclear. We discovered that both DYX1C1 and DCDC2 are involved in signalling pathways that are important in brain development; DYX1C1 is involved in estrogen signalling and DCDC2 is involved in ciliary signalling.

We found that the effect of DYX1C1 on estrogen signalling was concerted through its interaction with estrogen receptors (ERs) in in the presence of the endogenous ligand, 17β-estradiol. We observed that DYX1C1 regulates the degradation of ERs, resulting in decreased transcriptional responses to 17β-estradiol. Our findings suggest that the effects of DYX1C1 on brain development may be at least partially mediated by ERs and that hormonal factors may play a role in SRD. We also observed DYX1C1 and ERα complexes in the neurites of primary rat hippocampal neurons, which suggests a role for DYX1C1 in rapid non-genomic ER signalling.

The effect of DCDC2 on the ciliary signalling was such that the overexpression of DCDC2 was found to activate SHH signalling, whereas the downregulation of DCDC2 expression was found to enhance WNT signalling. We also observed that the DCDC2 protein localizes to the primary cilium in primary rat hippocampal neurons and is involved in regulating the length of the cilium through its role in stabilizing microtubules. DCDC2 was also found to interact with the ciliary kinesin-2 subunit KIF3A, a key molecule in function and maintenance of cilia. Consistent with a role in ciliary function, the overexpression of DCDC2 in *C. elegans* resulted in an abnormal neuronal phenotype that could only be observed in ciliated neurons. Our results were the first to suggest a role for DCDC2 in the structure and function of primary cilia. Later, others have reported more links between ciliary function and SRD candidate genes, most notably the putative role of DYX1C1 as a cytoplasmic assembly factor for ciliary dynein.

ROBO1 has been discovered as a SRD susceptibility gene in a large multi-generation family, in whom a rare haplotype in the broad genomic area of *ROBO1* is co-segregated with SRD. The expression of *ROBO1* has been shown

to be reduced from the SRD-associated haplotype, but the causal factor for the reduced expression was not known. In this thesis we have characterized genetic variation within the SRD-susceptibility haplotype by whole genome sequencing, aiming to identify variants that would increase our understanding of the altered expression of *ROBO1*. We found several novel variants in the SRD susceptibility haplotype and tested transcription factor binding to four of the variants by EMSA. We did not detect transcription factor binding to three of the variants. However, one of the variants was bound by the LIM homeobox 2 (LHX2) transcription factor with increased binding affinity to the non-reference allele. Knockdown of *LHX2* in lymphoblast cell lines extracted from subjects of the DYX5-linked family showed decreased expression of *ROBO1* supporting the idea that *LHX2* regulates *ROBO1*. Because the regulation of *ROBO1* is likely to be complex and the effect of the novel variants was at the most very subtle in our experiments, it remains unknown if any of them are causal factors for the SRD susceptibility.

The mouse ortholog of *ROBO1* has been shown to have many functions in brain development: it is involved in neuronal migration of interneurons and pyramidal cells and in axonal guidance of major nerve tracts. The role of ROBO1 in mouse brain led us to test two hypotheses on two human populations: 1) We tested whether *ROBO1* controls midline crossing of auditory pathways in the family with reduced expression of *ROBO1* and 2) we tested whether in the normal population *ROBO1* is involved in the development of the corpus callosum, the major axon tract connecting the cerebral hemispheres.

The axonal crossing of the auditory pathways was studied using a functional approach, based on magnetoencephalography and frequency tagging. We found impaired interaural interaction in the subjects that had reduced *ROBO1* expression supporting a defect in midline crossing of auditory pathways. Moreover, the deficit in interaural interaction depended on the *ROBO1* in a dose-dependent manner. Our results suggest that *ROBO1* controls midline crossing of the auditory pathways and were the first evidence of a SRD susceptibility gene being linked to a specific sensory function in the human brain.

The role of *ROBO1* in callosal development was assessed by studying whether polymorphisms in *ROBO1* correlate with variation in the white matter structure in the corpus callosum. By using data acquired by both structural magnetic resonance imaging and diffusion tensor imaging we found that five polymorphisms in the regulatory region of *ROBO1* were associated with white matter density in the posterior part of the corpus callosum. One of the polymorphisms, rs7631357, was also significantly associated with the probability of connections from the body of the corpus callosum to the parietal cortical regions. Our results suggest that the human *ROBO1* may be involved in the regulation of the structure and connectivity of the posterior part of the corpus callosum. Overall, our results support the

idea that similarly as in mice, the human *ROBO1* is likely to play many different roles in brain development.

In conclusion, the results of this study have advanced the field of SRD research by suggesting new functions for SRD candidate susceptibility genes in cellular and developmental pathways that are highly relevant in the context of brain development. More studies will be needed to clarify the role of genes in the etiology of SRD and in the neurobiology of reading, but our results have provided clues that may be worthwhile to be investigated.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Massinen S.***, Tammimies K.*, Tapia-Páez I., Matsson H., Hokkanen M.E., Söderberg O., Landegren U., Castrén E., Gustafsson J.A., Treuter E., Kere J. Functional interaction of DYX1C1 with estrogen receptors suggests involvement of hormonal pathways in dyslexia. *Hum Mol Genet*. 2009 Aug 1;18(15):2802-12.

II **Massinen S.***, Hokkanen M.E.*, Matsson H., Tammimies K., Tapia-Páez I., Dahlström-Heuser V., Kuja-Panula J., Burghoorn J., Jeppsson K.E., Swoboda P., Peyrard-Janvid M., Toftgård R., Castrén E., Kere J. Increased expression of the dyslexia candidate gene DCDC2 affects length and signaling of primary cilia in neurons. *PLoS One*. 2011;6(6):e20580.

III Lamminmäki S., **Massinen S.***, Nopola-Hemmi J.*, Kere J., Hari R. Human ROBO1 regulates interaural interaction in auditory pathways. *J Neurosci*. 2012 Jan 18;32(3):966-71.

IV **Massinen S.**, Wang J., Laivuori K., Bieder A., Tapia Paez I., Jiao H., Kere J. Genomic sequencing of a dyslexia susceptibility haplotype encompassing ROBO1. *J Neurodev Disord*. 2016 Jan 27;8:4.

V Darki F.*, **Massinen S.***, Salmela E., Matsson H., Peyrard-Janvid M., Klingberg T., Kere J. Human ROBO1 regulates white matter structure in corpus callosum. *Brain Struct Funct*. 2016 May 30.

*Authors contributed equally to the study

The publications are referred to in the text by their roman numerals. Publication III also appears in the thesis of Satu Lamminmäki (2012). The original publications are reproduced with the permission of the copyright holder.

ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
A	adenine
aa	amino acids
AC	asymmetry coefficient
ADHD	attention deficit hyperactivity disorder
BCE	before common era
bp	base pair
C	cytosine
C_T	threshold cycle
CC	corpus callosum
cDNA	complentary DNA
CGI	Complete Genomics Incorporation
Chr	chromosome
CNS	central nervous system
CNV	copy number variation
Co-IP	co-immunoprecipitation
CPN	callosal projection neuron
DNA	deoxyribonucleic acid
DNLL	dorsal nucleus of the lateral lemniscus
DRC	dual-route cascaded
DTI	diffusion tensor imaging
E2	17β-estradiol
EBV	Ebstein-Barr virus
EI	excitatory-inhibitory
EMSA	electrophoretic mobility shift assay
eQTL	expression quantitative trait locus
ER	estrogen receptor
ERE	estrogen responsive element
FA	fractional anisotropy
FFA	fusiform face area
G	guanine
GFP	green fluorescent protein
GWAS	genome-wide association study
H12	helix 12 of estrogen receptor
HLA	human leukocyte antigen
IC	inferior colliculus
IDA	inner dynein arm
IFT	intraflagellar transport
INDEL	insertin-deletion polymorphism
INM	interkinetic nuclear migration
IPC	intermediate precursor cell

IQ	intelligence quotient
IZ	intermediate zone
LGE	lateral ganglionic eminence
LGN	lateral geniculate nucleus
Mb	mega base pairs
MEG	magnetoencephalography
MGE	medial ganglionic eminence
MGN	medial geniculate nucleus
MRI	magnetic resonance imaging
mRNA	messenger RNA
NCS	neonatal sclerosing cholangitis
NEC	neuroepithelial cell
NGS	next generation sequencing
ODA	outer dynein arm
PCD	primary ciliary dyskinesia
PKD	polycystic kidney disease
PLA	proximity ligation assay
qRT-PCR	quantitative real-time polymerase chain reaction
QTL	quantitative trait locus
RAN	rapid automatized naming
RGC	radial glial cell
RNA	ribonucleic acid
RNAi	RNA interference
ROI	region of interest
SERM	selective estrogen receptor modulator
shRNA	small hairpin RNA
SLI	specific language impairment
SNP	single nucleotide polymorphism
SRD	specific reading disorder
SSD	speech sound disorder
SVZ	subventricular zone
T	thymine
TPR	tetratricopeptide repeat
V1	primary visual cortex
VWFA	visual word form area
WGS	whole genome sequencing

1 INTRODUCTION

Reading is the process of extracting information from written text. Although the earliest evidence of graphic communication comes from symbols found in cave paintings dated to more than 10 000 years ago, it was only later (at around 3000 BCE) that the Sumerians in Mesopotamia developed the first proper writing system. The emergence of writing was a turning point in human cultural and social evolution because it enabled information storage and transfer in a more reliable and accurate way than speech. In fact, the impact of writing has been so enormous that we divide the whole timescale of human existence into prehistorical era and historical era on the basis of its existence.

Since the beginning of writing, numerous writing systems have evolved that can be broadly classified into logographic, syllabic, and alphabetic systems. In addition to the various real-world writing systems even fictional writing systems have been created, most famous of which must be the one used for Tolkien's Elvish.

Even in the present-day society, reading is common in our daily lives. For example an average person in the US has been estimated to be exposed to 100 000 words per day (not including working life), of which roughly one third are in written form. The use of traditional print media has decreased substantially over the years, but nevertheless the amount of reading has increased, mostly because of the widespread use of digital media that contain an overwhelming amount of information in written form. (Bohn et al., 2009) With such high amounts of text consumed per individual, the role of efficient reading remains important.

Learning to read usually requires instruction as opposed to the development of speech, which is driven spontaneously in the developmental program of childhood. Even when adequate educational opportunities are provided, roughly 10% of children with otherwise normal cognitive abilities and senses experience persistent difficulty in learning to read. This type of learning impairment has been termed as specific reading disorder (SRD), previously most commonly referred to as developmental dyslexia. In this thesis I have adopted the newer term, SRD, because it so accurately emphasises the specificity of the deficit in reading.

Brain imaging studies have demonstrated SRD as a neurobiological entity and the partially hereditary nature of SRD has gained recognition from family and twin studies. Over the past two decades research has implicated more than twenty genes as SRD candidate susceptibility genes. Their neurodevelopmental functions have remained largely unknown, although some have been shown to be involved in axonal guidance and/or neuronal migration.

Because SRD is a hereditary disorder that affects brain development, the research demands for a multidisciplinary approach. In this thesis we have studied three SRD candidate susceptibility genes *DYX1C1*, *DCDC2* and *ROBO1* by using methods in genetics, molecular neurobiology and brain imaging. The aim of this thesis was to find molecular and neurodevelopmental pathways that may be involved in the etiology of SRD.

2 REVIEW OF THE LITERATURE

2.1 SPECIFIC READING DISORDER

2.1.1 DEFINITION OF SRD

SRD is a defined in the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) under the code F81.0. (World Health Organization, 2016):

"The main feature is a specific and significant impairment in the development of reading skills that is not solely accounted for by mental age, visual acuity problems, or inadequate schooling. Reading comprehension skill, reading word recognition, oral reading skill, and performance of tasks requiring reading may all be affected. Spelling difficulties are frequently associated with specific reading disorder and often remain into adolescence even after some progress in reading has been made. Specific developmental disorders of reading are commonly preceded by a history of disorders in speech or language development. Associated emotional and behavioural disturbances are common during the school age period."

SRD has also been referred to as developmental dyslexia, with the word dyslexia deriving from Greek and meaning "difficulty with words". Another type of reading disability, acquired dyslexia or alexia, can be caused by brain damage and is thus distinct from SRD.

2.1.2 SYMPTOMS OF SRD

The symptoms of SRD range from mild to severe and can vary depending on the age and reading level of the individual. Typically, SRD is characterized by early difficulties in learning to name letters and associate sounds with letters, subsequent difficulties in pronouncing pseudowords and real words without sentence context or meaning cues, and later problems with fluent reading and spelling. SRD usually manifests itself as a failure in learning to read in the first grades at school, but because poor readers and good readers tend to maintain their relative positions along the spectrum of reading ability (Shaywitz et al., 1999), reading problems often persist through the lifespan. Many adults with SRD can acquire very reasonable reading skills through developing alternative reading strategies to compensate for their problems, but often the disorder remains apparent through poor reading fluency (Lefly and Pennington, 1991).

The symptoms of SRD may also depend on the level of complexity of the orthography in the writing system. In transparent orthographies, in which the correspondence between letter units (graphemes) and sound units

(phonemes) is very straightforward, it is common that the major problem in SRD is impaired reading fluency. The more non-transparent the orthography is, the more reading accuracy tends to be impaired. (Landerl et al., 2013; Seymour et al., 2003)

Sensory correlates of SRD include subtle defects in visual and auditory processing. Cognitive deficits in SRD can be found in component reading and language related functions, such as phonological awareness (accessibility to the mental representations of the sound structure of words), verbal short-term memory and rapid automatized naming (RAN) (the ability to quickly name aloud familiar visual stimuli). (Ramus and Ahissar, 2012) The psychometric measures assessing the reading and language related functions have been shown to correlate with reading skills (Cirino et al., 2002) so they have been used as endophenotypes when studying SRD. Because individuals with SRD seem to fall into distinct subgroups in relation to the endophenotypes, several subtypes of SRD with distinct neurocognitive profiles have been suggested to exist. (Crews and D'Amato, 2009; King et al., 2007)

2.1.3 DIAGNOSIS OF SRD

SRD is most commonly diagnosed during the first school years. Reading skills, assessed by various psychometric measures, follow a normal distribution in the general population (Shaywitz et al., 1992) so the diagnosis of SRD is based on applying a threshold on the continuum of reading abilities in such a way that the lowest tail is classified as affected. The diagnosis of SRD usually takes into account personal history in reading problems and discrepancies between actual reading skills and expectations based on intelligence quotient (IQ) or chronological age. Typically a discrepancy of more than two standard deviations is considered as an indication of SRD. The rationale behind the IQ-discrepancy criterion is that it would be able to distinguish individuals with SRD from those who have poor reading skills due to other developmental problems. However, recent research suggests that SRD exists within the whole spectrum of intellectual abilities and thus does not support low IQ as a criterion for exclusion (Tanaka et al., 2011).

2.1.4 EPIDEMIOLOGY OF SRD

SRD is the most common learning disorder. Estimates of the prevalence of SRD are dependent on the diagnostic criteria used, but most commonly range from 5 % to 10% (Lyytinen et al., 2015; Rutter et al., 2004; Shaywitz et al., 1990). SRD has been found to exist in all writing systems studied, even including logographic writing systems (Navas et al., 2014). The gender ratio in affected individuals is such that SRD is roughly twice as common in males than females (Rutter et al., 2004).

2.1.5 COMORBIDITIES OF SRD

Several other disorders are comorbid with SRD, in other words they tend to co-occur with SRD. The most commonly reported comorbidities include attention deficit hyperactivity disorder (ADHD) (Mascheretti et al., 2016) and specific language impairment (SLI) (Paracchini, 2011), speech sound disorder (SSD) (Stein et al., 2004) and dyscalculia (Davis et al., 2014). The comorbidities have been suggested to share at least part of their risk factors and genetic background with SRD.

2.1.6 REMEDIES FOR SRD

When a child is diagnosed with SRD, special attention is needed to support the child in his or her studies in order to ameliorate the reading difficulty as much as is possible. A traditional remedy for poor reading skills has been phonics-based reading instruction. Recently a technology-based method, the GraphoGame has been shown to be effective in remedial reading intervention; children at risk for developing SRD benefit from training of grapheme-phoneme correspondence in the digital environment of the GraphoGame more than training by traditional methods. (Saine et al., 2011)

2.2 NORMAL READING

2.2.1 COGNITION OF READING

The cognitive tasks required for reading involve letter recognition, grapheme to phoneme conversion, semantics, syntax and reading comprehension. Cognitive functions can be studied by using computational models in developing and testing hypotheses. Current computational models of reading combine parallel and serial processing at several levels and include both feedforward and feedback mechanisms. The dual-route cascaded (DRC) model (Coltheart et al., 2001) and the triangle model (Plaut et al., 1996) are the most widely studied models on visual word recognition and reading aloud.

The DRC model assumes two different cognitive routes for reading words: the sublexical route and the lexical route. The lexical route can be further divided into lexical nonsemantic and lexical semantic routes. In the sublexical route of reading the word is assembled from the sublexical parts through grapheme to phoneme conversion. It is commonly used with new words or pseudowords (non-words that look like real words). The lexical route is based on the visual recognition of the whole word. If the word is familiar, the reader can retrieve the phonological information from memory. It is commonly used when reading irregular words (in which the grapheme to phoneme conversion strategy is difficult to use). The lexical semantic route is

not used in reading words in isolation, but comes to serve when reading multiple words together. It takes advantage of the semantic environment of the word in retrieving its meaning. The different reading routes can be used in parallel for example when reading a familiar word with regular orthography. (Coltheart et al., 2001)

The triangle model is based on a direct link between the representations of orthography and phonology, and also to their connections to and from semantic representations. The model highlights the importance of experience and feedback because all the connections between representations are learned. The triangle model differs from the DRC model in such respect that it allows all of the three representations to participate simultaneously. (Plaut et al., 1996)

2.2.2 PHYSIOLOGY OF READING

2.2.2.1 Visual pathways from the retina to the cortex

Reading begins with sensing of the written words by the photoreceptor cells of the visual system. The eye movement during reading includes repositioning the foveas (the area of the retina responsible for sharp vision) by fast and transient movements of both eyes simultaneously (saccades) flanked by short moments when the eyes halt and focus on one location (fixations). The visual information required for reading is acquired mainly during fixations. (Rayner, 1998)

The axons from retinal ganglion cells form the optic nerve and transfer the visual information from the retina through the optic chiasm, in which the axons coming from the nasal halves of the visual field cross the midline. After the optic chiasm, the axons are bundled in the optic tract and most of them make synaptic contacts in the lateral geniculate nuclei (LGN) in the thalamus. From the LGN the vast majority of visual information is transferred through the optic radiation to the primary visual cortex (V1) in the occipital lobe. Each hemisphere receives input predominantly from the contralateral visual field. (Figure 1) (Nassi and Callaway, 2009)

The processing of visual input begins already at the subcortical level, at which the retinal and thalamic cells are sensitive to local changes in stimulus contrast in their circular receptive fields. Different properties of the visual stimuli include the spatial and temporal frequencies as well as luminance and spectral contrasts. These properties are processed in parallel by distinct pathways in such a way that the parallel pathways remain segregated throughout the visual pathway from the retina to the cortex. The cells in the LGN can be divided coarsely into three categories that function in relaying different properties of the visual stimuli to the cortex: magnocells have high sensitivity to contrast and high temporal and low spatial frequencies and parvocells have low sensitivity to contrast but high sensitivity to high spatial

and low temporal frequencies. The function of the third type of cells, the koniocells, is still poorly known but they seem to have various response properties. In general, the magnocells receive achromatic signals whereas the parvocells receive red-green colour-opponent signal and the koniocells receive blue-yellow colour-opponent signal. The LGN also receives descending input from the primary visual cortex. (Nassi and Callaway, 2009)

In the V1, signals from the two eyes come together and the receptive fields of the neurons are elongated and responding strongly to rod-shaped stimuli and their orientation. In the visual cortex, the visual attributes such as motion shape and colour are inferred based on the incoming signals. (Nassi and Callaway, 2009)

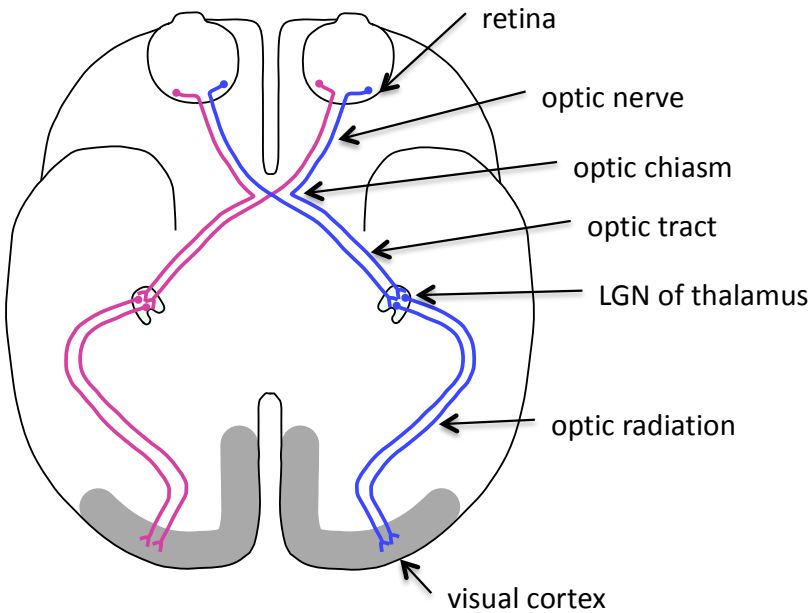

Figure 1 **A simplified presentation of the visual pathways**. The information in the ascending visual pathways (shown in magenta and blue) is transferred from the retina via the lateral geniculate nucleus (LGN) in the thalamus to the visual cortex. Each cortical hemisphere receives input mainly from the contralateral visual field. The figure is modified from the article by Solomon and Lennie. (Solomon and Lennie, 2007)

2.2.2.2 Cortical processing in reading

Most of what is currently known about cortical processing in reading is based on research on visual word recognition, i.e. the identification of single words when eyes are fixed. Natural reading is a serial process of visual sampling of words by saccadic eye movements and is more complex than visual word recognition as it involves the coordinated action of sensory and motor processes. The neural correlates of natural reading and their degree of

overlap with visual word recognition remain mostly unresolved. (Schuster et al., 2016)

The most prominent model of brain activation in visual word recognition consists of three circuits in the left hemisphere: the dorsal, ventral and anterior circuit. They are each hypothesized to play different roles in visual word recognition. (Pugh et al., 2000)

The anatomical areas involved in the dorsal (parieto-temporal) circuit are the angular and the supramarginal gyri, and parts of the temporal lobe, including the left superior temporal gyrus. The dorsal circuit is thought to function in grapheme-phoneme conversion, thus corresponding to the sublexical route of the DRC. The ventral (occipito-temporal) circuit includes the lateral exstrastriate, fusiform, and inferior temporal regions. The ventral circuit activates in the rapid and automatic identification of whole words, thus corresponding to the lexical route of the DRC. The anterior (inferior frontal) circuit includes the inferior frontal and precentral gyri. It is thought to function in motor coding of speech and semantics. (Jobard et al., 2003; Pugh et al., 2000) (Figure 2)

The reading networks are relatively consistent between different languages. (Paulesu et al., 2000) Interestingly, a recent study has suggested that the Exner's area, located in the left dorsal premotor cortex, is part of a separate reading subsystem for reading hand-written words that functions by using kinaesthetic gesture coding in identifying the letters. (Nakamura et al., 2012)

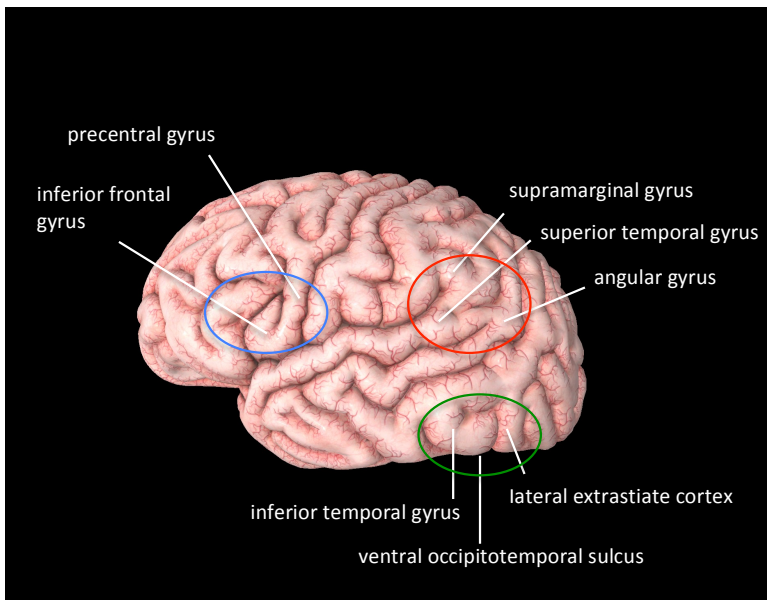

Figure 2 **The location of the reading circuits in the brain**. The approximate location of the dorsal (encircled in red), ventral (encircled in green) and anterior reading circuits (circled in blue). The image of the cerebral cortex is exported from Essential Anatomy 5 (3D4Medical).

2.2.2.3 The visual word form area

The visual word form area (VWFA) in the left ventral occipitotemporal sulcus belongs to the ventral reading circuit and is a key area in visual word recognition. The VWFA has been shown to activate in response to (single) written words independent of the language or orthography used. (Bolger et al., 2005) The VWFA does not function exclusively in word recognition, as it also activates during other tasks such as object and colour naming. (Starrfelt and Gerlach, 2007) Nevertheless, the activation has been shown to be typically higher for written words than for other visual stimuli for example chequerboards. Moreover, the activation of the VWFA is stronger to words or pseudowords than to consonant strings, indicating that the area gains specificity to the orthographical features of the language used. (Cohen et al., 2002) It is not fully known how the activation of the VWFA during word recognition differs from other types of VWFA activation. In fact, the VWFA has been suggested to be involved in the general integration of visual information with higher-level processing. (Vogel et al., 2014)

Neurons in the posterior part of the VWFA respond to individual letters and in the anterior part they respond to letter combinations (James et al., 2005), even whole known words (Glezer et al., 2009). Two different models have been proposed for the function of the VWFA. According to the local combination detectors hypothesis some neurons in VWFA are sensitive to the direction of lines or a combination of lines and initiate a mainly bottom-up driven process in reading (Dehaene et al., 2005). According to the interactive account hypothesis the neurons in VWFA are not specialized to letters but are sensitive to shapes and this model assumes top-down predictions from language areas. (Price and Devlin, 2011)

2.2.2.4 Subcortical structures in reading

Also subcortical structures outside of the visual pathways may contribute to reading. Banai et al. have suggested that reading skills are dependent on the integrity of the subcortical auditory mechanisms. (Banai et al., 2009) Corpus callosum (CC), the large axon tract connecting the cerebral hemispheres, may also be involved in reading by mediating interhemispheric interaction.(Vandermosten et al., 2012)

Reading may also depend on the function of the cerebellum, which is concerned with fine motor coordination and integration of sensory perception. (Marien et al., 2014) The superior colliculus in the midbrain is involved in controlling the eye-movements during reading. (Soetedjo et al., 2002)

2.2.2.5 The effect of literacy on the brain

Learning to read causes changes in the function and anatomy of the brain that are rather consistent among individuals despite the fact that the cultural invention of reading and writing is not old enough to have influenced the evolution of human brain. This has been explained by the neuronal recycling hypothesis proposing that new cultural inventions that are acquired through learning, such as reading, overrun evolutionarily older brain circuits and recycle them to be used for a new purpose. The anatomy and connections of the pre-existing circuits are never completely overwritten and thus they constrain the organisation of the recycled circuits, leading to limited interindividual variability. (Dehaene and Cohen, 2007)

Reading involves converting written text into language, so consequently, the main effect of the acquisition of literacy on the organisation of the brain is that the cortical areas involved in visual processing form reciprocal connections to language networks situated in the left-hemisphere temporal and inferior regions. Especially the activity and connections from the left VWFA are enhanced when literacy is acquired. The preferential activation of the WWFA to words is not seen in illiterates and its emergence positively correlates with reading ability. (Dehaene et al., 2010) The VWFA can develop rather fast: in 6-year old children only 8 weeks of training grapheme-phoneme connections on the GraphoGame was enough for word preference to begin. (Brem et al., 2010)

The VWFA is located next to the face recognition system of the left fusiform face area (FFA). During reading acquisition as the WWFA develops word preference, the response to faces is decreased in the left but increased in the right FFA, suggesting that there is competition between the recognition of symbols and faces in the left hemisphere. (Dehaene et al., 2010)

A cortical site overlapping with the VWFA is responsible for the mirror image consistency of faces and objects. A typical feature of alphabets is that not all the letters are symmetrical in shape and thus their mirror images should not be recognized as the same letter. Therefore in expert adult readers, the brain has developed mirror image inconsistency to words and letters. (Pegado et al., 2011)

Reading acquisition also enhances activation in cortical areas that are connected to the VWFA. There is an increased activation in early visual processing areas that feed visual information to the VWFA. (Szwed et al., 2011) The planum temporale is the putative brain area for storing phonemic representations in the brain and receives input from the VWFA. The activation of the planum temporale by speech is enhanced by literacy, most probably due to increased need for phonological analysis. (Dehaene et al., 2010)

The acquisition of literacy can also be associated with thickening of many of the cortical regions that have been associated with reading. This has been observed in bilateral angular, dorsal occipital, middle temporal, left

supramarginal and superior temporal gyri. (Carreiras et al., 2009) The acquisition of reading has also been suggested to alter both the intrahemispheric and interhemispheric cortical connectivity. Illiterate and literate adults have differences in the microstructure of the temporoparietal portion of the arcuate fasciculus that putatively connects the VWFA to other regions such as the planum temporale. The differences most probably reflect more connectivity in the literate adults and increased activation of the sublexical route of reading. (Thiebaut de Schotten et al., 2014) Moreover, when compared to illiterates, individuals who learnt to read during early adulthood had more white matter in the splenium or the isthmus of corpus callosum. The finding suggests that reading acquisition enhances interhemispheric connectivity of the angular and dorsal occipital gyri leading to more efficient transfer of phonological and/or visual information. (Carreiras et al., 2009)

2.3 NEUROBIOLOGY OF SRD

2.3.1 THEORIES OF THE ORIGIN OF SRD

Since the first reports of specific reading problems in the late 19th century various terms have been used to describe them, often reflecting the underlying assumptions about the neurobiological mechanisms involved. In 1878, Adolph Kussmaul, a German neurologist described his adult patients with reading difficulties as having “word blindness” with an unknown neurological cause. Later, reports of acquired forms of reading disability were published, enforcing a medical view on reading disabilities, according to which reading problems are caused by brain injury. In the early 1900s Dr. James Hinshelwood, a Scottish eye surgeon, suggested that specific reading difficulties were due to a deficit in visual memory for words and letters, which would explain the letter and word reversals that his patients made when writing. The letter reversals also prompted Dr. Orton, an American neurologist to introduce a new term, “strephosymbolia” (meaning twisted symbols) for his reading disabled patients. He hypothesized that reading disability was caused by a failure to establish the dominance of the left hemisphere in reading. Although a German ophthalmologist, Rudolf Berlin, had introduced the term dyslexia already in 1887, the term began to be more widely used from 1930s onwards when it became more widely accepted that the visual processing problems do not account for all of the problems in reading problems and that dyslexia is a learning disorder. (Lawrence, 2009)

The modern theories emphasise that SRD is a language-based disorder. The phonological theory of SRD emerged in 1970s (Bradley and Bryant, 1978) and states that SRD is caused by a deficit in the processing of phonemes. The phonological theory has gained wide acceptance, as a deficit

in processing phonemes has been the most robust and specific behavioural correlate of reading disability in many studies. (Melby-Lervag et al., 2012)

A major criticism of the phonological theory is that phonological problems alone are not sufficient to cause SRD. The double deficit theory postulates that a deficit in rapid automatized naming (RAN) of visually presented stimuli may cause SRD in combination with or in isolation from the phonological deficit. According to the theory, individuals with both of the deficits have a more severe form of SRD. (Wolf and Bowers, 1999) The speed of RAN predicts future reading fluency in pre-literate children and is thought to reflect the rapid integration of a wide range of cognitive skills that may also act as components of reading skill. (Melby-Lervag et al., 2012)

Also multiple deficit models for SRD have been proposed. These models take into account that there may be multiple cognitive factors contributing to reading ability that may interact in a probabilistic manner. The cognitive risk factors have also been proposed to explain some of the comorbidities of SRD. (Pennington, 2006)

Other suggested theories of the origin of SRD include the sluggish attentional shifting hypothesis (a multimodal deficit in processing transient stimuli) (Hari and Renvall, 2001), visual attention span hypothesis (decreased ability to process multiple distinct visual elements in parallel) (Bosse et al., 2007), magnocellular hypothesis (a deficit in the magnocellular processing of fast temporal visual stimuli) (Stein and Walsh, 1997), temporal sampling hypothesis (abnormal oscillations in the primary auditory cortex obscure speech perception) (Goswami, 2011). So far no theory has completely explained the full range of symptoms in SRD.

2.3.2 FUNCTIONAL AND ANATOMICAL STUDIES OF SRD

Post-mortem, electrophysiological, and brain imaging studies have started to accumulate information on the neuronal pathways that are dysfunctional in SRD, but much is still uncovered. The functional and anatomical studies of SRD in different languages have yielded some similar results thus supporting the notion that SRD is a neurobiological entity. (Paulesu et al., 2001) Furthermore newborns with a familial risk background for SRD who later develop SRD have been shown to differ from controls in auditory processing as measured by event related potentials to pure tones. (Leppanen et al., 2012) The presence of such early effects in SRD suggests that the brain circuits in SRD are most probably already altered before birth, emphasising the developmental nature of SRD.

Common findings in functional imaging studies of SRD include reduced activation of the parieto-temporal and occipito-temporal reading pathways in the left hemisphere. Compensatory activation (more activation compared to controls) has commonly been observed in the area of the left and right inferior frontal gyrus and in the right occipito-temporal area. The compensatory activation may be caused by recruitment of supporting brain

areas that results in accurate but not fluent reading. (Richlan et al., 2009; Shaywitz and Shaywitz, 2005)

The findings of disrupted reading networks in SRD suggest that there may be disturbances in the white matter pathways in the networks. Diffusion tensor imaging studies have indicated local white matter changes in the left arcuate fasciculus and corona radiata. Additionally, the white matter integrity in these tracts commonly correlates with reading skill. (Vandermosten et al., 2012) In callosal white matter pathways the integrity of the microstructure has been reported to be altered in individuals with SRD. (Frye et al., 2008)

Several studies have reported that individuals with SRD have reduced grey matter volume in bilateral temporoparietal and left occipitotemporal cortical regions when compared to age-matched controls. As an increase in the grey matter volume may be a consequence of practising a new skill, such as reading, it may be that the observed reduction in grey matter volume is due to less experience in reading. When the SRD group was compared to reading-level matched controls, only the right precentral gyrus had smaller grey matter volume in the SRD group, supporting the idea that the earlier observations were a consequence rather than a cause of the reading problems. (Krafnick et al., 2014) These findings also emphasise the need for more studies with reading-level matched controls.

Post-mortem studies have also suggested reduced asymmetry of the planum temporale in SRD, which is usually larger in the left hemisphere. (Adler et al., 2013) A recent brain imaging study by Altarelli et al. supports the hypothesis of reduced asymmetry of the planum temporale, but only in males. (Altarelli et al., 2014)

Diaz et al. have reported dysfunction of the auditory thalamus in SRD; more specifically they used functional magnetic resonance imaging (MRI) to measure responses to tasks that required processing of phonemes and observed that the activity of the medial geniculate nucleus (MGN) was abnormal. The results suggest that the MGN plays a role in the phonological processing in SRD. (Diaz et al., 2012) Post-mortem analyses of individuals with SRD have shown abnormalities in the cell distribution in the MGN. (Galaburda et al., 1994)

In addition to the structures with measurable differences in size and morphology, there may be differences at the level of microneuropathology in the brains of individuals with SRD. The reported abnormalities in post mortem histological analysis include ectopias (small neuronal congregations in an abnormal superficial layer location) and microgyri (an area that includes four cortical layers instead of the usual six layers). Both result from disturbances in neuronal migration. (Galaburda et al., 1985)

2.4 GENETICS OF SRD

2.4.1 HEREDITY OF SRD

The clustering of SRD in families was recognized already in the early 1900's. (Lawrence, 2009) In the 1950's, the first large-scale family study of SRD supported the familial nature of SRD. (Hallgren, 1950) Since then numerous studies have reported that family-risk is one of the strongest predictors of SRD. (Thompson et al., 2015)

Familial clustering of a trait is consistent with the involvement of genetic factors, but could also be explained by shared family environment. Wadsworth et al. conducted a study on adopted children and found no evidence of environmental transmission from parents to offspring. (Wadsworth et al., 2002) Environmental correlates of reading ability include parent's educational background, parent's library visits and the number of books at home (although it must be noted that they are most probably not independent of the parent's genotype). (Olofsson and Niedersoe, 1999) The relative contributions of genetic and environmental influences in SRD have been dissected by comparing concordance rates for monozygotic (70%) and dizygotic twins (41%), indicating a substantial genetic component. (DeFries and Alarcón, 1996; Willcutt et al., 2010) Indeed, the heritability (proportion of the phenotypic variance that is due to genetic variance) of SRD is estimated to be as high as over 60 %. (Hawke et al., 2006; Willcutt et al., 2010) Roughly equal heritability was estimated for reading ability, which was larger than the heritability estimate for intelligence. (Kovas et al., 2013)

In the general population, SRD usually follows a complex inheritance pattern, although in 20-30% of families with affected children the inheritance pattern follows autosomal dominant inheritance. (Pennington et al., 1991) Based on the occurrence pattern it has been estimated that there may be several common genetic variants with low penetrance as well as rare variants with high penetrance that confer susceptibility to SRD, reflecting underlying locus heterogeneity (genes at different chromosomal loci affecting the same phenotype) for SRD. As a consequence of the genetic heterogeneity, different families may harbour different combinations of risk factors.

It has also been suggested that different subgroups of SRD may have different genetic effects. For example the role of genes may be larger in those with high IQ. (Wadsworth et al., 2010)

2.4.2 SEARCHING FOR SRD SUSCEPTIBILITY GENES

The search for SRD candidate susceptibility genes has been ongoing for more than 30 years and has involved various strategies differing on their methods and sample sets. SRD is a difficult disorder for genetic studies because differences in diagnostic criteria between populations may lead to inconsistencies in the phenotype and in the genetic effects. An alternative

strategy for finding genes involved in SRD or general reading ability is to use in quantitative trait loci (QTL) analysis of continuous psychometric measures related to reading skill or its components.

Just as in the field of human genetics in general there has been a gradual shift in focus of the genetic studies of SRD from positional cloning by linkage analysis to genome-wide association studies (GWAS) and more recently to large-scale sequencing. Lately, also the role of copy-number variation (CNV) in SRD has been explored. (Veerappa et al., 2013a; Veerappa et al., 2013b; Veerappa et al., 2014) Nevertheless, much of what is currently know of the genetic susceptibility of SRD has roots in the early findings of genetic linkage and studies of individuals with chromosomal translocations.

The genetic studies on SRD have pinpointed to several candidate susceptibility loci, nine of which are listed by the HUGO Gene Nomenclature committee (*DYX1-DYX9*) (Gray et al., 2015). It has been suggested that the more biologically complex a phenotype is, the more locus heterogeneity (multiple genes affecting the trait) is involved. (McClellan and King, 2010) This seems to hold true in the case of SRD, as its etiology is by no means simple and an increasing number of genes have been suggested to be involved in the disorder or general reading ability (summarized in Table 1). The status of the majority of the suggested SRD candidate susceptibility genes is still unresolved, as no replication studies have yet been published. Some of them (such as *PCNT*, *DIP2A, S100B* and *PRMT2* (Poelmans et al., 2009)) are within the same genomic area and it is unclear whether only one, some of them or all of them or none of them are true susceptibility genes for SRD. Many suggested SRD genes are also involved in other language-related phenotypes, of which the most well known examples are *FOXP2* and *CNTNAP2* that are involved in the same pathway that regulates neurodevelopment of language and speech. (Vernes et al., 2008)

Although a sizeable group of genes have been suggested to be involved in SRD, the remaining section will review the genetic findings in SRD with an emphasis on the genes studied in the thesis. Also some findings on *KIAA0319* will be reviewed because of its close physical proximity and the possible genetic interactions with *DCDC2*. The focus is on the genetic findings that were published before the studies included in this thesis but also more recent findings will be discussed when not directly related to the results of this thesis

Table3 *Candidate genes for SRD or reading related traits, listed in the order of publication date from the oldest to the most recent*

Symbol	First report and replication or other support	Official gene name (in bold) and general description of a protein function(s) according to UniProtKB (Boutet et al., 2016)
DYX1C1	(Taipale et al., 2003). Further studies reviewed in the main text.	**Dyslexia susceptibility 1 candidate 1.** Involved in neuronal migration during development of the cerebral neocortex. May regulate the stability and proteasomal degradation of the estrogen receptors that play an important role in neuronal differentiation, survival and plasticity. Axonemal dynein assembly factor required for ciliary motility.
DRD4	Linkage to SDR (Hsiung et al., 2004)	**Dopamine receptor D4.** Dopamine receptor responsible for neuronal signalling in the mesolimbic system of the brain, an area of the brain that regulates emotion and complex behaviour. Its activity is mediated by G proteins, which inhibit adenylyl cyclase. Modulates the circadian rhythm of contrast sensitivity by regulating the rhythmic expression of NPAS2 in the retinal ganglion cells.
HRAS	Linkage to SDR (Hsiung et al., 2004)	**HRas proto-oncogene GTPase.** Ras proteins bind GDP/GTP and possess intrinsic GTPase activity.
KIAA0319	(Cope et al., 2005). Further studies reviewed in the main text.	**KIAA0319.** Involved in neuronal migration during development of the cerebral neocortex. May function in a cell autonomous and a non-cell autonomous manner and play a role in appropriate adhesion between migrating neurons and radial glial fibres. May also regulate growth and differentiation of dendrites.
ROBO1	(Hannula-Jouppi et al., 2005). Further studies reviewed in the main text.	**Roundabout guidance receptor 1.** Receptor for SLIT1 and SLIT2 that mediates cellular responses to molecular guidance cues in cellular migration, including axonal navigation at the ventral midline of the neural tube and projection of axons to different regions during neuronal development. Interaction with the intracellular domain of FLRT3 mediates axon attraction towards cells expressing NTN1. In axon growth cones, the silencing of the attractive effect of NTN1 by SLIT2 may require the formation of a ROBO1-DCC complex. Plays a role in the regulation of cell migration via its interaction with MYO9B; inhibits MYO9B-mediated stimulation of RHOA GTPase activity, and thereby leads to increased levels of active, GTP-bound RHOA. May be required for lung development.
DCDC2	(Meng et al., 2005b). Further studies reviewed in the main text.	**Doublecortin domain containing 2.** Protein that plays a role in the inhibition of canonical Wnt signalling pathway. May be involved in neuronal migration during development of the cerebral neocortex. Involved in the control of ciliogenesis and ciliary length.

GCFC2 (C2ORF3) and MRPL19	(Anthoni et al., 2007). Negative replication (Newbury et al., 2011; Paracchini et al., 2011; Scerri et al., 2011; Venkatesh et al., 2013). Positive replication to general cognitive ability (Scerri et al., 2012)	**GC-rich sequence DNA-binding factor 2 and mitochondrial ribosomal protein L19.** *GCFC2*: Factor that represses transcription. It binds to the GC-rich sequences (5'-GCGGGGC-3') present in the epidermal growth factor receptor, beta-actin, and calcium-dependent protease promoters. Involved in pre-mRNA splicing through regulating spliceosome C complex formation. May play a role during late-stage splicing events and turnover of excised introns.
KIAA0319L	Suggestive evidence (Couto et al., 2008)	**KIAA0319 like.** Possible role in axon guidance through interaction with RTN4R. Acts as a receptor for adeno-associated virus and is involved in adeno-associated virus infection through endocytosis system.
S100B	Chromosomal deletion cosegregating with SRD (Poelmans et al., 2009). Positive replication (Matsson et al., 2015)	**S100 calcium binding protein B.** Required for normal spindle assembly. Plays a key role in mother-centriole-dependent centriole duplication; the function seems also to involve CEP152, CDK5RAP2 and WDR62 through a stepwise assembled complex at the centrosome that recruits CDK2 required for centriole duplication. Reported to be required for centrosomal recruitment of CEP152; however, this function has been questioned. Also recruits CDK1 to centrosomes. Plays a role in DNA damage response. Following DNA damage, such as double-strand breaks (DSBs), is removed from centrosomes; this leads to the inactivation of spindle assembly and delay in mitotic progression.
DIP2A	Chromosomal deletion cosegregating with SRD (Poelmans et al., 2009). Positive replication (Kong et al., 2016)	**Disco interacting protein 2 homolog A.** May provide positional cues for axon pathfinding and patterning in the central nervous system.
PCNT	Chromosomal deletion cosegregating with SRD (Poelmans et al., 2009).	**Pericentrin.** Integral component of the filamentous matrix of the centrosome involved in the initial establishment of organized microtubule arrays in both mitosis and meiosis. Plays a role, together with DISC1, in the microtubule network formation. Is an integral component of the pericentriolar material (PCM). May play an important role in preventing premature centrosome splitting during interphase by inhibiting NEK2 kinase activity at the centrosome.

PRMT2	Chromosomal deletion cosegregating with SRD (Poelmans et al., 2009)	**Protein arginine methyltransferase.** Arginine methyltransferase that methylates the guanidino nitrogens of arginyl residues in proteins such as STAT3, FBL, histone H4. Acts as a coactivator (with NCOA2) of the androgen receptor (AR)-mediated transactivation. Acts as a coactivator (with estrogen) of estrogen receptor (ER)-mediated transactivation. Enhances PGR, PPARG, RARA-mediated transactivation. May inhibit NF-kappa-B transcription and promote apoptosis. Represses E2F1 transcriptional activity (in a RB1-dependent manner). May be involved in growth regulation.
DOCK4	Deletion associated with SRD (Pagnamenta et al., 2010)	**Dedicator of cytokinesis 4.** Involved in regulation of adherens junction between cells. Plays a role in cell migration. Functions as a guanine nucleotide exchange factor (GEF), which activates Rap1 small GTPase by exchanging bound GDP for free GTP.
NEDD4L	(Scerri et al., 2010). Positive replication (Mueller et al., 2014)	**Neural precursor cell expressed, developmentally down-regulated 4-like, E3 ubiquitin protein ligase.** E3 ubiquitin-protein ligase, which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly transfers the ubiquitin to targeted substrates. Inhibits TGF-beta signalling by triggering SMAD2 and TGFBR1 ubiquitination and proteasome-dependent degradation. Promotes ubiquitination and internalization of various plasma membrane channels such as ENaC, Nav1.2, Nav1.3, Nav1.5, Nav1.7, Nav1.8, Kv1.3, EAAT1 or CLC5. Promotes ubiquitination and degradation of SGK1 and TNK2. Ubiquitinates BRAT1 and this ubiquitination is enhanced in the presence of NDFIP1. Plays a role in dendrite formation by melanocytes.
MC5R	(Scerri et al., 2010)	**Melanocortin 5 receptor.** Receptor for MSH (alpha, beta and gamma) and ACTH. The activity of this receptor is mediated by G proteins, which activate adenylate cyclase. This receptor is a possible mediator of the immunomodulation properties of melanocortins.
DYM	(Scerri et al., 2010)	**Dymeclin.** Necessary for correct organization of Golgi apparatus. Involved in bone development.
DGKI	(Matsson et al., 2011)	**Diacylglycerol kinase iota.**
FOXP2	Association with reading related measures in SRD family trios (Peter et al., 2011)	**Forkhead box P2.** Transcriptional repressor that may play a role in the specification and differentiation of lung epithelium. May also play a role in developing neural, gastrointestinal and cardiovascular tissues. Can act with CTBP1 to synergistically repress transcription but CTPBP1 is not essential. Plays a role in synapse formation by regulating SRPX2 levels. Involved in neural mechanisms mediating the development of speech and language.

ZNF280D	Translocation associated with SRD (Buonincontri et al., 2011)	**Zinc finger protein 280A.** May function as a transcription factor.
TCF12	Translocation associated with SRD (Buonincontri et al., 2011)	**Transcription factor 12.** Transcriptional regulator. Involved in the initiation of neuronal differentiation. Activates transcription by binding to the E box (5'-CANNTG-3').
PDE7B	Translocation associated with SRD (Buonincontri et al., 2011)	**Phosphodiesterase 7B.** Hydrolyzes the second messenger cAMP, which is a key regulator of many important physiological processes. May be involved in the control of cAMP-mediated neural activity and cAMP metabolism in the brain.
CNTNAP2	Association with reading related measures in SLI families (Newbury et al., 2011). Positive replication (Peter et al., 2011). De novo CNV (Veerappa et al., 2013a)	**Contactin associated protein-like 2.** May play a role in the formation of functional distinct domains critical for saltatory conduction of nerve impulses in myelinated nerve fibres. Seems to demarcate the juxtaparanodal region of the axo-glial junction (By similarity)
CMIP	Association with reading related measures in SLI families (Newbury et al., 2011). Positive replication (Scerri et al., 2011)	**c-Maf inducing protein.** Plays a role in T-cell signalling pathway. Isoform 2 may play a role in T-helper 2 (Th2) signalling pathway and seems to represent the first proximal signalling protein that links T-cell receptor-mediated signal to the activation of c-Maf Th2 specific factor.
CYP19A1	(Anthoni et al., 2012)	**Cytochrome P450 family 19 subfamily A member1**. Catalyses the formation of aromatic C18 estrogens from C19 androgens.
PCDH11X	Copy number variation in individuals with SRD (Veerappa et al., 2013b)	**Protocadherin 11 X-linked.** Potential calcium-dependent cell-adhesion protein.
GABARAP	De novo CNV in SRD (Veerappa et al., 2013a)	**GABA type A receptor-associated protein.** Ubiquitin-like modifier that plays a role in intracellular transport of GABA(A) receptors and its interaction with the cytoskeleton. Involved in apoptosis. Involved in autophagy. Whereas LC3s are involved in elongation of the phagophore membrane, the GABARAP/GATE-16 subfamily is essential for a later stage in autophagosome maturation.
NEGR1	De novo CNV in SRD (Veerappa et al., 2013a)	**Neuronal growth regulator 1.** May be involved in cell-adhesion. May function as a trans-neural growth-promoting factor in regenerative axon sprouting in the mammalian brain.

ASIC2 (ACCN1)	De novo CNV in SRD (Veerappa et al., 2013a)	**Acid sensing ion channel subunit 2.** Cation channel with high affinity for sodium, which is gated by extracellular protons and inhibited by the diuretic amiloride. Also permeable for Li+ and K+. Activation by an extracellular pH drop is followed by a rapid pH-independent inactivation. Heteromeric channel assembly seems to modulate channel properties.
DCDC5	De novo CNV in SRD (Veerappa et al., 2013a)	**Doublecortin domain containing 5.**
CDK11B (CDC2L1)	(Luciano et al., 2013)	**Cyclin dependent kinase 11B**. Plays multiple roles in cell cycle progression, cytokinesis and apoptosis. Involved in pre-mRNA splicing in a kinase activity-dependent manner. Isoform 7 may act as a negative regulator of normal cell cycle progression.
CDK11A (CDC2L2)	(Luciano et al., 2013)	**Cyclin dependent kinase 11A.** Appears to play multiple roles in cell cycle progression, cytokinesis and apoptosis. The p110 isoforms have been suggested to be involved in pre-mRNA splicing, potentially by phosphorylating the splicing protein SFRS7. The p58 isoform may act as a negative regulator of normal cell cycle progression.
RCAN3	(Luciano et al., 2013)	**RCAN family member 3.** Inhibits calcineurin-dependent transcriptional responses by binding to the catalytic domain of calcineurin A. Could play a role during central nervous system development (By similarity).
ZNF385D	(Eicher et al., 2013)	**Zinc finger protein 385D.**
MYO5B	(Mueller et al., 2014)	**Myosin VB.** May be involved in vesicular trafficking via its association with the CART complex. The CART complex is necessary for efficient transferrin receptor recycling but not for EGFR degradation. Required in a complex with RAB11A and RAB11FIP2 for the transport of NPC1L1 to the plasma membrane. Together with RAB11A participates in CFTR trafficking to the plasma membrane and TF (transferrin) recycling in nonpolarized cells. Together with RAB11A and RAB8A participates in epithelial cell polarization. Together with RAB25 regulates transcytosis.
NSF	CNVs associated with SRD (Veerappa et al., 2014)	**N-ethylmaleimide sensitive factor , vesicle fusing ATPase.** Required for vesicle-mediated transport. Catalyses the fusion of transport vesicles within the Golgi cisternae. Is also required for transport from the endoplasmic reticulum to the Golgi stack. Seems to function as a fusion protein required for the delivery of cargo proteins to all compartments of the Golgi stack independent of vesicle origin. Interaction with AMPAR subunit GRIA2 leads to influence GRIA2 membrane cycling.
DRD2	(Chen et al., 2014)	**Dopamine receptor D2.** Dopamine receptor whose activity is mediated by G proteins, which inhibit adenylyl cyclase.

GNPTAB	(Chen et al., 2015)	**N-acetylglucosamine-1-phosphate transferase alpha and beta subunits.** Catalyses the formation of mannose 6-phosphate (M6P) markers on high mannose type oligosaccharides in the Golgi apparatus. M6P residues are required to bind to the M6P receptors (MPR), which mediate the vesicular transport of lysosomal enzymes to the endosomal/prelysosomal compartment.
NAGPA	(Chen et al., 2015)	**N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase.** Catalyses the second step in the formation of the mannose 6-phosphate targeting signal on lysosomal enzyme oligosaccharides by removing GlcNAc residues from GlcNAc-alpha-P-mannose moieties, which are formed in the first step. Also hydrolyses UDP-GlcNAc, a sugar donor for Golgi N-acetylglucosaminyltransferases.
CEP63	(Einarsdottir et al., 2015)	**Centrosomal protein 63.** Required for normal spindle assembly. Plays a key role in mother-centriole-dependent centriole duplication; the function seems also to involve CEP152, CDK5RAP2 and WDR62 through a stepwise assembled complex at the centrosome that recruits CDK2 required for centriole duplication. Reported to be required for centrosomal recruitment of CEP152; however, this function has been questioned. Also recruits CDK1 to centrosomes. Plays a role in DNA damage response. Following DNA damage, such as double-strand breaks (DSBs), is removed from centrosomes; this leads to the inactivation of spindle assembly and delay in mitotic progression.
GRIN2B	(Mascheretti et al., 2015)	**Glutamate ionotropic receptor NMDA type subunit 2B.** NMDA receptor subtype of glutamate-gated ion channels with high calcium permeability and voltage-dependent sensitivity to magnesium. Mediated by glycine. In concert with DAPK1 at extrasynaptic sites, acts as a central mediator for stroke damage. Its phosphorylation at Ser-1303 by DAPK1 enhances synaptic NMDA receptor channel activity inducing injurious Ca2+ influx through them, resulting in an irreversible neuronal death.
CTNND2	(Hofmeister et al., 2015)	**Catenin delta 2.** Has a critical role in neuronal development, particularly in the formation and/or maintenance of dendritic spines and synapses. Involved in the regulation of Wnt signalling. It probably acts on beta-catenin turnover, facilitating beta-catenin interaction with GSK3B, phosphorylation, ubiquitination and degradation. Functions as a transcriptional activator when bound to ZBTB33. May be involved in neuronal cell adhesion and tissue morphogenesis and integrity by regulating adhesion molecules.
NRSN1	(Skeide et al., 2016)	**Neurensin 1.** May play an important role in neural organelle transport, and in transduction of nerve signals or in nerve growth. May play a role in neurite extension. May play a role in memory consolidation.

2.4.3 THE *DYX1* LOCUS AND THE *DYX1C1* GENE

2.4.3.1 Mapping of the DYX1 locus

The first linkage study of SRD in 1983 revealed that heteromorphisms (detected as differences in staining properties in chromosome analysis) in chromosome (Chr) 15 showed linkage to SRD. (Smith et al., 1983) These findings led to the discovery of the first susceptibility locus for SRD (Smith et al., 1991). Later severalproperties in chromosome analysis) in chromosome (Chr) 15 showed linkage to SRD. (Smith et al., 1983) These findings led to the discovery of the first susceptibility locus for SRD (Smith et al., 1991). Later several independent linkage analyses have replicated the finding of genetic markers in Chr 15 being inherited together with SRD. (Grigorenko et al., 1997; Schulte-Korne et al., 1998) The most significant evidence for linkage has most commonly been near 15q21, which was named as the *DYX1* locus. Also the nearby locus 15q15 has been linked to SRD and it has been suggested that there may be more than one susceptibility gene in the *DYX1* locus. (Schumacher et al., 2008)

2.4.3.2 Discovery of DYX1C1

A key finding that led to the discovery of the susceptibility gene in *DYX1* was that in a Finnish family a translocation involving the *DYX1* locus was found to cosegregate with SRD. The translocation t(2;15)(q11;q21) was found in four family members: in the father and two children who have SRD and one child with overall cognitive performance below the normal range (and who thus did not fulfil the diagnostic criteria for SRD). (Nopola-Hemmi et al., 2000) In more detailed mapping of the translocation, the breakpoint could be refined to a region that included the exons 8 and 9 of a previously unknown gene. The gene disrupted by the translocation was named dyslexia susceptibility 1 candidate 1 (*DYX1C1*) and became the first candidate susceptibility gene for SRD. (Taipale et al., 2003)

The transcribed region of *DYX1C1* was also sequenced in 20 unrelated affected individuals, and the polymorphisms that were found were genotyped in a first sample set (35 additional cases and 113 controls) and a replication sample set (54 cases and 84 controls). Two single nucleotide polymorphisms (SNPs) were associated with SRD. The SNP 3G > A (rs3743205) in the promoter area was hypothesized to affect transcription factor binding (as was later confirmed by Tapia-Paez et al.). (Taipale et al., 2003; Tapia-Paez et al., 2008) The SNP 1249G > T (rs57809907) is a nonsense mutation that causes a premature stop codon that truncates the protein leaving out the four N-terminal amino acids. The odds ratios (a measure of association between the exposure and an outcome) were 3.2 (95% confidence interval 1.5 - 6.9) for -

3G>A and 2.3 (95% confidence interval 1.2 - 4.2) for 1249G>T, indicating that *DYX1C1* confers a modest risk for SRD. (Taipale et al., 2003)

2.4.3.3 DYX1C1 replication studies

The studies that have tried to replicate the association of *DYX1C1* with SRD have yielded inconclusive results. Some studies have reported positive associations, although sometimes a risk allele in one population may have been a protective allele in another population, suggesting differences in the haplotype structure between populations. (Brkanac et al., 2007; Dahdouh et al., 2009; Lim et al., 2011; Venkatesh et al., 2014; Wigg et al., 2004) Many studies have failed to detect association with SRD of any polymorphisms in or near *DYX1C1* (Marino et al., 2005; Meng et al., 2005a; Newbury et al., 2011; Scerri et al., 2004) but this may reflect the fact that most of the studies have had very limited statistical power (not enough samples or variants studied) to detect variants with such modest genetic effects as was suggested even by the original findings on *DYX1C1* (Taipale et al., 2003). In fact, there appears to be a tendency for positive replications more often in larger data sets (Kere, 2014). Two recent meta-analyses have assessed the role of the *DYX1C1* SNPs. They did not support association of -3G/A (Tran et al., 2013; Zou et al., 2012) or 1249G/T (Tran et al., 2013) with SRD.

Recently a group of single markers or haplotypes that had shown positive association with SRD were studied in the European cross-linguistic NeuroDys cohort that consists of altogether more than 900 individuals from eight European countries. Two SNPs in *DYX1C1* in the Dutch subsample reached nominal significance for association with SRD, but the results did not remain significant after correction for multiple testing. In fact, no marker or haplotype that was studied was significantly associated with SRD in a meta-analysis of the whole NeuroDys dataset. In the same study, also the associations with quantitative measurements of word reading or spelling were analysed. One marker within *DYX1C1* associated with spelling (and withstood correction for multiple testing) in the subsample from Switzerland sample set, but not in the whole NeuroDys cohort. (Becker et al., 2014)

Dahdouh et al have suggested a gender effect in the association of *DYX1C1* with SRD; they found evidence for a stronger role of *DYX1C1* in females (Dahdouh et al., 2009). In conclusion, the genetic studies support at most a weak effect for *DYX1C1* in susceptibility to SRD.

2.4.3.4 Expression and regulation of DYX1C1

DYX1C1 is expressed in various human tissues, with the most abundant expression in brain, lung, kidney and testis. The protein was found to localize in the nucleus when transiently transfected to the monkey kidney cell line COS-1. Human brain sections showed immunoreactivity for DYX1C1 in the

nuclei of a minority of cortical neuronal cells or white matter glial cells. The localization pattern was also studied in brains that had areas affected by ischemia, and in them DYX1C1 could also be found in the cytoplasm and neuronal processes and the fraction of positive cells appeared to be increased in ischemia. These results suggest that the distribution of DYX1C1 is regulated according to the functional state of the cell. (Taipale et al., 2003)

The molecular mechanisms in the regulation of *DYX1C1* have been studied in more detail by assessing transcription factor binding to the -3G/A SNP that is located in a putative promoter area of *DYX1C1*. The area of the SNP was found to be bound by a transcription factor complex formed by TFII-I, PARP1, and SFPQ. The transcription factors have a role in memory and brain development so their involvement in regulating *DYX1C1* is highly relevant when considering SRD. (Tapia-Paez et al., 2008)

2.4.3.5 Protein structure of DYX1C1

At the time when *DYX1C1* was identified as a susceptibility candidate gene for SRD it was a previously unidentified gene and its function was unknown. The transcribed region of *DYX1C1* consists of 10 exons, encoding for a 420 amino acids (aa) long protein. The DYX1C1 protein contains three tetratricopeptide repeat (TPR) domains in the C-terminus (Taipale et al., 2003) and a p23 domain in the N-terminus (Figure 3) (Wang et al., 2006).

The TPR domains are 34 aa long loosely conserved protein sequences usually arranged in tandem repeats of 3 to 16 domains. Each TPR domain consists of two anti-parallel alpha-helical subdomains. Together the TPR domains in a protein usually form amphipathic grooves that can bind target peptides. This feature of the TPR domains enables them to act as interaction scaffolds in protein complexes, an important event in many cellular processes. Indeed, TPR domain proteins have been reported to play a role in various cellular functions, for example in cell cycle regulation, protein degradation and protein translocation. (Allan and Ratajczak, 2011)

The p23 domains consist of seven beta-strands that are arrayed in a compact antiparallel beta-sandwich fold and are thought to be involved in protein-protein interactions or stabilizing protein folds. (Garcia-Ranea et al., 2002) The p23 domain of DYX1C1 can alternatively also be classified as a related protein module, CS domain (named after CHORD-containing proteins and SGT1). CS domains have been hypothesized to be co-chaperone interaction domains for heat shock protein 90 (Hsp90). (Lee et al., 2004) In fact, DYX1C1 is known to interact with Hsp90 through its p23 domain. Other interaction partners that bind to the p23 domain of DYX1C1 include heat shock protein 70 (Hsp70) and CHIP, an E-3 ubiquitin ligase. (Hatakeyama et al., 2004) Later, two other regions in the middle part of DYX1C1 were found to be highly conserved during evolution and were named as “DYX1C1 charged region” and the “DYX1 domain” (Figure 3). (Tammimies et al., 2013)

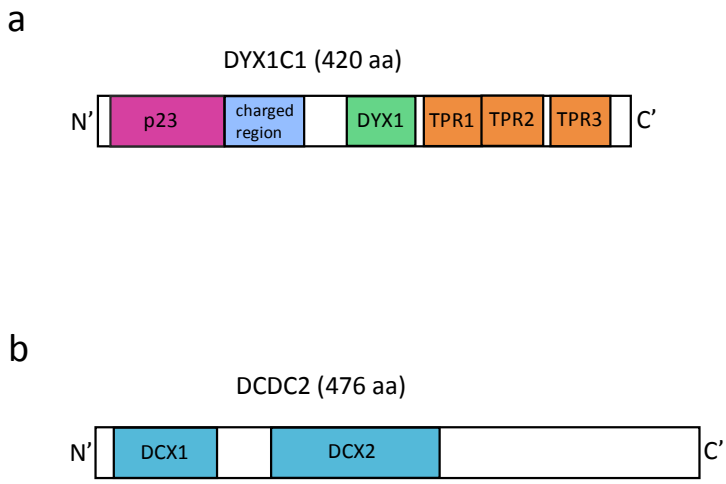

Figure 3 **A schematic illustration of the full-length protein structure of DYX1C1 and DCDC2.** a) DYX1C1 contains a p23 domain and three tetratricopeptide repeat (TPR) domains. (Taipale et al., 2003; Wang et al., 2006) The charged region and the DYX1 domain were discovered more recently (Tammimies et al., 2013) and were not studied in this thesis. b) DCDC2 contains two doublecortin (DCX) domains. (Reiner et al., 2006).

2.4.4 THE *DYX2* LOCUS AND THE GENES DCDC2 AND KIAA01319

2.4.4.1 Mapping of the DYX2 locus

The earliest observation of a SRD susceptibility locus on Chr 6 came from the study by Smith et al. in 1991. Using linkage analysis on less than 200 sib pairs from the US, they detected evidence for linkage of SRD to 6p21, near the HLA region. (Smith et al., 1991) The findings were further investigated on the same study sample using more informative markers and an additional independent sample of 50 dizygotic twin pairs. Taken together, the linkage analysis provided support for a QTL for SRD on 6p21.3. (Cardon et al., 1994)

Grigorenko et al. performed the first replication study for the 6p21 using linkage analysis on six extended families from the US. The most significant linkage was for the reading related phenotype phonological awareness. (Grigorenko et al., 1997) Later many studies have replicated linkage of SRD to the Chr 6 susceptibility locus that was named *DYX2*, and some have also found association. (Deffenbacher et al., 2004; Fisher et al., 2002; Fisher et al., 1999; Gayan et al., 1999; Kaplan et al., 2002; Marlow et al., 2003). Some studies have failed to find linkage to the region of *DYX2* (Field and Kaplan, 1998; Schulte-Korne et al., 1998). Nevertheless, it is the most replicated

susceptibility locus for SRD. Recently the *DYX2* has also been reported to be involved in speech sound disorder, which is one of the comorbidities of SRD. (Eicher et al., 2015)

2.4.4.2 Finemapping of DYX2 and candidate susceptibility genes

Deffenbacher et al. performed finemapping of the *DYX2* locus using linkage analysis on an extended sample from US, Colorado (1559 individuals from 349 families). They were able to refine the QTL for SRD into a 3.24 Mb interval that included 12 genes. Further association analysis showed association of a cluster of five closely spaced genes that all are expressed in the CNS: Neurensin 1 (*NRSN1*), Doublecortin domain containing 2 (*DCDC2*),, *KIAA0319*, Tyrosyl-DNA phosphodiesterase 2 (*TDP2*) and acyl-CoA thioesterase 13 (*ACOT13*). (Deffenbacher et al., 2004)

Subsequent studies have pointed to *DCDC2* and *KIAA0319,* situated within 200 kb from each other, as being the most likely susceptibility genes in *DYX2*. Initial findings of association with SRD were contradictory; two independent studies reported evidence for association of *DCDC2* but not of *KIAA0319* (Meng et al., 2005b; Schumacher et al., 2006), whereas a third and a fourth independent study both reported that *KIAA0319* showed much stronger association with SRD than *DCDC2* (Cope et al., 2005; Francks et al., 2004). In the NeuroDys association study, one SNP and one haplotype in *DCDC2* reached nominal association with SRD in the Hungarian subsample and four markers within *KIAA0319* showed nominal association with spelling as a quantitative trait, but none of the associations remained significant after correction for multiple testing. (Becker et al., 2014) Nonetheless, recent meta-analyses have gained support for *DCDC2* (Zhong et al., 2013) or *KIAA0319* (Shao et al., 2016; Zou et al., 2012) or both of them (Muller et al., 2016) as SRD susceptibility candidate genes. The results suggest that both *DCDC2* and *KIAA0319* may play a role in the susceptibility to SRD. Recently also a nonsynonymous coding SNP in *DCDC2* showed association with spelling performance in German families. (Matsson et al., 2015)

Certain susceptibility haplotypes of *KIAA0319* and *DCDC2* have been found to have genetic interactions with each other that result in non-additive genetic effects to the susceptibility to SRD. (Powers et al., 2016) The molecular mechanism of the interaction involves the regulatory element associated with dyslexia 1 (READ1), a compound short tandem repeat in intron 2 of *DCDC2* that can be bound by the transcriptional repressor ETV6. (Powers et al., 2013) The Different alleles of the READ1 may regulate the expression of KIAA0319 through a direct physical interaction with the genomic area of the KIAA0319 susceptibility haplotype. (Eicher et al., 2015) Alleles of READ1 can also regulate the DCDC2 promoter activity in luciferase experiments. (Meng et al., 2011) These findings support the idea that

KIAA0319 and *DCDC2* are involved in a common pathway that may be involved in SRD.

2.4.4.3 Structure and cellular function of DCDC2

The human DCDC2 protein is 476 aa long and has two doublecortin (DCX) domains that have a ubiquitin-like tertiary structure (Figure 3) (Kim et al., 2003) and are thought to function in microtubule dynamics. (Coquelle et al., 2006) The doublecortin domain usually either exists as a single copy or as tandem repeats, as is the case for DCDC2. (Reiner et al., 2006) The prototype of the family of the doublecortin superfamily is the x-chromosomal gene Doublecortin (*DCX*), the deletions of which cause disorders of severely disturbed neuronal migration, more specifically lissencephaly in males and subcortical laminar heterotopia in females. (des Portes et al., 1998) Also the retinitis pigmentosa 1 (autosomal dominant) (*RP1*) gene that has been found mutated in retinitis pigmentosa (a hereditary form of blindness) contains two doublecortin domains. (Sullivan et al., 1999)

Prior to this study, the mouse DCDC2 has been show to bind to and stabilize microtubules, and to induce their polymerization. Consistent with the role in microtubule dynamics, the overexpressed mouse DCDC2 has been observed to localize to the microtubule bundles of the cytoskeleton in a subpopulation of COS-7 cells. When DCDC2 was overexpressed, the cellular distribution of microtubules was such that concentric circles around the nucleus were observed to a varying degree. Nevertheless, the majority cells were reported to exhibited nuclear and nucleolar localization. In addition to the interaction with microtubules, the DCDC2 protein interacted with the JIP-1 and JIP-2 proteins that are involved in the c-Jun N-terminal kinase (JNK) signalling pathway. (Coquelle et al., 2006)

2.4.4.4 The structure and function of KIAA0319

The KIAA0319 protein contains a motif at the N-terminus with seven cysteines (MANSC), five polycystic kidney disease (PKD) domains and a single transmembrane domain. Alternative splicing is known to produce multiple protein isoforms of KIAA0319 (Velayos-Baeza et al., 2007), the longest of which is a transmembrane protein on the plasma membrane whereas at least one of the shorter isoforms can be secreted. (Velayos-Baeza et al., 2008) The secreted isoform as well as some extracellular fragments proteolytically cleaved and shed from the extracellular domain of KIAA0319 (Velayos-Baeza et al., 2010) have been suggested to act as signalling molecules. The membrane-bound KIAA0319 can also be internalized through the clathrin-mediated endocytic pathway, suggesting that the amount of KIAA0319 on the cell surface is regulated by endocytosis. (Levecque et al., 2009) The intracellular portion of KIAA0319 may also be released by

proteolytic cleavage and it may translocate to the nucleus where it has been suggested to affect gene expression. (Velayos-Baeza et al., 2010)

2.4.5 THE *DYX5* LOCUS AND THE *ROBO1* GENE

2.4.5.1 Mapping of the DYX5 locus

DYX5 on 3p12-q13 was identified as a SRD susceptibility locus in a genome-wide linkage study of a Finnish family (hereafter referred to as the DYX5-linked family) that constitutes the largest pedigree so far reported to show simple autosomal dominant segregation for SRD. In the linkage analysis, 21 affected family members from three generations were genotyped, of which 19 shared copies of Chr 3, identical by descent. Genetic recombination in one individual limited the shared haplotype into a region of 33 Mb within the pericentric region of Chr 3. (Nopola-Hemmi et al., 2001)

In neuropsychological tests, the affected members from the older generations were most commonly classified as severe SRD and the younger as mild or compensated SRD. This most probably reflects a change in the school system in Finland (better overall quality of education and identification and special education of children with learning difficulties). In general, the SRD phenotype in the family was such that the affected persons showed poor performance in tests of phonological awareness, verbal short-term memory, and RAN (Nopola-Hemmi et al., 2002).

More support for the *DYX5* locus was gained in a genome-wide linkage study in which QTL were mapped for SRD. Markers in 3q13 were among the most significant multipoint linkage results in a sample consisting of 119 families from the Colorado twin study of reading disability. (Fisher et al., 2002) Furthermore, in a study of 77 families from USA ascertained through a preschool child with SSD, markers within the *DYX5* showed linkage to SSD-related quantitative traits in an analysis targeted on the pericentromeric region of Chr 3. The results suggested that the *DYX5* locus has pleiotropic effects affecting both SRD and SSD. The model of inheritance for the *DYX5* susceptibility haplotype was estimated to be additive (suggesting recessive inheritance) for SSD in contrast to the dominant effects in SRD. (Stein et al., 2004)

2.4.5.2 Discovery of ROBO1

At the time of the original linkage study of the DYX5-linked family, Chr 3 was still sequenced with modest resolution. The 5-hydroxytryptamine receptor 1F (HTR1F) was known to reside in the linkage region and therefore its coding sequence was Sanger sequenced but no variation was found in two dyslexic

subjects from the DYX5-linked family. The effect of the gene on SRD could not be excluded but the authors concluded that it was likely that another gene was responsible for the susceptibility to SRD. (Nopola-Hemmi et al., 2001)

A clue that led to the identification of the susceptibility gene in the *DYX5* locus was discovered when an individual with SRD (unrelated to the DYX5-linked family) was found to carry a chromosomal translocation t(3;8)(p12;q11) with a breakpoint within the *DYX5*. Finemapping of the translocation revealed that the breakpoint disrupted the roundabout guidance receptor (*ROBO1*) gene. More specifically, the translocation breakpoint was situated between the first and second coding exons of the longest transcript variant *ROBO1a* (NM_002941) and upstream of the other major transcript variant *ROBO1*b (NM_133631). (Hannula-Jouppi et al., 2005)

This finding prompted the question whether the *ROBO1* gene was also the susceptibility gene in the DYX5-linked family. The transcribed region of the *ROBO1* gene and the exon-intron boundaries and 1 kb upstream of the first transcribed exon (NM_002941) were Sanger sequenced in a subset of the DYX5-linked family but no variants were found exclusively in individuals with SRD. Instead, consistent with the earlier linkage findings, a specific haplotype was found to segregate with SRD. This haplotype was not found in other unrelated families, suggesting that the haplotype is rare. In order to define the effect of the SRD susceptibility haplotype on *ROBO1* expression, lymphoblasts from the DYX5-linked family were studied. By comparing allelic peak heights from the Sanger sequenced *ROBO1* cDNA, the expression of *ROBO1* from the susceptibility haplotype was found to be on average 66% of the expression in controls. This finding provided indirect evidence for *ROBO1* as the SRD susceptibility gene in *DYX5* locus, especially since no such allelic suppression was observed for neighbouring genes. (Hannula-Jouppi et al., 2005)

Recently, a SNP (rs331142) located near a putative enhancer of *ROBO1* was significantly associated with SRD in family-based association analysis in two Canadian sample sets. Moreover, the over-transmitted allele correlated with low *ROBO1* expression in frontal cortex (Tran et al., 2014), coinciding with the reduced expression of *ROBO1* observed in the DYX5-linked family. In another recent study in an unselected Australian sample of twins and their siblings, 21 out of the 144 SNPs tested in *ROBO1* were found to associate with test scores in a non-word repetition task, reflecting phonological buffer capacity. Only one SNP showed nominally significant association with reading and spelling ability. The results suggest that *ROBO1* may be an important gene for language acquisition through its role in phonological buffering. (Bates et al., 2011) In contrast, in a sample of 493 nuclear families from Italy, Mascheretti et al. found no evidence for association between *ROBO1* SNPs and SRD, but instead they found association for mathematics abilities. (Mascheretti et al., 2014)

ROBO1 has been found to be deleted with a few neighbouring genes in a 15-Mb deletion. The phenotype of the deletion carrier included developmental delay and craniofacial dysmorphism (Petek et al., 2003). This finding supports an important developmental role for the genomic area of *ROBO1*.

2.5 CNS DEVELOPMENT

2.5.1 GENERAL DEVELOPMENT OF THE CEREBRAL CORTEX

2.5.1.1 Neurulation and neurogenesis

The CNS starts to develop at neurulation from cells originating from the ectodermal embryonic layer, taking place already at 3-4 weeks of gestation. At neurulation the neural plate is folded into the neural tube, which will eventually develop into the brain and the spinal cord. In the developing cerebral cortex neuroepithelial cells (NECs) attach to the ventricular surface and the pial lamina with long processes spanning the entire thickness of the cortex. They undergo symmetrical cell divisions to generate enough progenitor cells to account for all the neurons of the neocortex. (Figure 4a) (Jiang and Nardelli, 2016)

In the beginning of neurogenesis, the NECs develop into radial glial cells (RGCs). The RGCs are capable of symmetric cell divisions for the purpose of maintaining the cell pool, but most commonly the RGCs drive neurogenesis forwards through asymmetric cell divisions. In the direct route of neurogenesis, one division produces a new RGC and a neuron, whereas in the indirect route one division produces a new RGC and an intermediate precursor cell (IPC), which usually divides symmetrically into two neurons. Both NECs and RGCs engage in interkinetic nuclear migration (INM) in which the nucleus moves periodically in apical and basal directions along the length of the cell in a series of events that are coordinated with cell divisions. The purpose of the interkinetic movements is not well understood, but nevertheless it is important because the failure of INM disturbs neurogenesis. The inhibitory and excitatory neurons of the cortex are derived from distinct lineages of RGC cells. (Jiang and Nardelli, 2016)

2.5.1.2 Radial neuronal migration

The glutamatergic pyramidal neurons of the cortex are born in the subventricular zone (SVZ) of the pallium and then migrate radially through the intermediate zone (IZ) to the cortical plate. The RGCs provide a scaffold along which the neurons migrate and assemble once they have reached their

destinations. In the lower IZ, the newly born migrating neurons adopt a multipolar morphology for a short period while they explore the environment for extrinsic cues that guide their migration. The next phase of neuronal migration involves a transition to bipolar morphology with a leading process oriented towards the pial surface and a trailing process oriented towards the ventricle. The six-layered laminar organisation of the neocortex is formed by sequential waves of migrating neurons. The cortical layers form from the inside out: the neurons on the most internal layers arrive first and the subsequent waves of migratory neurons travel past them. (Jiang and Nardelli, 2016) (Figure 4)

2.5.1.3 Tangential neuronal migration

Tangential neuronal migration is performed primarily by the GABAergic interneurons of the cerebral cortex. (Marin, 2013) In tangential migration the migrating neurons migrate parallel to the pial surface over long distances. Most GABAergic interneurons are derived from progenitors in the lateral and medial ganglionic eminences (LGE and MGE respectively) of the subpallium. In the first phase of tangential migration, the cells migrate to the pallium. The cells are guided by attracting and repulsive cues and on the way to the pallium they avoid the striatum and the preoptic area. The leading process of the migrating neuron branches in response to external cues, which appears to serve as the mechanism that guides the migration. Once the migrating neurons have crossed the subpallium-pallium boundary, the cells become responsive to repelling cues from the subpallium so they do not return. The second phase of tangential neuronal migration is the intracortical dispersion, which involves the spreading of interneurons into the cerebral cortex along migratory streams through the marginal zone, subventricular zone or the subplate. In the last phase of tangential migration, when the migrating interneurons approach their final destination on the cortex, they switch from tangential to radial migration and subsequently adopt their correct laminar position. (Marin, 2013) (Figure 4b)

2.5.1.4 Post-migratory development of neurons

After the neurons have found their correct position in the cortex they further differentiate into various neuronal subtypes. In order to form neural circuits, the neurons extend axons and dendrites to form synaptic contacts with other neurons. The interneurons usually connect locally within the neocortex while the pyramidal projection neurons may extend their axons to more distal targets. Axonal pathfinding is guided by repulsive or attracting cues that can act over long distances (secreted cues) or short distance (cues on the surface of other cells). The receptors for the guidance cues are usually expressed on the surface of the growth cone of the axon. Receptor activation

can trigger intracellular signalling cascades that induce reorganization of the cytoskeleton and thereby direct movement. (Jiang and Nardelli, 2016)

When the axon reaches the target cell, the growth cone is transformed into the presynaptic terminal and thus synaptogenesis is initiated. The generation of synapses continues actively even after birth. In early childhood, until to roughly 2 years of age, excitatory synapses are overproduced in the cortex by activity-dependent synaptogenesis. The inhibitory synapses are slower to develop. During development, the neural circuits are fine-tuned by eliminated some synapses while enforcing other synapses. The development of the cerebral cortex is not complete until roughly 20-25 years of age, even after which the brain continues to show considerable plasticity in the synapses and neuronal connections. The myelination of axons also contributes to the maturation of the neural circuits. (Jiang and Nardelli, 2016)

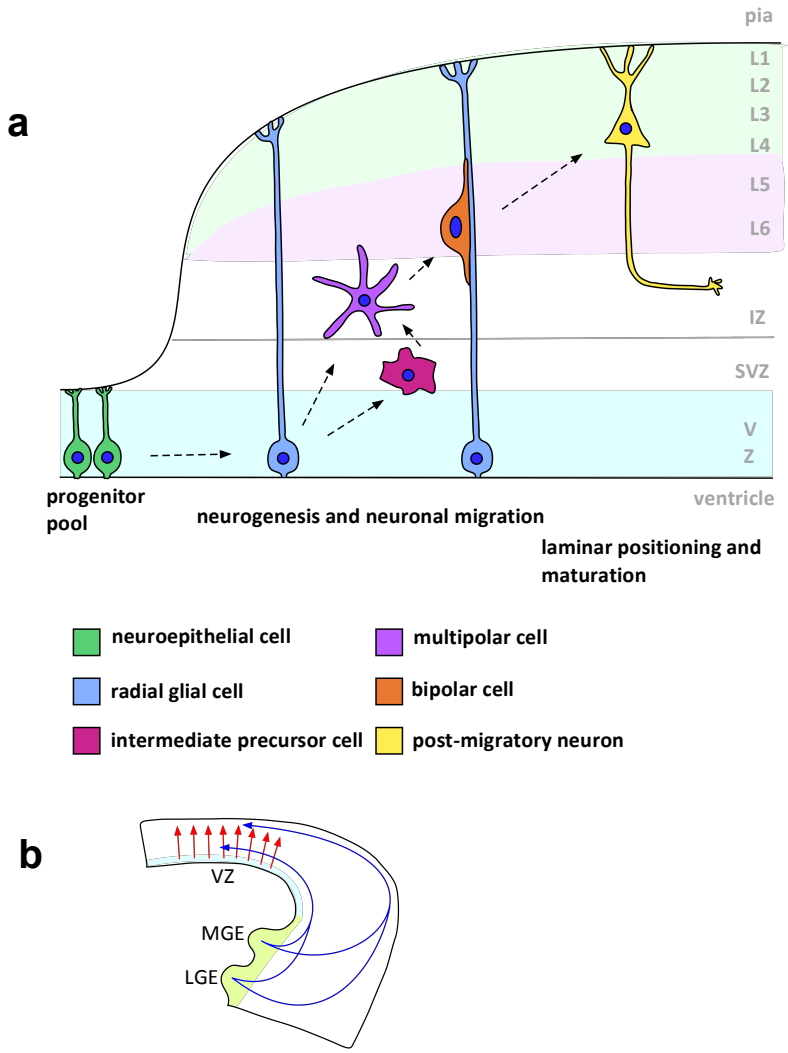

Figure 4 **Summary of the development of the cerebral cortex.** a) The neuroepithelial cells (NECs) proliferate in the VZ to create the neuronal progenitor pool. The NECs can differentiate into radial glial cells (RGCs) and further into neurons either directly or through intermediate precursor cells (IPCs). The newly born neurons migrate from the subventricular zone (SVZ) to the intermediate zone (IZ) and adopt a multipolar morphology. Next, bipolar neurons migrate radially on the RGC scaffold until they arrive at their correct position in the cortical plate (layers L1 – L6) and undergo neuronal maturation. Figure modified from the article by Guo et al. (Guo et al., 2015) b) The glutamatergic neurons of the cerebral cortex originate in the ventricular zone (VZ) in the pallium and undergo radial migration (red arrows). The GABAergic neurons of the cerebral cortex originate in the medial and lateral ganglionic eminences (MGE and LGE respectively) in the subpallium and undergo tangential migration (blue arrows). Figure modified from a review by Luhmann et al. (Luhmann et al., 2015)

2.5.2 CORPUS CALLOSUM STRUCTURE AND DEVELOPMENT

The corpus callosum (CC), consisting of over 200 million axons, is the largest axon tract in the brain and connects the two hemispheres of the cerebral cortex thus being able to function in higher-level cognitive tasks that require efficient interaction between the hemispheres. The CC is formed by the axons from callosal projection neurons (CPN) that have their cell bodies in layers II/III, V and VI of the neocortex. The CPN extend an axon through the CC to connect to homotopic areas in the contralateral hemisphere. In addition to the homotopic callosal projections a subpopulation of in CPNs in the deep cortical layers can send a second projection to other contralateral or ipsilateral brain regions. A second subpopulation of CPNs in the superficial cortical layers may participate in local circuitry within the contralateral and ipsilateral hemispheres. (Fame et al., 2011)

The CC is classically divided into five anatomical regions: genu, rostrum, body, isthmus and splenium. (Figure 3) The regions differ in their axonal architecture; the posterior part of the splenium, the isthmus and the body are composed of fast-conducting large diameter fibres that transfer mainly sensory information, whereas the rostrum, genu and anterior splenium are composed of thinner and slow-conducting fibres that transfer mainly information between association cortical areas. (Fabri et al., 2014)

Two different models have been proposed for the function of the CC in the interaction between the two hemispheres. According to the (I) excitatory model, the main role of the CC is to facilitate interhemispheric transfer of information, which increases the balance of activation between the two hemispheres. According to the (II) inhibitory model, the CC functions by mediating the inhibition of the contralateral hemisphere by the dominant hemisphere, and thus increasing the imbalance of activation between the two hemispheres. Most probably the CC has both excitatory and inhibitory effects. (Bloom and Hynd, 2005)

During the development when the callosal axons cross the midline they are guided by specific populations of glial and neuronal cells including the indusium griseum, the glial wedge, the subcallosal sling and the midline zipper glia. (Fame et al., 2011) The development of the CC takes place in two parts: most of the fibres of the CC develop during the embryonic and early postnatal period in humans, except for the splenium which mostly develops later, starting from the perinatal period and continuing to adolescence. The majority of CC axons are myelinated, onwards from roughly 6 months of postnatal age. The myelination proceeds from the posterior fibres that convey sensory information to the anterior fibres that connect associative areas. (Fabri et al., 2014)

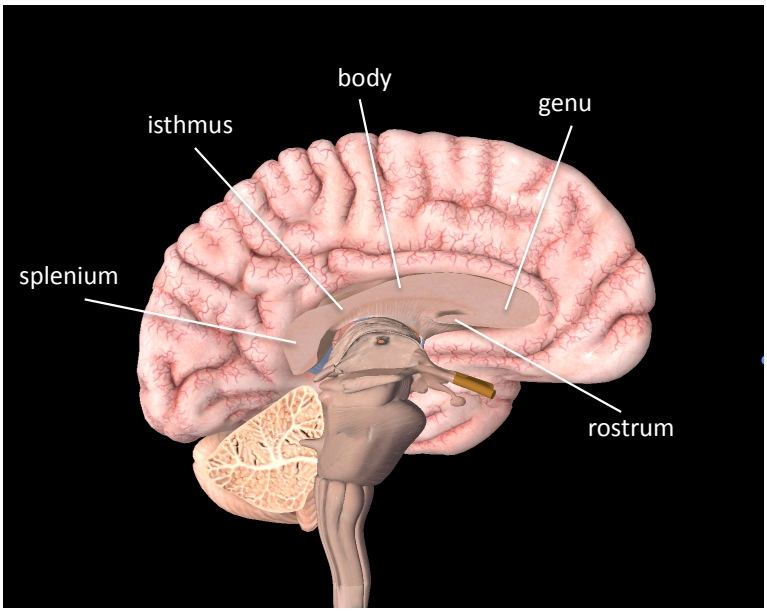

Figure 5 **The corpus callosum in a midsagittal view of the brain**. The CC is situated below the cerebral cortex and above the thalamus. The five classical anatomical regions of the CC are presented in the figure. The image of the brain is exported from Essential Anatomy 5 (3D4Medical).

2.5.3 AUDITORY PATHWAYS AND THEIR DEVELOPMENT

The auditory pathways include specific structures in the inner ear, brainstem and thalamus as well as the cerebral cortex. The ascending auditory pathway transfers the auditory information from the cochlear cells of the inner ear to the primary auditory cortex via several intermediate relay points. The ascending auditory pathway involves multiple overlapping and parallel pathways, and in general their anatomy is better known than their function in auditory processing.

The hair cells in the cochlea respond to auditory stimuli and activate the auditory nerve fibres. When the auditory nerve enters the brainstem it is divided into anterior and posterior branches. The anterior branch gives rise to the ventral auditory pathway that is responsible for sound-localization and the posterior branch gives rise to the dorsal auditory pathway that is involved in the analysis of complex stimuli. Axons from both of the pathways make synaptic contacts to separate regions on the cochlear nucleus. The axons arriving at the cochlear nucleus are arranged in a tonotopic organization according to their origin at the cochlea, and the organization is maintained in the ascending pathway all the way up to the cortex. (Pickles, 2015)

From the cochlear nucleus the ventral pathway ascends to specific nuclei in the ipsilateral and contralateral superior olivary complex; thus the

superior olivary complex is the first area of the ascending auditory pathway that receives input from both ears. The axons from the ventral pathway make synaptic contacts in the nuclei of the lateral lemniscus and from there the pathways connect to the inferior colliculus. The dorsal pathway ascends from the cochlear nucleus mainly directly to the contralateral inferior colliculus. From the inferior colliculus, the ascending auditory pathways lead to the medial geniculate body of the thalamus that relays the auditory information to the primary auditory cortex. (Figure 4) There are several ascending and descending connections between the brainstem nuclei with several connections also crossing the midline. Moreover descending pathways from the cortex participate to auditory processing at the subcortical level. (Pickles, 2015) Subcortical processing contributes to various aspects of auditory perception including the sensing of frequency and location (Moerel et al., 2015) as well as the processing of speech sounds (Krizman, 2010).

Steady-state responses at the auditory cortex to sounds from one ear are weaker during binaural than monaural listening, indicating binaural suppressive interaction that requires convergence of the inputs from both ears somewhere along the auditory pathways. The suppression is typically larger for ipsilateral than contralateral sounds. (Fujiki et al., 2002) The binaural suppressive interaction is part of normal binaural listening, which plays a role in sound localization and speech perception (Akeroyd, 2006).

During human development, the basic structure of the auditory pathways develops during the embryonic period, but the different levels of the auditory pathways mature at different times. The auditory pathways in the brainstem mature during the perinatal period, the thalamocortical projections mature in early childhood, and finally cortical auditory processing matures in later childhood (age 6-12 years). (Moore and Linthicum, 2007)

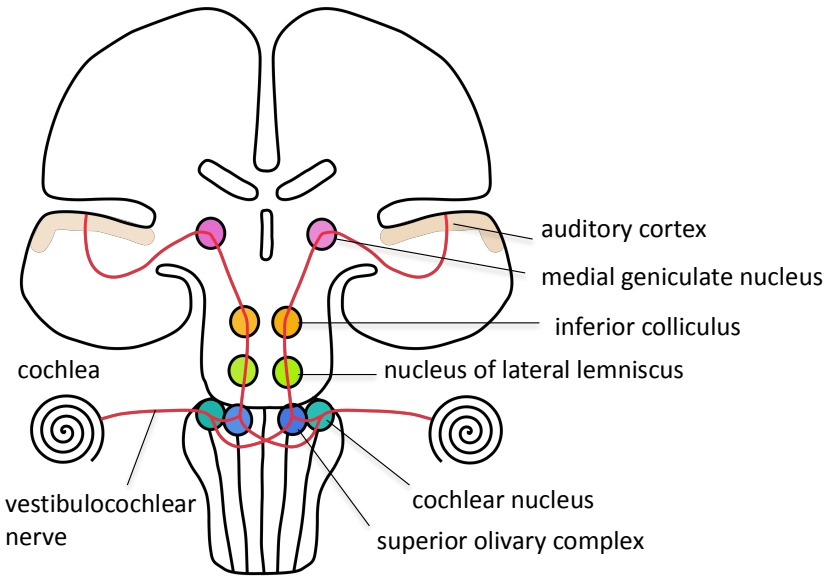

Figure 6 **A highly simplified presentation of the auditory pathways**. The information in the ascending auditory pathways (shown in red) is transferred from the cochlea to the processing stations in the brainstem, thalamus and the cortex. In this figure only the lowest possible level of midline crossing is presented but in reality the auditory pathways can cross the midline at multiple levels. The figure is modified from the article by Patel et al. (Patel and Iversen, 2007)

2.6 ANIMAL MODELS OF SRD CANDIDATE SUSCEPTIBILITY GENES

2.6.1 THE NEURONAL MIGRATION HYPOTHESIS

Based on the observed anomalies in neuronal migration in the microanatomical post mortem studies of SRD (Galaburda et al., 1985), a neuronal migration hypothesis has been postulated. According to the hypothesis impaired neuronal migration during development is a risk factor for SRD by leading to changes in the grey matter and white matter of reading circuits. To test this hypothesis, the role of SRD candidate susceptibility genes in neuronal migration has been studied in knockout or knockdown or overexpression animal models. *In utero* RNA interference (RNAi) is a method for embryonic knockdown of the target gene in migrating neurons. More specifically, short hairpin RNA (shRNA) plasmid vectors are microinjected into the lateral cerebral ventricles of rat or mouse embryos and they move inside the neurons when electroporated with a pulse. After the animals have developed for the desired period, they are investigated. (Bai et al., 2003)

Recently the shRNA method has been shown be prone to off-target effects in migrating neurons, so the studies should be interpreted with caution. (Baek et al., 2014) Although it is currently not known how much off-target effects contribute to the neuronal migration phenotypes of SRD candidate genes, overall the studies of SRD candidate genes in animal models support the neuronal migration hypothesis, but suggest that other mechanisms, such as altered axonal guidance or synaptic connections may also be involved. In humans the neuronal migration hypothesis is supported by the presence of specific reading difficulties in periventricular nodular heterotopia, which is a neuronal migration disorder. (Chang et al., 2005; Felker et al., 2011)

2.6.2 *Dyx1c1* IN MURINE MODELS

The role of DYX1C1 in neuronal migration has been studied using *in utero* RNAi in murine models. Normally radial migration takes place from the ventricles to the embryonic neocortex, but when *Dyx1c1* is knocked down at gestational day 14, the cells do not migrate normally but instead they accumulate at the multipolar stage of migration. In rescue experiments, the deficit in neuronal migration could be overcome by simultaneous overexpression of human DYX1C1 or a truncated version of DYX1C1 containing the C-terminal TPR domains. (Wang et al., 2006) Another population of the migrating *Dyx1c1* shRNA treated cells has also been reported to migrate past their expected location in the cortex. Moreover, the overexpression of DYX1C1 alone in migrating rat neurons has not been associated with a defect in neuronal migration. (Currier et al., 2011)

Another study concluded that *in utero* RNAi of *Dyx1c1* in rats resulted in neuronal migration defects that were similar to the ones observed in dyslexic humans. The migration defects were found both in the neocortex and in the hippocampus. (Rosen et al., 2007) The knockdown of *Dyx1c1* in migrating neurons has also been associated with a more indirect effect in the rat cortex; some untransfected GABAergic neurons accumulated with the heterotopic collection of transfected neurons, suggesting non-cell autonomous effects for DYX1C1. (Currier et al., 2011) The knockdown of *Dyx1c1* did not alter the volume of the cortex, or the hippocampus or the midsagittal area of the CC, but the anatomy of the MGN in the thalamus was changed in such a way that the neurons were smaller than normally. (Szalkowski et al., 2013a)

The behavioural phenotype of the *Dyx1c1*-knockdown rats has been studied in their postnatal life. The rats performed normally in a basic auditory discrimination task but showed abnormal auditory processing for more complex auditory stimuli. Heterotopias (small clusters of neurons that have migrated to the wrong destination) that were found in the hippocampus were associated with impairments in spatial learning. (Threlkeld et al., 2007) Also subtle impairments in the working memory (Szalkowski et al., 2011) and visual attention (Szalkowski et al., 2013a) of the *Dyx1c1* RNAi-treated rats have been reported.

Taken together the anatomical and behavioural consequences of the knockdown of *Dyx1c1* in rats resemble the impairments found in humans with SRD, and thus they strengthen the role of *DYX1C1* as a plausible candidate susceptibility gene for SRD.

2.6.3 *Dcdc2* IN MURINE MODELS

Similarly as for *Dyx1c1*, the effects of knocking down the *Dcdc2* gene in migrating neurons have been studied using *in utero* RNAi in murine models. The knockdown of *Dcdc2* in rat embryos resulted in migrating cells with typical bipolar morphologies, but that did not reach their target areas in the cortex. (Meng et al., 2005b) In another study, the *Dcdc2* knockdown rats were analysed postnatally. Anatomical findings included periventricular heterotopias and migration abnormalities in the hippocampus (in 25% of the rats). In this study a subset of the transfected neurons migrated past their target areas in the cortex. Overexpression of the human DCDC2 together with the *Dcdc2* shRNA vectors could rescue the periventricular heterotopia phenotype, but did not rescue the phenotype of the overmigrating neurons. The overexpression of DCDC2 alone did not have an effect on neuronal migration. (Burbridge et al., 2008)

The phenotype of the *Dcdc2* knockdown rats included abnormal ability to discriminate speech sounds from continuous streams. Also the responses in the auditory cortex were recorded and the responses were different in *Dcdc2* knockdown rats than control rats after training on a variety of speech sound discrimination tasks. The results suggest altered neuronal plasticity in the *Dcdc2* knockdown rats. (Centanni et al., 2016)

Contrarily to the findings in rat, the homozygous *Dcdc2* knockout mice with a targeted deletion of *Dcdc2* had structurally normal brains and displayed normal neocortical neurogenesis and neuronal migration. Differences in the *Dcdc2* knockout mice compared to wildtype mice became only apparent when simultaneous *in utero* RNAi was performed to knockdown *Dcx*; after the *Dcx* knockdown the disturbance in neuronal migration was then greater in the *Dcdc2* knockouts than in wildtype mice. The results suggest that in mice DCX and DCDC2 may have at least partially overlapping functions. (Wang et al., 2011)

Although the brain anatomy of the *Dcdc2* knockout mice appeared normal, both heterozygous and homozygous mutant mice had impairments in visual discrimination abilities, visuo-spatial memory and long-term memory. (Gabel et al., 2011) Truong et al. have also reported that the homozygous *Dcdc2* knockout mice have defects in working memory, reference memory and rapid auditory processing. (Truong et al., 2014) Che et al. studied the physiological properties of pyramidal cells in the neocortex of *Dcdc2* knockout mice. They measured action potential rate and timing in the neurons and found impaired spike-timing precision in *Dcdc2* knockout mice, suggesting poorer signalling capabilities of the neurons. The effect was

mediated through increased expression of Grin2B subunit of the N-methyl-D-aspartate receptor (NMDAR). (Che et al., 2014) Another alteration at the cellular level found in the *Dcdc2* knockout mice was increased spontaneous and evoked glutamatergic synaptic transmission, suggesting increased functional excitatory connectivity. (Che et al., 2016)

2.6.4 *Kiaa0319* IN MURINE MODELS

Kiaa0319 has been shown to be involved in neuronal migration in rats by *in utero* RNAi. The specific role of Kiaa0319 in neuronal migration has been suggested to be in mediating the adhesion between neurons and glial cells through the PKD domains that are thought to be involved in cell-cell adhesion. (Paracchini et al., 2006) The findings in the rats with embryonic knockdown of Kiaa0319 also include abnormalities in the growth and differentiation of dendrites (Peschansky et al., 2010) and a reduction in the midsagittal area of the CC. (Szalkowski et al., 2013b) The adult *Kiaa0319* knockdown mice have impairments in auditory processing (Centanni et al., 2014; Szalkowski et al., 2012) and spatial learning (Szalkowski et al., 2012). In contrast to the findings in rat, the knockout of Kiaa0319 in mice results in normal neuronal migration and no major abnormalities at anatomical or behavioural level or in the electrophysiology of neurons. (Martinez-Garay et al., 2016)

2.6.5 *Robo1* IN ANIMAL MODELS

2.6.5.1 Roundabout gene in drosophila

The roundabout (*robo*) gene was originally identified in fruit fly (*Drosophila melanogaster*) in a mutant screen targeting genes that control axonal midline crossing in the CNS (Tear et al., 1993) and belongs to the immunoglobulin family of transmembrane receptors. In *robo* mutant flies two populations of neurons are affected, those with longitudinal axons and those with commissural axons; the longitudinal axons normally do not cross the midline but in the *robo* mutants they cross once, whereas the commissural axons normally cross the midline only once but in the *robo* mutants they cross multiple times. (Kidd et al., 1998) The midline was hypothesized to contain a repellent guidance molecule, which later proved to be Slit, the ligand for the roundabout receptor. (Kidd et al., 1999) The amount of Robo receptor on commissural axons needs to be appropriately regulated so that axons crossing the midline are not responsive to the repelling signal by Slit, but after crossing they must again become responsive in order to not cross again. The surface levels of Robo are regulated by Commissureless (Comm) that regulates the delivery of Robo to the growth

cone in vesicles. (Keleman et al., 2005) The ligand binding induces Clathrin-mediated endocytosis of Robo, which is needed for the receptor activation and recruitment of downstream signalling molecules. (Chance and Bashaw, 2015) Robo signalling can lead to remodelling of the actin filaments in the cytoskeleton by several downstream pathways. One pathway involves Abelson tyrosine kinase (Abl) and Enabled (Ena) (Bashaw et al., 2000) Another pathway involves forming a complex, in which the intracellular domain of ligand-activated Robo interacts with Son of sevenless (Sos) through the adaptor protein Dreadlocks (Dock) (Yang and Bashaw, 2006). The downstream signalling pathway of Robo has been suggested to be shared with other repulsive axon guidance receptors Unc5 and Derailed (Drl), probably through the involvement of the guanine nucleotide exchange factor Trio. (Long et al., 2016)

2.6.5.2 Robo1 in mice

In vertebrates there are usually four homologous *Robo* family genes and three homologous *Slit* genes. In mice *Robo1*, *Robo2*, and *Robo3* are expressed in the developing nervous system, although not exclusively. In contrast, the expression of *Robo4* is limited mostly to endothelial cells (Park et al., 2003). ROBO1 binds to all of the Slit ligands in mice (Mambetisaeva et al., 2005; Mommersteeg et al., 2013) but SLIT1 and SLIT2 are the most important ligands for ROBO1 in the development of the CNS. (Andrews et al., 2006)

Homozygous *Robo1* knock-out mice (*Robo1* $^{-/-}$) that express no *Robo1* mRNA or protein die at birth and display severe defects in axonal pathfinding, including dysgenesis of the CC and hippocampal commissure. In the *Robo1* $^{-/-}$ mice, the misrouted callosal and hippocampal commissural axons terminate in the septum without crossing the midline. (Andrews et al., 2006) However some of the callosal axons in *Robo1* $^{-/-}$ mice crossed the midline and when they were analysed by tractography they were shown to form normal homotopic connections in the contralateral hemisphere (Unni et al., 2012). Moreover the hippocampal commissure that is normally separate from the CC was partially mixed together with the CC in the *Robo1* $^{-/-}$ mice. (Andrews et al., 2006; Unni et al., 2012) An additional defect in axonal pathfinding in the *Robo1* $^{-/-}$ embryos was that the developing corticothalamic and talamocortical axons reached their destination earlier than in wild type embryos. (Andrews et al., 2006)

Long et al. have described a different *Robo1* knockout mouse strain with very little expression of a mutant allele of *Robo1*. (Long et al., 2004) The phenotype of the homozygous mutant mice was different from the strains described by Andrews et al. (Andrews et al., 2008; Andrews et al., 2006); the Long et al. strain was viable and no major defects in axon guidance were found. The differences in the degree of the knockout most probably explain the discrepancy. (Lopez-Bendito et al., 2007)

ROBO1 has also been shown to be involved in the regulation of thalamocortical tracts. Spontaneous electrical activity and its associated calcium transients upregulate *Robo1* transcription, which results in a decrease in the rate of thalamocortical axon growth. (Mire et al., 2012)

The mouse ROBO1 has been shown to affect the development of both pyramidal neurons and interneurons in the cortex. The homozygous *Robo1* knockout mice had more interneurons in the cortex, suggesting a role for ROBO1 in the migration of interneurons (Andrews et al., 2006) but the observation may at least partially caused by increased proliferation. (Andrews et al., 2008) Also the route of the migration had changed in the *Robo1* $^{-/-}$ mice; normally the interneurons are repelled by the striatum but in the knockout mice they migrated through it. (Andrews et al., 2006; Hernandez-Miranda et al., 2011) Moreover, the interneurons in *Robo1* $^{-/-}$ mice had mean increased total process length and number of neurites and number of branches, suggesting that ROBO1 also regulates the morphology of the interneurons. (Andrews et al., 2008)

The homozygous *Robo1* knockout mice have been found to have excessive numbers of both early- and late-born pyramidal neurons in the cortex, which indicates that Robo1 regulates the proliferation and generation of pyramidal neurons. (Yeh et al., 2014) Moreover, the laminar positioning of the pyramidal neurons in the supragranular layers (II/III) of the neocortex was also affected in the Robo1 knockout mice in such a way that the cell density was larger than in the wild-type mice. Also the Knockdown of *Robo1* by *in utero* RNAi resulted in delayed radial migration and loss of the normal laminal inside-out pattern of the layers II/III. These findings support a role for ROBO1 in neuronal migration of the pyramidal neurons as well as in the termination of migration and in determining the final position. (Gonda et al., 2013)

Robo1 has also other developmental roles in mice, for example it has been shown to guide the axons from the retinal ganglion cells in the visual pathways. (Plachez et al., 2008)

2.7 CELL BIOLOGICAL ASPECTS OF THE THESIS

The result of this thesis indicated that DYX1C1 regulates estrogen receptor (ER) signalling and that DCDC2 is involved in the function of the cilium. This chapter will provide a brief introduction into ERs and cilia.

2.7.1 ESTROGEN RECEPTORS

The classical estrogen receptors (ERs) are Estrogen receptor alpha (ERα) and Estrogen receptor β (ERβ), encoded respectively by the *ESR1* and *ESR2* genes. They belong to the nuclear receptor superfamily and contain the protein domains that are characteristic of the superfamily: N-terminal (A/B)

domain that contains sites for transactivator binding, DNA-binding (C) domain, hinge (D) and a ligand binding (E) domain containing 12 α-helices (H1-H12). (Robinson-Rechavi et al., 2003) The H12 of the E domain is involved in the binding of ER co-regulators. (Brzozowski et al., 1997) The ERs additionally contain a domain in the C-terminus (F) that contributes to receptor activity by modifying the receptors interactions with coactivators. (Skafar and Zhao, 2008)

ERα and ERβ can have different affinities and specificities for their ligands. The endogenous agonists for ERs (endoestrogens) include estrone (E1), 17β-estradiol (E2) and estriol (E3), of which E2 has the highest affinity for both ERs. Several other compounds can also have antagonistic and/or agonistic effects on the ERs: phytoestrogens (plant-derived substances that have chemical and structural similarities to estrogen), xenoestrogens (exogenous compounds that bind to ERs), metalloestrogens (some heavy metal ions that bind to ERs) and selective estrogen receptor modulators (SERMs; partial agonists of ERs). (Farooq, 2015) The best-known example of a SERM is tamoxifen, which can be used to treat estrogen-receptor positive breast cancer. (Jordan, 2006)

ER signalling can follow two distinct pathways: the genomic and non-genomic pathways (Figure 7). In the genomic pathway the ligand-activation induces a conformational change in the ERs that enables the ERs to dissociate from chaperone proteins. The ERs then dimerize and can either bind directly the DNA in estrogen responsive elements (EREs) in the regulatory region of the downstream target gene or the ERs can bind to regulatory regions through protein-protein interactions with other transcription factors. The ERs also recruit coactivators and corepressors, which can interact with the receptor and modulate its transcriptional activities. The genomic pathway of ER signalling is relatively slow; the time from initial hormone stimulus to the specific outcome in cells can range from hours to days. (Marino et al., 2006)

The non-genomic ER signalling (also called rapid ER signalling) can have fast effects on the target cells, even within seconds from the stimulus. The non-genomic effects are mediated by membrane-bound forms of ERα or ERβ, but also by a third type of membrane estrogen receptor, G-protein coupled estrogen receptor 1 (*GPER1*). In non-genomic ER signalling, the activated receptors activate cellular kinases in specific signalling cascades that may have effects that are either dependent or independent of transcription. (Marino et al., 2006)

The activation of the ER signalling pathway affects various physiological processes, such as female reproductive functions, skeletal homeostasis, metabolism, cardiovascular system and function and development of the CNS. (Nilsson and Gustafsson, 2011)

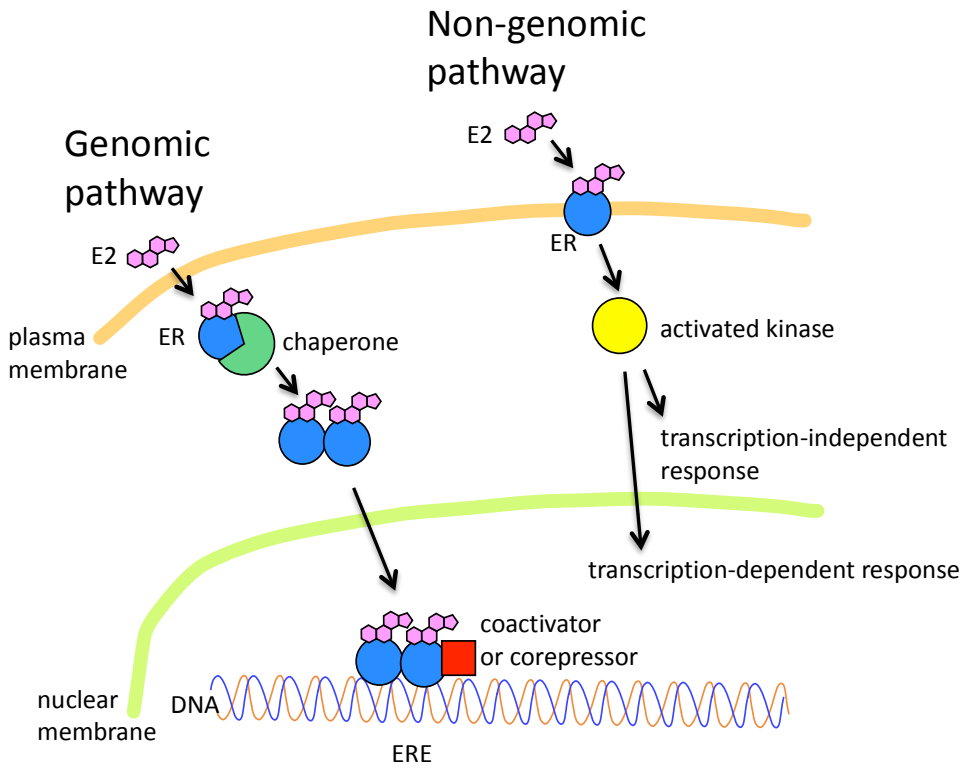

Figure 7 **Estrogen receptor (ER) signalling pathway.** In the genomic pathway the ligand-activation by 17β-estradiol (E2) leads to the dissociation of ER from chaperone proteins, which enables the ERs to dimerize and bind directly to DNA in estrogen responsive elements (EREs). Alternatively, the ER can indirectly bind to DNA through protein-protein interactions with other transcription factors (not shown in the figure). The ERs may also interact with coactivators and/or corepressors to activate or repress target genes. In the non-genomic pathway the ligand activates membrane-bound ERα, ERβ or GPER1, leading to the activation of specific kinases and signalling cascades. The final responses can be dependent or independent of transcription.

2.7.2 PRIMARY AND MOTILE CILIA

Most types of human cells, even neurons contain a cilium, a cell organelle that is usually projected from the surface of the cell body. The structure of cilia is conserved in evolution and consists of a microtubule-based core, called the axoneme, which is ensheathed by the ciliary membrane, an extension of the plasma membrane. (Gerdes et al., 2009) The basal body, formed by the mother centriole, nucleates the axoneme. (Kobayashi and Dynlacht, 2011) (Figure 8a)

Cilia can be broadly classified as motile or non-motile, and these two classes generally differ on their structure. The motile cilia contain a "9+2" microtubule arrangement in their axoneme, consisting of a central pair of single microtubules and nine microtubule pairs surrounding them. Additionally, the motile cilia contain structures that function in the movement of the cilium: outer and inner dynein arms (ODA and IDA respectively), radial spokes and central pair projections. (Figure 8c) The axoneme of the immotile primary cilia usually contains the "9+0" arrangement, in which the central pair is absent. (Figure 8b)(Ibanez-Tallon et al., 2003) In both motile and nonmotile cilia, the most distal axoneme of the cilium is usually formed by microtubule singlets instead of the doublets in the proximal axoneme. (Silverman and Leroux, 2009) Specialized types of cilia include the connecting cilium of rod and cone photoreceptor cells and the kinocilium of vestibular and cochlear hair cells. Moreover olfactory sensory neurons have specialized cilia that are responsible for olfaction. (Falk et al., 2015)

The presence of the cilium is linked to the cell cycle in such a way that the cilium is present normally in cells that are quiescent or terminally differentiated, but not in dividing cells. The formation of the cilium (ciliogenesis) and disassembly according to the stage of the cell cycle are tightly regulated. (Sanchez and Dynlacht, 2016) Moreover when the cilium is present, its correct length is important for the appropriate ciliary function, and is dynamically regulated by the balance of ciliary assembly and disassembly. (Ishikawa and Marshall, 2011)

Ciliary proteins are synthesized in the cytoplasm and endoplasmic reticulum. The transition zone, at the base of the cilium, regulates the entry of molecules into the cilium. (Reiter et al., 2012) Protein transport within the cilium is carried out by a system called intraflagellar transport (IFT) that includes motor proteins, kinesins and dyneins moving along the axoneme. Kinesins are responsible for the anterograde transport (towards the tip) and dyneins are responsible for the retrograde transport (towards the base). IFT is important for the biogenesis and maintenance of the cilium. (Goetz and Anderson, 2010)

The primary cilium is involved in several signalling pathways and it has been thought to act as the cells antenna, sensing and transducing signals from the environment. (Fuchs and Schwark, 2004) The signalling pathways associated with primary cilia include Wingless (WNT) (Corbit et al., 2008), Sonic Hedgehog (SHH) (Rohatgi et al., 2007), Transforming growth factor β (TGF-β) (Clement et al., 2013), Notch (Lopes et al., 2010) and Receptor tyrosine kinase signalling (RTK) (Christensen et al., 2012). The ciliary signalling pathways contribute to the regulation of various cellular processes such as cell cycle control, differentiation, migration, polarization, neurotransmission and metabolism.

Motile cilia are able to generate flow of fluids such as the moving of mucus in the respiratory tract (Ikegami et al., 2010) and the cerebrospinal

fluid in the CNS (Faubel et al., 2016). Even the movement of the gametes and the embryo in fallopian tube is at least partially generated by motile cilia in the tube epithelium. (Lyons et al., 2006) During embryonal development the nodal cilia that beat in a rotating motion have a critical role in the establishment of left-right asymmetry. (Nonaka et al., 1998)

With such diverse functions for the primary and motile cilia it has become evident that defects in cilia may cause a wide variety of diseases, together termed as ciliopathies. The organs commonly affected by ciliopathies include kidney, liver, skeleton, the eye and the brain.

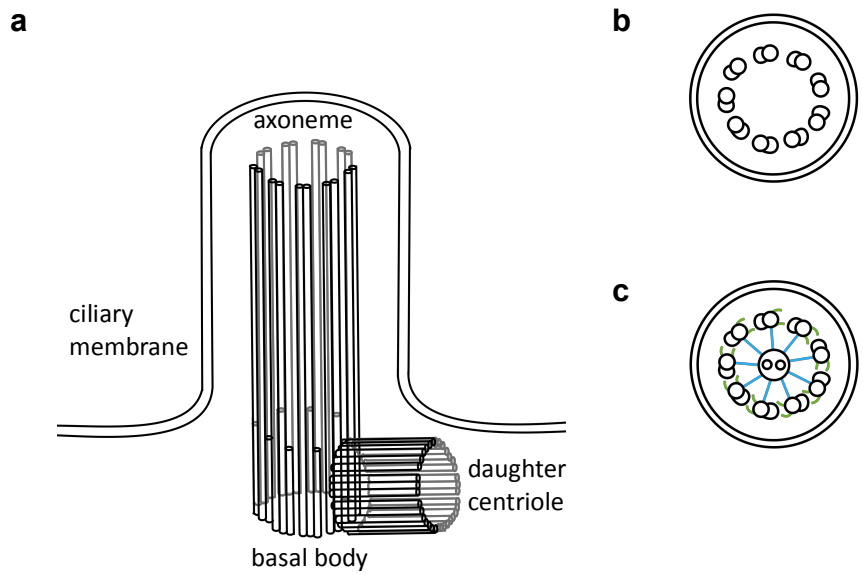

Figure 8 **Ciliary structure**. a) Nine microtubule pairs form the axoneme. At the base of the axoneme the basal body anchors the cilium. b) A cross-section of a primary cilium shows the nine microtubule pairs in the 9+0 arrangement. c) A cross-section of a motile cilium shows the nine microtubule pairs in the 9+2 arrangement. Motile cilia include a central pair of microtubule singlets, radial spokes (shown in blue) and outer and inner dynein arms (shown in green). The figure is modified from the article by Ainsworth et al. (Ainsworth, 2007)

3 AIMS OF THE STUDY

The general aim of this thesis was to gain more detailed information of the function of the SRD candidate susceptibility genes and to find neurodevelopmental and molecular pathways, in which they might be involved. The function of the SRD candidate susceptibility genes may give us information about the etiology of SRD, or the neurobiology of reading ability in general. The specific aims were to:

1) Study the cell-biological and molecular function of DYX1C1 (I) and DCDC2 (II)
2) Search for a causal factor for the dysregulation of *ROBO1* in DYX5-linked family (IV)
3) Investigate the role of ROBO1 in axonal crossing of the midline in auditory pathways in the DYX5-linked family with reduced *ROBO1* expression (III) and in callosal development in a population of normally developing children and adolescents (V)

4 MATERIALS AND METHODS

In this section I describe the main features of the materials and methods, and their purpose of use in this thesis. A more thorough description of the materials and methods can be found in the original publications (I -V)

4.1 HUMAN SUBJECTS (III-V).

In study III, ten individuals from the DYX5- linked family who were affected with SRD participated in the brain imaging study along with ten age and sex matched control subjects (normal readers). RNA samples extracted from Epstein-Barr virus (EBV) –transformed lymphocyte (lymphoblastoid) cell lines from the same individuals from the DYX5-linked family were also analysed for *ROBO1* gene expression. RNA samples extracted from isolated peripheral blood mononuclear cells from 10 anonymous blood donors recruited from Finnish Red Cross Blood Service were used as controls.

In study IV, all of the 19 affected members of the DYX5-linked family who share the SRD susceptibility haplotype (Hannula-Jouppi et al., 2005; Nopola-Hemmi et al., 2001) were included as subjects in the genomic sequencing. Moreover, lymphoblastoid cell lines from six affected DYX5-linked family members and three control individuals were used to study the effects of knockdown of the *LHX2* gene.

In study V, brain imaging and genotyping were carried out on 76 typically developing children and young adults, aged between 6 to 25 years, all from Nynäshamn, Sweden. (Soderqvist et al., 2010)

All of the subjects gave their informed consent. In study V, the parents provided the informed consent if the children were below 18 years old. The studies had previous approval from the local ethics committee.

4.2 METHODS IN MOLECULAR BIOLOGY

4.2.1 EXTRACTION OF BIOLOGICAL SAMPLES (I-V)

The extraction of DNA from blood or saliva, and the extraction of RNA or proteins from cellular material were done using standard procedures.

4.2.2 METHODS IN CELL BIOLOGY

4.2.2.1 Cell cultures (I-IV)

Cells extracted from various tissues can be manipulated to grow and proliferate ex vivo. In this thesis cell lines or primary cell cultures have been used as a source for RNA in gene expression studies (III) and also as models in protein localization and protein interaction studies (I, II) and as models in studying the effect of overexpression or silencing of genes of interest (I, II, IV).

The cells (Table 2) were cultured under standard conditions. In the experiments that assessed the interaction of DYX1C1 with estrogen receptors (I), the cultured cells were treated with the estrogen receptor agonist 17β-estradiol (E2) or selective estrogen receptor modulator 4-hydroxytamoxifen (4-OHT) or the proteasome inhibitor MG-132 when applicable. Ethanol (EtOH) or dimethylsulfoxide (DMSO) was used as control.

Table3 *The cell lines and primary cell cultures used in the thesis*

Cell-line	Origin	ATCC (American Type Culture Collection) number	The study/studies
MCF7	Human breast cancer	HTB-22	I
COS-7	African green monkey kidney	CRL-1651	I, II
SH-SY5Y	Human neuroblastoma	CRL-2266	I,II
NIH/3T3	Mouse fibroblast	CRL-1658	II
HEK-293	Human embryonic kidney	CRL-1573	IV
hTERT RPE-1	Human retinal pigment epithelium	CRL-4000	IV
Rat primary hippocampal or cortical neurons	Hippocampus or cortex of rat fetuses (embryonal day 17)		I, II
DYX5-linked family and control lymphoblasts	EBV-transformed lymphocytes		III, IV

4.2.2.2 Plasmid constructs (I, II, IV)

Plasmid constructs were used to overexpress or downregulate genes in cultured cells. Some of the plasmid constructs were prepared for these studies while other plasmid constructs were made by others previously. The preparation of constructs was performed using PCR-based strategies, using as a template appropriate cDNA from a tissue in which the gene of interest in expressed. The details of the plasmid constructs can be found in the respective studies (I, II, IV).

4.2.2.3 Transfections (I, II, IV)

In cell transfections, the cells are made permeable to exogenous DNA. In this thesis we have used lipofections that are based on creating small particles containing the plasmid constructs covered with lipid bilayer. The particles fuse with the cell membrane releasing the plasmid inside the cells. The transfections were performed using lipofectamine or Fugene according to standard protocols.

4.2.2.4 Transductions (III, IV)

Cell transductions rely on virus vectors to insert the exogenous DNA into cells. In study III and IV transductions with EBV have been used to immortalize lymphocytes. Moreover in study IV adenoviral vectors have been used to transduce the lymphoblast cell lines with shRNA vectors in order to knock-down the gene of interest (*LHX2*).

4.2.2.5 Immunocytochemical stainings (I, II)

In immunocytochemical stainings cells grown on coverslips were fixed, permeabilized, and stained with primary and secondary antibodies in order to examine the localization of proteins in the cells (studies I and II). The immunocytochemical stainings were also used in ciliary or neuronal morphology analysis (study II). The details of the primary and secondary antibodies used can be found in the respective studies.

4.2.2.6 Microscopy (I, II)

Confocal laser scanning microscopy and conventional optical microscopy were used to obtain images of the cells according to standard procedures.

4.2.3 METHODS FOR ANALYSING PROTEIN QUANTITY AND INTERACTIONS

4.2.3.1 Western blot (I, II, IV)

Western blot analysis can be used to identify proteins in a sample. First, the proteins are separated in gel electrophoresis, based on their molecular weight. Secondly, the proteins are transferred onto a membrane, and then detected with specific antibodies on the membrane. We have used western blot analysis in combination with other methods to detect the proteins of interest (I, II) and to analyse the effect of DYX1C1 protein on the protein levels of estrogen receptors (study I).

4.2.3.2 Co-Immunoprecipitation (I, II)

In co-immunoprecipitation (Co-IP), an antibody is used to capture the protein of interest (bait) together with possible interacting partners (prey) from a solution of mixed proteins, usually a cell lysate. Subsequently, the bait-prey complex is isolated and the interacting partners can be detected for example by Western blotting. We have used Co-IP to study the possible interactions between DYX1C1, ERs and CHIP (study I), and to study the possible interaction of DCDC2 and KIF3A (study II).

4.2.3.3 Microtubule binding assay (II)

We have used a microtubule binding assay to study if the full-length or deletion constructs of DCDC2 bind to microtubules. The assay is based on the feature of microtubules to pellet from a solution of proteins when centrifuged at 100 000 x g, while other proteins usually stay in the supernatant unless they bind to microtubules and pellet with them.

4.2.3.4 In situ poximity ligation (I, II)

In situ proximity ligation assay (PLA) can be used to identify the existence and localization of protein complexes at native levels with high sensitivity and specificity in fixed cells or tissues. It allows the detection of transient interactions as opposed to for example co-immunoprecipitation, which is most useful in detecting rather stable interactions. The *in situ* PLA protocol is briefly as follows. First, primary antibodies raised in different species bind to the proteins of interest. Secondly, species-specific oligonucleotide conjugated secondary antibodies (PLA probes) bind to the primary antibodies. Subsequently, a connector oligonucleotide is annealed to

complementary regions in PLA probes. If the PLA probes are situated within close proximity in the cellular environment, the connector oligonucleotides can be ligated to form a circular DNA molecule that can remain hybridized to the PLA probes and act as a template in in vitro DNA synthesis by rolling circle amplification. The amplified DNA can be detected with a fluorescent DNA probe with complementary sequence. (Fredriksson et al., 2002; Soderberg et al., 2006)

The *in situ* PLA has been used in study I to study the interaction between DYX1C1 and ERα, and in study II to study the interaction between DCDC2 and KIF3A.

4.2.3.5 The EMSA assay (IV)

The electrophoretic mobility shift assay (EMSA) can be used to study protein binding to nucleic acids and therefore has been widely used in studying the binding of transcription factors to promoter areas. In the assay, labelled oligonucleotide probe and protein sample are mixed and subjected to electrophoresis under native conditions. The unbound probe is used as control. The interaction can be seen as retardation of the band on the gel as the protein-DNA complex will be larger and migrate slower on the gel than the unbound probe. The bound protein can be identified by adding an antibody against it to the assay, producing a "supershift" as the protein-DNA-antibody complex is even slower to migrate on the gel than the DNA-protein complex. (Hellman and Fried, 2007)

The EMSA assay has been used in the study V to verify binding between LHX2 and a possible regulator sequence of *ROBO1*. (study IV) Moreover we studied the specificity of the binding by adding an unlabelled competing probe that contained a previously known binding site for LHX2.

4.2.4 METHODS FOR ANALYSING THE QUANTITY AND REGULATION OF GENE EXPRESSION

4.2.4.1 Quantitative real-time polymerase chain reaction (I, II, III and IV)

Quantitative real-time polymerase chain reaction (qRT-PCR) can be used for measuring steady-state mRNA levels and thereby obtain an estimation of gene expression levels. In qRT-PCR, the amplification of DNA molecules by polymerase chain reaction (PCR) is monitored in real-time using fluorescent reporters. The method can take advantage of either non-specific fluorescent dyes (such as SYBR Green) that bind to any double-stranded DNA or sequence-specific DNA probes (such as Taqman) that contain a fluorophore and a quencher group that is designed to allow fluorescence only when the

probe binds to its target. Both methods are used this thesis to study the expression levels of the genes of interest (I, II, III and IV).
The analysis of the data produced in qRT-PCR can be done in several ways. One of the most widely adopted methods is the comparative threshold cycle method (Schmittgen and Livak, 2008), which is based on the assumption that the PCR product amount is doubled in every cycle. The threshold cycle (C_T) is the number of amplification cycles required to reach an arbitrary threshold. The C_T-value is usually normalized by subtracting the C_T-value to one or preferably to a mean of several control genes that have a steady-rate of expression in the cell or tissue in question (house-keeping genes), resulting in ΔC_T –values. The $\Delta\Delta C_T$ value can be calculated by comparing all of the samples to one sample chosen as an internal control. The relative difference or the fold-change of the other samples to the internal control sample can be calculated by $2^{\wedge} \Delta\Delta C_T$.

4.2.4.2 Gene expression microarrays (II)

Gene expression microarrays are a powerful tool for the gene-expression profiling of cell populations or tissue samples. They allow the measurement of steady-state mRNA levels of a multitude of genes at the same time. In the microarrays, thousands of species of synthetic oligonucleotides are immobilized and dotted onto a slide, acting as probes to the fluorescently labelled samples. Then if the sample contains specific transcripts that can hybridize to the probe, a fluorescence signal is produced at that location of the slide. A picture is taken, and analyzed. The results have to be carefully normalized accounting for different biases, such as cross reactivity between a probe and a transcript that does not perfectly match that probe by complementary sequence.

In study (II) we have studied the gene expression in rat hippocampal neurons that overexpress DCDC2-V5 using the Rat Gene 1.0 ST array that constitutes more than 27,000 gene-level probe sets.

4.2.4.3 Luciferase assay (I, II and V)

Luciferase assays are diverse group of techniques that use luciferase enzymes as a reporter. Most common applications include studying the effect of regulatory regions and signalling pathways on transcriptional activity. When in contact with an appropriate substrate, the luciferase enzyme catalyses a chemiluminescent reaction and the released photon can be detected. The light produced by the luciferase enzyme is comparative to the steady-state mRNA level of the luciferase gene. The luciferase assay is usually performed

as a dual-assay in which one control luciferase is used to control for the transfection efficiency.

We have tested if our genes of interest are involved in certain signalling pathways by cotransfecting overexpression or silencing constructs of our genes of interest with luciferase reporter vectors containing the luciferase gene together with a concensus binding site for known transcription factors of the signalling pathways. More specifically we tested the effect of DYX1C1 on ER signalling (study I), and the effect of DCDC2 on SSH and WNT signalling pathways (study II). Moreover, we studied the effect of a novel polymorphism in a possible regulatory region of *ROBO1* on transcriptional activity by inserting the flanking area of the SNP into a luciferase construct with a constitutive promoter. (V).

4.2.5 FUNCTIONAL STUDIES IN C. ELEGANS (II)

In study II the function of human DCDC2 was studied *in vivo* in Caenorhabditis elegans (*C. elegans*) in transgenic animals that overexpressed DCDC2. The transgenic worm lines were generated by standard microinjection procedures. We studied also transgenic worms overexpressing ZYG-8, an endogenous *C. elegans* protein that belongs to the doublecortin family of proteins. (Gonczy et al., 2001)

4.2.6 GENOTYPING

4.2.6.1 SNP microarray (V)

Thousands of SNPs can be studied on the microarray technology, in which usually small nucleotide probes are immobilized on a microchip, hybridized with the sample (that is commonly fluorescently labelled) and imaged. The SNP arrays contain allelic probes and based on the binding of the study sample onto the two alleles, the genotype can be estimated. In study V we have performed genotyping on the Affymetrix Genome-wide Human SNP array 6.0 (Thermo Fisher Scientific Inc.), including more than 906600 SNPs and more than 946000 probes for detecting copy number variation.

4.2.6.2 Whole genome sequencing (IV)

Next generation sequencing (NGS) technologies have advanced remarkably over the last ten years or so and allow the rapid sequencing of whole genomes or whole exomes. There are two main categories of NGS techniques: short-read sequencing and long-read sequencing. The first step of short-read sequencing is usually the preparation of DNA library by fragmentation of the template, followed by ligation to common adaptors and clonal expansion of

the DNA. The sequencing reactions in short-read sequencing are generally based on either sequencing by ligation or sequencing by synthesis. In paired-end sequencing both ends of a fragment are sequenced. Long-read sequencing methods can be divided into single-molecule real-time sequencing or synthetic approaches in which the library for short-read sequencing is constructed using barcodes that allow the assembly of larger fragments in silico. (Goodwin et al., 2016)

In study IV we have studied the SRD-susceptibility haplotype in the DYX5-linked family by whole-genome sequencing (WGS). Whole genome sequencing of the pooled DNA sample from affected individuals was performed on an Illumina HiSeq 2000 (Illumina Inc.) as paired end reads to 100 bp. Whole genome sequencing of two dyslexic male individuals were performed by Complete Genomics Incorporation (CGI). The two methods used are both based on short-reads, but the Illumina method uses sequencing by synthesis and the CGI method uses sequencing by ligation.

4.2.6.3 Sanger sequencing (I, II, IV)

The Sanger method (Sanger et al., 1977) is a more traditional, but much less robust, sequencing method than the NGS methods. It is based on the random addition of chain terminators into the nascent DNA chain in polymerase chain reaction, and the following size-based separation of the created DNA fragments on electrophoresis. We have used Sanger sequencing to validate novel variants discovered by WGS and also to confirm the identity of the plasmid constructs built for studies I and II. Sanger sequencing was performed using dye-terminator chemistry and automated sequencers (Applied Biosystems) according to normal protocols.

4.3 BRAIN IMAGING METHODS

4.3.1 MAGNETOENCEPHALOGRAPHY (III)

Magnetoencephalography (MEG) is a method for detecting brain activation with high temporal resolution but relatively low spatial resolution. It is based on detecting the weak magnetic fields, generated by the small electrical currents that arise when there is neuronal signalling in the brain. The MEG device includes superconducting quantum interference devices (SQUIDs) that are extremely sensitive gradiometers that measure changes in the magnetic fields. The location of the source for the magnetic field is estimated from the acquired data. Although approximately 50 000 active neurons are needed to generate a detectable signal, the magnetic fields are so weak (in the order of femtoteslas) that a magnetically shielded room is required to protect from background magnetic fields, including the earth's magnetic field.

In study III we used a special application of MEG, frequency tagging (Fujiki et al., 2002), to study binaural interaction in auditory pathways in the DYX5-linked family and control subjects. In frequency tagging the inputs to both ears are amplitude modulated at different frequencies and consequently the responses to both ears at the auditory cortex can be extracted from each hemisphere by means of the modulation frequencies.

4.3.2 MAGNETIC RESONANCE IMAGING (V)

Magnetic resonance imaging (MRI) is an imaging technique for studying brain anatomy. It is based on the phenomenon of nuclear magnetic resonance, in which nuclei in a magnetic field are capable of absorbing and emitting electromagnetic radiation. The images acquired by MRI are often with high resolution and good contrast. Another advantage of MRI is that it is a noninvasive and safe method.

Diffusion tensor imaging (DTI) is a MRI technique that is based on detecting the diffusion of water molecules. In the brain, water does not diffuse freely in all directions but there are several factors that restrict diffusion such as cell membranes. As water can most easily diffuse in the direction of the axons, measuring the amount and the direction of diffusion by DTI is a good technique for studying the microstructure and the architecture of axons and the white matter tracts that they form in the brain.

In study V we have used structural MRI techniques to investigate the structure of the CC and the thickness of the cortex in normally developing children and young adults.

4.4 BIOINFORMATIC METHODS

4.4.1 ANALYSIS OF BRAIN IMAGING DATA

4.4.1.1 MEG data analysis (III)

In study III several bioinformatic processing steps were performed in order to extract measures of binaural suppression from the MEG measurements. The workflow included averaging of the MEG signals in order to improve the signal-to-noise ratio and to obtain the sinusoidal steady-state responses. The average from the vector sums of four adjacent gradiometer pairs, including the one with the largest steady-state response, was used to quantify activation at the auditory cortex. The binaural suppression was calculated based on the response strengths during binaural and monaural listening.

The binaural suppression was also quantified by additional source analysis, in which the magnetic field was modelled with equivalent current

dipoles and the strength of the response was calculated from peak-to-peak values from the source waveforms.

4.4.1.2 MRI and DTI data analysis (V)

The structural images acquired by MRI in study V were processed using Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL) method (Ashburner, 2007), which segmented the image into grey matter, white matter and cerebral spinal fluid. Also cortical thickness was measured using automatic longitudinal stram in FreeSurfer. (Reuter et al., 2012)

Various quantitative indices can be calculated from the data produced by DTI, for example fractional anisotropy (FA) with the value ranging from 0 (total isotropy, meaning unrestricted diffusion) to 1 (total anisotropy, meaning diffusion restricted to one direction). The preferred direction of diffusion can be used to perform white matter fibre tracking, also called tractography. Usually a seed or region of interest (ROI) is used as a starting point for the tracking of the white matter tracts. In the study V we have used probabilistic fibre tracking to find the CC white matter fibres and to segment the CC based on its connections to cortical areas. Moreover, the probability of connection to cortical areas and the mean FA values were calculated for the callosal segments.

4.4.2 ANALYSIS OF GENETIC DATA

4.4.2.1 Microarray data-analysis (II)

The pre-processing, normalization and statistical analysis of the microarray data was done using R statistical program and the statistical packages Affy (Gautier et al., 2004) and Limma (Smyth, 2004). The list of differentially expressed genes was subjected to gene ontology and pathway enrichment analysis on the WebGestalt bioinformatics resources (Zhang et al., 2005).

4.4.2.2 SNP chip data-analysis (V)

In study V, the genotype data was extracted from the SNP chip using Birdsuite (Korn et al., 2008) and the quality control was performed using PLINK (Purcell et al., 2007). For further analysis we chose the SNPs in the region that contains the exons and introns of *ROBO1* as well as roughly 300 kb upstream and 10 kb downstream of the longest transcript variant. We used Haploview to construct a haplotype map of the selected region and used the Tagger algorithm to select tagging SNPs for the haplotype blocks that were found. (Barrett et al., 2005)

4.4.2.3 WGS data analysis (IV)

In study IV the sequencing reads from the pooled sample were first aligned to the reference sequence, which was National Center for Biotechnology Information (NCBI) human reference genome build 37 (GRCh37). The alignment was performed using Burrows-Wheeler Aligner (Li and Durbin, 2009). Genome Analysis Toolkit (GATK) (McKenna et al., 2010) was used for variant calling, and subsequently the variants were annotated using ANNOVAR software (Wang et al., 2010). Data from the two individual samples that were sequenced at CGI was analysed using the Complete Genomics Analysis Pipeline which included mapped, performed the variant calling and annotation. (Carnevali et al., 2012; Drmanac et al., 2010), Our variant filtering strategy aimed at finding variants that belong to the SRD susceptibility haplotype in the DYX5-linked family; we kept variants that were found in all three samples (both individually sequenced samples and the pooled sample) and were heterozygous and novel. Finally, we used various software and databases to predict transcription factor binding to the candidate variants.

4.4.3 OTHER STATISTICAL ANALYSES (I - V)

Student's t-test was used to test for statistically significant differences between two data sets; In study I DYX1C1 overexpressing cells were compared to control cells in the ERE-luciferase assay; in study II the ciliary length in transfected cells was compared to the length in untranscefted cells; in study IV the insert-containing vector was compared to the empty vector in the luciferase promoter assay.

Analysis of variance (ANOVA) was used to test for statistically significant differences between the cells transfected using different overexpression or knockdown constructs in the WNT-luciferese and SHH-luciferase assays. In study III different forms ANOVA tests were used to compare the MEG results from study subjects from the DYX5-linked family to the control subjects. Additionally Pearson's correlation coefficient was calculated to measure correlation between ipsilateral suppression and *ROBO1* expression levels. In study IV Pearson's correlation coefficient was used to assess if *ROBO1* expression levels correlate with the severity of SRD.

In study V higher level statistical parametric mapping analysis with a flexible factorial design was used to study the associations between SNPs and morphological variation in the CC and thickness of the cortex.

5 RESULTS

In this thesis we have studied the function of two SRD candidate susceptibility genes *DYX1C1* (I) and *DCDC2* (II) at the cellular level. We have also characterized genetic variation in a previously identified SRD susceptibility haplotype in the broad genomic region of *ROBO1* (IV) and studied the role of *ROBO1* in human brain development (III and V).

5.1 STUDIES ON DYX1C1 AND DCDC2 (I AND II)

5.1.1 SUBCELLULAR LOCALIZATION STUDIES (I AND II)

When studying proteins whose functions are poorly known, a popular starting point is to study the subcellular localization of the endogenously or exogeneously expressed protein. We used cultured rat hippocampal neurons from embryonal day 17 as neuronal cell models. The MCF-7 cell line was chosen because it endogenously expresses ERα and the NIH/3T3 cell line was chosen because the non-dividing cells are ciliated.

5.1.1.1 Localization of DYX1C1 (I)

We overexpressed epitope tagged DYX1C1 in the COS-7 cell line (Figures 9a and 9b) and in the MCF-7 cell line and detected DYX1C1 in the nucleus and in the cytoplasm. Our findings were in line with earlier reports of nuclear and cytoplasmic localization of the human DYX1C1 (Taipale et al., 2003) or its rat homolog (Wang et al., 2006). In the *in situ* PLA experiment we also detected the endogenous DYX1C1 protein in the neurites of primary rat hippocampal neurons.

5.1.1.2 Localization of DCDC2 (II)

Prior to this thesis the function of the human DCDC2 at the cellular level was unknown. We performed transient transfections of DCDC2 expression vectors to rat primary hippocampal neurons and showed that the overexpressed epitope-tagged DCDC2 localizes to the neurites and the cytoplasm and surprisingly also strongly to the primary cilium. (Figure 9c) We also performed an *in situ* PLA experiment using an antibody against DCDC2, which verified the ciliary localization of endogenous DCDC2 in the primary rat hippocampal neurons. Also in the mouse fibroblast cell line NIH/3T3 there was a ciliary localization of overexpressed human DCDC2 in ciliated cells. In the non-ciliated cells (African green monkey kidney cell line

COS-7 or dividing NIH/3T3-cells) the overexpressed DCDC2 protein accumulated in the cytoplasm on microtubule networks near the centrosome. We did not detect nuclear or nucleolar localization of the overexpressed human DCDC2 in COS-7 cells in contrast to previous findings of overexpressed mouse DCDC2 (Coquelle et al., 2006).

5.1.2 PROTEIN INTERACTION STUDIES (I, II)

The vast majority of proteins function together with other proteins during cellular events, through transient or stable protein-protein interactions. Thus the interactions of a protein are essential for understanding its function.

5.1.2.1 Interactions of DYX1C1 (I)

It was previously known that DYX1C1 interacts with the E3 ubiquitin-protein ligase CHIP that targets certain proteins for proteosomal degradation. (Hatakeyama et al., 2004) CHIP was also known to be involved in the regulation of ERs (Fan et al., 2005; Tateishi et al., 2006). Because of these previously reported interactions and because the ERs are known to function in brain development we wanted to find out whether DYX1C1 could be involved in the same molecular protein network as CHIP and ERs. We used several distinct methods to study the possible interaction of DYX1C1 with ERα or ERβ: co-localization analysis, *in situ* PLA, Co-IP and protein-level analysis. At the time of our study we did not have access to a reliable antibody for ERβ and therefore the methods that required an antibody (*in situ* PLA and Co-IP) could be performed only for ERα.

In the co-localization analysis of overexpressed DYX1C1 and ERs in COS7-cells we showed, that in the presence of the ER agonist E2 a punctuate pattern could be observed, in which DYX1C1 co-localized with the ER under study (ERα or ERβ) (Figure 9a), whereas without ligands a diffuse pattern could be observed in the nucleus for DYX1C1 and ERs (Figure 9b). The pattern was similar to the E2 dependent colocalization pattern between ERα and RIP140, which is a known co-regulator for ERα (Cavailles et al., 1995). In the presence of the SERM 4-OHT, the co-localization pattern was not seen. The observed co-localization pattern suggested a possible interaction of DYX1C1 with E2-activated ERs.

We also used *in situ* PLA to test the possibility for interaction between endogenous DYX1C1 and ERα in primary rat hippocampal neurons. We found that ERα and DYX1C1 could be found within close proximity in the neurites of the cells. The amount of positive signals increased when the cells were treated with E2. These results also suggest that E2 is essential for the possible interaction between DYX1C1 and ERα.

To verify the that DYX1C1 and ERα can be found in the same protein complexes, we performed Co-IPs using protein extracts from SH-SY5Y cells

overexpressing epitope tagged DYX1C1 and ERα. We pulled down with an antibody against ERα and were able to detect DYX1C1 in the precipitate, supporting the idea that DYX1C1 interacts with ERα. Moreover we performed the Co-IP also using protein extracts from cells overexpressing DYX1C1 and CHIP. When we pulled down with an antibody against CHIP, we could observe DYX1C1 in the precipitate, verifying the interaction between DYX1C1 and CHIP.

In the protein-level analysis we overexpressed varying levels of DYX1C1 in the MCF7 cell-line, in which ERα is expressed endogeneously. We found that overexpression of DYX1C1 decreased ERα levels in a dose-dependent manner. The effect of DYX1C1 was greater in E2-treated cells, but in 4-OHT treated cells the effect was diminished. The proteasome inhibitor MG132 blocked the downregulation of ERα by DYX1C1, which suggests that the proteasome is needed for the downregulation. The effect of increasing amounts DYX1C1 on ERβ levels was studied in similar protein-level analysis in SH-SY5Y cells but with exogenously expressed ERβ. We were able to show downregulation of ERβ in the presence of E2 and in the absence of ligand, suggesting that DYX1C1 can downregulate also ERβ.

We also observed that the overexpression of DYX1C1 led to a decrease in the amount of ERα-staining in immunofluorescencently labelled MCF7-cells, supporting a role for DYX1C1 in regulating ERα.

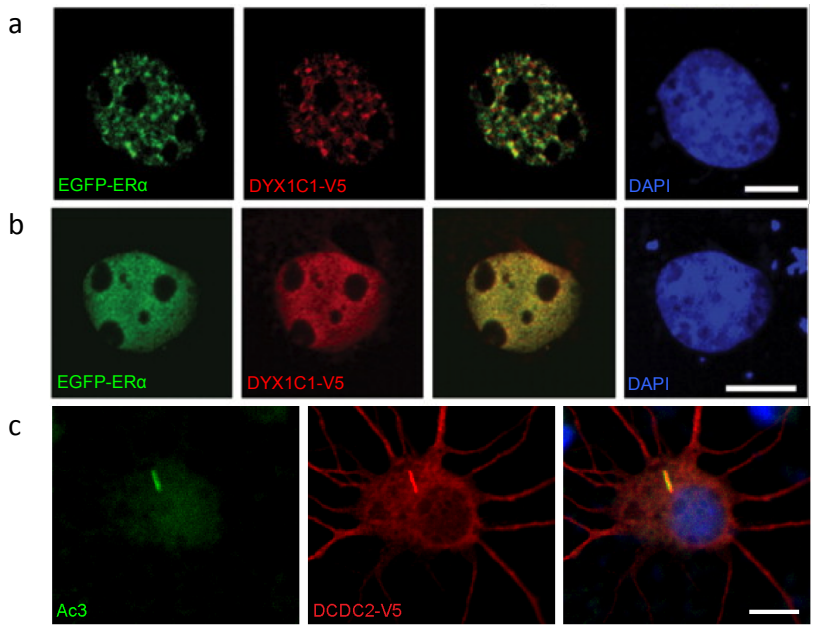

Figure 9 **Overexpressed DYX1C1 and DCDC2 in cell models.** a) Epitope-tagged ERα and DYX1C1 were overexpressed in COS-7 cells. The merged image shows a punctate co-localization pattern for ERα and DYX1C1 in the presence of E2. b) Without E2 the punctate pattern could not be observed (ethanol was used as control). c) Epitope-tagged DCDC2 was overexpressed in primary rat hippocampal neurons. The merged image shows that DCDC2 co-localizes with Ac3, a marker for neuronal cilia.

5.1.2.2 Interactions of DCDC2 (II)

We confirmed that, as suggested by the presence of the two DCX domains, the DCDC2 protein binds microtubules in the microtubule pelleting assay. Moreover, we found that DCDC2 possibly interacts with KIF3A that is a subunit of the IFT motor protein kinesin-2 and is important in the formation and maintenance of the cilium. (Corbit et al., 2008) Using *in situ* PLA in primary rat hippocampal neurons we found that endogenous KIF3A and DCDC2 can be found within close proximity in the cilium. A positive signal was also detected in the cytoplasm, suggesting that the possible interaction between DCDC2 and KIF3A is not spatially limited only to the cilium. In order to verity that DCDC2 and KIF3A can be found in the same protein complex we also performed Co-IP using protein extracts from rat hippocampal neurons. We were able to detect KIF3A in the precipitate when DCDC2 was used as the bait, supporting the idea of an interaction between KIF3A and DCDC2.

5.1.3 DCDC2 AND CILIARY LENGTH (II)

We hypothesized that DCDC2 could be involved in the regulation of the length of the cilium by stabilizing the microtubules in the ciliary axoneme and thus making microtubule assembly more favourable than disassembly. Consistent with our hypothesis we observed that overexpression of DCDC2 increased ciliary length; the average length of the primary cilium was roughly doubled in the hippocampal neurons or NIH/3T3 cells that overexpressed DCDC2. In contrast, the knockdown of *Dcdc2* by shRNA did not affect the length of the cilium in either in rat hippocampal neurons or NIH/3T3 cells. Both the ciliary membrane and the microtubule axoneme were extended in the abnormally long DCDC2 overexpressing cilia as shown by staining with markers specific for the axoneme and the ciliary membrane.

5.1.4 STUDYING SIGNALLING PATHWAYS (I, II)

Cellular processes are usually regulated by various signalling pathways. The interaction of DYX1C1 with ERs suggested involvement of DYX1C1 in the ER signalling pathway (I) and the localization of DCDC2 in the cilium suggests possible involvement of DCDC2 in ciliary signalling pathways (II).

5.1.4.1 DYX1C1 and estrogen signalling (I)

Because of our finding that DYX1C1 can downregulate the protein levels of both ERα and ERβ, we investigated the effect of DYX1C1 on the ER signalling pathways; a decrease in ER protein levels would be expected to result in decreased transcriptional activity of the ER signalling pathway in response to E2. We used transfection based luciferase reporter assays with a reporter vector that contains an estrogen response element (ERE). The MCF7 cell line with endogenous expression of ER was co-transfected with luciferase reporter and DYX1C1 overexpression construct. ERβ overexpression construct was co-transfected with the reporter vector and DYX1C1 overexpression construct into SH-SY5Y cells. In both cases the transcriptional activity of the ERE was decreased in the presence of DYX1C1 when compared to controls. These findings indicate that DYX1C1 can act as a repressor for both the ERα and ERβ signalling pathways.

5.1.4.2 DCDC2 and ciliary signalling (II)

The DCDC2 interaction partner KIF3A is known to be a key molecule in ciliary signalling pathways, more specifically in SHH (Huangfu et al., 2003) and WNT (Corbit et al., 2008) signalling. Because of the ciliary localization of DCDC2 and the interaction between DCDC2 and KIF3A, we wanted to study whether the overexpression or downregulation of *DCDC2* in primary cortical neurons would affect SHH and WNT signalling pathways.

We used luciferase reporter vectors for the WNT and SHH pathways in combination with overexpression of DCDC2 or knockdown of *Dcdc2* in rat primary cortical neurons. We found that knockdown of *Dcdc2* enhanced WNT signalling but the overexpression of DCDC2 had no effect on WNT signalling. In contrast, the overexpression of DCDC2 overactivated SHH signalling but the knockdown of *Dcdc2* did not have an effect on SHH signalling.

We also analysed the effect of DCDC2 overexpression in primary rat hippocampal neurons using a gene expression microarray. A group of 54 genes were differentially regulated between the DCDC2-overexpressing cells and control cells. Pathway analysis using Wikipathways, showed significant enrichment of genes associated with cell cycle and Hedgehog signalling pathway, which was consistent with our results on the SHH luciferase assay. Gene ontology analysis showed that the list of differentially expressed genes was enriched for terms associated with microtubule cytoskeleton and cell cycle.

5.1.5 THE ROLE OF SPECIFIC PROTEIN DOMAINS (I, II)

We studied deletion constructs of DYX1C1, DCDC2 and ERα in order to gain insight into the role of the specific domains in the proteins.

5.1.5.1 The role of DYX1C1 protein domains (I)

Previously, a different subcellular localization pattern had been observed for deletion constructs of rat DYX1C1 in COS7-cells; when the TPR-domains were deleted DYX1C1 localized predominantly in the nucleus, whereas when the p23-domain was deleted DYX1C1 located predominantly in the cytoplasm. (Wang et al., 2006) We made comparable deletion constructs of the human DYX1C1 protein: one with a deletion of the three TPR domains (DYX1C1-ΔTPR) and the other with a deletion of the p23 domain (DYX1C1-Δp23). In contrast to previous findings, we observed that both of our deletion constructs were localized in the nucleus as well as the cytoplasm when overexpressed in cell lines.

We wanted to know which domains in DYX1C1 are important in the interaction with the ERs so we tested the DYX1C1 deletion constructs in co-localization analysis and Co-Ip. In the co-localization experiments, the DYX1C1-Δp23 construct did not show similar co-localization pattern with E2-activated ERs as the full-length DYX1C1 and the DYX1C1-ΔTPR construct. Thus, the results suggest that the p23 domain is responsible for mediating the interaction between DYX1C1 and ERs. Consistently, the DYX1C1-Δp23 construct showed less interaction with ERα in Co-IP than the full-length DYX1C1 and the DYX1C1-ΔTPR construct. Moreover, the DYX1C1-

Δp23 construct did not bind to CHIP in Co-IP, whereas the DYX1C1-ΔTPR showed similar interaction as the full-length DYX1C1.

Stability of endogenous ERα was also studied by immunofluorescence in MCF-7 cells transfected with full-length and deletion DYX1C1 constructs. As with the full-length DYX1C1, the staining of ERα was diminished in DYX1C1-ΔTPR expressing cells. The amount of ERα staining appeared normal in DYX1C1-Δp23 expressing cells, which suggests that p23 is needed for the proteasomal downregulation of ERα.

5.1.5.2 The role of ERα protein domains (I)

The H12 in the ligand binding E domain of ERs is involved in interactions with ER co-activators and co-repressors. (Brzozowski et al., 1997) We studied the involvement of the H12 in the interaction between DYX1C1 and ERα by examining the co-localization pattern of overexpressed DYX1C1 and a construct of ERα, in which the H12 was deleted (ERαΔH12). The co-localization of ERαΔH12 with DYX1C1 in COS7-cells was only partial when compared to the full-length ERα. The results suggest that DYX1C1 shares features with a variety of established ER co-regulators.

5.1.5.3 The role of DCDC2 protein domains (II)

In order to study the role of the DCX domains in DCDC2, we made two deletion constructs of DCDC2; one with a deletion of the first DCX domain (DCDC2ΔDCX1) and another with a deletion of the second DCX domain (DCDC2ΔDCX2). In contrast to the full-length DCDC2, both of the DCDC2 deletion constructs failed to localize to the cilium in primary hippocampal neurons or NIH/3T3-cells, which indicates that both doublecortin domains are needed for the ciliary localization. Nevertheless the deletion constructs did not have a dominant negative effect on the full-length DCDC2, because the full-length DCDC2 still localized to the cilium when co-expressed with either of the deletion constructs. Moreover, the overexpression of either of the deletion constructs had no effect on the ciliary signalling pathways studied (WNT and SHH).

5.1.6 FUNCTIONAL STUDIES OF DCDC2 IN C. ELEGANS (II)

Our studies in cultured cells indicated a role for DCDC2 in ciliary structure and function *in vitro* but we also wanted to study the role of DCDC2 in ciliated cells *in vivo*. Therefore we created transgenic *C. elegans* strains overexpressing DCDC2 or ZYG-8, the only endogenous member of the doublecortin protein family in *C. elegans*. The ZYG-8 has been shown to be involved in the regulation of microtubule dynamics in cell division (Gonczy et al., 2001) and throughout the cell cycle (Bellanger et al., 2007).

The DCDC2 and ZYG-8 expression constructs contained a promoter that drove the expression of the inserted genes in three neurons, of which two are ciliated (AQR and PQR) and one is non-ciliated (URX) in wild-type *C. elegans*. The overexpression of human DCDC2 in *C. elegans* caused ectopic branching in the cell soma and dendrites of AQR and PQR neurons but the morphology of URX neurons was normal. A similar phenotype was seen when ZYG-8 was overexpressed. Moreover we analysed the ciliary morphology in the AQR and PQR neurons and observed that the cilia in DCDC2 and ZYG-8 overexpressing neurons were absent, although they were normally present in control animals. The fact that only neurons that are ciliated in the wild-type *C. elegans* had altered morphology when DCDC2 or ZYG-8 was overexpressed suggests that the overexpression may disturb normal ciliary function and lead to the phenotype with ectopic branching. Correspondingly, in the cells that normally are non-ciliated the morphology was normal even when DCDC2 or ZYG-8 were overexpressed.

We wanted to know whether DCDC2 overexpression can lead to changes in neuronal morphology also in mammalian cells, so we analysed primary hippocampal neuronal cultures transiently transfected with the DCDC2 expression vector. We observed increased branching of neurites in the DCDC2 overexpressing cells, but the total length of the neurites was not significantly changed.

5.2 STUDIES ON ROBO1 (III, IV, V)

We have studied *ROBO1* in two subject groups with different properties:

1) In the Finnish DYX5-linked family we have characterized genetic variation in a previously identified rare SRD susceptibility haplotype in the broad genomic region of *ROBO1* (IV). We have also investigated the consequences of the reduced *ROBO1* expression in the DYX5-linked family by using MEG to measure functional crossing of the auditory pathways. (III)

2) We have studied the role of *ROBO1* in the development of the CC in a population of normally developing children and young adults. (V)

5.2.1 SEARCHING FOR A CAUSAL VARIANT IN THE DYX5-LINKED FAMILY (IV)

Prior to our studies it was not known why the expression of *ROBO1* is reduced in the DYX5-linked family. Moreover, none of the genetic variation that had been attributed to the SRD susceptibility haplotype was unique to the DYX5-linked family, but could be also found in controls as different haplotype combinations. We assumed that the underlying genetic factor for the SRD susceptibility could be found in the area of the susceptibility

haplotype, but in an area that had not been covered by the initial studies of the DYX5-linked family. (Hannula-Jouppi et al., 2005)

5.2.1.1 Sequencing of the SRD susceptibility haplotype (IV)

Because the SRD susceptibility haplotype in the DYX5-linked family is large (Nopola-Hemmi et al., 2001) and covers roughly 1% of the human genome, we decided to use WGS to characterize genetic variation within the susceptibility haplotype. Our study design consisted of sequencing a pooled DNA sample on Illumina HiSeq platform, as well as two individual samples separately at CGI.

To form the pooled sample, we combined equal amounts of DNA from all of the 19 affected individuals from the DYX5-linked family who share the SRD susceptibility haplotype. The rationale behind using the pooled sample was that it would be a cost-effective way to identify the variants that belong to the susceptibility haplotype. Because in the genomic area of the SRD susceptibility haplotype all of the pooled DNA samples contain one copy of Chr 3 that is identical, the collection of sequencing reads from the pooled sample would be expected to contain half of the reads coming from the susceptibility haplotype and the other half from different chromosomes. This ratio would not be affected by errors in pipetting or DNA quantification that may sometimes cause differential representation of individuals in the pooled sample, a possible limitation of pooled studies. (Schlotterer et al., 2014)

We obtained a 19-fold average read depth of the pooled sample, which amounts to only one read per individual. The relatively low coverage was acceptable, because we were focusing on the shared haplotype instead of the individuals. Moreover, in the two samples sequenced separately by CGI the average read depth was more than 50-fold, compensating for the low coverage of the pooled sample.

5.2.1.2 Filtering of the variants (IV)

WGS can produce numerous false positive variants, especially at such relatively low sequencing depths as we had for the pooled sample. By comparing the results from the two individually sequenced samples and the pooled sample we were able to robustly filter out the variants that were not likely to belong to the susceptibility haplotype or were probable artefacts. By using two different sequencing platforms, we further reduced the effect of technical errors. Roughly half of the SNPs found in the pooled sample were not found in both of the individually sequenced samples, and thus were discarded.

The downside of our efficient filtering strategy is the possibility that we may have discarded true variants. For example we filtered out some variants that were found in both of the individually sequenced samples but not in the

pooled sample. With the low coverage in the pooled sample there is a risk that those were true variants but remained undetected in the pooled sample.

After the comparison between the samples there were 288 intronic heterozygous SNPs, 374 intergenic heterozygous SNPs, and 242 small insertion-deletion polymorphisms (INDELs) in the area that includes introns and exons of *ROBO1* and 1 Mb upstream of the transcription start site. We were able to confirm two of the previously published exonic variants in *ROBO1*: one insertion in the *ROBO1* 3' untranslated region (rs113692951) and one exonic SNP (rs7616243). All of the other previously reported exonic variants are most probably sequencing artefacts from the initial study. (Hannula-Jouppi et al., 2005)

Because the susceptibility haplotype in the DYX5-linked family is rare and it has not been detected in any other individuals studied, we were most interested in variants that were not annotated in public databases. We found several novel variants: one intronic SNP and three intergenic SNPs and 4 small deletions and 34 small insertions. The intronic SNP (SNP1) was situated between the first non-coding exon and the first coding exon of *ROBO1a*, upstream of *ROBO1b*. The intergenic SNPs were situated 94 kb (SNP2) and 196 kb (SNP3) upstream from the *ROBO1a* promoter. All of the novel INDELs were located in mononucleotide or dinucleotide repeat regions and are unlikely to have functional consequences as they appear as typical microsatellite repeats. We also searched for novel variants in the region 5 Mb upstream from the *ROBO1a* transcription start site because SNPs that can regulate gene expression have been shown to be enriched in the region covering 5 Mb from the transcription start site. (Kirsten et al., 2015) We found 13 more novel SNPs.

In public databases such as the Database of Genomic Variants, many large structural variants can be found in the region of *ROBO1*. This suggests that neutral SVs can exits in the region. In line with those observations, we were able to found some structural variation in our studies, but none appeared to be specific to the SRD susceptibility haplotype.

It must be noted that it is possible that any of the 167 other refseq genes than *ROBO1* within the SRD susceptibility haplotype could have an effect on the SRD phenotype. We therefore screened the whole susceptibility haplotype for rare coding variants (minor allele frequency below 5% in public databases). We found two rare coding SNPs in zinc finger protein 717 (*ZNF717*) but no other rare coding variants.

5.2.1.3 Transcription factor binding analysis and EMSA of the novel variants (IV)

We chose SNP1, SNP2 and SNP3 (Table 3) for further analysis because they were located most proximally to the *ROBO1* gene. We found that SNP1 was located in an enhancer region listed in the FANTOM5 promoterome atlas. (Andersson et al., 2014) In addition, SNP1, SNP2 and SNP3 were situated in

predicted binding sites for transcfription factors. We tested the binding of proteins to the area of the SNPs by EMSA, using nuclear extracts from RPE-1 cells but the results did not support transcription factor binding to SNP1, SNP2 or SNP3.

One of the more distantly located (4,8 Mb from the *ROBO1* transcription start site) novel SNPs (SNP4) (Table 3) caught our attention because it was situated in a conserved regulatory element identified in a comparative analysis of the genomes of 29 mammals. (Lindblad-Toh et al., 2011) The SNP4 was confirmed to be shared heterozygously in the 19 affected DYX5-linked family members by Sanger sequencing. Upon closer examination we saw that the SNP4 was situated within close proximity to a TAATTA element, which is a high-affinity binding site for the family of homeodomain (Hox) transcription factors (Affolter et al., 2008). One member of the Hox family, LIM homeobox 2 (*Lhx2*) has previously been reported to repress *Robo1* expression in mice. (Marcos-Mondejar et al., 2012) Furthermore several transcription factors, including LHX2, were predicted to bind to the area of the SNP4 according to the UniPROBE database, which contains 8-mer binding profiles for transcription factors. (Newbury et al., 2011) Interestingly, the DYX5-linked family specific T-allele was predicted to create two more 8-mer binding sites for LHX2 when compared to the reference C-allele, suggesting that the DYX5-linked family allele would have enhanced binding properties. This finding led us to hypothesize that LHX2 would bind to the DNA sequence in the area of the SNP4, and that the higher affinity of LHX2 for the allele in the DYX5-linked family could explain the suppressed expression of *ROBO1* in the DYX5-linked family.

We tested the hypothesis of differential binding of LHX2 to the area of the C and T alleles of SNP4 using EMSA with nuclear extracts from *LHX2* overexpressing HEK293 cells. We were able to detect protein binding to the area of the SNP4 for both the C and the T alleles. There was more protein bound using the LHX2 overexpressing cell extracts when compared to control cell extracts, which suggests that it indeed was LHX2 that bound to our probes. Adding an antibody to the EMSA can help confirming the identity of the protein bound by the probes. Sometimes a supershift can be seen when the antibody bound to the protein makes the protein-DNA-antibody complex migrate slower on the gel than only the protein-DNA complex would. We did not detect such a supershift but instead, the binding of LHX2 to the allelic probes was weakened when antibodies were added. This could be explained by competitive binding to the same site in LHX2 by the antibody and the probes. The addition of LHX2 antibodies to the GFP control sample did not alter binding. In conclusion, the results supported the binding of LHX2 to our allelic probes.

We also studied the specificity of the LHX2 binding to our probes by testing whether we could compete away the binding with an unlabelled cold-probe containing a known binding site for LHX2 from the CYP19A1 gene promoter (Honda et al., 2012). We found that the cold probe slightly

weakened the binding, with the DYX5-linked family specific T-allele being more resistant to the effect. The results supported our hypothesis of the higher binding affinity of LHX2 to the T-allele.

Table3 *The functionally studied SNPs.*

Variant	Position	Reference allele	Alternative allele	Region	Distance to ROBO1 (bp)
SNP1	79667838	A	G	ROBO1 intron	+149,221
SNP2	79911063	G	T	Intergenic	−94,004
SNP3	80013510	T	C	Intergenic	−196,451
SNP4	84674201	C	T	Intergenic	−4,857,142

5.2.1.4 Further functional studies of SNP4 (I)

We aimed to find more evidence for allelic differences in SNP4 so we studied the effect of the alleles on transcriptional activity using a luciferase promoter assay. We inserted the alleles and some of their surrounding base pairs into luciferase reporter vectors upstream from a constitutive promoter. In the luciferase assay, the cells were cotransfected with LHX2 overexpression vector or with GFP overexpression vector as control. We detected increased luciferase activity of both of the alleles in the LHX2 overexpressing cells when compared to the vector without inserts, suggesting that both of the SNPs enhance transcription, possibly through LHX2 binding. We detected enhanced transcriptional activity of the allelic constructs compared to the vectors without inserts also in GFP overexpressing cells, possibly because there may be some endogenous factors binding to the SNPs. The binding of endogenous transcription factors to the allelic constructs would be expected because the allelic inserts contain the TAATTA motif that is a binding site for many transcription factors. (Affolter et al., 2008) The orientation of the SNPs did not have an effect, nor could we find any differences between the alleles.

5.2.1.5 The regulation of ROBO1 by LHX2 in humans (I)

We wanted to study if LHX2 is involved in the regulation of *ROBO1* in humans because of the reported regulation of *Robo1* by LHX2 in mice. (Marcos-Mondejar et al., 2012) Therefore we tested the effect of the knockdown of *LHX2* on *ROBO1* expression levels. By using lentiviral shRNA vectors we were able to reduce the expression levels of *LHX2* to on average 50% of control levels in lymphoblastoid cell lines. We had lymphoblastoid cell lines from affected members of the DYX5-linked family and from anonymous blood donor controls. In both of these groups, the expression of

ROBO1 decreased when *LHX2* was knocked down. We could not observe a difference between the DYX5-linked family members and controls, possibly because our sample size was small or because the cell line model used may have different regulation of *ROBO1*. Nevertheless, our findings showed that that LHX2 may also regulate *ROBO1* in humans. However, the direction of the regulation was not as we expected because in mice Lhx2 represses the expression of *Robo1* (Marcos-Mondejar et al., 2012). We therefore studied if *ROBO1* and *LHX2* are co-expressed, using information of their expression in 22 human brain areas from the FANTOM5 database (Forrest et al., 2014). There was a positive correlation of *ROBO1* and *LHX2* expression in both adult and fetal brain tissues, indicating that LHX2 may also act as a positive regulator of *ROBO1*.

5.2.2 STUDYING THE FUNCTION OF ROBO1 IN THE DYX5-LINKED FAMILY (III)

The DYX5-linked family is the only reported example of inherited *ROBO1* deficiency in humans. Thus they provided us with a great opportunity to study the function of *ROBO1* in human brain development.

5.2.2.1 The crossing of auditory pathways in the DYX5-linked family (III)

Because SRD is often associated with various deficits in auditory processing (Hamalainen et al., 2013) and because the orthologs of *ROBO1* are known to affect the midline crossing of axons in animal models (Andrews et al., 2006; Kidd et al., 1998; Long et al., 2004; Unni et al., 2012) we decided to study functional crossing of auditory pathways in the DYX5-linked family. We hypothesized that deficient crossing of axons as a result of inadequate levels of *ROBO1* expression would be seen as decreased binaural suppressive interaction. More specifically, we were interested in the extent of the binaural suppression of the ipsilateral responses because the auditory pathway from the ipsilateral ear to the cortex does not cross the midline, but instead the suppressive signal from the contralateral ear crosses the midline. We used MEG and frequency tagging, a method based on amplitude modulations, to separate the cortical responses to sounds heard by the left and the right ears and by comparing monaural and binaural responses we were thereby able to measure the extent of ipsilateral suppression.

We found that in line with our hypothesis, the affected members from the DYX5-linked family showed less ipsilateral suppression than control individuals. Contralateral suppression did not differ between the groups, as expected (the auditory pathway from the contralateral ear to the cortex crosses the midline, but the suppressive signal does not).

5.2.2.2 Correlation between ROBO1 expression and the ipsilateral suppression (III) or severity of SRD (IV)

We wanted to investigate whether the reduced *ROBO1* expression in the DYX5-linked family could be the causal factor behind the reduced ipsilateral suppression. Hence we tested if there was correlation between the amount of ipsilateral suppression and the level of *ROBO1* expression in lymphoblastoid cell lines from the same DYX5-linked family members who participated in the MEG study. Indeed, we found that the less *ROBO1* was expressed, the less ipsilateral suppression there was. The results suggest that ROBO1 regulates axonal crossing of the midline in the auditory pathways. (III)

In study IV we also studied the relationship between *ROBO1* expression and the severity of SRD phenotype. We found that the less *ROBO1* was expressed, the more problems the subject had in phonological coding.

5.2.2.3 The expression of ROBO1 and its isoforms (III)

Lymphoblastoid cell lines from anonymous blood donors were used as controls in the gene-expression measurements of total *ROBO1* expression. We were not able to detect a difference in total *ROBO1* expression levels between the affected members from the DYX5-linked family and the controls in qRT-PCR. It is possible that our method for quantifying gene expression was not sensitive enough to detect the differences. In the initial study of *ROBO1* expression levels in the DYX5-linked family, the expression from the SRD susceptibility haplotype was on average 66% of control levels (Hannula-Jouppi et al., 2005), which would correspond to 88% (166/200) of normal biallelic expression levels.

We were interested in finding out if we could attribute the deficit in ipsilateral suppression to either of the most common transcript variants, *ROBO1a* or *ROBO1b*. We found that *ROBO1a* was not expressed in the lymphoblastoid cell lines at detectable levels, and thus we were not able to clarify which isoform of *ROBO1* or if both of the isoforms are attributable to the phenotype in the DYX5-linked family. We also found that *ROBO1b* was the major isoform in commercially available human brain cDNA samples in all brain areas studied. Both isoform had their highest expression in fetal brain. Our results are in line with previous observations, in which the mouse *Robo1b* has been shown to have more widespread expression than *Robo1a*, both temporally and spatially. (Clark et al., 2002)

5.2.3 STUDYING THE FUNCTION OF ROBO1 IN NORMAL POPULATION (V)

ROBO1 has been shown to be involved in callosal development in mice (Andrews et al., 2006; Unni et al., 2012). We hypothesized that if ROBO1

affects callosal development also in humans, we might see an association of genetic variation in *ROBO1* with variation in the structure of the CC in the normal population. Thus we performed an association analysis in which we assessed the effects of common SNPs in the *ROBO1* locus to morphological variation of the CC in a group of 76 normally developing children and young adults. Moreover, we analysed the association of *ROBO1* SNPs with the thickness of the cortex, since the mouse ROBO1 has been shown to regulate the migration of pyramidal neurons and their laminar distribution in the cortex. (Gonda et al., 2013)

5.2.3.1 ROBO1 SNPs and callosal morphology (V)

We first searched for the CC white matter fibres; all of the individuals' DTI data was subjected to probabilistic fibre tracking using the body of the CC as the seed region. A group level map of all individuals was constructed that consisted of tracts that were present in 90% of the individuals. This area was used as the region of interest (ROI) in the association analysis.

The association analysis was conducted in two stages with the aim of optimizing the number of SNPs; using more SNPs would capture the variation more efficiently but would also result in less power to detect subtle genetic effects because of multiple testing burden. In the first phase we chose 20 tagging SNPs within the genomic area of the *ROBO1a* transcript, including roughly 300 kb downstream and 10 kb upstream of the gene. When we assessed the association of the SNPs with the white-matter segmented structural MRI images in the area of the ROI we found that two of the SNPs (rs17396958 and rs1393375) were significantly associated with white matter density in the posterior part of the CC.

The first phase of the association study covered less than 50% of SNP variation in the *ROBO1* locus. Therefore we wanted to confirm and refine the genetic association in the second round of the association study, in which we selected more SNPs within and between the haplotype blocks that were associated with CC structure in the first phase. There were 28 SNPs in total in the second phase. We found that five SNPs (rs6770755, rs7631357, rs7637338, rs7651370, rs9853895) were significantly associated with white matter density in the right posterior part of the CC, connecting the parietal and occipital cortical regions. (Figure 10) Together the results of the association studies suggest that similarly as in mice, the human ROBO1 functions in callosal development.

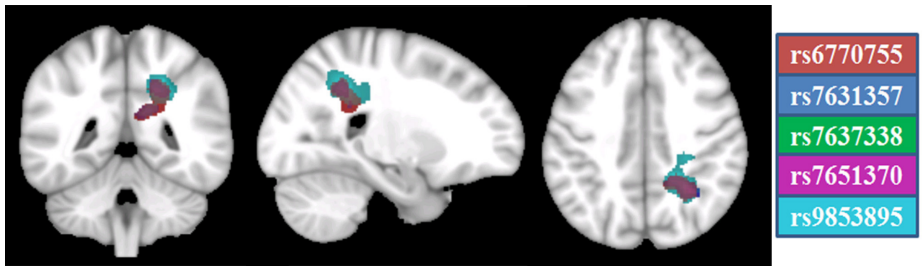

Figure 10 **Association of ROBO1 SNPs with callosal morphology.** Five SNPs in the putative regulatory region of ROBO1 were significantly associated with white matter volume in the right posterior part of the corpus callosum.

5.2.3.2 ROBO1 SNPs, probability of connection and FA (V)

The CC was further divided into smaller segments based on the bilateral connections to five different cortical regions of interest (anterior frontal, superior frontal, parietal, temporal, occipital cortex); the five cortical regions were used as target regions of probabilistic fibre tracking with the body of the CC used as the seed region. Next, we computed two indices for the five segments of the CC: probability of connection and fractional anisotropy. Probability of connection indicates the structure of the white matter pathways (such as number, thickness, size and the myelination of axons). FA reflects the organization and packing of axons as well as myelination. The indices were analysed for association with the same five SNPs that showed association with white matter density within CC. The mean probability of connection of the body of the CC to the parietal areas significantly correlated with the SNP rs7631357. There was no correlation between the FA values and genotypes in the 5 SNPs.

5.2.3.3 ROBO1 SNPs and cortical thickness (V)

We further analysed the five *ROBO1* SNPs that showed association with white matter density within CC. We performed an association analysis of the SNPs with the thickness of the cortex that was measured from the structural MRI data. Three SNPs (rs6770755, rs7631357 and rs7651370) showed a trend for association with cortical thickness of the left parietal region, but did not remain significant after correcting for multiple testing. The interhemispheric asymmetry was also studied by calculating the asymmetry coefficient (AC) of thickness of the five cortical regions of interest. Four SNPs showed association with AC of parietal region (rs rs6770755, rs7631357, rs7651370 and rs9853895) and remained significant after correcting for multiple testing.

6 DISCUSSION

6.1 ER AND CILIARY SIGNALLING PATHWAYS

6.1.1 ESTROGEN RECEPTOR PATHWAY AND SRD

The results of our study (I) suggest that the protein encoded by the SRD candidate susceptibility gene *DYX1C1* interacts with both ERα and ERβ and is involved in the ER signalling pathway through regulating the proteasomal degradation of ERs.

6.1.1.1 DYX1C1 and ERs in the brain

The connection of *DYX1C1* to ERs is interesting because of the well documented effects of ERs on brain development and neuronal processes such as neuronal differentiation, survival and plasticity.

It has been proposed that the division of labour between the two main types of ERs is such that ERα mainly drives sexual differentiation in the brain whereas ERβ plays a role in brain morphogenesis by affecting cortical layering and interneuron migration. (Fan et al., 2010) Mice with a homozygous deletion of ERβ show increased apoptosis in the ventricular zone and delayed neuronal migration of neurons destined to the most superficial layers of the cortex. Moreover, the processes of RGCs show abnormalities in the ERβ knockout mice, suggesting impairment in their ability to guide neuronal migration. (Wang et al., 2003) Thus the interaction between DYX1C1 and ERβ may provide one mechanism by which DYX1C1 is involved in neuronal migration.

We detected DYX1C1 and ERα complexes in the neurites in the primary rat hippocampal neurons. This suggests a function for DYX1C1 in the rapid non-genomic effects of estrogen. In addition to the membrane-bound forms of the classical ERs, also GPER1 participates in rapid estrogen signalling in the brain but so far it is not known whether DYX1C1 also interacts with GPER1. Rapid estrogen signalling may activate specific signalling cascades that regulate synapse structure and function, thus reorganizing neural circuits. (Alexander et al., 2016; Fan et al., 2010)

ERs have been implicated in spatial learning and memory functions of the hippocampus in mice. (Boulware et al., 2013) These findings suggest that the spatial learning deficit in the *Dyx1c1* knockout rats (Szalkowski et al., 2011) may be explained at least in part by the interaction of DYX1C1 with ERs. Moreover, the suppression of ERα and the subsequent persistent downregulation ERα target genes has been reported to be needed for memory formation after contextual fear conditioning in mice; certain

downstream targets of ERα signalling were suggested to act as "memory suppressor genes" that must be downregulated when a memory is formed. (Cho et al. 2015)

The effect of estrogen on auditory processing has been studied in various animal models including songbirds, mice and zebrafish. In Zebrafinch, the local experience-dependent synthesis of estrogen in the neurons of the caudomedial nidopallium (an analogue of the mammalian auditory association auditory cortex) has been shown to enhance the coding efficiency of the neurons possibly by synaptic modulation and result in increased ability to discriminate auditory signals. (Tremere and Pinaud, 2011) Estrogen also modulates the response properties of neurons throughout the ascending auditory pathway in songbirds. (Caras, 2013) The involvement of ERs in auditory perception suggests that the abnormalities in auditory processing in the *Dyx1c1* knockout rats could be mediated through the interaction of DYX1C1 and ERs. There is suggestive evidence that estrogen also affects auditory perception in humans as several studies have reported differences in auditory perception between males and females. Moreover, in females, changes in estrogen levels have been associated with differences in auditory function during the menstrual cycle, pregnancy and menopause. (Caras, 2013)

As the observed gender ratio for SRD is such that roughly twice as many men than women are affected (Rutter et al., 2004), a hypothesis for the etiology of SRD that involves hormonal factors is appealing. In the future, more thorough studies of *DYX1C1* or other SRD candidate genes in relation to the ER pathway may help explaining why males have a higher risk for SRD. Moreover, Dahdouh et al. have reported that *DYX1C1* may be a stronger risk factor for SRD in females than in males (Dahdouh et al., 2009). The observed gender-specific association of *DYX1C1* with SRD may in part be explained by the finding by Tammimies et al. that ERβ regulates the expression of *DYX1C1* (Tammimies et al., 2012).

After our findings on the role of *DYX1C1* in regulating ERs also another gene that functions in the ER pathway, Cytochrome P450 family 19 subfamily A member 19 (*CYP19A1*), has been suggested to be involved in SRD. Anthoni et al. reported that a translocation t(2;15)(p12;q21) in an individual with SRD disrupted the complex promoter region of *CYP19A1*. Moreover, haplotypes in the region of *CYP19A1* moderately associated with SRD as a categorical trait in three out of five family-based cohorts studied. Also in two cohorts, SNPs in *CYP19A1* were associated with speech and reading related quantitative traits. (Anthoni et al., 2012)

CYP19A1 encodes for the aromatase enzyme, which catalyses the chemical reaction in which androgens are converted to estrogens. It is the rate-limiting step in the pathway of estrogen biosynthesis. In the brain the aromatase enzyme contributes to ER signalling through local synthesis of estrogen. Anthoni et al. also studied homozygous aromatase knockout mice and reported abnormalities in cortical development with increased neuronal

density detected both in embryos and full-grown mice. In primary rat hippocampal neuron cultures the addition of testosterone or E2 enhance neurite outgrowth, but adding letrozole, an aromatase inhibitor, could block the effect of testosterone. (Anthoni et al., 2012)

Although the role of *CYP19A1* as a SRD susceptibility gene remains somewhat uncertain as no positive or negative replication studies have yet been reported, the finding that *CYP19A1* functions in the same estrogen receptor pathway as *DYX1C1* further supports the hypothesis that hormonal factors may be involved in SRD. In fact, a high level of intrauterine testosterone (that may be converted to estrogen by aromatase) has been hypothesized to increase the risk for SRD and other neurodevelopmental disorders that are more common in males, such as ADHD and autism spectrum disorders (James, 2008). Moreover, the transcription factor *LHX2* that we suggested to be a possible regulator of *ROBO1* is also known to regulate *CYP19A1* (Honda et al., 2012), suggesting that *CYP19A1* and *ROBO1* may share regulatory factors during development.

6.1.2 THE CILIUM AND SRD CANDIDATE GENES

The results of this thesis were the first direct indication ever of a ciliary localization for DCDC2 or any of the protein encoded by SRD susceptibility candidate genes. After our findings on DCDC2 also other connections between SRD candidate genes and cilia have been reported.

Even before this thesis Li et al. had performed a comparative genomic screen identifying the ciliary/flagellary and basal body proteome in eukaryotes and demonstrated significant overrepresentation of proteins with TPR domains. They identified DYX1C1 as one of the proteins that were likely to be involved in basal body and ciliary biogenesis. (Li et al., 2004) More recently, Ivliev et al. constructed gene coexpression networks by analysing large-scale gene-expression datasets from human tissues that harbour motile cilia, and based on the networks they predicted genes to be involved in ciliary function. Among the predicted ciliary genes were *DYX1C1*, *KIAA0319* and *DCDC2*. (Ivliev et al., 2012) Moreover, Hoh et al. compared the transcriptional profile of multiciliated mouse tracheal epithelial cells to nonciliated cells and found that DYX1C1 is upregulated during ciliogenesis. (Hoh et al., 2012) Recently, Tammimies et al. showed that *DCDC2* and *DYX1C1* are regulated by Regulatory Factor X transcription factors (RFXTFs), a gene family known for regulating ciliary genes through binding to promoter areas that contain X-box motifs. (Tammimies et al., 2016)

The KIAA0319 protein contains four polycystic kidney disease domains (PKD). The PKD domains were first identified in the polycystin-1 protein (Bycroft et al., 1999), which is involved in the mechanosensory functions of the primary cilium in kidney cells (Nauli et al., 2003). Thus it would not be surprising to see that KIAA0319 is also involved in the function of the cilium.

One SRD candidate gene, *CEP63*, has also been implicated in the function of the centrosome, an organelle that is functionally coupled to the cilia during cell cycle. A mutation in CEP63 that segregated with SRD in a Swedish family was found by exome sequencing and was predicted to cause an amino acid substitution that would be damaging for the function of the protein. CEP63 is required for centriole duplication, which is an important event in cell cycle progression. (Einarsdottir et al., 2015) Moreover, a deletion that cosegregates with SRD in a small family was reported to include the centrosomal genes, Pericentrin (*PCNT*) and S100 calcium binding protein B (*S100B*) along with two other genes. (Poelmans et al., 2009) Interestingly, PCNT has been suggested to be required for the assembly of primary cilia in vertebrate cells. (Jurczyk et al., 2004) Also the S100B has been shown to localize to the centrosome in U251 glial cells. (Sorci et al., 1998)

6.1.3 DCDC2 AND THE CILIUM

6.1.3.1 Novel mutations in DCDC2 in ciliopathies

Recent whole exome sequencing studies have identified novel mutations in *DCDC2* in renal hepatic ciliopathy (Schueler et al., 2015), nonsyndromic deafness (Grati et al., 2015) and neonatal sclerosing cholangitis (NSC) (Girard et al., 2016; Grammatikopoulos et al., 2016). A common factor in the phenotypes is the involvement of the cilium in the pathogenesis.

Renal-hepatic ciliopathy is manifested as varying degrees of hepatic fibrosis and renal dysfunction. Schueler et al. reported that in two unrelated patients the disease was caused by biallelic mutations in *DCDC2*, including a nonsense mutation, a frameshift and an obligatory splice site mutation, all of which resulted in truncation of the DCDC2 protein. In addition Schueler et al. re-analysed the homozygous *Dcdc2* knockout mice and showed that they had liver abnormalities that resembled the human phenotype. (Schueler et al., 2015)

NSC is a severe disease of the bile ducts, the draining system of the liver. Some patients, but not all, also have defective kidneys. Two independent studies have reported mutations in *DCDC2* in NSC patients; Girard et al. found homozygous mutations in *DCDC2* (a missense mutation in first doublecortin domain and an in frame deletion of 14 amino acids in the second doublecortin domain) in altogether 4 children with NSC in two families (Girard et al., 2016) and Grammatikopoulos et al. found that seven out of 24 individuals with NSC carried biallelic, protein truncating mutations in *DCDC2* (Grammatikopoulos et al., 2016).

Non-syndromic deafness is a hereditary form of deafness with no other associated symptoms. Grati et al. found a homozygous missense mutation (Gln424Pro) in *DCDC2*, which cosegregated with non-syndromic deafness in a Tunisian Family. The mutation was situated in a conserved region the C-

terminal region of DCDC2 suggested to contain residues that engage in interactions with partners involved in cell signalling. (Grati et al., 2015)

Although one coding SNP in *DCDC2* has been found to associate with SRD (Matsson et al., 2015), in general it appears that SRD may be caused by misregulation of the normal version of the DCDC2 protein or rather neutral missense mutations, whereas more damaging alterations in the protein structure are associated with more severe consequences to the individual that involve hearing loss, hepatic or renal dysfunction or a combination of those.

6.1.3.2 Recent findings on the localization of DCDC2 and its effect on ciliary structure

All of the studies that reported novel mutations in the coding area of *DCDC2* also confirmed the ciliary localization of DCDC2 *in vivo*; in humans DCDC2 has been detected in the primary cilium of cholangiocytes (the cells that form the biliary system in the liver) (Girard et al., 2016; Grammatikopoulos et al., 2016; Schueler et al., 2015) and the primary cilium of renal tubule cells of the kidneys (Schueler et al., 2015). In the mouse brain, DCDC2 has been detected in multiciliated ependymal cells and pia mater cells. (Schueler et al., 2015) In rats DCDC2 has also been shown to localize to the kinocilia (specialized cilia that function in morphogenesis and/or mechanotrasduction in the inner ear) of inner, outer and vestibular hair cells and to the primary cilia of all supporting cell types. (Grati et al., 2015)

The ciliary localization of DCDC2 has also been confirmed *in vitro* in hTERT-RPE1 cells (Schueler et al., 2015; Tammimies et al., 2016) and in MDCK-II cells (Schueler et al., 2015). Schueler et al. showed that the localization of DCDC2 varies in a cell-cycle dependent manner. In anaphase and metaphase DCDC2 localizes to spindle microtubules, in late telophase/diakinesis to the abscission structure, and during interphase to the cilium in cells that were ciliated. These findings fit well with our observation that the human DCDC2 binds to microtubules. Nevertheless there were also some microtubule containing structures related to cell cycle that DCDC2 was excluded from, such as the basal body, mitotic spindle poles and the midbody. (Schueler et al., 2015)

The mutations associated with NSC or renal-hepatic ciliopathy resulted in protein that failed to show the normal ciliary localization (Girard et al., 2016; Schueler et al., 2015) or could not be detected in the cells at all (Grammatikopoulos et al., 2016). Because all of the patient-associated mutations that failed to localize to the cilium were such that disrupted either or both of the doublecortin domains, these findings were in agreement with our finding that both functional DCX domains are needed for the ciliary localization of DCDC2 in primary rat hippocampal neurons and NIH/3T3 cells. Using hTERT-RPE1 cells, Schueler et al also overexpressed deletion constructs of DCDC2 in which either of the DCX domains were deleted and

replicated our findings of their inability to localize to the cilium. (Schueler et al., 2015)

The NCS associated mutations resulted in less cilia (Girard et al., 2016) or absence of normally constructed primary cilia in the liver tissue of the patients (Grammatikopoulos et al., 2016), suggesting a defect in ciliogenesis. Schueler et al. also showed that loss of *Dcdc2* function by siRNA knockdown disturbs renal epithelial ciliation in 3D cultures. The defect could be rescued by expressing human wild type DCDC2, but not the DCDC2 constructs with renal-hepatic ciliopathy associated mutations. (Schueler et al., 2015)

Grati et al. studied the effect of DCDC2 overexpression in early postnatal rat inner ear organotypic cultures and observed that the kinocilia and primary cilia were elongated in both hair cells and supporting cells. These results are consistent with our finding that DCDC2 overexpression increases the length of the cilium in primary rat hippocampal neurons and NIH/3T3 cells. Interestingly, the overexpression of the nonsyndromic deafness associated Gln424Pro mutant in the rat inner ear cultures caused a 2-3 fold increase in ciliary length when compared to the wild type protein. Also several ciliary abnormalities could be observed: branching, duplication or triplication of the cilium. These results suggest that in contrast to the other novel *DCDC2* mutations that appear to cause a loss of function, the Gln424Pro may cause a gain-of function. (Grati et al., 2015)

6.1.3.3 Recent findings on DCDC2 and ciliary signalling

We showed that the knockdown of *Dcdc2* in rat hippocampal neurons resulted in activation of the canonical WNT signalling pathway, consistent with the role of the primary cilium in restraining canonical WNT signalling. Also in other cases when the function of the cilium has been disrupted, for example by loss of Kif3a, an increase in WNT signalling has been observed (Corbit et al., 2008). In agreement with our findings in primary rat hippocampal cells and NIH/3T3 cells, Schueler et al. reported that knockdown of *Dcdc2* in NIH/3T3 cells resulted in increased activation of the canonical WNT pathway. They observed also that overexpression of DCDC2 reduced the activation of canonical WNT signalling, which was an effect that could not be seen in our experiments. (Schueler et al., 2015) The discrepancy could be due to possible differences in the efficiency of the overexpression of DCDC2 or differences in the reporter assays used.

We found that DCDC2 interacts with the ciliary kinesin subunit KIF3A, which functions in WNT signalling by inhibiting the phosphorylation of Dishevelled (DVL) by Casein kinase I (CKI). When DVL is phosphorylated, beta-catenin is stabilized and is able to activate gene-expression through TCF/LEF transcription factors. (Corbit et al., 2008) Schueler et al. gained more insight into the molecular interactions of DCDC2 by showing in Co-IP experiments that DCDC2 interacts with all of the three homologous dishevelled proteins in humans (DVL1, DVL2 and DVL3). The human

mutations associated with renal-hepatic ciliopathy did not abolish the interaction with DVL3, nor did deletion of either of the two DCX domains but the construct with both DCX domains deleted did not interact with DVL3. Schueler et al. also showed by immunoprecipitation that DCDC2 also interacts with JIP1, a regulator of the JNK signal transduction pathway. One of the renal-hepatic ciliopathy associated mutations produced a mutated form of DCDC2 that failed to interact with JIP1. (Schueler et al., 2015)

In this thesis we also reported that the overexpression of DCDC2 activates SHH signalling in primary rat hippocampal cells and NIH/3T3 cells. The receptor for SHH is the Patched1 (PTCH1), which is localized in the cilium and prevents smoothened (SMO), another transmembrane protein from access to the ciliary membrane. The binding of SHH to PTCH1 results in the inactivation of PTCH1, which allows the KIF3A mediated translocation and accumulation of SMO into the ciliary membrane. The activated SMO triggers a signalling cascade that controls the balance between activator and repressor activities of GLI transcriptional regulators. (Rohatgi et al., 2007) The interaction between DCDC2 and KIF3A suggests that the mechanism by which the overexpression of DCDC2 increases activation of SHH signalling pathway may be the enhancement of KIF3A mediated translocation of SMO into the cilium.

6.1.4 DYX1C1 AND THE CILIUM

6.1.4.1 DYX1C1 mutants and ciliary phenotypes

Recently, several studies have implicated *DYX1C1* and its vertebrate orthologs in ciliary function. Two independent studies have assessed the effect of knockdown of the zebrafish (*Danio rerio*) ortholog of *DYX1C1* using antisense morpholino oligonucleotides. Both studies reported that the *dyx1c1* morphants displayed a phenotype similar to knockdown of other cilia genes: body curvature, hydrocephalus, kidney cysts and situs inversus (reverse lateralization of the body patterning). (Chandrasekar et al., 2013; Tarkar et al., 2013) Tarkar et al. showed additionally that Dyx1c1 was needed for the left-sided expression of zebrafish nodal gene southpaw (*spaw*), which is required for normal left-right axis development. (Tarkar et al., 2013)

Tarkar et al. also reported that homozygous mutant mice with deletion of exons 2-4 of *Dyx1c1* displayed a phenotype that was very similar to primary ciliary dyskinesia (PCD). PCD is a disorder caused by abnormal ciliary motility, with typical findings including defective mucociliary airway clearance, defective tails in sperm (manifested as male infertility), and situs inversus. Most homozygous mutants were embryonic lethal, but those that survived after birth had hydrocephaly. Consistently with the situs inversus phenotype, DYX1C1 was detected in the embryonic node, where the left-right

patterning is established at the early embryonal stage. Moreover, in an independent mouse mutant screen for congenital heart defects a missense mutation of *Dyx1c1* was found. The mutation was located in the start codon of *Dyx1c1* resulting in a N-terminally truncated protein and the associated phenotype also included defective body patterning. (Tarkar et al., 2013)

Consistently with the observed phenotype of *Dyx1c1* knockout mice, mutations in *DYX1C1* can also cause PCD in humans. Tarkar et al. reported a mutation resulting in loss of exon 7 of *DYX1C1,* which was found in altogether six families with PCD. The also found other mutations in *DYX1C1* in PCD patients, including truncating mutations in 7 affected individuals. (Tarkar et al., 2013) Moreover, Raidt et al. have reported two PCD patients with biallelic mutations in *DYX1C1*, one patient with a homozygous nonsense mutation and the other with two distinct nonsense mutations. (Raidt et al., 2014) Also Marshall et al. reported a biallelic mutation in *DYX1C1* in PCD; one patient had the exon 7 deletion in one chromosome and a rare missense variant (predicted to be disease causing) in the other chromosome. (Marshall et al., 2015)

6.1.4.2 The effect of DYX1C1 on ciliogenesis, ciliary structure and ciliary function

Chandrasekar et al. reported a reduction in the number of cilia in several tissues in the *dyx1c1* morphant zebrafish, including the Kupffer's vesicle (a transient structure essential for specifying organ laterality), the pronephros and olfactory placode. Moreover, all of the above-mentioned tissues also had a varying degree of reduction in ciliary length. Also spinal cord cilia had dramatically reduced ciliary length. (Chandrasekar et al., 2013) Conversely Tarkar et al. did not report alterations in the distribution or the length of cilia in *dyx1c1* morphant zebrafish or *Dyx1c1* knockout mice. (Tarkar et al., 2013) Nevertheless, taken together the observations that both DYX1C1 and DCDC2 can affect ciliary length suggest that *DYX1C1* and *DCDC2* may together be involved in a network of proteins that regulates ciliary length. In line with this hypothesis, Tammimies et al have later shown that the DYX1C1 and DCDC2 proteins can interact with each other. (Tammimies et al., 2016)

The ultrastructure of the cilia were studied by transmission electron micrographs of tracheal cilia of the mouse *Dyx1c1* knockouts (Tarkar et al., 2013) and pronephric cilia in zebrafish *dyx1c1* morphants (Chandrasekar et al., 2013). In both cases the ODA and IDA of the ciliary axoneme were absent, suggesting a defect in ciliary motility. Also human respiratory epithelial cells isolated from PCD patients with biallelic *DYX1C1* mutation have been shown to have defects in IDA and ODA. (Marshall et al., 2015; Tarkar et al., 2013)

Consistently with the defect in ODA and IDA there is a defect in the motility of the cilia in *Dyx1c1* knockout mice. Ependymal tissue from the knockout mice was studied ex vivo as preparations. Ciliary beating was

absent from the preparations that were from *Dyx1c1* knockout mice, whereas those from wild-type mice continued to have ciliary beating. (Tarkar et al., 2013) Ciliary motility was also analysed in nasal brush biopsies from PCD patients with *DYX1C1* mutations and the results indicated that the functional consequence of *DYX1C1* mutations can range from ciliary immobility to only mildly decreased ciliary beating frequency. (Raidt et al., 2014; Tarkar et al., 2013) Taken together, the results suggest that DYX1C1 is essential for the movement of the motile cilia. (Chandrasekar et al., 2013)

6.1.4.3 Recent findings on the interactions of DYX1C1

The dynein arm protein complexes of the ODA and IDA are pre-assembled in the cytoplasm before they are transported to the cilium. Tarkar et al. showed that DYX1C1 interacts with dynein axonemal assembly factor 2 (DNAAF2), one of the previously known cytoplasmic preassembly factors. (Omran et al., 2008; Tarkar et al., 2013) They suggested that DYX1C1 functions as a cytoplasmic axonemal dynein assembly factor.

Centrosomal localization has been reported for overexpressed DYX1C1 in SH-SY5Y cells (Tammimies et al., 2013) and the endogenous DYX1C1 in hTERT-RPE1 cells (Tammimies et al., 2016). The overexpressed rat DYX1C1 has been detected in the centrosome and to a lesser extent in the primary cilium in NIH/3T3 cells. (Hoh et al., 2012) Tarkar et al. reported cytoplasmic localization of endogenous DYX1C1 in mouse nasal epithelial cells. (Tarkar et al., 2013) The observed localizations of DYX1C1 fit well with the hypothesis of DYX1C1 being a preassembly factor for ciliary dynein.

The protein interactome of DYX1C1 has been studied in SH-SY5Y cells (Tammimies et al., 2013) and in mouse trachea (Tarkar et al., 2013) by co-immunoprecipitation followed by mass-spectrometry. Both studies found enrichment of proteins involved in chaperone functions such as protein folding and response to unfolded protein, which was in line with earlier reports of the interaction of DYX1C1 with CHIP and heat shock proteins. (Hatakeyama et al., 2004) Tammimies et al. also reported that the DYX1C1 interactome was enriched with cytoskeletal proteins and suggested that DYX1C1 regulates the cytoskeleton during neuronal migration. Tammimies et al. also studied the effect of stable overexpression or transient knockdown of *DYX1C1* to global gene expression in SH-SY5Y cells using gene expression arrays. Among the differentially expressed genes were many involved in neuronal migration and cell cycle. (Tammimies et al., 2013)

Boldt et al. have recently characterized the ciliary proteome and the interactions of the ciliary proteins and the complexes that they form. Among the protein complexes that they reported was a complex formed by DYX1C1 and DNAAF2 and ubiquitin protein ligase E3D (UBE3D). (Boldt et al., 2016) Ubiquitinylation has been shown to be important in initiating the extension of the axoneme at the early stage of ciliogenesis (Kasahara et al., 2014) and in controlling ciliary length (Huang et al., 2009; Maskey et al., 2015). For

example the ODA intermediate chain subunit IC2 has been shown to be a substrate of the ubiquitin conjugating system. (Huang et al., 2009) Based on these findings and the results of this thesis in which DYX1C1 is implicated in proteasome-dependent regulation of ERs, it would be tempting to hypothesize that DYX1C1 functions in regulating the ubiquitinylation of ciliary proteins through interaction with molecular chaperones and the E3 ubiquitin ligases (possibly *UBE3D*). The proteasome at the ciliary base is also involved in regulating ciliary signalling. For example in SHH signalling the proteasome partially degrades the full-length Gli2 and Gli3 transcriptional regulators to convert them to the repressor forms (Pan and Wang, 2007) and in WNT signalling the proteasome mediates the degradation of cytoplasmic beta-catenin. (Gerdes et al., 2007)

6.1.4.4 DYX1C1 and the Cilium and ERs

Our findings suggest that DYX1C1 is involved in the ER signalling pathway but recent research points towards a role for DYX1C1 in ciliogenesis. The findings prompt the question of whether estrogen receptors are involved in ciliary function. The effect of estrogen to ciliary function has mostly been studied in relation to the beating of motile cilia in epithelial tissues of the oviduct and the airways. In vitro assays using cultured primary human airway epithelial cell have shown that another sex-hormone progesterone decreases ciliary beating, with the effect inhibited by coadministration of E2. (Jain et al., 2012). E2 also plays a role in the beating of motile cilia that transports the oocyte towards the uterus in the mouse oviduct; E2 downregulates IL6Rα via ERalpha resulting in increased beating frequency. (Shao et al., 2009) These effects suggest that ERs and DYX1C1 may both regulate motile cilia at least in some circumstances.

6.1.4.5 Cilia and the development of cerebral cortex

The earliest hints that cilia are involved in brain development came from observations on ciliopathies. Mutations in more than 80 genes have been associated with human ciliopathies and the vast majority of the mutations have been reported to affect neurodevelopment or neurobehaviour. The neurodevelopmental abnormalities reported in ciliopathies include a defect in neural tube closure, defects in neuronal migration and the inability of the cerebellar peduncles to cross the midline resulting in the molar tooth sign that can be seen in MRI. Also agenesis of the CC and hydrocephalus has been reported. (Guo et al., 2015)

As further evidence for a ciliary role for SRD candidates is accumulating and the causal factor in SRD is hypothesized to be a mild defect in neuronal migration it is highly relevant to ask what the role of the cilium is in neuronal migration and general development of the cerebral cortex. At the time of our

study on DCDC2 the function of the cilium in brain development was poorly characterised, but recently several studies have reported important roles for ciliary proteins in brain development.

Guo et al. have studied the role of 30 ciliopathy-related genes in the development of the cerebral cortex by *in utero* RNAi in mice. They reported that the effects of knockdown of the ciliary genes include various effects at distinct stages of development. They found that the knockdown of ciliary genes may disrupt the organization of the apicobasally polarized radial glial scaffold and the proliferative niche. Also decreased progenitor cell division was observed. The knockdown of ciliary genes was also shown to affect radial neuronal migration. More specifically the observed disturbances included delayed transition from the multipolar to the bipolar stage, delayed migration and altered morphology or number of the processes. Consistently with the observed defect in migration, many of the ciliary genes impaired laminar organization of the cortex, especially of the neurons with upper layer identities. (Guo et al., 2015) These results suggest that the reported involvement of DYX1C1 and DCDC2 in neuronal migration is fully compatible with their observed role in ciliary function.

The primary cilium has also been shown to function in tangential migration. Tangentially migrating neurons (future cortical GABAergic interneurons) assemble a primary cilium, which transduces signals through the SHH pathway. The SHH signalling was needed for the tangentially migrating neurons to leave their tangential stream and re-orient toward the cortical plate. A conditional knockout of *Kif3a* in MGE cells resulted in abnormal distribution of the tangentially migrating neurons. Interestingly, the length of the primary cilium of the migrating neurons varied according to the stage of migration (Baudoin et al., 2012).

The knockdown of ciliary genes has also been shown to affect post-migratory neuronal differentiation in the cerebral cortex, including axonal growth, axonal guidance, neurite extension and arborization. (Guo et al., 2015) Consistently with the deficit in axonal pathfinding primary cilia have been shown to be involved in the development of the CC. More specifically SHH signalling through the primary cilium controls the GLI transcriptional regulators and is essential for the correct positioning of the guidepost cells that guide the pathfinding of callosal axons. (Laclef et al., 2015)

Primary cilia are also found in neurons in adults. The role of cilia in the cerebral cortex in adults remains poorly understood, but recently the primary cilium has been shown to function in adult-born mouse hippocampal neurons in regulating their synaptic integration and thus their assembly into functional neuronal circuits through a mechanism involving WNT signalling. (Kumamoto et al., 2012)

6.1.5 *ROBO1* IN THE DYX5-LINKED FAMILY

6.1.5.1 Novel variants in the SRD susceptibility haplotype in the DYX5-linked family

We have identified several novel variants belonging to the SRD susceptibility haplotype that may help explaining why the expression of *ROBO1* is reduced in the DYX5-linked family. We studied whether the novel SNPs could affect transcription binding such as an expression quantitative trait locus (eQTL; a genetic variant that affects gene-expression). A common mechanism for eQTL is allele-specific binding of transcription factors that can lead to differences in histone modifications, DNA methylation or mRNA expression. (Albert and Kruglyak, 2015) Thus we tested the effect of SNP1, SNP2, SNP3 and SNP4 on transcription factor binding on EMSA, but the results did not support transcription factor binding to the area of SNP1, SNP2 or SNP3. We can of course not exclude the possibility that some transcription factors that are not present in the nuclear lysates from hTERT RPE-1 cells could regulate *ROBO1* by binding to the SNP1, SNP2 or SNP3 and thus we can not completely reject the hypothesis that these SNPs regulate the expression of *ROBO1*.

We observed that the area of the SNP4 was predicted to be bound by LHX2, with higher affinity for the allele that was specific to the SRD susceptibility haplotype. We showed that the SNP4 was bound by LHX2 in EMSA. Moreover we were able to show a slight allele-specific effect when we added a competitive probe with a previously known LHX2 binding site; the SRD susceptibility haplotype allele was more resistant to the effect of the competing probe, which was in line with the suggested higher binding affinity. Conversely, we were not able to detect any allelic difference in gene-expression in the luciferase promoter assay, which could be due to insufficient sensitivity of our assay to detect any subtle effect. Taken together we suggest that SNP4 may be a distant regulator of *ROBO1*, but with such mild effects in the functional studies we cannot be certain whether the SNP4 is the causal factor behind SRD susceptibility in the DYX5-linked family. Another possibility is that we have missed the true causative variant in our studies and that the SNP4 could be a modifying factor for SRD susceptibility. For example the rare SNPs in the coding area of *ZNF717* may be interesting as encodes a transcription factor that may be part of a network of transcription factors that coordinate major gene expression differences in human and chimpanzee brain. (Nowick et al., 2009)

It is a common phenomenon for complex phenotypes that it is difficult to confirm the causality of the associated variants. The majority of common risk variants identified in human GWAS are situated in non-coding regions and are expected to be involved in gene regulation (Maurano et al., 2012) but very few of them have been functionally validated. Rare variants detected using NGS technologies could be expected to involve more penetrant risk alleles

(Cirulli and Goldstein, 2010), but as in our study, pinpointing the causal variant is not always straightforward.

6.1.5.2 LHX2 and ROBO1

We showed that LHX2 may bind to SNP4 with higher affinity. Moreover our results suggest that LHX2 may also act as a positive regulator of *ROBO1* during brain development in contrast to previous findings in mice in which LHX2 is a repressor of *Robo1* (Marcos-Mondejar et al., 2012). The seeming discrepancy in the findings suggests complex cellular context-dependent regulation of *ROBO1* by LHX2.

LHX2 is an attractive candidate for explaining the reduced expression of *ROBO1* in the DYX5-linked family, because in mice LHX2 is involved in the development of thalamocortical connections by regulating *Robo1* expression. Specific conditional deletion of *Lhx2* in the thalamus alters projections from the MGN, from which the auditory pathways connect to the auditory cortex. (Marcos-Mondejar et al., 2012) MGN has been suggested to be involved in the processing of phonemes and its abnormal activation has been observed in individuals with SRD. (Diaz et al., 2012) An unusual pattern of cell-size distribution within the MGN (Galaburda et al., 1994) and abnormal thalamo-cortical connectivity has been observed in individuals with SRD. (Fan et al., 2014) Interestingly, also the knockout mice of the mouse ortholog of another suggested SRD candidate susceptibility gene *CNTNAP2* have abnormalities in auditory processing and display abnormalities MGN, including decreased number and density of neurons as well as altered size distribution. (Truong et al., 2015) Also the Dyx1c1 knockdown rats had abnormalities in their MGN (smaller neurons than usually). (Szalkowski et al., 2013a)

Another important role for LHX2 in mouse brain development is that it controls the timing of neurogenesis and is required for WNT signalling in maintaining cortical progenitor proliferation. (Hsu et al., 2015) LHX2 has also been shown to regulate the patterning of sensory areas in the cortex. (Zembrzycki et al., 2015)

6.1.5.3 Further support for ROBO1 as the SRD susceptibility gene in the DYX5-linked family

Hannula et al. have hypothesized that haploinsufficiency of *ROBO1* causes SRD in the DYX5-linked family. We were not able to confirm some of the previously reported SNPs in *ROBO1,* which were partially the same SNPs that were used in the allelic expression study showing reduced *ROBO1* expression from the SRD-associated allele. (Hannula-Jouppi et al., 2005) We therefore sought additional evidence that *ROBO1* is the susceptibility gene in the DYX5-linked family. We investigated the relationship between *ROBO1* expression levels and the severity of SRD and found a tendency that the

lower *ROBO1* is expressed, the more severe deficit in phonological coding the person has. We also showed a role for ROBO1 in the development of auditory pathways. These findings support the role of *ROBO1* as the susceptibility gene in the DYX5-linked family.

6.1.5.4 ROBO1 in axonal crossing of the auditory pathways

As the ipsilateral suppression is dependent on midline crossing of the axonal tract that conveys the inhibitory signal from the contralateral hemisphere, our results show that ROBO1 plays a role in the axonal crossing of auditory pathways in humans. Because at several levels in the ascending auditory pathways there are axonal tracts that cross the midline, it is difficult to pinpoint to the exact region in the auditory pathways where ROBO1 functions in midline crossing. One possibility is that small abnormalities along the auditory pathways accumulate. Nevertheless our results were the first time that a SRD susceptibility gene was linked to a specific sensory function of the human brain. It would have been interesting to know whether the degree of ipsilateral suppression also correlates with *ROBO1* expression in healthy control subjects in a similar manner as in the affected members of the DYX5-linked family, but unfortunately we did not have blood samples from the control individuals used in the MEG study.

The ascending auditory pathways contain a special type of cells, the excitatory-inhibitory (EI) neurons that are thought to function in the localisation of sounds by detecting interaural intensity differences. As the EI neurons are capable of enhancing contralateral input and suppressing the ipsilateral input, their function would be well suited for mediating ipsilateral suppression during binaural listening. The lowest level brainstem nucleus where EI neurons have been detected is the lateral superior olive, which connects to the dorsal nucleus of the lateral lemniscus (DNLL) and the inferior colliculus (IC). Also the DNLL and IC contain EI cells. (Li and Pollak, 2013) As the EI cells can suppress ipsilateral input, it would be tempting to hypothesize that ROBO1 controls axonal pathfinding or neuronal migration of these cells. In line with this hypothesis, in the rat embryos *Robo1* is expressed in IC. (Marillat et al., 2002)

SRD is often associated with subtle impairments along the auditory pathways. Banai et al. have suggested that reading skills are dependent on the integrity of the subcortical auditory mechanisms. (Banai et al., 2009) Phonological processing abilities have been shown to correlate with reading skills, with poorer abilities consistently linked to SRD. It is not known if the phonological processing deficits in SRD are a consequence of deficits in lower level auditory processing (Goswami, 2011) or results from a deficit in phonological representations or access to them (Ramus and Szenkovits, 2008).

ROBO1 has also been suggested to be involved in another aspect of auditory processing; a study has found suggestive genetic linkage of auditory

cortical activation strength to the 3p12 locus, with *ROBO1* situated in the area of the linkage peak. (Renvall et al., 2012)

6.1.6 ROBO1 IN CALLOSAL DEVELOPMENT

We found that five SNPs in the putative regulatory region of ROBO1 correlate with white matter density in the posterior part of the CC (splenium), which connects the temporal, parietal, and occipital cortices. We also found that one SNP (rs7631357) was significantly associated with the probability of connections of the body of the CC to parietal cortical regions of the both hemispheres. Although the white matter indices do not directly explain the biophysical factors such as size, diameter, membranes, myein thickness, or packing of axons, our results are the first report of a role for ROBO1 in the general population in the development of a specific structural feature of the human brain. More specifically our results suggest that ROBO1 may be involved in regulating the development of the CC. Unfortunately we did not report the direction of the associations, for example if the most common allele was associated with higher or lower probability of connection. Moreover, our sample set is considered small for a genetic study and although we did not perform actual power analysis, our study is likely to have limited statistical power to detect subtle genetic effects. Nevertheless, our findings can be considered credible as they are convergent with earlier observations of the role of ROBO1 in callosal development in mice. (Andrews et al., 2006; Unni et al., 2012) We did not find associations with FA values of the posterior CC, which is in line with a recent study that estimated that the additive genetic effects on FA values of the posterior CC are not significantly different from zero. (Blokland et al., 2012)

The role of CC in reading or SRD is not entirely clear, although several studies have shown alterations in morphology or microstructure of the white matter in the posterior CC in individuals with SRD. A morphometric study by Rumsey et al. suggested that the splenium and isthmus of the CC are larger in male adults with SRD. (Rumsey et al., 1996) Robichon et al. found that especially the isthmus of the CC was thicker in adults with SRD. (Robichon and Habib, 1998) von Plessen et al. observed that the posterior midbody region of CC was shorter in children with SDR than a controls (von Plessen et al., 2002)

In many studies the integrity of the microstructure of the posterior CC has been reported to be higher in poor readers than in normal readers. Dougherty et al. studied children with different reading abilities and reported that normal readers have lower radial diffusivity and higher FA in the posterior CC than poor readers, possibly reflecting that normal readers have a higher proportion of thick axons and fewer axons in total in this area. Moreover, the FA values correlated negatively with phonological awareness. (Dougherty et al., 2007) Odegrad et al. replicated the negative correlation

between phonological awareness and FA values in the posterior CC and also found a similar effect for decoding skills. (Odegard et al., 2009)

Also positive correlations of reading-related measures with white matter integrity have been reported. In adults who are normal readers the volume of the isthmus and rostrum of the CC positively correlated with phonological decoding efficiency, whereas the white matter integrity of the anterior midbody correlated positively with reading comprehension. (Welcome and Joanisse, 2014) In another study the splenium and isthmus of the CC have been shown to have more white matter in individuals who learnt to read during early adulthood when compared to illiterates. In anatomical analyses of normal readers who learned to read during childhood, the area in the CC that showed increased white matter in late-literates was shown to harbour interhemispheric connections between the angular gyri and dorsal occipital gyri. In functional connectivity analysis, reading was shown to enhance the connectivity between the left and right angular gyri. (Carreiras et al., 2009)

Frye et al. have reported differential associations of white matter organization with subcomponents of reading: orthographic processing skills showed negative correlated and the skills associated with phonological awareness showed positive correlation. (Frye et al., 2008)

Different explanations for the role of CC in SRD have been proposed. According to a hypothesis proposed by Rumsey et al. the more efficiently connected posterior CC may reflect deficient lateralisation of the language networks. (Rumsey et al., 1996) According to this model, the left cortex reading networks inhibit the corresponding areas in the right hemisphere through the white matter tracts in the posterior CC. Therefore abnormal development of the CC would lead to underdevelopment of the left regions and overdevelopment of the right hemisphere. According to an alternative hypothesis a more efficiently connected CC could better mediate compensatory activation of the right hemisphere in SRD. The posterior CC has also been suggested to connect the angular as well as dorsal occipital gyri leading and function in the efficient transfer of phonological and/or visual information during reading. (Carreiras et al., 2009)

Recently, Chechlacz et al. have shown that the CC plays a role in controlling the allocation of spatial attention by regulating interhemispheric dynamics. (Chechlacz et al., 2015) This suggests that alterations in the CC could at least partially explain the deficits in visual attention (Bosse et al., 2007) that are often associated with SRD.

We also observed a trend for association of three *ROBO1* SNPs with cortical thickness of the left parietal region. Our finding suggests that if *ROBO1* controls neocortical lamination in humans as the orthologous gene does in mice (Gonda et al., 2013), the environmental influences are larger on cortical thickness than the effect of *ROBO1*. Four SNPs in *ROBO1* showed association with the AC of parietal region. Asymmetries in the structure of the cerebral cortex have been observed in SRD but they may reflect a consequence of less reading experience. (Bishop, 2013)

6.1.7 OTHER FINDINGS RELATED TO CC IN IMAGING GENETICS OF SRD CANDIDATE GENES

Other studies in imaging genetics have been published that have studied SRD candidate susceptibility genes in the same group of normally developing children and adolescents as we did in study V. In a two-part study Darki et al. found that SNPs in *DYX1C1* (rs3743204), *DCDC2* (rs793842), *KIAA0319* (rs6935076) affected white matter volume in left temporoparietal regions and that the overlap area for the three SNPs included parts of the superior longitudinal fasciculus and the posterior part of the CC. In tractography, the overlap area was found to connect to the left middle temporal gyrus to the angular gyrus and the supramarginal gyrus, as well as to bilateral lateral occipital cortex. The *DCDC2* SNP also showed correlation to reading ability. (Darki et al., 2012; Darki et al., 2014)

Two tagging SNPs in the genomic area of *CEP63* were also studied in the same dataset as in study V. One SNP (rs7619451) was significantly associated with white higher matter volume in the right hemisphere overlapping with right superior longitudinal fasciculus and posterior part of CC. In the right temporo-parietal region, the significant area in the right hemisphere partly overlapped with a region that was previously found to associate with SNPs in *DYX1C1* and *KIAA0319*. The rs7619451 was also significantly associated with reading ability. (Einarsdottir et al., 2015)

Also an imaging genetics study on individuals with SRD and controls has shown a correlation of a deletion in the second intron of the *DCDC2* gene with FA values in the left arcuate fasciculus and splenium of the CC irrespective of their reading abilities. (Marino et al., 2014)

Taken together with the study V, these studies suggest that DYX1C1, DCDC2, KIAA0319 and ROBO1 and CEP63 may all be involved in the development of the posterior CC, possibly reflecting a common pathway in SRD.

7 CONCLUSIONS

In this thesis we have made progress in elucidating the function of three SRD candidate susceptibility genes: *DYX1C1*, *DCDC2* and *ROBO1*. In study I we revealed that DYX1C1 may be involved in regulating ERs. After our studies also the *CYP19A1* gene (that encodes for the aromatase enzyme) has been suggested to be involved in SRD (Anthoni et al., 2012). Together these findings suggest that it may be worthwhile to investigate whether hormonal factors play a role in SRD.

In study II we found a role for DCDC2 in ciliary signalling pathways and in controlling microtubule dynamics within the cilium. After our studies other findings have firmly established the role of DCDC2 and also DYX1C1 in ciliary function. Some SRD candidate susceptibility genes have been shown to function in the centrosome, which is functionally coupled to the cilia during the cell cycle. Based on these findings Chandrasenkar et al. have proposed that SRD could be considered as a ciliopathy. (Chandrasekar et al., 2013)

In study III we detected several novel variants belonging to the SRD susceptibility haplotype in the DYX5-linked family. We also suggested a possible mechanism for the reduced expression of *ROBO1* in the DYX5-linked family; the LHX2 transcription factor may bind to a distant regulatory sequence of *ROBO1* with higher affinity to the allele specific to the SRD susceptibility haplotype. The possible regulation of *ROBO1* by LHX2 is interesting because in mice LHX2 has been shown to regulate *Robo1* in thalamocortical axonal guidance of the auditory pathways. (Marcos-Mondejar et al., 2012)

We also studied the function of ROBO1 in brain development. In study IV we showed that ROBO1 is involved in regulating axonal midline crossing of the auditory pathways in the DYX5-linked family, and in study V we showed that ROBO1 is involved the development of the CC in the normal population. Both of these results fit well with the observations of ROBO1 in animal models that have shown that ROBO1 has several roles in brain development, especially in axonal pathfinding.

The major limitation of our molecular studies is that the cell line models that we have used may not correspond well to circumstances in the developing brain. Perhaps in the future induced pluripotent stem cells differentiated into neurons may serve as better models for research. (Robinton and Daley) The major limitation of our brain imaging studies is their small size, and thus there is a demand for independent replication of our findings. (Button et al., 2013)

During the early stages of this thesis it was thought that there may be at least two separate molecular pathways in the etiology of SRD; the role of *ROBO1* in axonal guidance was considered to be possibly separate from the

other SRD candidate genes (*DYX1C1, DCDC2* and *KIAA0319*) involved in neuronal migration. Nowadays ROBO1 has also been shown to function in neuronal migration of both gabaegic and glutamatergic neurons in the cortex. Moreover, the connection of DYX1C1 and DCDC2 to cilia has widened the perspective on the possible function of DYX1C1 and DCDC2 as the cilium has been shown to have diverse roles in brain development, including axonal guidance. Consistently there is increasing evidence for the involvement of DYX1C1, DCDC2, KIAA0319 and ROBO1 in common neurodevelopmental or molecular pathways.

In murine models *Dyx1c1*, *Dcdc2* and *Kiaa0319* knockouts have displayed various abnormalities in auditory processing. In study III we have showed that also ROBO1 has a specific function in auditory pathways, more specifically regulating midline crossing of the axonal tracts that convey inhibitory information from the contralateral side to the ipsilateral side during binaural listening.

It has been hypothesized that SRD is a consequence of disruption in the neural circuits that function in reading. A disruption in neural circuits can be caused by disturbances at several levels of cortical development: progenitor development, neuronal migration, and neuronal connectivity. SRD candidate susceptibility genes have been shown to influence all of these stages. A consequence of the altered neuronal circuits in SRD has been proposed to be abnormal brain oscillations leading to disturbances in auditory sampling and phonological processing. (Lehongre et al., 2013)

There are considerably more suggested candidate genes now than there were in the early days of this thesis. It remains to be seen which of the many suggested SRD candidate susceptibility genes will enforce their relationship to SRD and which will not. Future studies will most probably give more information on what the most essential molecular networks are in relation to the etiology of SRD. A common feature of the findings in imaging genetic studies of the SRD candidate susceptibility genes DYX1C1, DCDC2, KIAA0319, ROBO1 and CEP63 is the involvement of the posterior CC. This suggests that they may be involved in the same pathway affecting callosal development.

SRD is an important disorder to study as the reading skills of an individual have many social aspects. Reading problems starting at school age may cause that the child is not able to keep up with his/her peers, which may have many negative consequences. If the problems persist into adulthood they may hamper many opportunities for example in working life. SRD research is also interesting from an evolutionary point of view as language is one of the major differences in humans from species that are near on the evolutionary tree. Thus learning about the genes involved in reading and language networks in general may shed light into the human evolution.

SRD is an intriguingly specific disorder; it is a somewhat counter-intuitive phenomenon that certain genetic variants can influence susceptibility to a deficit in reading without affecting overall cognitive performance. This aspect

in the etiology of SRD remains still largely unexplained but the functional characterization of the SRD susceptibility candidate genes may provide clues that may help us understand the process. The results of this thesis have contributed to the research towards better understanding of SRD.

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