SUSCEPTIBILITY GENES AND NEURODEVELOPMENTAL MECHANISMS IN DYSLEXIA

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Helsinki 2007

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ACADEMIC DISSERTATION

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"The real voyage of discovery consists not in seeking new landscapes but seeing with different eyes"

Marcel Proust

"Excuse me, can I have a pair of leather wings?"

Arttu Ahola, four years old,
at the supermarket

To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Kaminen N**, Hannula-Jouppi K, Kestilä M, Lahermo P, Muller K, Kaaranen M, Myllyluoma B, Voutilainen A, Lyytinen H, Nopola-Hemmi J and Kere J, 2003: A genome scan for developmental dyslexia confirms linkage to chromosome 2p11 and suggests a new locus at 7q32. J. Med. Genet. 40:340-345.

II Taipale M, **Kaminen N***, Nopola-Hemmi J*, Haltia T, Myllyluoma B, Lyytinen H, Muller K, Kaaranen M, Lindsberg P, Hannula-Jouppi K and Kere J, 2003: A candidate gene for developmental dyslexia encodes a nuclear tetratricopeptide repeat domain protein dynamically regulated in brain. Proc. Natl. Acad. Sci. USA 100:11553-11558.

III Hannula-Jouppi K, **Kaminen-Ahola N**, Taipale M, Eklund R, Nopola-Hemmi J, Kääriäinen H and Kere J, 2005: The axon guidance receptor gene ROBO1 is a candidate gene for developmental dyslexia. PLoS Genetics 1(4):467-474.

IV Wang Y*, Paramasivam M *, Thomas A, Bai J, **Kaminen-Ahola N**, Kere J, Voskul J, Rosen G. D, Galaburda A. M. and LoTurco J. J. 2006: DYX1C1 functions in neuronal migration in developing neocortex. Neuroscience 143:515-522.

V Linkai R*, **Kaminen-Ahola N***, Tatlisumak T, Elomaa O, Kere J and Lindsberg P. Expression of Dyx1c1 following cerebral ischaemia and heat shock – relation to heat shock proteins 70 and 90. Submitted.

* equal contribution
In addition some unpublished data are presented.
The publications are referred to in the text by their roman numerals.

ABSTRACT

Developmental dyslexia is a specific reading disability, which is characterised by unexpected difficulty in reading, spelling and writing despite adequate intelligence, education and social environment. It is the most common childhood learning disorder affecting 5-10 % of the population and thus constitutes the largest portion of all learning disorders. It is a persistent developmental failure although it can be improved by compensation. According to the most common theory, the deficit is in phonological processing, which is needed in reading when the words have to be divided into phonemes, or distinct sound elements. This occurs in the lowest level of the hierarchy of the language system and disturbs processes in higher levels, such as understanding the meaning of words.

Dyslexia is a complex genetic disorder and previous studies have found nine locations in the genome that associate with it. Altogether four susceptibility genes have been found and this study describes the discovery of the first two of them, *DYX1C1* and *ROBO1*. The first clues were obtained from two Finnish dyslexic families that have chromosomal translocations which disrupt these genes. Genetic analyses supported their role in dyslexia: *DYX1C1* associates with dyslexia in the Finnish population and *ROBO1* was linked to dyslexia in a large Finnish pedigree. In addition a genome-wide scan in Finnish dyslexic families was performed. This supported the previously detected dyslexia locus on chromosome 2 and revealed a new locus on chromosome 7.

Dyslexia is a neurological disorder and the neurobiological function of the susceptibility genes *DYX1C1* and *ROBO1* are consistent with this. *ROBO1* is an axon guidance receptor gene, which is involved in axon guidance across the midline in *Drosophila* and axonal pathfinding between the two hemispheres via the corpus callosum, as well as neuronal migration in the brain of mice. The translocation and decreased *ROBO1* expression in dyslexic individuals indicate that two functional copies of *ROBO1* gene are required in reading. *DYX1C1* was a new gene without a previously known function. Inhibition of *Dyx1c1* expression showed that it is needed in normal brain development in rats. Without Dyx1c1 protein, the neurons in the developing brain will not migrate to their final position in the cortex.

These two dyslexia susceptibility genes *DYX1C1* and *ROBO1* revealed two distinct neurodevelopmental mechanisms of dyslexia, axonal pathfinding and neuronal migration. This study describes the discovery of the genes and our research to clarify their role in developmental dyslexia.

ABBREVIATIONS

ADHD	attention-deficit hyperactivity disorder
BAC	bacterial artificial chromosome
bp	base pairs
CD/CV	common disease/common variant
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COS cells	African green monkey kidney cells
CP	cortical plate
DNA	deoxyribonucleic acid
EEG	electroencephalography
ERP	event-related potential
FISH	fluorescence *in situ* hybridisation
fMRI	functional magnetic resonance imaging
HEK	human embryonic kidney
HLA	human leucocyte antigen
IBD	identical by descent
IBS	identical by state
IZ	intermediate zone
kb	kilobase pairs (1kb = 1000bp)
LD	linkage disequilibrium
LOD	logarithm of odds
MEG	magnetoencephalography
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MZ	marginal zone
NPL	non-parametric linkage
PCR	polymerase chain reaction
PET	positron emission tomography
OR	odds ratio
QTL	quantitative trait loci
RACE	rapid amplification of cDNA ends
RR	relative risk
RNA	ribonucleic acid
RNAi	interference ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction

shRNA	short hairpin ribonucleic acid
SLI	specific language impairment
SNP	single nucleotide polymorphism
SSD	speech-sound disorder
TDT	transmission disequilibrium test
VZ	ventricular zone
YAC	yeast artificial chromosome

INTRODUCTION

Language is a mental skill that is needed for communication. It consists of sounds and symbols, which are used to express concrete things and abstract thoughts. Reading and writing are essential parts of communication, and impairment in these skills affects the everyday life of as many as every tenth individual. This disorder, called developmental dyslexia, can cause difficulties in reading due to letters that are seen backwards or errors with double consonants. Writing may be slow and handwriting illegible, and there may be clumsiness and deficits in co-ordination. The mild form of dyslexia just complicates the life of the carrier, but it can also be a reason for educational and social problems and displacement from society.

Dyslexia is a neurological disorder. It is associated with visual and auditory deficits and deficits in motor functions. Brain imaging studies have revealed altered activation in the brain regions associated with language between normal and dyslexic individuals during reading tasks. Also structural alterations in the dyslexic brain have been observed. Previous family and twin studies have shown that there is a strong genetic component behind dyslexia. Dyslexia is a complex genetic disorder: several loci in the genome have been associated with it and the influence of the environment is indisputable. Four dyslexia susceptibility genes have been found, opening up a new era in dyslexia research.

Genes have been described as offering a molecular window into the human brain (Fisher & Marcus 2006). Thus molecular genetic research could be considered the frames of those windows. The view depends on the identification of susceptibility genes, understanding the function of them and finding molecular pathways that connect these genes. In our research, we have opened a new window by finding the first two interesting susceptibility genes for dyslexia and investigating their function in brain development. The aim of our research has been to reveal the biological basis of this common disorder. It has not been resolved yet, but we have taken a great leap forward.

REVIEW OF THE LITERATURE

1. Developmental dyslexia

1.1. Definition of developmental dyslexia

Dyslexia research was started at the end of the 19th century by Doctors Kussmaul, Morgan, Kerr and Hinshelwood, who brought out a syndrome called 'word-blindness', which perfectly described the first belief of dyslexia being a visual deficit (Pennington 1990, Stein and Walsh 1997, Shaywitz and Shaywitz 2003). This "word-blindness" was later called dyslexia [in Greek dys- = impaired, lexis = word] and individuals who suffered from it were described as persons with normal or high intelligence. Some of them were found to be talented in mathematics, but all of them had problems in reading or spelling, or in both (Pennington 1990). Over the years, the description has been sharpened: currently dyslexia is defined as a specific reading disability, which is characterised by unexpected difficulty in reading, spelling and writing despite adequate intelligence, education and social environment (World Health Organization 1993). It is designated as developmental dyslexia, which emphasises the endogenous aetiology of the disorder and separates it from acquired dyslexia, which could be a consequence of an injury or a distinct disease. Developmental dyslexia is a persistent failure to acquire efficient reading skills, despite possible improvement of reading skills by compensation and training (Hayes et al. 2003, Shaywitz and Shaywitz 2003).

By definition, the word 'specific' separates dyslexia from other learning disabilities among which dyslexia constitutes the largest part, 80% (Lerner 1989, Lyon 1995). The other learning disorders are dysgraphia with a deficit in physical writing (Sandler et al. 1992), dyscalculia with difficulties in mathematics (Shalev and Gross-Tsur 2001), dyspraxia with disability of co-ordination and movements and leading to difficulties in speech (Hurst et al. 2006), and dysphasia with difficulties in articulation, expression by words and understanding speech (Gopnik and Crago 1991). Symptoms of language-related disorders such as speech-sound disorder (SSD) which is defined by difficulties in articulation, phonology and cognitive linguistic processes (Shriberg et al. 1999) and specific language impairment (SLI) with poor expressive and receptive oral language (Leonard et al. 2006), overlap with each other but also with dyslexia (Gopnik and Crago 1991, Vargha-Khadem et al. 1995). SSD, as well as speech and language disorder

(SPCH), which is also known as developmental verbal dyspraxia, are connected more intensively to articulation instead of input and processing of language. Attention-deficit hyperactivity disorder (ADHD) is often associated with learning disorders, but actually it is an externalising or behavioural disorder, not a language disorder (Pennington 2006). However, it co-occurs with dyslexia more frequently than expected by chance; as many as 25-40% of children with dyslexia have ADHD (Dykman and Ackerman 1991) and therefore they most probably share some aetiological risk factor.

1.2. Prevalence and heritability

Dyslexia is the most common childhood learning disorder affecting 5-10% (Pennington 1990, Shaywitz et al. 1992, Snowling 2000) or as many as 17,5% (Shaywitz 1998) of the population. The number of dyslexic individuals is remarkable and it has been discussed whether reading ability occurs along the continuum and dyslexia represents the lower end of the normal distribution (Shaywitz et al. 1992). The wide range of prevalence is partly caused by inconsistent diagnostic criteria for variable dyslexia phenotypes. In addition, the frequency of dyslexia differs between languages; among Finnish adults the prevalence is about 6% (Lyytinen et al. 1995). It has been assumed that dyslexia in different languages has a universal biological origin and that the prevalence depends on the orthography of the language. Furthermore, a language with transparent orthography like Finnish is easier to read than a language with deep orthography like English, because in Finnish each letter corresponds more accurately to a certain sound (Paulesu et al. 2001). However, research among English-speaking Caucasians and Chinese-speaking Asians has shown that the origin of dyslexia is dependent on writing systems (e.g. alphabetic and Chinese logographic system) (Siok et al. 2004), which suggests that letters and logographic symbols are processed by different methods in the language regions of the brain. It has been traditionally thought that dyslexia is more common among males than females. Recent studies have indicated that there most likely exists some gender-specific factor, which is not as strong as had been thought (Pennington et al. 1991, Rutter et al. 2004, Liederman et al. 2005).

In the beginning of the 20th century it became evident that dyslexia is clustered in certain families (Thomas 1905, review by Pennington 1990). The familial and heritable character of dyslexia was proven in 1950, when the first large-scale family study was carried out by Hallgren (Hallgren 1950, reviewed by Pennington et al. 1991). Family and twin studies have shown that there is a strong genetic component behind dyslexia. The concordance in

the diagnosis of developmental dyslexia is higher in monozygotic (68%) than dizygotic (38%) twins (DeFries and Alacron, 1996). According to previous studies it is estimated that heritability is between 40-70% (Gayan and Olson, 2003), and family history is one of the most important risk factors (Shaywitz and Shaywitz 2003). Clinical studies have shown that up to 50% of children of dyslexic parents, 50% of siblings of dyslexics and 50% of parents of dyslexic children are affected (Finucci 1976). In addition to familial and inherited nature, Hallgren also presented that the inheritance of dyslexia is autosomal dominant, which was disproved afterwards. Due to genetic heterogeneity, dyslexia has turned out to be a complex trait without classical Mendelian inheritance with some exceptions.

1.3. Theories of developmental dyslexia

1.3.1. The phonological theory

The effective use of language requires the interaction of memory with sensory input and motor output systems. The principal types of memory required for language are phonological (the sound of words), orthographic (the spelling of words) and semantic (our knowledge of the words) (Price 2000). The hierarchy of these components forms a language system. At higher levels there are neural systems engaged in processing like semantics, syntax and discourse. In other words, understanding the meaning of words, grammatical structures and connecting words to the sentences appears there. At the lowest level there is the phonologic module dedicated to processing the distinctive sound elements that constitute language. The functional unit of the phonological module is the phoneme, defined as the smallest discernible segment of speech. For example, the word "bat" consists of three phonemes: /b/ /æ/ /t/. Speech is a natural and inherent skill and a child only has to be in an environment where the language can be heard. To speak a word, the speaker retrieves the word's phonemic constituents from his or her internal lexicon, assembles the phonemes, and utters the word. Instead of that, reading ability is acquired and must be taught. To read a word, the reader initially must divide the word into its underlying phonemes (Shaywitz et al. 2003). Most evidence suggests that deficits in dyslexia are in the lowest level of the hierarchy, in phonological processing (Shaywitz et al. 1999, Ramus et al. 2003).

1.3.2. The magnocellular theory

Sensory input to phonological, ortographic and semantic memories can occur via auditory processing for spoken words and sounds, visual processing for written words and signs, or tactile processing for braille used by blind readers (Price 2000). Deficits in visual and auditory systems, visual discrimination (Livingstone et al. 1991) and weakness in auditory segmenting (Stein and Walsh 1997), have commonly been associated with dyslexia. This supports the magnocellular theory, in which dyslexia is caused by impaired sensory processing due to either abnormal auditory or visual magnocellular pathways, or both. Anomalies in the thalamus of dyslexic individuals have been observed (Tallal et al. 1990, 1993; Eden et al. 1996, Stein and Walsh 1997) and the thalamus is known to be the co-ordinator between sense organs and the cortex for all sensory pathways, except sense of smell (Bear et al. 2007). Magnocells of magnocellular layers in the thalamus have been observed to be significantly smaller in dyslexic than control readers (Galaburda et al. 1985). In addition to a deficit in hearing and seeing, dyslexia is associated with motor symptoms such as clumsiness and delayed motor milestones (Fawcett and Nicholson 1999, Stoodley et al. 2005).

The phonological, visual, auditory and motoric deficits are combined into the general magnocellular theory. According to that, the dysfunctions of cells in the magnocellular pathway affect all sensory modes and also the posterior parietal cortex and the cerebellum (Stein and Walsh 1997). The cerebellum is known to have an important role in sensory perception and motor output (Bear et al. 2007). The cerebellar deficit hypothesis has combined deficits in motor skills, balance and automation in dyslexic individuals to alterations in cerebellum, such as decreased cerebellar activation in brain imaging studies (Nicolson et al. 2001).

1.3.3. Combining the two theories

All these symptoms which are associated with phonological and magnocellular theories have been combined in a hypothesis of impaired temporal processing, which suggests that dyslexic individuals may be unable to process fast incoming sensory information adequately in any domain in the brain (Stein and Walsh 1997). This theory is challenged by the finding of a relatively small number of dyslexics who have sensory deficits (Ramus 2004). The phonological and magnocellular theories are also combined in a hypothesis, which suggests a common mechanism in brain development causing a variety of symptoms depending on the place of abnormality (Ramus 2004). According to the bottom-

up theory, the alterations in the thalamus lead to the abnormal structure of certain cortical regions. This scenario could explain how auditory deficits cause phonological deficits and how visual deficits cause visual-spatial attentional problems, as described in the magnocellular theory (Ramus 2004). In the scenario called top-down, the causal direction is opposite and anomalies in the development of the cortex generate thalamic anomalies. This theory has been supported by animal studies (Herman et al. 1997, Peiffer et al. 2002), which suggests that the primary deficit in dyslexia is in phonological processing whereas the sensory impairments are secondary alterations.

The magnocellular and phonological theories are also combined in the disconnection theory, due to alterations in the brain in particular regions (Paulesu et al. 1996, Klingberg et al. 2000). According to the disconnection theory, the connections between the different components of the language system are weak. This could be caused by alterations in the common mechanisms in brain development, as hypothesised before by Ramus (2004).

1.3.4. Other theories

In the GBG hypothesis Doctors Geschwind, Behan and Galaburda suggest that autoimmune disorders like coeliac disease and learning disorders like dyslexia are more common among left-handed individuals (Geschwind and Behan 1982, Geschwind and Galaburda 1985a, b, c). According to that hypothesis, the high level of testosterone during foetal development delays the development of the left hemisphere and causes learning disabilities, whereas left-handedness has been associated with a larger and more dominant right hemisphere. The high testosterone level has an effect on the thymus where T-cell maturation occurs and that could cause exposure to the autoimmune disorders. This theory has been received with a lot of disagreement (Gilger et al. 1998, Segalowitz et al. 1994). The association between autoimmune disorders and dyslexia is still topical due to significant positive correlations between them (Pennington 1987, Hugdahl 1990, Tonnessen et al. 1993) and the dyslexia candidate locus in HLA-region on chromosome six (Smith et al. 1991, Cardon et al. 1994, 1995).

Dyslexia has been proposed to be linked to fatty acid metabolism and diet of omega-3 and omega-6, especially of highly unsaturated fatty acids (HUFA), but no significant results have been found (Baker et al. 1985, Richardson and Ross 2000, Richardson et al. 2003, Richardson 2006). However, biological facts of the importance of fatty acids in brain development (Das and Fams 2003, do Nascimento and Oyama 2003) as well as suggestive

results with learning disorders (Richardson 2006, Cyhlarova et al. 2007) support the role of fatty acids in dyslexia. Whether the role is as an environmental factor such as diet or whether it involves a deficit in fatty acid metabolism, and how crucial it really is, remains to be seen.

The most recent theory, the perceptual noise exclusion hypothesis suggests that the deficit in dyslexia is in the ability of distinguishing visual signals despite the disturbing factors around. This implicates non-optimal tuning of neurons which have been speculated to be caused by some impairment in GABA neurotransmitter system in the cortex (Sperling et al. 2005, 2006).

1.4. Testing for dyslexia

In order to distinguish real dyslexic individuals from poor readers due to social or educational reasons, adequate tests have to be performed. There are no universally accepted thresholds or definitions for diagnosis of developmental dyslexia and that complicates the analysing of research results. Commonly dyslexia is defined as two years retarded reading ability compared to chronological age (Williams and O'Donovan 2006). The phonological deficit of dyslexics is the main definition for developmental dyslexia, and includes three main components. Phonological awareness is the ability to access and manipulate speech sounds consciously. It is tested by rhyming, syllable counting and sounding out pseudo-words (Temple et al. 2003, Ramus 2004) like "vaappo" or "esmeri" in Finnish and "melk" in English. Lexical retrieval reveals how quickly a person introduces the word's phonemic constituents from their short-term memory and it is tested by rapid naming tasks. The third component of phonological processing is a verbal short-term memory, which is tested by digit span or non-word repetition (Ramus 2004). Orthographical skills are needed for changing letters to phonemes. It is tested for example by two visually presented letters and the tested individuals should recognise if they are the same or not (Temple et al. 2001). Poor orthographical letter processing, also called poor grapheme-phoneme mapping is considered the fourth component of phonological deficit and also as a consequence of poor phonological awareness (Ramus 2004). After different tests of dyslexia the results are compared to cognitive skills as determined by estimation of general intelligence.

2. The neural basis of dyslexia

2.1. Dyslexia as a neurodevelopmental disorder

The human brain consists of two hemispheres, where language function is associated predominantly with the left (perisylvian) cortex, in the association region of the parietal-temporal-occipital complex (Sun et al. 2003, Bear et al. 2007). The auditorial association region is in the temporal region and the visual association region is in the posterior region of the brain. The neural system was first associated with reading by a French neurologist Dejerine who suggested, at the end of the 19th century, that left posterior brain regions, parietotemporal and occipitotemporal areas, are critical for reading (Dejerine 1891, 1892 reviewed by Shaywitz and Shaywitz 2003). Post-mortem studies of dyslexic brains have also shown structural changes, ectopias and microgyri. Ectopias, small nests of abnormally placed neurons, have been found in the cortex and are considered neuronal migration anomalies during foetal development in the neocortex (Galaburda et al. 1985, Chang et al. 2005, Sokol et al. 2006). These neurons have missed their target in the cortex, altered the normal six-layer structure of it and are associated to the regions, which are involved in the reading process. Microgyri are more severe alterations in the organisation of all cortical layers (Galaburda et al. 1985). Recent neuroimaging studies of developmental dyslexia have indicated that dyslexic brains have a variety of structural alterations and functional disruptions. Due to the wide spectrum of techniques, many subtle methodological variances and differences in methods in selecting and testing participants, results of the studies have often been inconsistent. After all, the network of three regions, parietotemporal and occipitotemporal regions and inferior frontal gyrus, all primarily in the left hemisphere, were found to be essential for the reading process (figure 1, Shaywitz et al. 2002, Turkeltaub et al. 2003).

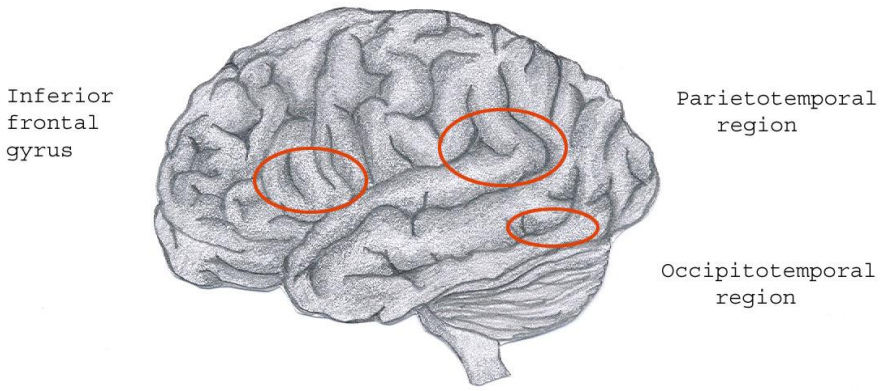

Figure 1. Language regions in the left hemisphere.

2.1.1. Parietotemporal region

Neuroanatomic lesions in the parietotemporal area were described in research on acquired inability to read, alexia (Shaywitz and Shaywitz 2003). There have also been changes in the symmetry of left planum temporale, which is a triangular region on the upper surface of the temporal lobe, between post-mortem dyslexic and the normal reader's brain (Geschwind and Levitsky 1968, Heim and Keil 2004). Typically this brain area is larger in the left hemisphere, but this asymmetry is not seen in the dyslexic brain. This could be a result of decreased cell death in the right planum temporale during foetal development, which affects the neuronal connections (Galaburda et al. 1987, Paul et al. 2006). These studies are criticised because of the small number of samples and technical details concerning post-mortem dyslexic brain samples like long storage period (Shapleske et al. 1999, Heim and Keil 2004).

Magnetic resonance imaging (MRI) technique gives anatomical information based on nuclear magnetic resonance. It does not have enough resolution to identify histological anomalies in subcortical or cortical regions, but it is however capable of providing information of disorders affecting brain structures. The MRI studies have revealed similar morphological alterations in the reduced or reversed asymmetry of parietotemporal language regions in the dyslexic brain, as was found in post-mortem research (Hynd et al. 1990, Larsen et al. 1990, Duara et al. 1991, Kushch et al. 1993, Dalby et al 1998, Robichon et al. 2000), but there are also negative findings (Grigorenko 2001, Leonard et al. 1993, 2001). Interestingly, also some evidence has been found showing that reduction

of asymmetry is caused by alterations in interhemispheric pathways, which go through the corpus callosum to the perisylvian language regions (Galaburda et al. 1990, Filipek 1995). In addition, voxel-based morphometry (VBM) technique, which is used to characterise regional cerebral volume and tissue concentration differences in structural MRI, has shown reduced grey matter in the parietotemporal region of dyslexic brain (Brown et al. 2001, Silani et al. 2005, Vinckenbosch et al. 2005). Furthermore, a novel MRI technique called diffusion tensor imaging (DTI), which provides information about white matter microstructure *in vivo*, has indicated alterations in the left parietotemporal region in dyslexic readers. The severity of these microstructural alterations in white matter tracts correlated with reading ability and the alterations are suggested to reduce the communication between cortical areas involved in visual, auditory and language processing (Klingberg et al. 2000). This supports the disconnection theory in developmental dyslexia.

Positron emission tomography (PET) and functional magnetic resonance imaging method (fMRI) measure the changes in blood flow that are induced within large populations of neurons. Several functional neuroimaging studies have shown altered activation in dyslexic brains so that most consistently dyslexia has been associated with reduced or absent activity in the left temporal-parietal regions (Paulesu et al. 1996, Rumsey et al. 1997, Shaywitz et al. 1998, 2002; Brunswick et al. 1999, Paulesu et al. 2001, Temple et al. 2001). Studies with dyslexic children have revealed that parietotemporal region could be considered a core of phonological deficit. Dysfunction has been there from early childhood and it is not a consequence of poor reading (Shaywitz et al. 2002).

2.1.2. Occipitotemporal region

The occipitotemporal region has been also linked to dyslexia in several brain imaging studies (Horwitz et al. 1998, Brunswick et al. 1999, Temple et al. 2001, Paulesu et al. 2001, Shaywitz et al. 2002, 2003). This region is considered an interface between processing of visual information like written words from visual cortex and language domains in the parietotemporal region and inferior frontal gyrus (Tarkiainen et al 1999, Devlin et al. 2006). The occipitotemporal region is activated by real words and pseudo-words in a similar way, which suggests that stored visual information is pre-lexical, just combinations of letters (Cohen et al. 2000, 2002).

2.1.3. Inferior frontal gyrus

Dyslexia has been associated with unusual or enhanced activity in left frontal-lobe language regions in dyslexic adults (Paulesu et al. 1996, Salmelin et al. 1996, Rumsey et al. 1997, Shaywitz et al. 1998, Brunswick et al. 1999, Paulesu et al. 2001, Tallal et al. 2001). Instead of reading, this frontal-lobe language region, also known as Broca's area, is associated with generating word associations, which is a necessary part of sentence production (Price 2000). This indicates the region's role in the higher levels of the linguistic system. Interestingly, the positive correlation between age and activation in inferior frontal gyri of dyslexic children has been found, which indicates that frontal sites attempt to compensate for the dysfunction of the posterior regions (Brunswick et al. 1999, Shaywitz et al. 2002, Temple et al. 2003). Furthermore, this supports the model of interactive compensation, which suggests that well developed higher level reading subskills can compensate for weaker lowel level subskills and thus cause individual differences in reading achievement (Chiarello et al. 2006). In addition to activity in frontal gyri, increased activity of the right occipitotemporal region was also detected in compensation of poor readers (Shaywitz et al. 2000).

In addition to these three main regions affected in dyslexics, the reduction of activation or altered structure of the thalamus (Brunswick et al. 1999) and left cerebellum (Brunswick et al. 1999, Leonard et al. 2001, Eckert et al. 2003) have also been observed, which supports the magnocellular theory and cerebellar deficit hypothesis, respectively.

2.1.4. Electrophysiological studies

Brain processes with high temporal resolution are detected by electrophysiological recording techniques like electroencephalography (EEG) and magnetoencephalography (MEG). Results from both fMRI and MEG studies have suggested the significance of the parietotemporal region in dyslexia and indicated that dysfunction in posterior cortical regions leads to compensation in frontal-lobe systems (Salmelin et al. 1996, Shaywitz et al. 1998, Brunswick et al. 1999). Event-related potentials (ERPs) are measured by EEG and they can be used to detect the brain activity associated with speech sound processing in dyslexia studies (Leppänen and Lyytinen 1997). The auditory ERP and MEG studies have indicated that neural processing of rapid auditory stimuli is disrupted in dyslexia (Kraus et al. 1996, Nagarajan et al. 1999, Temple 2002). The results prove that there are differences in brain electrical activation even in six-month-old children with or without a

risk for dyslexia (Leppänen and Lyytinen 1997, Pihko et al. 1999, Leppänen et al. 2002). Differences are detected also in newborns, and therefore auditory ERPs could be considered a predictive measurement for later language and neurocognitive outcomes (Guttorm et al. 2005). According to electophysiological studies, the problems observed with phonological analysis among dyslexic individuals could be derived from impairment in low-level auditory transient processing, which is suggested to be derived from magnocellular deficit of auditory magnocells in the thalamus (Stein and Walsh 1997). It has been shown that in addition to visual magnocells, auditory magnocells have also been abnormally smaller in the dyslexic brain (Galaburda et al. 1994). Interestingly, the distinct auditory ERPs have also been detected in a mouse line which has an increased incidence of ectopias, very similar to those found in the dyslexic brain, compared to normal mice (Frenkel et al. 2000).

2.1.5. Remediation and neuroplasticity in dyslexic readers

The remediation of reading ability can occur, in addition to compensation by other brain regions, also by neuronal changes in regions important in the reading process. This kind of plasticity has been evoked by training programmes, which include exercises to improve temporal processing and phonological awareness skills of learning-impaired children (Hayes et al. 2003). The results of these exercises among dyslexic children have been measured in electrophysiological (Kraus et al. 1995, Kujala et al. 2001), MEG (Simos et al. 2002) and fMRI (Temple et al. 2003) studies. According to studies, the reading skills of both dyslexic children (Kujala et al. 2001, Temple 2003, Strehlow et al. 2006) and adults (Eden et al. 2004) have been improved by training. Audiovisual training without linguistic material suggested that the deficit in dyslexia occurs not only in phonological processing but also in the dysfunction of general sensory discrimination (Kujala et al. 2001). Studies of neuronal plasticity have also indicated that in addition to attention and motivation, an adequate type of programme is needed for neural changes in the brain (Ahissar et al. 1992, Singer 1995, Kujala et al. 2001, Strehlow et al. 2006). The central nervous system has higher plasticity in early developmental stages (Singer 1995) and thus remediation should be started as early as possible.

2.2. Neuronal migration in brain development

In order to understand the structural and functional alterations in brain development, phenomena such as morphology, development and cell migration should be understood.

The neural plate of the ectoderm, the most external of the three embryonic layers, will eventually form the central nervous system (CNS). At first the neural plate forms a neural tube, which differentiates into two major parts, spinal cord and the brain. The brain differentiates into three parts: brain stem, forebrain or cerebrum and cerebellum. The forebrain consists of left and right hemispheres, which are specialised for distinct cognitive and behavioural functions (Sun et al. 2003, Bear et al. 2007). For example, the language function is predominantly localised in the left perisylvian cortex in 97% of right-handers and ~60% of left-handers (Galaburda et al. 1978, Geschwind and Miller 2001). Both of the hemispheres have a grey cortex with mainly somas of neurons and white matter consisting of axons of neurons surrounded by myelin sheaths of glia cells. Due to the distinct functions of the two hemispheres, their structure, the types of cells, neurotransmitters and receptors are distributed in an asymmetric way. The cortical regions are joined by association comissures. The hemispheres are linked by the corpus callosum, which is the largest fibre tract in the brain. It consists of commissural axons, whose function is to transfer information between two hemispheres to co-ordinate their localised functions (Richards et al. 2004).

2.2.1. Migration of cortical neurons

The development of the forebrain depends on radial and tangential cell migrations, which are critical for the formation of the complex structure of the brain. Cell migration takes place from their site of origin to their final destination and the best characterised is radial migration of projection neurons, from the neural tube to the surface of the brain (Pilz et al. 2002). Cells proliferate near the centre of the neural tube, in a region called the ventricular zone (VZ, figure 2A). Cortical development begins when cells migrate radially and tangentially to form a preplate, which is a horizontal neuronal network. When the preplate is established, migration is continued and radially oriented neurons from the VZ form a cortical plate (CP). The cortical plate divides the preplate population in two horizontal cell layers: the uppermost layer is called the superficial layer or the marginal zone (MZ), and under the cortical plate is the subplate (figure 2B). These three layers form a thin, grey layer of the forebrain. Between the grey layer and the VZ remains a wide layer of white matter called the intermediate zone (IZ). Subsequently, the cortical plate grows and six separate layers can be detected in it. The first migrating cells stay in the deeper layers and later born cells will migrate a longer way to the surface (Lambert de Rouvroit and Goffinet 1998, Pilz et al. 2002). In an evolutionary aspect, these cortical layers are the part of the brain developed latest and uniquely mammalian (Ayala et al. 2007).

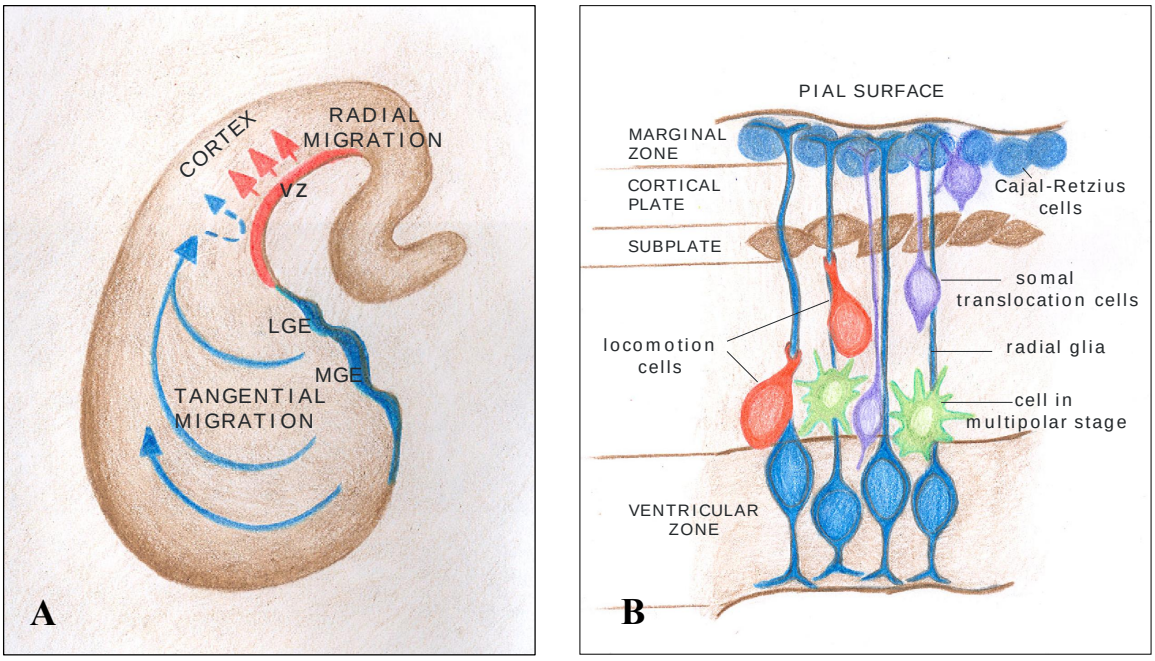

Figure 2. A: Routes of radial (red) and tangential (blue) neuronal migration in neuronal tube from ventricular zone, medial- and lateral ganglionic eminence (MGE, LGE) to the cortex. **B:** Radial organization of the cortex. Projection neurons are born from the radial glia cells in VZ and migrate along radial glial fibers toward the pial surface. The first cohort of neurons constitutes the preplate. The subsequental waves of migrating neurons split the preplate into two layers: the superficial marginal zone with Cajal-Retzius cells and the deeper subplate. The cortical plate, which forms six cortical layers, will be formed between these zones. Two modes of radial migration, somal translocation and locomotion and also neurons in multipolar stage are shown in the picture (modified from Ayala et al. 2007).

Radial migration

Three models of radial migration for cortical neurons, which form the cortical plate, have been detected. In the locomotion model, entire cells migrate and are guided by radial fibres of radial glial cells. In the nuclear translocation model, the cells first extend leading processes in the direction of migration and then move the nucleus through the elongated process to its destination (Nadarajah et al. 2001, figure 2B). In addition to these two models, a new model was recently found, multipolar migration (Tabata and Nakajima 2003). Multipolar migration differs from the previously detected locomotion and nuclear translocation models in many ways: by morphology, the changing direction and the rate of migration and sometimes by even staying in the same position (Tabata and Nakajima 2003). This model of migration is also considered more like a particular developmental

stage for cortical neurons, which indicates that migrating neurons in the brain development undergo a series of morphological transitions (LoTurco and Bai 2006, Kriegstein and Noctor 2004). After a start as bipolar progenitors and precursors in the ventricular zone (VZ), cells become multipolar and branched as they leave the VZ and enter the lower intermediate zone (Tabata and Nakajima 2003, Kriegstein and Noctor 2004). Within the IZ cells they re-establish a bipolar morphology as they migrate towards and into the CP (Noctor et al. 2002). In radial migration, the transition into and out of the multipolar stage is a sensitive state for disruptions in migration of neocortical neurons (Nagano et al. 2004, Tsai et al. 2005, LoTurco and Bai 2006).

Tangential migration

Tangential cells migrate orthogonally to the direction of radial migration and they have different mechanisms of cell guidance (Marin and Rubenstein 2003). The role of tangential migration in CNS development is still less than clear, but it is performed by interneurons, which are mainly inhibitory neurons containing gamma-aminobutyric acid (GABA) neurotransmitter. In contrast, the neurons from radial migration are mainly excitatory pyramidal cells. Both inhibitory and excitatory neurons are involved in information processing in the nervous system as well as in signalling between the nervous system and other systems (Zhu et al. 1999). Interneuronal cells originate in the VZ (Tan et al. 1998) or extracortical ganglionic eminence in the basal forebrain (Anderson et al. 1997, Zhu 1999), and they turn to radial migration when needed (figure 2A). The place of birth of the neuron determines its route of migration, while the developmental state of the brain determines the phase of migration, which is defined to occur along either the marginal zone, or subventricular zone, and the intermediate zone (Anderson et al. 1997, Parnavelas 2000, Anderson 2001). Eventually, the neurons reach their final position within the correct cortical layer (Anderson 2001, Kriegstein and Noctor 2004) using a mechanism that is still unknown.

2.2.2. Axon guidance in migration

Normal development of the cortex in the brain consists of neuronal migration and layer-targeted determination, and requires activity-dependent signalling and refinement (Sur and Rubenstein 2005, LoTurco and Bai 2006). The process of migration is interactive and there are several groups of molecules that guide axons and regulate cell migration: receptors, transcription factors, adhesion molecules, extracellular matrix proteins,

diffusible and intracellular signalling molecules, and components of associated signalling cascades (Kriegstein and Noctor 2004). These molecules are also required for proper axonal pathfinding between the cortices of the left and right hemispheres, through the corpus callosum. Growth cones of migrating neurons are attracted or repelled by guidance molecules, such as *robo* and *slit* (Li et al. 1999, Yuan et al. 1999). Nervous systems of insects and vertebrates are bilaterally symmetric and have special midline structures, which establish a partition between the two mirror image halves (Kidd et al. 1998). *Robo* (*roundabout*) was first found in *Drosophila*, when a large-scale mutation screening was used to identify genes that control the decision by axons to cross that midline. A mutation in *robo* caused some axons, instead of staying on their own side of the midline of central nervous system, or crossing the midline just once, to now cross it multiple times (Seeger et al. 1993, Kidd et al. 1998). This disrupts the structure of axon comissures, which link two sides of the nervous system together.

The Drosophila gene *robo* and its ligand *slit* have several counterparts in mammals, named *Robo1* to *Robo3* and *Slit1* to *Slit3,* respectively, in rodents. The orthologous human genes are *ROBO1* to *ROBO4* and *SLIT1* to *SLIT3* (Aruga et al. 2003a, 2003b). The function of *Robo1* in brain development has been studied in a mouse model. As in *Drosophila,* it is known as an axon guidance receptor gene, which encodes a receptor on the surface of axon growth cone. A secreted extracellular matrix protein Slit was observed to be a chemorepulsive ligand for *Robo1* and together they form a repulsive guidance system, which regulates axon branching and commissural axon pathfinding (Yan et al. 1999). In addition to the formation of the corpus callosum and hippocampal comissure, Slit/Robo signalling also regulates tangential cell migration (Hu 1999, Wu et al. 1999, Zhu et al. 1999, Andrews 2006). *Robo1* mRNA has been detected in the developing neocortex and in the proliferative zone of basal forebrain (Marillat et al. 2002). Furthermore, *Robo1* expression in the cortex was identified in well-defined routes of tangentially migrating GABAenergic interneurons. In *Robo1* knockout mice increased number of interneurons enter the cortex and reach the target earlier compared to normal control mice (Andrews et al. 2006). Observations on the *Slit1* and *Slit2* knockout mice suggest that Slits are guides for callosal, corticothalamic and thalamocortical axons in pathfinding (Bagri et al. 2002). *Slit1/2* and *Slit2* knockout mice show no defect in interneuron migration into the cortex. Furthermore, *Slit* knockout mice have malformations in thalamocortical axons, whereas in *Robo1* knockout mice these axons reach their destination earlier than control mice (Bagri et al. 2002, Andrews et al. 2006). However, differences between *Robo1* and *Slit* knockout mice indicate that the signalling is more complex and additional mechanisms are involved.

2.2.3. Deficits in neuronal migration

Development of CNS is an extremely complex process, where cascades of intracellular and extracellular processes are interconnected. Based on genetically engineered or spontaneously occurred mouse models, these unclear mechanisms are beginning to be better understood. Developmental brain disorders can be caused by molecules in two main categories: molecules in actin and microtubule cytoskeletal stability and remodelling, or molecules in migratory guidance (Pilz et al. 2002). The gene *Reelin* was the first component of a signalling pathway which guides cells to the correct location in the cortex. It was found by chance, due to a behavioural deficit of mice (Alter et al. 1968) and the neuropathology of it has been studied. In Reeler mutants, the normal order of cortical layers has been turned upside-down (Caviness 1982). The *Reelin* gene encodes a putative extracellular matrix protein which is found extracellulary in layer I in the cortex (D'Arcangelo et al. 1997). It binds to a number of receptors in migrating cells and is considered a stop-signal for migration when the target is α3β1-integrin (Dulabon et al. 2000).

Genetic studies in mice and humans have revealed more molecular determinants of neuronal migration. Mutations in two genes, *LIS1* and *doublecortin X* (*DCX,* named for its X chromosomal locus), have revealed deficits in microtubule cytoskeletal stability and have been found in individuals with disorders called type 1 lissencephaly and subcortical laminar heterotrophia (SCLH) or double cortex (DC). Individuals with type 1 lissenchepaly typically have severe mental retardation and intractable epilepsy. Instead of six cortical layers, there are four unorganised layers of neurons and smooth surface on the brain. Individuals suffering from SCLH display milder mental retardation than in lissencephaly, less-severe epilepsy and affected males have a more severe phenotype than females (Berg et al. 1998, Gleeson and Walsh 2000). In females with mutated *DCX,* the migration is arrested halfway to the cortex. Most of the point mutations in these syndromes have been detected in the conserved doublecortin peptide motifs of *DCX* (Meng et al. 2006). These mutated genes are suggested to cause disorganisation of both pyramidal and interneurons and thus affect both radial and tangential migration (Meyer et al. 2002, Kappeler et al. 2006, Bai et al. 2003, Koizumi 2006). *Lis1* and *DCX* encode microtubule-associated proteins (Sapir et al. 1997, Gleeson et al. 1999), of which Dcx is involved in coupling between the nucleus and centrosome during neuronal migration, and thus it has an essential role in translocation of the nucleus and the bipolar shape of neuroblasts in adult mouse forebrain (Koizumi et al. 2006). When expression is inhibited, the migration occurs in a less organised manner, with shorter nuclear jumps and shorter,

more unstable branches (Kappeler et al. 2006). Although the migration speeds and routes seem to be the same, as well as the response to guidance cues, fewer neurons find their way to the correct final position (Kappeler et al. 2006, Koizumi et al. 2006).

The mutation in *RELN,* human homologue of *Reelin*, is associated with autosomal recessive type 1 lissencephaly with severe abnormalities of the cerebellum, hippocampus and brainstem (Hong et al. 2000). Furthermore, a *Very low density of lipoprotein gene* (*VLDLR*), which is associated with cerebellar ataxia, mental retardation, cerebellar hypoplasia and cerebral gyral simplification (Schurig et al. 1981), has been found to be a part of the *Reelin* signalling pathway, which guides neuroblast migration in the cortex (Boycott et al. 2005). Mutations in all *DCX*, *LIS1, RELN* and *VLDLR* cause deficits in the laminar architecture of the cortex and thus are a link between these genes. Together they are responsible for 70% of the cases of type 1 lissenchepaly (Leventer 2005, Keays et al. 2007). Recent studies have indicated the role of *DCX* as a microtubule regulator (Moores et al. 2006) and furthermore the presences of mutations in *TUBA3*, a human homologue of α-1 tubulin, are present in two patients with type 1 lissenchepaly. Interestingly, one of the mutations locates in the region of *TUBA3,* which probably interacts with *DCX* (Keays et al. 2007).

Findings on these genes, the biological functions, which associate to cytoskeleton and axonal guidance in neural migration during brain development, represent successful discoveries in the research of developmental disorders. Identification of the genes and finding out their functions will reveal new information on the disorders, possibly new treatments in the future and it also will reveal the complex molecular mechanisms behind the development of the brain.

3. Gene discovery in complex disorders

Gene discovery has undergone a huge change at the beginning of the millennium, when a draft sequence of the whole human genome was reported (Lander et al. 2001, Venter et al. 2001). The number of predicted protein coding genes has decreased from 30,000 to 20,000-25,000, which consist of coding regions covering just 1.2% of the whole sequence of the genome (International Human Genome Sequencing Consortium 2004). The importance of regulatory elements, transcription factors and splice variants for the proper functioning of the genome has been understood, and the relatively new field of epigenetics has emerged in bioscience. Large-scale projects like The Human Epigenome Project

(Bradbury 2003) and HapMap Project (International HapMap consortium, 2005) produce new information about the genome. Researchers have moved from working on lab benches to more work with computers and databases, and new information has raised new questions. Research with Mendelian diseases has become easier with the completion of the genomic sequence, but the mystery of complex diseases has not disappeared.

3.1. Challenges in searching for genes of a complex disorder

3.1.1. Environmental factors

In contrast to monogenic or Mendelian disorders and diseases, complex diseases have a multifactoral genetic and environmental aetiology. In addition to the effect of an unknown number of genes, the role of one's environment is also important. It is evident, considering the results from genetic studies with identical twins; only one of them could be affected with a particular disease despite an identical genome. The environmental factor could be, for example, smoking, which might bring out the asthma phenotype (Ikäheimo et al. 2006), while smoking, as well as unhealthy eating habits, could reveal arteriosclerosis (Talvia et al. 2006). The factor could also be in the environment of the uterus; for example the nutrition of the mother could affect the epigenetic state of the developing foetus (Waterland et al. 2006). If we think of the effect of the environment as a continuum, in which there is a broken leg at one end and at the other end there is a purely genetic disorder like AGU disease (Ikonen et al. 1991), dyslexia appears to be in the middle of the continuum due to its heritability of 40-70% (Gayan and Olson 2003, Bates et al. 2007). Although genetic and environmental factors both contribute to complex disorders (Caspi et al. 2003), the effect of one's environment on a phenotype is often difficult to discern and thus genetic factors may be a fruitful starting point for studies.

3.1.2. Genetic factors

To find genes that contribute to a complex disease is a challenge due to the presence of many causal factors and their variable effects on the overall heritability and phenotype. The problem in research is often locus heterogeneity, which implies the presence of variable disease loci and susceptibility genes in affected individuals. Complex diseases are likely to be caused by several susceptibility genes that lead to a similar phenotype. This

makes genetic mapping studies challenging, as do incomplete penetrance and phenocopies. In incomplete penetrance not all individuals who have the predisposing allele will manifest the disease. More harmful for genetic mapping efforts are phenocopies, which by chance have the similar disease phenotype but not the same predisposing allele. This variation causes errors, especially in genetic analysis in which only affected individuals are used (Lander and Schork 1994).

As stated above, a variety of risk alleles can lead to a disease phenotype. There can be many rare alleles or a few common alleles in the genetic background of a disease. According to the common disease-common variant (CD/CV) theory, there are only a few predominating risk alleles at each of the major underlying disease loci (Reich and Lander 2001). The alternative to the CD/CV hypothesis is the genetic heterogeneity hypothesis, in which there are several risk alleles, each of which occurs on a low frequency in a population (Smith and Lusis 2002). In reality, the range of aetiologies of diseases is likely to be wide. For example two genes for breast cancer, *BRCA1* and *BRCA2*, have been shown to have a variety of causative alleles in the high-risk families (Wang and Pike 2004). If the CD/CV theory is true, it creates a challenge for mapping studies. Common disease-causing alleles in the population are likely to be hard to find due to the small effect of one common allele on the phenotype (Lander and Schork 1994). For example Crohn disease, a complex inflammatory bowel disease, has a susceptibility allele on chromosome 5q31 with a population frequency of 37%. Heterozygosity for this allele was first found to increase the risk for Crohn disease 2-fold and homozygosity 6-fold (Rioux et al. 2001). However, this was a result from a Canadian population, and in a replication study with samples from European patients, the disease risk conferred by this locus was only 1.49 in homozygotes (Mirza et al. 2003). The low risk of this haplotype was consistent with the results of linkage analysis in British and German populations, which did not show linkage to this region (Hampe et al. 1999). This illustrates well how the effect of genes varies between populations and why the replication studies in complex disease are challenges.

Relative risk (RR) values are considered the most important epidemiological parameters for genetic epidemiological studies. Relative risk is defined as the risk of disease in exposed individuals compared with unexposed individuals (Lander and Schork 1994, Hemminki et al. 2006). A good example of RR is offered by lung cancer studies among smokers: a relative risk of lung cancer in active smokers is about 20 and the RR of lung cancer in non-smokers who are married to a smoker is 1.2-1.3 compared to non-smoking couples (IARC 2004 according to Hemminki et al. 2006).

3.2. Advantages of isolated populations in genetic research

Finland is an advantageous place for genetic research. It has been isolated mostly for geographical, but also for geopolitical reasons. There was an early migration wave of eastern Uralic speakers some 4,000 years ago, and smaller groups arrived 2,000 years ago from the south over the Gulf of Finland. These relatively small immigrant groups have established a limited gene pool in Finland and this lead to the founder effect. The Finnish gene pool has been modified by bottlenecks, after which the population has expanded relatively rapidly. Genetic drift has also had an effect, especially in the 16th century, when internal migration caused isolated rural populations. These small populations had been stable until industrialisation and offered a place for a limited amount of alleles from an already limited gene pool to become enriched (Peltonen et al. 1999). This reduction of allelic diversity can be seen in overrepresentation of some rare, mostly autosomal-recessive Mendelian disorders in Finland. The isolated genetic history of a population may be an advantage, in addition to mapping Mendelian disorders, also to mapping complex disorders. A high level of linkage disequilibrium (LD), a restricted amount of alleles and homogeneity of mutations due to founder mutations relieve the research. In addition to the isolated population, the well-recorded population history by church records since the 15th century, homogenous environment in regard to education, lifestyle and healthcare as well as the population's positive attitude towards research, have been a great advantage for Finnish genetic research.

3.3. Discovery of genes

There are two ways to search for genes, which cause a disease or disorder: genome-wide scanning and candidate gene studies. A genome-wide scan is performed in order to identify genomic chromosomal loci and subsequently genes and alleles, which link or associate to the disease in affected individuals more often than expected. Candidate gene studies are based on a prior hypothesis that a certain gene is a plausible candidate for a given disorder and thus worth studying (Hirschhorn and Daly 2005). There are two main statistical methods involved in gene hunting: linkage and association analyses. In the mapping of complex diseases these methods are often combined. Linkage analysis is applied in studies with families while association analysis is more often used in the case of independent population samples, family trios or sib-pairs. Linkage analysis is optimal for detecting rare alleles with high impact, whereas association analysis is more appropriate and powerful for identifying common disease alleles with only modest disease risk (Rich and Merikangas, 1996). Furthermore, linkage analysis is a tool for finding a certain locus

in the genome and association analysis can be used to directly find an allele that associates to a disease phenotype.

3.3.1. Linkage analysis

Linkage analysis is a statistical method for genetic studies using families. It is based on estimation of the distance between two loci. If they are very close to each other, it is unlikely that they are separated by recombination and thus they will be inherited together. Recombination fraction (θ) is the measure, which defines the genetic distance between the loci. When two loci are independent and for example they are on different chromosomes, the recombination fraction is 0.5, which means that there is no linkage between them. The recombination fraction is zero if the loci are very close to each other and 1% recombination is defined to correspond to 1 centiMorgan (cM) or about 1 megabase in a physical gene map (Strachan and Read 1999, Gyapay et al. 1994).

Instead of two loci of genes, linkage analysis is performed between a putative disease locus, which segregates in families and a polymorphic genetic marker. Commonly used markers are microsatellites, which have highly polymorphic, short repeated sequences varying in length in populations. The overall likelihood of linkage between each marker and disease locus is calculated. The ratio of two likelihoods, that based on the observed data with the given recombination fraction and that based on the chance occurrence of linkage, gives the odds of linkage. The logarithm of the odds is called the lod score and a positive lod is evidence of linkage. A lod score greater than 3 is considered a limit for statistical significant linkage in genome-wide scans of Mendelian characters (Lander and Schork, 1994), which means that the likelihood that the linkage occurs by chance is less than 1 in 1,000 for pointwise analysis and 0.05 when corrected for multiple testing over the entire genome. In complex disorders, the threshold of reliable linkage is different. A linkage is significant if according to statistical evidence the linkage occurs 0.05 times in the genome scan (p-value $< 4.9 \times 10^{-5}$ and LOD >3.3) (Lander and Kruglyak 1995). The given LOD thresholds are for fully informative markers and families; in practice, the thresholds can be recalculated for each study by a randomisation procedure.

Parametric analysis

In a standard LOD score analysis the precise genetic model with mode of inheritance, gene frequencies and penetrance of each genotype is required and thus it is called

parametric analysis. Two-point parametric analyses are used to estimate the linkage between a locus and marker. In a multipoint analysis the locus is compared simultaneously to several linked markers, which extracts the information of all the markers and may give a more pinpointed location for the linkage. Parametric analysis can be performed by programmes like Linkage and Genehunter, depending on the structure of pedigrees and the amount of markers (Kruglyak et al. 1996). A parametric analysis is suitable for simple Mendelian traits, but with complex disorders it requires more caution and understanding of the underlying statistical assumptions of the applied models. Use of parametric analysis can be of great advantage because of its higher statistical power compared to non-parametric analysis. However, linkage analysis of complex disorders could fail for risk alleles with only a modest effect on phenotype, as was the case in Crohn disease (see 3.1.2.).

Typically, the markers in genome-wide linkage analysis are about 10 cM apart from each other. The number of markers required depends on the sample; closely related individuals and families share the same large regions inherited from common ancestors and therefore, relatively small numbers of markers are needed. After the genome-wide linkage analysis, genetic fine-mapping is performed with a dense set of markers and with an aim to narrow the candidate region in size. This depends on the amount of recombination and variations in the analysed DNA, which correlates with the number, sizes of pedigrees and genealogical origin. After fine mapping, analysis of regional candidate genes is often performed (Figure 3).

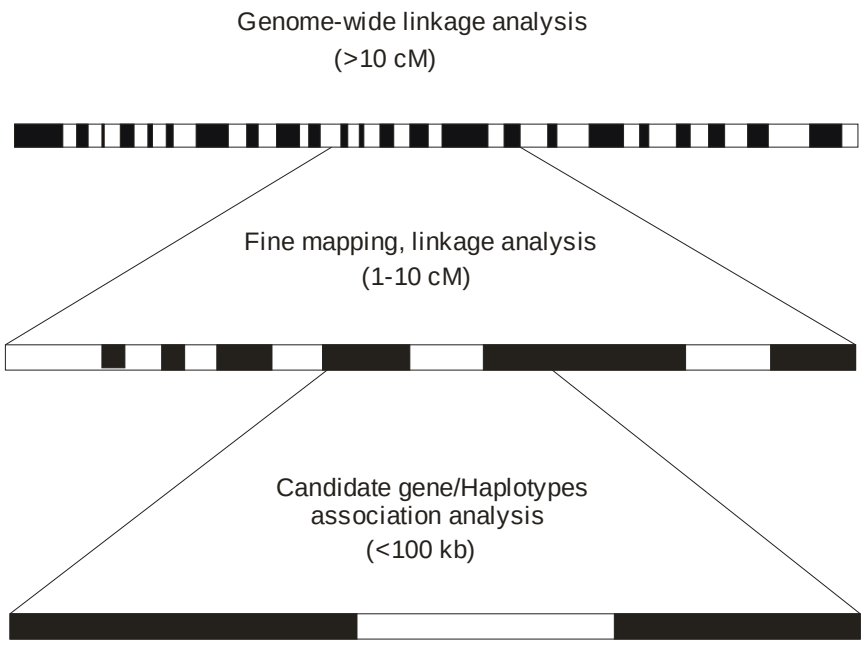

Figure 3. Scheme of mapping strategy: three steps that are commonly used in genetic analyses.

Non-parametric analysis

Non-parametric or model-free analysis is a tool for mapping genes of complex disorders. In this method the model of inheritance is not defined and it is often considered a better method for diseases with unknown levels of heterogeneity and oligogenicity. The idea is to find alleles or chromosomal regions that are shared between relatives of the same phenotype, and therefore the methods are also called allele-sharing methods. The basic hypothesis of this strategy is that individuals with a given phenotype also share their alleles identical-by-descent (IBD; in other words, similar alleles that are inherited from a common ancestor) rather than identical-by-state (IBS; in other words, similar alleles but inherited from different ancestors). A pair of siblings shares about 50% of their alleles identical-by-descent owing to random segregation. Non-parametric analysis tests which chromosomal regions show elevated IBD allele sharing in related individuals with the same phenotype (Fisher and DeFries 2002). In IBS the common ancestor of the allele at issue is not known due to missing parental samples or to the same allele in both parents. In IBD the parental samples with different alleles are needed in order to find the IBD between children. In general, IBS implies IBD when detecting rare alleles and two distinct origins are unlikely (Lander and Schork 1994, Strachan and Read 1999, Ferreira 2004).

The simple version of non-parametric analysis is the affected sib-pair method (ASP), in which unrelated small nuclear families are used (Strachan and Read 1999).

Quantitative non-parametric approaches (QTL methods)

Commonly used qualitative non-parametric approaches are based on all-or-nothing classification of affection status. When investigating quantitative traits, such as weight or length - or many language-related traits – quantitative non-parametric approaches, which take into account the correlation between genetic and phenotypic similarity of related individuals in the chromosomal region of interest, are more appropriate (Fisher and DeFries 2002). Regression analysis in sib-pairs is used to assess phenotype-genotype relationships, like in Haseman-Elston regression analysis of sib-pairs and DeFries-Fulker regression analysis of selected sib-pairs (Cardon and Fulker 1994, Fisher and DeFries 2002). Variance-components analysis involves partitioning the total variability into major-gene, polygenic and environmental factors (Amos 1994). The challenges in QTL mapping method are in the data collection, in the reliable measures and accurate phenotypic information. Clearly, traits that show large variation in an individual with time or from one test to another are not appropriate for these methods.

3.3.2. Association analysis

Association studies compare the allele frequency of a polymorphic marker in affected and unaffected individuals in a population. The study reveals markers that differ significantly between these two groups and may give a hint of which part of the suspected genome region associates with the phenotype. Single nucleotide polymorphisms (SNP) are commonly used markers today. Individually, they are less informative than microsatellite markers, but they are more common in the genome and technologically easier to genotype in large numbers. About 5 million SNPs have been registered to the GeneBank (NCBI, SNP database) and the number will rise even up to 11 million. The association between allele and phenotype is detected using a standard χ^2-test, in which the observed allele or genotype frequency distributions in cases and controls are considered. The statistical significance is evaluated using a p-value, and nominally a p-value <0.05 is regarded as significant (Lalouel and Rohrwasser, 2002). However, in genome-wide association studies or studies where numerous SNPs are tested for association, multiple testing corrections are needed and thus alter the nominal threshold. The odds ratio is often used in case-control studies, in which the risk of disease in exposed individuals is compared with unexposed

individuals. For rare diseases the odds ratio corresponds to the relative risk, but in cases of medium and high probabilities the distinction is important and thus the odds ratio is used (Hemminki et al. 2006).

Association analysis is often used right after fine-mapping of linkage analysis in candidate gene studies. If the Common Disease/Common Variant theory is proven the most common case, the association analysis will be an optimal tool (Risck and Merikangas 1997). The growing amount of information of polymorphic SNPs and the structure of the genome will enable studies on larger regions and even whole genome-wide analyses. Estimation of linkage disequilibrium plays an important role. Association analysis is divided roughly into direct and indirect strategies (Collins et al. 1997). In direct methods, polymorphic markers locate in the coding region of genes, they are common functional variants and they could be the direct reason for altered phenotype. The indirect method includes markers also in noncoding DNA and that is challenging because of the huge number of the markers that are needed if the whole genome is to be analysed (Collins et al. 1997). In practice, these strategies are combined and SNPs are often chosen in a case-specific manner, considering the aims of the study and also economical aspects.

Population stratification, which means that the expected allele frequency is altered for some other reason than caused by association to the phenotype one is interested in, can skew the result of association analysis. That is possible to avoid by carefully selecting the cases and controls for the studies (Cardon and Bell 2001). Isolated populations have been considered good case-control sample sets because of homogenous background (Peltonen et al. 2000), but isolation could also be a disadvantage due to the substructure of the population (Kere 2001). One solution is using family-based controls in association-based family studies like transmission disequilibrium test, TDT (Spielman et al. 1993). It is based on transmissions of a certain allele from heterozygous parents to their affected offspring and it is precluded that the association is caused by linkage disequilibrium or population stratification (Spielman and Ewens 1996).

Linkage disequilibrium

Alleles close to each other tend to be inherited together. When they are close enough their association can be detected in the population level and this phenomenon is called linkage disequilibrium (LD). The number of mutations and recombinations during the meiosis and the localisation of the hotspots (Jeffreys et al. 2001) define the sizes of the chromosomal regions, which are in linkage disequilibrium in the population. These regions, also called

haplotype blocks, are thought to be an advantage for genome-wide gene hunting, contributing common alleles behind common disorders. Due to these haplotype blocks, it is possible to select a smaller group of informative SNPs, so called tag SNPs, from the total number of SNPs. Therefore, instead of 11 million common SNPs, a few hundred thousand well-chosen SNPs are estimated to be needed to provide the information about the most common variation of the genome. The history of the population has an effect on the haplotype construction and also on study design. For example blocks in Africa, the region of human origin, have an average length of 11 kb, compared to blocks in Europe which are about 22 kb (Gabriel et al. 2002). The older the population is, the more variation and the lower LD it has. The advantages or disadvantages of long linkage disequilibrium blocks depend on the aims and the phase of a particular study. The LD between two separate loci will be weaker with time and at the same time, the higher amount of recombination enables establishing of more exact localisation of mutation (Greenwood et al. 2004).

3.3.3. Candidate gene studies

Positional candidate genes

In the positional candidate strategy, the gene is suggested based on its location in an interesting region in the genome. Most commonly the chromosomal region for a candidate gene is found by linkage analysis and fine-mapping has suggested some very likely genes contributing to the disease phenotype. Positional candidate gene studies are performed by resequencing or association analysis (Rich and Merikangas 1996, Cardon and Bell 2001, Tabor et al. 2002). In the former, the sequences of candidate genes are compared in patients and controls and possible novel disease-causing variants are detected. It is a laborious and still quite expensive method. Therefore the cheaper and more simple association analysis of previously establish polymorphisms (SNPs) is also used (Tabor et al. 2002). The simplest way to perform the association analysis is by comparing the allele or genotype frequency in disease cases and controls.

Functional candidate genes

The functional candidate gene strategy is based on a hypothesis that a gene due to its function could be the gene causing the disorder. The study of congenital autosomaldominant exercise-induced hyperinsulinism (EIHI) offers a plausible example for that. In this syndrome, physical exercise leads to inappropriate insulin release and

hypoglycaemia. The most likely disease gene is the major monocarboxylate transporter, monocarboxylate transporter 1 (MCT1) (Otonkoski et al. 2003). MCT1 is the transporter of pyruvates on the cell surface, but normally the expression is low on the membrane of insulin-producing pancreatic β-cells (Zhao et al. 2001). The low expression is considered a protection mechanism, which inhibits the insulin secretion induced by pyruvate during exercise (Ishihara et al. 1999) and that failure was detected in EIHI patients (Otonkoski et al. 2003).

Chromosomal aberrations

Translocations or other chromosomal aberrations like deletions or inversions are notable signs when hunting for a potential candidate gene. If a chromosomal aberration disrupts a gene, most likely the protein which is encoded by this allele is truncated, the expression is altered or totally absent. A fine example is reciprocal translocation t(9;22)(q34.1;q11.21), which caused the creation of a small acrocentric chromosome named Philadelphia (Nowell and Hungerford 1960 review by Geary 2000). Patients with Philadelphia chromosome suffered from chronic myeloid leukaemia, which was later found to be caused by the disruption of two genes, *BCR* and *ABL*, and the creation of the fusion gene *BCR-ABL,* encoding a constitutively active cytoplasmic tyrosine kinase (Sawyers 1999). However, translocation can offer only a plausible candidate gene that always has to be confirmed by genetic analyses or functional studies.

4. Genetic studies of dyslexia

Genetic factors play a significant role in the manifestation of dyslexia as is seen in family and twin studies. Therefore genetics is a way to approach the aetiology of this complex neurological disorder. Due to the continuum of reading skills, dyslexia can be considered a quantitative trait and thus each dyslexia locus is "a locus important in determining the phenotype of a continuous character (Strachan and Read 1999)". However, dyslexia is more complex and in some of the studies, the phenotype is distributed into components, of which each can be measured as a distinct quantitative trait. The number of the traits and genes contributing to dyslexia is obscure. Furthermore, whether there really are genes involved in the anomalies of certain brain regions or certain phases of the embryonal development, and thus cause a specific trait, needs to be found out. The state of dyslexia research by the year 2001, the beginning of this thesis project, is described in this chapter

(table I). The rate of genetic findings has been accelerated during the last years and the current situation of dyslexia research can be seen later in the Discussion-section (table II).

4.1. Mode of inheritance

Despite the consensus that there is a strong genetic component behind dyslexia, the mode of inheritance has been a topic of discussion. Data from family studies argue against X-linked or simple autosomal recessive transmission, but are consistent with an additive or dominant autosomal major locus effect due to high and similar recurrence rates in parents and siblings (Pennington et al. 1991). For now, a large number of genetic analyses have been performed which prove that dyslexia is indeed a complex disorder and does not generally show classical Mendelian inheritance. However, there could still be a few major genes in the multifactorial background, which explain the findings from the family studies (Pennigton et al. 1991).

4.2. Genetic analyses

Altogether six dyslexia loci had been revealed in the genome by the year 2001. The research was at first performed mainly by targeted linkage analysis. Limited chromosomal regions were chosen based on structural alterations, like in the case of centromeric heteromorphism on chromosome 15 (Smith et al. 1983) and on association between functioning of the immune system and developmental dyslexia, such as HLA region on chromosome 6 (Smith et al. 1991, Cardon et al. 1994) as well as a Rhesus factor locus on chromosome 1 (Rabin et al. 1993). Altogether, two genome-wide scans and one notable association analysis had been performed by the year 2001. The genome–wide analyses have revealed loci on chromosome 2p15-p16 (Fagerheim et al. 1999) and chromosome 3p12-q13 (Nopola-Hemmi et al. 2001). Family-based association analysis had confirmed that a region on chromosome 15q15 associates with dyslexia (Morris et al. 2000). In addition, two translocations on chromosome 15q21-q22 from Finnish dyslexia families (15A and 15B in figure 4) have been found (Nopola-Hemmi 2000).

4.3. Dyslexia phenotypes

In genetic analysis the dyslexia phenotype is treated as global or it is divided into components. In this context, global means the phenotype is evaluated using general diagnoses or quantitative analyses of overall indices of severity, for example using

standardised tests of word recognition or spelling ability. The distribution into distinct but related phenotypic components is based on theories of the nature of the reading process, and it is supported by cognitive-psychological and psychometric studies (Grigorenko et al. 1997, Fisher et al. 1999, Wijsman et al. 2000, Raskind et al. 2000). Specific tests have been developed to define for example single word reading, phoneme awareness, phonological decoding and rapid automised reading. Both ways to treat the phenotype, global and division into components can be analysed as quantitative traits. Different methods are used for phenotype testing in genetic analyses and this is likely to eventually complicate the genetics of this complex disorder.

4.4. Genetic findings in dyslexia until the year 2001

4.4.1. DYX1 on chromosome 15q21

The genetic linkage analyses for finding dyslexia loci were started as early as 1983. Smith et al. performed a targeted linkage study on chromosome 15 by considering a centromeric heteromorphism, which is natural variation in the shape or staining pattern of a chromosome. However, this linkage was found only in 20% of the studied families and furthermore the finding was excluded in a Danish study (Bisgaard et al. 1987). The following targeted linkage studies revealed a new chromosomal region on 15q15-q21 (Smith et al. 1991, Grigorenko et al. 1997, Schulte-Körne et al. 1998, Nöthen et al. 1999), which was supported later by association analysis of 178 parent-proband trios (Morris et al. 2000).

There are also two interesting translocations found in two Finnish dyslexic families (Nopola-Hemmi et al. 2000). In the first family, a balanced reciprocal translocation t(2;15)(q11;q21) disrupts chromosomes 2 and 15 and co-segregates with dyslexia (figure 4A, family 15A). The boy with translocation (II, 3) has low overall performance and therefore his phenotype for dyslexia is unknown, but two of his three sisters have translocation and dyslexia. In the other family, a translocation t(2;15)(p13;q22) associates with dyslexia in one family member, who is the only female sibling with translocation in the family (figure 4B, family 15B). These findings supported the role of chromosomal region 15q21-q22 as a dyslexia locus.

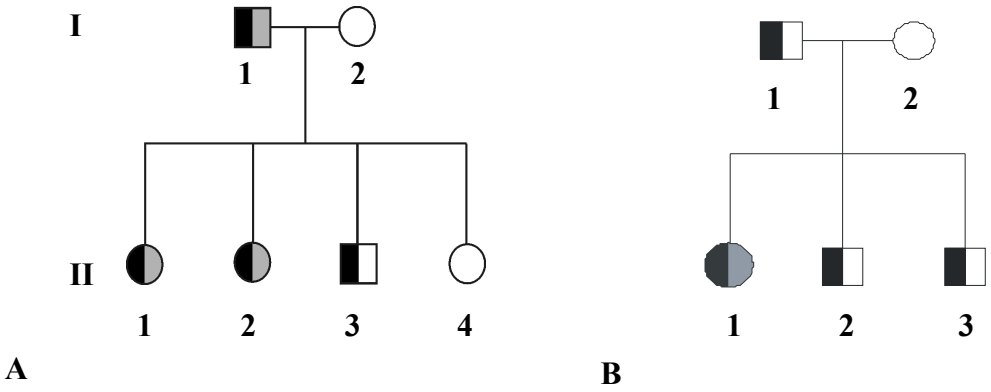

Figure 4. Families with dyslexia and translocations. A: family 15A with translocation t(2;15)(q11;q21) B: family 15B with translocation t(2;15)(p13;q22). Black fill denotes translocation, grey fill dyslexia (Nopola-Hemmi et al. 2000).

4.4.2. DYX2 and DYX4 on chromosome 6p21-22 and 6q11-12

Due to phenotypic co-occurrences between learning disabilities, immune disorders and left-handedness, it was thought in the 1980s that they could share a common aetiology (see GBG theory in 1.4.4., Geschwind and Behan 1982, Geshwind and Galaburda 1985, Gilger et al. 1997). This led research to the human histocompatibility antigen (HLA) region on the short arm of chromosome 6, which was considered a possible quantitative trait locus (QTL) influencing dyslexia (Smith et al. 1991, Cardon et al. 1994 and 1995). Although the causality between autoimmune disturbances and developmental dyslexia has not been proved, the linkage to chromosome 6p21-p22, known as dyslexia locus 2 (*DYX2*) has been replicated several times (Grigorenko et al. 1997, Fisher et al. 1999, Gayan et al. 1999, Grigorenko et al. 2000). However, there are also groups that have been unable to replicate the linkage to this region (Bisgaard et al. 1987, Field and Kaplan 1998, Smith et al. 1998, Schulte-Körne et al. 1998, Petryshen et al. 2000).

Dyslexia locus 4 (*DYX4*) is located in the centromeric region of the long arm of chromosome 6. Two-point parametric analysis indicated, and multipoint parametric and non-parametric analyses supported, the targeted linkage between phonological coding and chromosomal region 6q11.2-q12 (Petryshen et al. 2001).

4.4.3. DYX3 on chromosome 2p15-p16

The first genome-wide scan was performed by Fagerheim et al. in 1999 using one large Norwegian pedigree in which dyslexia was inherited as an autosomal dominant trait. Both two-point parametric and multipoint non-parametric analyses showed evidence for linkage between the dyslexia phenotype and chromosome 2p15-p16 (Fagerheim et al. 1999). This result was supported later by targeted linkage analysis in 97 Canadian families (Petryshen et al. 2000).

4.4.4. DYX5 on chromosome 3p12-q13

The second genome-wide scan was based on a large Finnish pedigree where dyslexia was segregated in autosomal dominant way (Nopola-Hemmi et al. 2001). The parametric linkage analysis indicated significant linkage between the dyslexia phenotype and chromosomal region 3p12-q13. Two positional candidate genes, 5-hydroxytryptamine receptor 1F (*5HT1F*), which has a role in learning and memory (Meneses 1998) and the dopamine D3 receptor gene (*DRD3*), which has been suggestively associated previously with schizophrenia (Griffon et al. 1996), were investigated. The coding region of *5HT1F* was sequenced without interesting findings and *DRD3* was excluded from the linkage region by radiation hybrid mapping.

4.4.5. Suggestive dyslexia loci on chromosome 1p22 and 1p34-p36

Chromosomal region of rhesus blood group CcEe antigen locus and two other DNA markers on chromosome 1p34-p36 have been associated with dyslexia in targeted linkage studies (Rabin et al. 1993). At the same time a balanced translocation t(1;2)(p22;q31) between chromosomes 1 and 2 was detected (Froster et al. 1993). Translocation co-segregated with delayed speech development and dyslexia in a single family. In addition to that, the targeted linkage analysis showed suggestive evidence of linkage to a wide region on chromosome 1p using eight families (Grigorenko et al. 2001).

Table I. Dyslexia loci until 2001. The following abbreviations have been used: TLNG: targeted linkage, GWS: genome-wide scan, HE: Haseman –Elston regression analysis, DF: DeFries-Fulger regression analysis and VC: variance-component analysis. QTL indicates that the phenotype has been treated as a quantitative trait.

LOCUS	AUTHORS	METHOD	PHENOTYPE	MATERIAL families, country	FINDINGS
DYX1	Smith et al. 1983	TLNG,param	global	9, USA	LINKAGE:LOD 3.24 chrom.15p11.1-q11.1
Chrom. 15	Bisgaard et al. 1987	TLNG,param	global	5, Denmark	EXCLUSION: cen15 (15p11.1-q11.1), LOD -3.42
	Smith et al. 1991	TLNG,HE	global,QTL	19, USA	LINKAGE: 15q15-15qter, p=0.009 / 15q15-15qter, p=0.03
	Rabin et al. 1993	TLNG,param	global	9, USA	EXCLUSION:proximal region of 15q
	Grigorenko et al. 1997	TLNG,param/NPL	global	6, USA	LINKAGE:LOD 3.15 in 15q21 for single word reading/significant in 15q11
	Schulte-Körne et al. 1998	TLNG,param/NPL	global	7, Germany	LINKAGE: 15q21multipoint p=0.0042 /15q21 multipoint p=0.03
	Morris et al. 2000	association	global	178 trios, UK	ASSOCIATION: 15q15, DD-three marker haplotype p<0.001
	Nopola-Hemmi et al. 2000	translocations	global	2, Finland	TRANSLOCATIONS: t(2;15)(q11;q21), t(2;15)(p13;q22)
DYX2&DYX4	Smith et al. 1991	TLNG,HE	global,QTL	19, USA	LINKAGE: 6p21.3, p<0.02 / 6p21.3, p<0.0001
Chrom. 6	Cardon et al. 1994,1995	TLNG,DF	global,QTL	19,46 DZ pairs,USA	LINKAGE: 6p21.3, p=0.04 / 6p21.3, p=0.009
	Grigorenko et al. 1997	TLNG,param/NPL	global	6, USA	LINKAGE: no significant / 6p22.3-21.3, multipoint p=<0.005 strongest for phoneme awarenessp<0.000001, weakest for single-word reading p<0.005
	Schulte-Körne et al. 1998	TLNG,param/NPL	global	7, Germany	no significant linkage
	Field and Kaplan, 1998	TLNG,param/NPL	global	78, Canada	absence of linkage in 6p22, LINKAGE: 6q13-q16.2 for phonological decoding
	Fisher et al. 1999	TLNG,HE,VC	components,QTL	82, UK	LINKAGE: 6p21.3,multipoint, phonological decoding p=0.007, orthographic coding p=0.006/ 6p21.3,multipoint, phonological decoding p=0.004, orthographic coding p=0.007
	Gayan et al. 1999	TLNG,DF	components,QTL	79 twin-based families, USA	LINKAGE: 6p21, LOD for phoneme awareness 1.46, phonological decoding 2.42 and orthographic coding 3.1.
	Petryshen et al. 2000	TLNG,HE,VC	components,QTL	79, Canada	no significant linkage / weak linkage 6p23-21.3, phon. decoding, rapid autom.naming, spelling
	Grigorenko et al. 2000	GWS,NPL	global	8, USA	LINKAGE: 6p21.3 for a variety of phenotypes
DYX4	Petryshen et al. 2001	GWS,NPL	components,QTL	100 affec.sib-pairs	LINKAGE: 6q
DYX3	Fagerheim et al. 1999	GWS,NPL	global	1, Norway	LINKAGE: 2p15-16
Chrom. 2	Nopola-Hemmi et al. 2000	translocations	global	2, Finland	TRANSLOCATIONS: t(2;15)(q11;q21), t(2;15)(p13;q22)
DYX5 Chrom. 3	Nopola-Hemmi et al. 2001	GWS,param	global	1, Finland	LINGAKE:3p12-q13

4.5. Other interesting loci-related language disorders

Speech-sound disorder loci (SSD)

Speech-sound disorder is a complex disorder, characterised by a speech-sound production error which is associated with a deficit in articulation, phonologic processes, such as phonological memory and speech-sound coding, and cognitive linguistic processes. It is prevalent in childhood and the prevalence among three-year-old children is 16% and has fallen to 3.8% by age 6 (Shriberg et al. 1999). It is cormorbid with dyslexia and it has been suggested that early developmental problems in spoken language predict dyslexia in children from high-risk families (Pennigton and Lefly 2001). Based on the overlapping of these two disorders and the dyslexia *DYX5* locus on chromosome 3p12-q13 (Nopola-Hemmi et al. 2001), the association between SSD and DYX5 was tested. The measures of phonologic memory linked strongly to the pericentromeric region of chromosome 3 (Stein et al. 2004). Furthermore, an association with chromosome 15q14-q21 and SSD has also been detected (Stein et al. 2006), as well as an association with 15q11-q13 and with Prader-Willi syndrome (PWS) and Angelman syndrome (AS). PWS and AS also belong to a group of disorders that have deficits in speech and language skills (Moncla et al. 1999, Dykens et al. 2004).

Specific language impairment loci 1-3 (SLI1-3)

Specific language impairment (SLI) quantitative trait loci 1-3 have been mapped on chromosomes 16q, 19q and 13q21, respectively (SLI consortium 2002, Barlett et al. 2002). SLI is a congenital and multifactorial disorder with approximately 7% prevalence among children entering school. Those children fail to develop language in a normal manner and any explanations such as neurological disorders, auditory or visual impairments or lack of opportunity cannot be found (SLI consortium 2002).

Speech and language disorder locus (SPCH1)

The definition of severe speech and language disorder is partly overlapping with the symptoms of milder disorder SLI. Speech and language disorder is described as a disorder with impairments in articulation, expressive speech, grammar and phonological processing (Vargha-Khadem et al. 1995). Although it is rare, it is a notable disorder in language disorder research. By mapping the gene in a large origin pedigree “KE family” suffering from speech and language disorder and by finding an individual with a similar speech and language disorder and a translocation (5;7)(q22;q31.2), the first gene associated with

speech and language development was found. It is a transcription factor FOXP2 (forkhead box p2) on chromosome 7q31 (Lai et al. 2001). In a fMRI study the affected family members had alterations in brain activity during tasks like word repetition and voxel-based morphometry revealed alterations in the amount of grey matter in several brain regions. Interestingly, the inferior frontal cortex was underactivated in the affected members of the KE family. Instead of normal activation in typical language regions, the activation was increased in a number of untypical parts of the brain, suggesting compensation (Liegeois et al. 2003). The result of repetition of the words and pseudo-words tests indicated that the deficit is more likely in the system, which is responsible for sequential articulation than phonological processing (Watkins et al. 1999). These findings suggest that *FOXP2* has a significant role in the development of neural systems that mediate speech and language.

Autism and loci of language disorders

Locus 7q31 has been associated also with another neurodevelopmental disorder, autism. According to the American Psychiatric Association (1994), autistic individuals suffer from repetitive and ritualistic behaviours, impairment in social interactions and facial expression as well as communication that in some cases is very difficult to separate from SLI or more severe language impairments (Lord et al. 1994). Linkage studies (Ashley-Koch et al. 1999, Barrett et al. 1999, Philippe et al. 1999) and a translocation t(7;13)(q31.3;q21) carried by an autistic individual (Vincent et al. 2000) have indicated that *FOXP2* is a plausible gene for the aetiology of both, language impairment and autism. However, absence of any mutation or association evidence in a study of 169 multiplex families with autism (857 individuals) and of 43 families with SLI (210 individuals) suggested that *FOXP2* does not play a major role in these disorders (Newbury 2002). Interestingly, also chromosomes 15q11-q13 (*AUTS4*, Baker et al. 1994, Shao et al. 2003) and 6p21.3-p21.2 (Junaid et al. 2004) have been linked to autism.

AIMS OF THE STUDY

This study was carried out to reveal the genes behind developmental dyslexia. By means of understanding these genes and their functions we wanted to gain understanding on those neurodevelopmental mechanisms associated with reading ability, and which also make every human brain unique.

The aims of the study were

A to reveal genes associated with developmental dyslexia by genome-wide scan, translocations found in dyslexic individuals and association analyses

B to characterise function of the susceptibility gene *DYX1C1* by clarifying its protein synthesis and localisation in the cell during metabolic challenge and studying its role in the neuronal migration in brain development

C and thus strengthen our understanding of the molecular genetics underlying reading disability

MATERIALS AND METHODS

This study has been approved by the ethical review board of the Helsinki University Central Hospital and informed consent was obtained from the participants. Animal protocols in study IV were approved by the University of Connecticut Institutional Animal Care and Use Committee and in study V by the County Administrative Board of South Finland.

1. Samples

1.1. DNA samples for dyslexia study (I, II, III)

Eleven families with 97 individuals (97 subjects of whom 40 were dyslexic, 39 were non-dyslexic and 18 were uncertain) were recruited, blood samples were collected and DNA was extracted for genome-wide scan (I) thorough testing for dyslexia from the Department of Paediatric Neurology at the Hospital for Children and Adolescents, University of Helsinki (9 families) and the Central Hospital of Central Finland, Jyväskylä (2 families), Finland. From these eleven families, 75 individuals were available for linkage studies.

For genotyping and association analysis (study II, III), 58 dyslexic and 61 non-dyslexic individuals from 20 unrelated families were recruited by the Department of Paediatric Neurology at the Hospital for Children and Adolescents, University of Helsinki and 3 families and 33 unrelated dyslexic and non-dyslexic couples by the Child Research Centre, Jyväskylä, Finland. Additional population controls consisted of 100 anonymous blood donors (II and III). Non-dyslexic individuals in families were tested for dyslexia, except in two families for linkage analysis (families 10 and 11) where dyslexia testing was performed in three of the non-dyslexic subjects with normal results, and a further eight subjects reporting normal reading performances were also classified as unaffected.

The segregation of risk haplotype in a large four-generation family (study III, figure 8) was detected by genotyping 21 dyslexic, two non-dyslexic and two unverified dyslexic individuals. The family was recruited by the Department of Paediatric Neurology at the Hospital for Children and Adolescents, University of Helsinki (Nopola-Hemmi et al. 2001).

The diagnostic criteria for dyslexia included remarkable deviation (depending on age, at least 2 years) in reading skills compared to chronological age and normal performance

intelligence quotient (PIQ >85). The diagnosis and degree of dyslexia were determined by Finnish reading and spelling tests designed for children (Häyrinen et al. 1999) and adults (Leinonen et al. 2001). Intelligence was estimated by Wechsler tests for children (WISC-R; Wechsler 1984) and for adults (WAIS-R; Wechsler 1992). Eight subtests covering verbal and visual skills were used: Information, Digit Span, Vocabulary, Similarities, Picture Completion, Picture Arrangement, Block Design, and Coding. In addition, reading-related neurocognitive skills (phonological awareness, rapid automatised naming, and verbal short-term memory) were assessed by neuropsychological tests (Korkman et al. 1997, Dencla and Rudel 1976, Christensen 1982, Wolf 1986).

1.2. Family 15A with translocation t(2;15)(q11;q21) (II)

A family segregating t(2;15)(q11;q21) and dyslexia phenotype was studied to identify a positional candidate gene for dyslexia in the translocation breakpoint (figure 4A, family 15A). The translocation was found in a family, which had had several miscarriages. Karyotypes of translocated chromosomes were obtained from phytohaemagglutin-stimulated blood lymphocytes with G-banding following standard procedures. The father of the family had a history of profound reading and writing difficulties in school and his two daughters with translocations had been diagnosed with dyslexia at Helsinki University Hospital. The son was diagnosed with specific difficulty in reading-related skills; however, his overall performance was also slightly below normal (Nopola-Hemmi et al. 2000).

1.3. A dyslexic individual with translocation t (3;8) (p12;q11) and his family (III)

A balanced reciprocal translocation t(3;8)(p12;q11) in a dyslexic individual was revealed by infertility investigations in which he was diagnosed with oligoteratozoospermia. He has three siblings, and all four children have been neuropsychologically evaluated at a specialist hospital (figure 5). The translocation carrier and one of his sisters were diagnosed with severe dyslexia and the other two siblings had subnormal intelligence, but not dyslexia. The mother was reported as a good reader, but no information on reading performance was available on the deceased father. The other three siblings have normal karyotypes whereas the parents were not available for karyotyping. Most likely the translocation has arisen *de novo,* as knowing the infertility of the index case, while no such history or miscarriages were recorded for his mother. In addition to DNA, also RNA was available from the individual with translocation from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines.

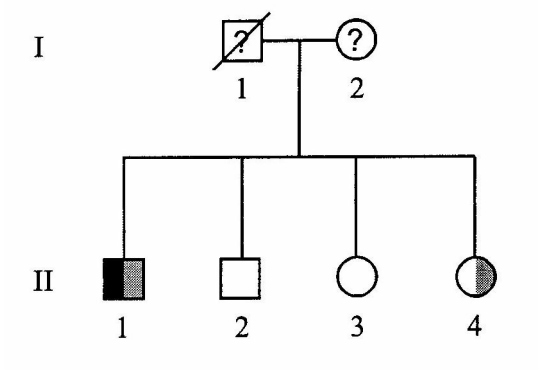

Figure 5. Family with dyslexia and translocation t(3;8)(p12;q11). Black fill denotes translocation and grey fill dyslexia (study III).

1.4. Human brain samples (II)

In order to detect the expression of *DYX1C1* in normal and ischaemic human brain, brain tissue from six deceased individuals was collected. The patients had died of cardiac arrest or ischaemic stroke. The post-ischaemic time before death varied from 15 to 60 h and samples were obtained at rapid autopsies with post-mortem delays varying from 10 to 40 h. Tissue blocks with cortical and some subcortical tissues were obtained from the core or an area close to the core of the infarction. Control samples from an individual who died immediately after sudden cardiac arrest were dissected from homologous contralateral locations for comparison.

1.5. Rats for *in utero* electroporation and focal brain ischaemia studies (IV, V)

In utero electroporation and RNAi methods were applied to embryonic Wistar rats (Charles River laboratories, Wilmington, MA). Thirty-five male adult Wistar rats were used as an animal model of local ischaemic stroke for detecting *Dyx1c1* expression in metabolic stress.

2. Genetic analyses

2.1. Genotyping with microsatellite makers (I)

A genome-wide scan was performed to find out the linkage between the dyslexia phenotype and genomic regions in eleven Finnish families. The scan was carried out at the Finnish Genome Centre, University of Helsinki, using 376 microsatellite markers from the Applied Biosystems Linkage Mapping set MD-10. The average distance between markers was 10 cM (the median was 9.2 cM and the range from 0.63 to 28.0 cM). The electrophoresis was run using a Megabase 1000 capillary electrophoresis instrument (Molecular Dynamics, Sunnyvale, CA) and the alleles were visualised using Genetic Profiler 1.5 software (Molecular Dynamics).

2.2. Linkage analysis (I)

The genome scan data were analysed by non-parametric multipoint and parametric two-point linkage analysis using Genehunter and MLINK softwares. Genehunter performs reconstruction of haplotypes and complete multipoint analysis based on estimation of allele sharing identical-by-descent (IBD) among all affected family members at each location in the genome (Kruglyak et al. 1996). For non-parametric analysis, the affected-only mode of analysis was used. Common recessive alleles in the population can cause inheritance patterns that are reminiscent of autosomal dominant inheritance and so parametric linkage analysis was performed using both dominant and recessive inheritance models. In the dominant model the disease allele frequency was 0.0001, and the penetrances for homozygous normal, heterozygous, and homozygous affected were 0.06, 0.95 and 0.95. In the parametric recessive model, the disease allele frequency was 0.1 and the corresponding penetrances for affected were 0.02, 0.04 and 0.8. Female and male recombination rates were equal in both models.

2.3. Genotyping with SNPs (II, III)

Single nucleotide polymorphisms (SNP) were detected for association analysis. DNA was extracted from blood samples and SNPs were genotyped after polymerase chain reaction (PCR) by direct sequencing or using restriction enzymes. In the absenceof an appropriate restriction site, it was produced for the sequence in the PCR by using mutated primers. After digestion, PCR products were electrophoresed on agarose gel.

For direct sequencing the standard PCRs were carried out containing 10–50 ng of genomic DNA, 1 x DyNAzyme II buffer, 1.5 mmol/l $MgCl_2$, 160 µmol/l dNTPs, 0.6 µmol/l of each primer, 0.6 U of DNA polymerase (DyNAzyme II, Finnzymes, Espoo, Finland), and 0–4% DMSO. In case of poor amplification, the DNA polymerases Ampli*Taq* Gold (Perkin Elmer, Roche Molecular Systems Inc) or DyNAzyme EXT (Finnzymes) were used under similar conditions. Amplifications were performed with an initial denaturation at 94°C for two minutes (Ampli*Taq* Gold 10 min), followed by 35–40 cycles each of 35 seconds at 94°C, 35 seconds at 55–62°C, and one minute at 72°C, with a final elongation at 72°C for ten minutes. Purified PCR products (PCR purification kit, Gel extraction kit, Qiagen) were either directly sequenced or cloned before sequencing (TOPO TA Cloning Kit, pCR 2.1-TOPO vector, Invitrogen). Sequencing was performed with ABI 377 and ABI 3100.

2.4. Association Analysis (II)

The statistical significance between cases and controls was evaluated using χ^2-test. Fisher's exact test was used when the number of expected observations was less than five. The Bonferroni correction was applied to reduce type I error (significance was accepted at the 5% level). Odds ratios (OR) [$OR= f_{(aff)}/(1-f_{(aff)}:f_{(contr)}/(1-f_{(contr)}]$ with 95% confidence intervals (95% CI) were calculated for each association.

2.5. Mutation screening by direct sequencing (I, II, III)

The exons of *FOXP2*, *DYX1C1* and *ROBO1/DUTT* were screened in the case of mutations by direct sequencing. Primers flanking each exon were designed using the Primer3 programme (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3, Rozen and Skaletsky 2000). Standard PCRs were used and sequencing was performed in both directions. Genomic sequences corresponding to exons were identified through BLAST searches of GeneBank (NCBI). The promoter regions of *DYX1C1* and *ROBO1/DUTT,* about 1 kilobase upstream of the gene, were also screened.

3. Gene discovery

3.1. Fluorescent *in situ* hybridisation (II, III)

Fluorecent *in situ* hybridisation (FISH) was used to refine the translocation breakpoint, which was seen in karyotype analysis, within the bacterial artificial chromosome (BAC) clone RP-11-178D12. For FISH studies, metaphase spreads were obtained from EBV-transformed lymphoblastoid cell lines derived from the individual with the translocation. Genomic yeast artificial chromosome (YAC) and BAC clones were obtained to probes and DNA was labelled with biotin-14-dATP using nick translation. After denaturation, both slides with metaphase spreads and probes were hybridised in a humid chamber at 37°C for two days. Labelled DNA was detected with avidin-conjugated fluorescein isothiocyanate (FITCH). Chromosomes were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). Signals were visualised with a Zeiss Axioplan 2 fluorescent microscope (Nopola-Hemmi et al. 2000).

3.2. Southern blot analysis (II, III)

Southern blotting was used to study exact localisation of translocations t(2;15)(q11;q21) and t(3;8)(p12;q11). Genomic DNA were digested with *Bam*HI, *Eco*RI, *Hind*III, *Bsa*AI, *Pst*I, or *Sph*I (study II) and *Bam*HI, *Bgl*II, *Ear*I, *Eco*RI, *Hae*II, *Hind*III, *Nco*I, and *Pst*I (study III), subjected to electrophoresis in an agarose gel, and transferred to Hybond N+ membrane with alkaline blotting. Probes were produced by standard PCR methods from human genomic DNA and the purified PCR products were labelled with [α -32P] dCTP. Hybridisation was performed overnight and filters were autoradiographed with a PhosphorImager.

3.3. Structure prediction (II)

Translocation t(2;15)(q11;q21) revealed a novel gene in clone 178D12, now known as *DYX1C1*. It was predicted by GENSCAN and FGENES software and confirmed by sequencing RT-PCR products. The promoter region of *DYX1C1* was predicted with TSSG and TSSW software (available at http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html) and neural network promoter prediction (NNPP) software (available at www.fruitfly.org/seq_tools/promoter.html). Transcription factor binding sites were predicted by TESS (at www.cbil.upenn.edu/tess), MATINSPECTOR (at www.gsf.de/biodv/matinspector.html) and TFSEARCH (at www.cbrc.jp/research/db/TFSEARCH.html).

3.4. Rapid amplification of 5' complementary DNA ends (5′ RACE) (III)

5′ RACE was performed in order to discover new exons in the upstream of the known *ROBO1* gene. Human brain mRNA was converted to cDNA using TaqMan kit (Applied Biosystems) and SMART RACE cDNA Amplification kit (Clontech) was used for amplifying ROBO 5' sequences of the corresponding mRNAs. 5′ cDNA ends were amplified with the Universal Primer A and a *ROBO1* gene specific primer, followed by nested PCR with the Nested Universal Primer A. PCR products were purified and directly sequenced (ABI).

3.5. Cloning (II)

DYX1C1 gene was cloned according to the gene predictions and RT-PCR analysis to the pcDNA3.1/V5-6XHis (C-terminal V5 epitope and a polyhistidine tail) expression vector (Invitrogen) with and without STOP codon, which means that the protein could be expressed with and without the V5-6XHis tag. Standard PCR was performed and products were cloned to vector using TOPO TA cloning kit (Invitrogen). Plasmids were multiplied by chemical transformation in *Escherichia coli* and purified using QIAprep Spin Miniprep Kit (Qiagen). Inserts and insert-vector boundaries were verified by sequencing (ABI).

4. mRNA expression studies

4.1. Reverse Transcriptase (RT)-PCR (II, III)

In order to detect gene expression in the mRNA level, RT-PCRs were done. The expression of novel gene *DYX1C1* in different human tissues was analysed by RT-PCR from multiple-tissue cDNA panels 1 and 2 (Clontech). RT-PCR reaction in haploinsufficiency analysis for detecting expression of *ROBO1* alleles, was started by converting the lymphocyte mRNA to cDNA. cDNA synthesis was performed with MultiScribe Reverse Transcriptase and Random Hexamers for 10 min at 25°C, 30 min at 48°C and 5 min at 95°C (TaqMan kit, Applied Biosystems). After conversion, standard PCRs were performed using cDNA as a template. Due to the huge amount of template, fewer cycles in PCR were required.

4.2. Northern blot analysis (II)

In order to study the expression, size and potential splice variants of *DYX1C1* mRNA in different human tissues, the Northern blot analysis was performed. A cDNA probe corresponding to bases 40–630, spanning exons 2–5 of *DYX1C1* coding sequence, was hybridised to multiple-tissue Northern blots I and II (CLONTECH) according to the manufacturer's instructions.

4.3. Real-time PCR (TaqMan) (V)

Real-time PCRs were done in order to detect potential changes in the expression of *DYX1C1* caused by stress. In addition to *DYX1C1,* the expression of *HSP70* was also detected to confirm effective heat shock. Predesigned primer and probe sets for *DYX1C1* and *HSP70* (DYX1C1:Hs00370049, HSP70:Hs00359163, TaqMan Gene Expression Assay, Applied Biosystems) were used according to the manufacturer's protocols. GADPH labelled with VIC reporter dye (Applied Biosystems) was used as an endogenous control gene. Quantitative RT-PCR was performed using ABI PRISM 7700 sequence detector system (Applied Biosystems). Reaction conditions were programmed on a power Macintosh 7200, linked directly to the sequence detector. PCR amplifications were performed according to the manufacturer's recommendations.

4.4. Haploinsufficiency analysis (III)

To assess the allele-specific expression a standard method was used (Pastinen et al. 2003). The assay is based on the comparison of allelic peak heights (in arbitrary units) in cDNA sequence and genomic sequence from each individual. An allelic ratio is calculated for each sequence (e.g. height of allele A per height of allele C). Because the allelic ratio in a genomic sequence is by definition 1 (each allele is present as one copy per diploid genome), but the actual value may differ from 1 (because of chemical properties of the sequencing reactions), the cDNA allelic ratio values are normalised by dividing by the genomic allelic ratio in each experiment. To assess whether the normalised cDNA allelic ratios differed in dyslexic patients compared to controls, the values from replicated experiments were compared between the groups by two-tailed t-test. To estimate the degree of attenuation of one allele in dyslexic patients, the average cDNA allelic ratio in dyslexic patients was divided by the average cDNA allelic ratio in controls. Standard deviation of the measurements was calculated based on replicated experiments.

4.5. RNA extraction from HEK 293A cells (V) and lymphocytes (III)

Total cellular RNA was extracted from control and heat shocked human embryonic kidney (HEK) 293A cells for real-time PCR using the RNeasy Mini Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's protocol. Quantification was performed using spectrophotometry (GeneQuant II/Pharmacia Biotech). RNA for haploinsufficiency analysis was extracted from Epstein-Barr virus (EBV)-transformed lymphocyte cell lines from dyslexic and normal readers by Ficoll gradient centrifugation (Qiagen RNeasy purification kit).

5. Protein expression studies

5.1. Antibodies (II, IV, V)

To generate the antibody, the peptide sequence CATEAKAAAKREDQK, corresponding to amino acids 114-128 in *DYX1C1*, was used as an antigen for immunising rabbits (Sigma-Genosys). The serum was affinity purified using GST-DYX1C1 fusion protein columns in study V, whereas in studies II and IV the serum was unpurified. Additional primary antibodies, an anti-V5 antibody (Invitrogen), an anti-GFP antibody (Chemicon), anti-myc antibody (Abcam), an anti-flag antibody (Sigma) and anti-BrdU antibody (Accurat), were also used. An antibody against the C-terminus of *DYX1C1*, peptide sequence CKIRNVIQGTELKS, was generated to correspond to amino acids 408-420 (Thermo) and was used as an antigen for immunising goat (Everest Biotech). The serum was purified using columns of Sulfolink Gel (Pierce Biotechnologies). The mouse anti-Hsp70 monoclonal antibody (Stressgen) and Hsp90 α/β (F-8) mouse monoclonal antibody (Santa Cruz, CA, USA) were used in study V.

Secondary antibodies used in either immunocytochemistry or western blot analysis were TRITC-conjugated goat anti-mouse IgG (Sigma-Aldrich), Alexa Fluro 568 Goat anti-rabbit IgG (H+L) (Molecular Probe), Alexa Fluro 568 Goat anti-mouse IgG (H+L) (Molecular Probe), Alexa Fluro 568 Donkey anti-goat IgG (H+L) (Molecular Probe), Alexa Fluro 568 Goat anti-rat IgG (H+L) (Molecular Probe), Donkey anti-rabbit (H+L) HRP labelled secondary antibody (Research Diagnostics), Donkey anti-mouse (H+L) HRP labelled secondary antibody (Research Diagnostics) and Donkey anti-goat (H+L) HRP labelled secondary antibody (Research Diagnostics).

5.2. Cell cultures (II, IV, V)

The African green monkey kidney COS-1 and COS-7 cells, and HEK 293A cells were grown in DMEM with sodium pyruvate, 10% foetal calf serum, 50U/ml and 50µg/ml of penicillin and streptomycin at 37°C in humidified incubator at 5% CO_2.

5.3. Constructs (II, IV, V)

Full length *DYX1C1, HSP70* (also known as *HSPA1A* or *HSP72*, accession number in GenBank: BC009322), *HSP90α* (NM_005348) and *HSP90β* (NM_007355) cDNAs were amplified with gene-specific primers were designed according to the structure of TOPO vectors from human brain cDNA (Clontech). The inserts were cloned into pcDNA3.1/CT or/and NT-GFP-TOPO (Invitrogen) vectors. *DYX1C1* was cloned into a pcDNA3.1/V5-6xHis TOPO-expression vector (Invitrogen).

In study IV, plasmids pU6DyxHPB (bases 980-999) and pU6DyxHPE (bases 798-821) code short hairpin RNAs (shRNAs) for blocking the expression of *DYX1C1* gene. They were constructed from synthesised oligonucleotides (Invitrogen) and cloned into the mU6pro vector (Yu et al, 2002). Two control plasmids were also used: pU6DyxHPBm3, which had three mutations mismatched to pU6DyxHPB, and pU6-BT4HP1, which is an RNAi effective against neuronal β-3-tubulin (Yu et al., 2002). The eGFP expression plasmid pLZRS-CAgapEGFP (Okada et al. 1999) was used to mark transfected cells by co-electroporation.

Dyx1c1 rat construct was amplified by PCR (Platinum Taq DNA polymerase, Invitrogen) from rat embryonic (E14) brain cDNA. It was cloned to pGEMT-Easy vector (Promega), amplified using 5' primers incorporating myc-epitope-tagged sequences and subcloned into a pCAGGS vector to create pCA-Dyx. In order to investigate the role of *DYX1C1* domains for the localisation of protein in the cell, the full length and both N-terminal (p23 domain) and C-terminal (TPR domains) truncated *DYX1C1* constructs were done using pCAGGS vector. eGFP sequence from the plasmid pLZRS-CAgapEGFP were amplified and ligated to a pCAGGS vector in C-terminal end of full length and truncated DYX1C1 construct to form GFP fusion proteins.

5.4. Transient transfections (II, IV, V)

For transfection, COS-1 cells were plated on sterile glass coverslips in 12- and 24-well plates, and they were transfected by 60-70% confluent with FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's protocols. In study V, COS-1 and COS-7 cells were co-transfected by lipofectamine (Invitrogen) according to the manufacturer's protocol. In RNAi validation experiments in COS-7 cells, the ratio of shRNA to pCA-Dyx-eGFP was 30:1.

5.5. Immunofluorescence stainings (II, V)

In order to prepare cell samples for microscopy, COS-1 cells were fixed with paraformaldehyde and made permeable with Triton X-100/PBS. Cells were incubated first 30 min in BSA/PBS to block unspecific binding of the antibody. Then cells were incubated with V5-antibody (Invitrogen) for a period of 45 min followed by TRITC-conjugated goat anti-mouse IgG (Sigma-Aldrich). Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and in the end coverslips were mounted on glass slides. The detection of cells was performed by Zeiss Axioplan 2 Imaging fluorescence microscope equipped with an ISIS digital image analysis system (MetaSystems, Altlussheim, Germany).

5.6. Western blot analyses (II, IV, V)

The specificity of anti-V5 antibody of DYX1C1-pcDNA3.1/V5-6xHis-construct in study II and specificity of anti-GFP antibody of HSP70-, HSP90α- and HSP90β-GFPN/C constructs in study V were confirmed with standard western blotting methods. Denatured proteins were separated in SDS-polyacrylamide gel and blotted using standard protocols. In study IV, western blot analysis was performed to detect the size of DYX1C1 protein using both N-terminal and C-terminal antibodies.

6. Functional studies

6.1. DYX1C1 expression in stress, studies with cells *in vitro* (V)

In order to detect the effect of stress on the expression of DYX1C1, COS-1 cells for immunofluorecence stainings and HEK 293A cells for real-time PCR were stressed by heat shock. After transfection and a recovery period of 24 h, the transfected COS-1 cells

were heat shocked by placement incubator at 42°C for an hour, followed by incubation at 37°C when a recovery period was included. The first set of cells were fixed and stained immediately after the heat shock and the second set after a 3 h recovery period. HEK 293A cells were also heat shocked for TaqMan assay by placement incubator at 42°C for an hour and instead of fixing, the RNA of cells were extracted in the same time points than with COS-1 cells. Controls in both experiments were incubated at 37°C and fixed or extracted the same amount of time as the first set of heat shocked cells.

6.2. DYX1C1 expression in stress, studies with rat brain tissue *in vivo* (V)

Ischaemia is stressful for the cells like heat shock, and so *in vivo* studies with induced focal cerebral ischaemia for rat brain tissue were performed. Post-mortem human brain tissues from normal and ischaemic brains were also used in immunohistological stainings.

6.2.1. Focal cerebral ischaemia for rats and experimental groups (V)

Permanent focal cerebral ischaemia was induced during anaesthesia by the suture occlusion model, where normal blood flow was prevented with ligations in the carotid arteria, as described in the work of Liu et al. 2001. The sham controls underwent the same procedure, but in addition to a slightly different place of the block, it was withdrawn one minute later. Animals were divided into groups depending on the length of the post-ischaemia period. The controls (n=9) underwent cardiac perfusion-fixation 24 h after the sham occlusion. The animals exposed to ischaemia underwent the same procedure 6 h (n=7), 24 h (n=5), 3 days (n=7) and 7 days (n=6) after permanent ischaemia. The rats were terminated (Liu et al. 2001) and immediately after the cardioperfusion, the brains were removed and immersion-fixed in 20% formaldehyde solution. After fixation the brains were dissected, blocked in paraffin and stored until cut with a microtome (Leica SM2000) into 5 μm sections for histological stainings.

6.2.2. Immunohistochemistry (II, V)

In order to detect localisation of DYX1C1 protein in normal and ischaemic human brain (study II), brain tissues were fixed in formalin and embedded in paraffin, and standard immunohistochemical methods for post-mortem autobiopsy material were used (Lindsberg et al. 1996). Immunohistochemical stainings for normal and ischaemic rat brain tissues (study V) were also performed using standard methods and in addition to Dyx1c1, Hsp70 and Hsp90 were detected using mouse anti-Hsp70 monoclonal antibody

(Stressgen) and Hsp90 α/β (F-8) mouse monoclonal antibody (Santa Cruz, CA, USA). In both studies, an unpurified anti-DYX1C1 antiserum was used and as a control, all stained sections were compared with adjacent tissue sections incubated with the pre-immune serum in identical conditions and dilutions.

6.2.3. Light microscopy (II, IV)

Light microscopy of tissue sections in study II was performed with Leitz Laborlux D microscope (Leitz, Wetzlar) equipped with Nikon Coolpix 995 digital camera (Nikon). Morphologic analysis of the tissue sections and cell counting in study V were performed by a light microscope (Axioplan II, Zeiss, Germany) to determine the proportion of neuronal structures stained for Dyx1c1. Counting was performed systematically in three locations in the ipsilateral (infarcted) hemisphere: the infarction core area, the surrounding "penumbral" area and in the more remote parasagittal cortical area. Homologous areas were examined in the contralateral (non-infarcted) hemisphere. At magnification of 400x, 5 randomly selected fields (area of 0.299 mm^2 per field) were examined within each area and the number of positive nuclei and cytoplasms were recorded.

6.2.4. Statistical analysis for immunohistochemical samples (V)

Data are presented as mean ± SE for the indicated number of animals. Differences between the sham and experimental groups in each brain area were analysed with one-way analysis of variance (ANOVA), followed by Holm-Sidak post hoc test. Data sets that were not normally distributed were analysed with Kruskall-Wallis test followed by Dunn`s post hoc test. A *p*-value <0.05 was considered statistically significant.

6.3. *In utero* RNAi (IV)

Pregnant Wistar rats gestational day 14 were anaesthetised and microinjections of plasmid mixtures into the lateral cerebral ventricles of E14 embryos were made. shRNA plasmid vector, Dyx1c1-eGFP expression vector, and/or 0.5 μg of eGFP expression vector were injected into the VZ of each embryo. After injection of the plasmid, a single 75V pulse was delivered across the head of the embryo within the uterus. After 1-4 days the position and morphology of transfected cells were analysed by fluorescence microscopy. *In utero* transfection and RNAi methods were described in detail previously (Bai et al. 2003).

6.3.1. Histology and image analysis

All brains were sectioned and processed for image analysis as previously described (Bai et al. 2003). Briefly, brains were fixed with 4% paraformaldehyde and sectioned with a vibratome (Leica VT 1000S) and processed for immunocytochemistry. For BrdU labelling injections were made 1 hour prior to sacrifice and nuclei were labelled with TOP-PRO-3 (Molecular Probes). Leica TCS SP2 confocal microscope system and Photoshop 7.0 were used for acquiring and adjusting the images.

In order to quantify migration distances of all transfected cells in an image (200-1400 cells/section) the shortest distance from the ventricular zone surface to the soma of the transfected cells was measured. Migration distances were determined 4 days following transfection unless indicated otherwise. An automated particle analyses macro in ImageJ and Excel were used for analysis. A single mean migration distance was computed for each section (2-3 sections/embryo) and data from 3-6 embryos was used for comparisons of mean migration distance in different transfection conditions. To test for significant differences in the migration distances between the transfection conditions ANOVA was used. However, all phenotypic differences described were discernable qualitatively and were consistent from brain to brain.

7. Evolutionary analysis (II, III)

In order to compare the *DYX1C1* sequence between human and mouse, the mouse Dyx1c1 was constructed from two overlapping EST sequences (GenBank accession nos. BG242087 [GenBank] and AK005832 [GenBank]) and verified by comparing it to all available mouse *Dyx1c1* EST sequences. cDNA sequences of *Dyx1c1* and *DYX1C1* were aligned with CLUSTALX. The alignment was improved manually, and shaded with BOX-SHADE. The secondary structure of the T+A-rich region was predicted with MFOLD (available at www.bioinfo.rpi.edu/applications/mfold/old/dna/) with default parameters.

To study differences in *DYX1C1* and *ROBO1* sequences between human and non-humanprimates (chimpanzee, pygmy chimpanzee, gorilla, and orangutan), primate DNA samples were obtained from the Coriell Institute (Camden, New Jersey, Primate Panel PRP00001). The genomic sequences of non-human primates corresponding to all exons were determined by direct sequencing after PCR amplification with human-specific intronic primers. A likelihood ratio test was performed with the Codeml programme of the

PAML package (Clark et al. 2003). The likelihood ratio test was used to analyse variation in selective pressure in ROBO1 sequence in the different lineages. Non-synonymous and synonymous (dN/dS) ratio was counted. A smaller ratio than 1 is considered as purifying Darwinian selection, whereas ratio higher than one is regarded as a sign of positive selection.

RESULTS

1. The genome-wide scan

1.1. Linkage analysis (I)

In order to find genes which associate with the dyslexia phenotype in the Finnish population, a genome wide scan with microsatellite markers was performed for 88 subjects from 11 families, with 38 dyslexic members. Analyses revealed two loci, which linked to dyslexia, the previously defined region on 2p corresponding to *DYX3* and a novel locus on 7q32 corresponding to *SPCH1*. On chromosome 2, the highest NPL score was 2.55 for marker D2S2216 on 2p11 ($p = 0.004$), with a single high NPL 3.02 ($p = 0.03$) in family 2. Marker D2S2216 is approximately 34 cM centromeric from the *DYX3* locus, based on marker locus information from the Marshfield and DeCode maps. On chromosome 7, the highest NPL score was 2.77 ($p = 0.003$) for marker D7S530 on 7q32, with one family showing an NPL of 4.21 ($p = 0.03$, figure 6). In addition to non-parametric analyses, parametric analysis in the autosomal dominant model showed a significant two-point lod score of 3.01 for marker D2S286 (2p12). No evidence for linkage above background to the previously reported dyslexia loci on chromosomes 15q21, 6p21.3, 3p12-q13, and 18p11.2 was observed.

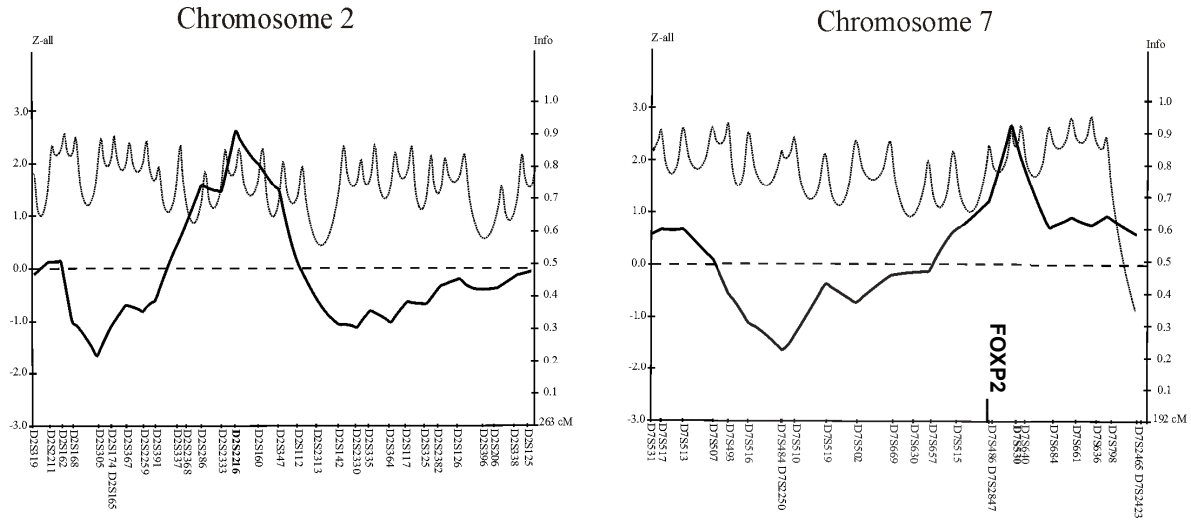

Figure 6. Linkage result of the genome-wide scan, two highest peaks on chromosomes 2 and 7, and *FOXP2* on chromosome 7.

1.2. Mutation screening of positional candidate gene FOXP2

The *FOXP2* (forkhead box P2) on 7q31 is the first gene, which has been associated with speech and language development (Lai et al. 2001). It is located within the peak of the linkage region, approximately 15 Mb centromeric from marker D7S530 and was considered here as a positional candidate gene. The entire coding sequence of candidate gene *FOXP2* was sequenced from three controls and six dyslexic subjects from different families, including the two highest scoring pedigrees, but no mutations were found.

2. The first dyslexia candidate gene *DYX1C1* (II, V)

2.1. Discovery of new gene *DYX1C1*

The karyotype analysis revealed a translocation, which cosegregated with dyslexia in a Finnish family (family 15A, figure 4A). The translocation breakpoint was refined within the BAC clone RP-11-178D12 (AC013355 [GenBank]) using fluorescent *in situ* hybridisation. To identify the exact breakpoint, PCR-amplified non-repetitive genomic DNA fragments from the BAC clone RPCI-11-178D12 were used as probes in Southern hybridisation. With a probe and six restriction enzymes the breakpoint was pinpointed between restriction sites for *Pst*I and *Hin*dIII (figure 7). This region of 3229 base pairs includes exons 8 and 9 of a novel gene, first called *EKN1* (for the Finnish words En Keksi Nimeä 1, deposited in January 2001 to GenBank as AF337549), and later officially renamed *DYX1C1,* dyslexia candidate gene 1 in DYX1 locus. There was also a 301-bp A+T-rich region with an almost complete 72-bp inverted repeat in the translocation region, suggesting a repeat-induced mechanism for the translocation. A+T-rich repeats are known to occur at many chromosomal rearrangement sites (Edelmann et al. 2001).

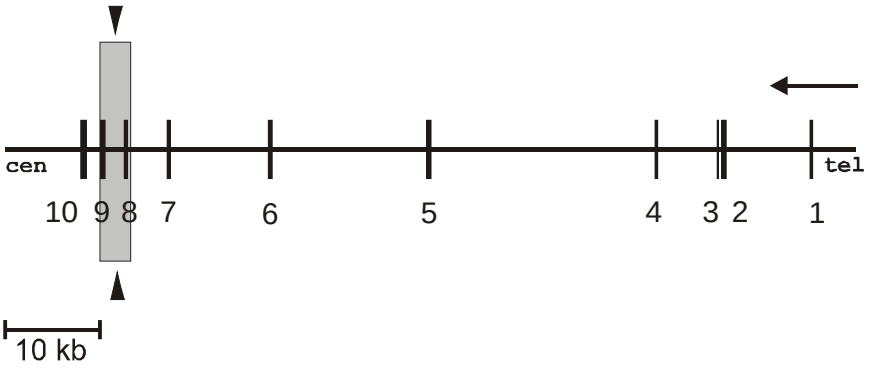

Figure 7. Physical map of the translocation breakpoint region in *DYX1C1* (two arrows). Black bars denote exons.

2.2. Characterisation of DYX1C1 gene structure

The coding sequence of *DYX1C1* was predicted from the genomic sequence of BAC clones RP-11-178D12 and CTD-2137J4, and the exon–intron boundaries were confirmed by RT-PCR. According to predictions, the length of *DYX1C1* mRNA is 1,993 bp, and it spans about 78 kb of genomic DNA. It encodes a predicted protein of 420 amino acids, consists of 10 exons and there are three tetra-trico peptide repeat domains (TPR) in the C-terminal (amino acids 290–323, 324–357, and 366–399). The promoter was identified by three promoter prediction programmes precisely before the 5' end of the cDNA obtained by RT-PCR. The putative promoter of *DYX1C1* has a TATA box (TATAAAT) at position 31. The start codon is located in exon 2, 369 bp from the predicted transcription initiation site. *DYX1C1* mRNA appears to exist in several different splice forms: exons 2 and 9 can be omitted, and there is an alternative acceptor splice site in intron 2. All these arrangements, however, alter the reading frame, leading to truncated protein products.

2.3. Genotyping with SNPs

To study potential association between certain alleles of *DYX1C1* and the dyslexia phenotype, polymorphisms were screened in 55 dyslexic individuals from 20 families and 113 controls (including both family-based and population controls; no significant differences were observed between them in allele frequencies) by direct sequencing. Because the first set included subjects from only 20 families, a replication set with 54 dyslexic and 82 control individuals was recruited. Eight SNPs were found: four of the SNPs (4C>T, 270G>A, 572G>A, and 1259C>G) were in the coding region and resulted in amino acid substitutions, whereas three (–164C>T, –3G>A, and –2G>A) resided in the 5' untranslated region. The eighth SNP is a G-to-T transversion at position 1249 of the *DYX1C1* mRNA, which results in the substitution of a glutamic acid for an ochre stop codon at amino acid position 417 and the deletion of the C-terminal tetrapeptide Glu-Leu-Lys-Ser.

2.4. Association between DYX1C1 allele and dyslexia phenotype

Combining data from two sample sets, the –3A allele frequency in dyslexic subjects was 0.085 (18/212 chromosomes) and 0.028 in controls (11/388 chromosomes), yielding an odds ratio of 3.2 (95% confidence interval 1.5–6.9, p = 0.002). The 1249T allele frequency in dyslexic subjects was 0.117 (25/214 chromosomes) and 0.054 (21/386 chromosomes) in controls, yielding an odds ratio of 2.3 (95% confidence interval 1.2–4.2, p = 0.006). Association tests for –3G>A and 1249G>T remained significant after

Bonferroni correction (p = 0.016 and 0.048, respectively). The other SNPs did not show significant differences.

A common haplotype of –3A and 1249T was seen in 14 dyslexic subjects from eight families but only in 4 normal readers from three families and six population controls. The –3A/1249T haplotype frequency in dyslexic subjects was 0.13 (14/106 cases) and 0.05 (10/192) in controls, yielding an odds ratio of 2.8 (95% confidence interval 1.2–6.5, p = 0.015). Also TDT in informative trios (n = 9) was performed. There were five transmissions and zero non-transmissions of the risk haplotype and zero transmissions and five non-transmissions of other haplotypes (p = 0.025).

2.5. DYX1C1 studies at mRNA level

In order to detect expression of DYX1C1 mRNA, RT-PCRs in adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocytes), were performed. *DYX1C1* was most widely expressed in the brain, lung, kidney, and testis. Northern hybridisation revealed about 2-kb transcript, corresponding to the predicted size of *DYX1C1* mRNA in all tissues studied. In addition, about 1-kb and 5-kb transcripts were seen in skeletal muscle but not in any of the other tissues studied.

2.6. DYX1C1 studies on protein level

2.6.1. DYX1C1 localisation in the cell

The cellular localisation of DYX1C1 protein was studied in transiently transfected COS-1 cells using immunofluorescence. The fusion protein, DYX1C1 with C-terminal V5 epitope and a polyhistidine tail, showed a staining pattern similar to 4',6-diamidino-2-phenylindole (DAPI) staining, suggesting that DYX1C1 is a nuclear protein. The result was similar with a construct including a deletion of the last four amino acids. Afterwards it was observed that transfection of full-length rat Dyx1c1 into COS-7 cells or to neurons resulted in protein concentrated in the cytoplasm. This raised the possibility that *DYX1C1* may be differentially localised and processed within different cell types. The cellular localisation following transfection of full-length *DYX1C1* in COS-1 and COS-7 cells were compared. Approximately 50% of COS-1 cells showed localisation to the nucleus while fewer than 10% of COS-7 showed nuclear localisation.

In order to explain different localisation of DYX1C1 depending on the cell type, the role of p23 and TPR domains in localisation were also determined. The N-terminus without the TPR domains but containing the p23 domain localised predominantly to the nucleus, and the C-terminal TPR domains localised predominantly to the cytoplasm. To investigate whether COS-1 cells produce N-terminal fragments of DYX1C1 protein, Western blot analysis of COS-1 cells transfected with human *DYX1C1* was performed. Protein was detected using antibodies directed to N-terminus (amino acids 114-128) and a C-terminus, and in both studies 48 kD and 24 kD products were observed. In immunocytochemistry of transfected COS-1 cells the N-terminal antibody identified cells with both nuclear and cytoplasmic localisation, while the C-terminal antibody identified cytoplasmic protein. These results indicate that there are two 24 kD proteins which were produced by cleavage of the 48 kD protein, and that the N-terminal fragment localises to the nucleus and cytoplasm, and the full length and C-terminal fragment localises predominatly to the cytoplasm. This result is contradictory to the earlier result with DYX1C1construct with C-terminal V5 epitope.

2.6.2. DYX1C1 expression in normal and ischaemic human brain

Expression of DYX1C1 protein in human brain was studied using immunohistochemical stainings of post-mortem brain tissue. Light microscopy of normal human brain sections revealed a strikingly nuclear expression pattern for DYX1C1 immunoreactivity, consistent with the first transfection results with DYX1C1-V5/6xHis construct. In both cortical neuronal cell populations and white matter glial cells, a minority of cell nuclei expressed DYX1C1 immunoreactivity. Characteristically, even neighbouring, identical-appearing, cells had different expression, which together with the lack of staining obtained with pre-immune serum, supports the specificity of immunoreactivity.

DYX1C1 immunoreactivity was also studied in individuals who died soon after the onset of acute ischaemic stroke. In contrast to the typically nuclear expression in the normal brain, also cytoplasmic expression was observed in ischaemic brain areas. In cortical areas representing early ischaemic morphology, the fraction of positive cell nuclei or cytoplasms appeared increased compared with non-ischaemic brain or contralateral hemispheres. In most ischaemic sections studied, also structures corresponding to neuronal processes were frequently found positive for DYX1C1. Quantitative or statistical analysis of expression was not attempted.

2.7. Functional studies of DYX1C1

The results from ischaemic brain tissue and new information of interactions between *Hsp70*, *CHIP* (*STUB1*) and *DYX1C1* (Hatakeyama et al. 2004) led us to perform stress reactions *in vivo* and *in vitro* and investigate potential interactions and the function of *DYX1C1*. In *in vitro* studies, after the heat shock, DYX1C1 relocates to small particles in the cytosol. Real time PCR was used to examine the expression of DYX1C1 and HSP70 in heat shocked and control HEK 293A cells. DYX1C1 expression did not change during the stress treatment in contrast to HSP70, whose expression level rose and proved that heat shock really happened.

In vivo studies the light microscopy field of normal rat brain tissue observed that a small proportion of neuronal and glial cell nuclei and a minority of neuronal cytoplasms were stained. Experiments with ischaemic rat brain indicate that there was a significantly increased fraction of Dyx1c1 immunoreactive cell nuclei in the infarct core at all time points (6 h, 24 h, 3 days and 7 days) after infarction compared to non-ischaemic brains. Expression was also significantly elevated at 24 h and 7 days compared to controlateral hemispheres. The density of cells with immunoreactive cytoplasms was also elevated, but not as high as the number of positive nuclei. The trend seems to be that the elevated density of positive nuclei was followed by more restrained but still elevated density of positive cytoplasms, which is consistent with the result of *in vitro* studies. In the border region the density of positive nuclei was much higher than the density of cytoplasms and the same trend was detected in the core. The elevation in density of positive cytoplasms occurs after delay and is more restrained across the board. The number of positive nuclei and cytoplasms was clearly smaller than in the core. In the distant region the situation change and the density of positive cytoplasms was higher (significantly elevated in time points 6 h and 24 h compared to non-ischaemic brain) than positive nuclei and the number of positive nuclei was lower than in core and border regions. Interestingly, although the density of positive cytoplasms in the distant region was lower than in the core, it was higher than the density of positive cytoplasms in the border region.

2.7.1. DYX1C1 and stress proteins HSP70 and HSP90

In order to study potential interactions co-localisation experiments were performed. In *in vitro* studies DYX1C1 and HSP70 were localised mostly in the nuclei and slightly in the cytoplasms. After heat shock treatment they were both observed to stay in the cytoplasm and to co-localise in small particles there. The amount of HSP70 was increased also in the nucleus, which is typical for that protein (Murphy et al. 1996, Wagstaff et al. 1996). Three

hours after heat shock, they were found at strikingly separate locations: DYX1C1 located mainly in the cytoplasm and HSP70 in nuclei. HSP70 was seen strongly after heat shock also in the nucleolus. The co-localisation of DYX1C1 and heat shock proteins 90α and β was also investigated. HSP90 has two isoforms with 86% similarity of amino acid sequence, but the isoforms' specific functions are not known. Before heat shock both HSP90s appeared mainly in the cytoplasm of the cell. The amount of them increased promptly after heat shock in the cytoplasm and also in the nucleus, and after 3 h it seems to be found also on cell membranes or cytoskeleton. The expression of HSP90s in the cytoplasm was similar to DYX1C1 and they co-localised in small particles right after the heat shock.

Stress response *in vivo* was found to be most prominent 24 h after ischaemia, and so the co-localisation of Dyx1c1 and stress proteins was examined at this time point. Neuronal Hsp70 expression was detected in the hippocampus and in the cerebral cortex at the border zone in ipsilateral hemisphere, but mainly in the perikarya. No neuronal staining was observed in the infarct core, contrary to Dyx1c1, whose elevated expression was observed in both the nucleus and cytoplasms. Compared to Hsp70 induction in pyramidal neuronal cells, no similar Dyx1c1 expression could be seen in the hippocampal area. The expression of Hsp90 was widespread 24 h after ischaemia: in both hemispheres in hippocampus, cortex, stratium and cerebellum, ependyma and choroid plexus (data not shown). The expression was predominantly detected in the perikarya, but also in the nuclei and dendrites. In all localisations where Dyx1c1 expression was increased, Hsp90 immunoreactivity was also enhanced.

3. DYX1C1 in neuronal migration (IV)

3.1. Interference of *DYX1C1* expression disrupts neuronal migration

To study the function of *DYX1C1* in neuronal migration *in vivo*, shRNA vectors capable of decreasing heterologously expressed Dyx1c1 protein by 30-70% in COS-7 cells were produced. By *in utero* electroporation and RNAi method cells in the rat embryo ventricular zone (VZ) surface were transfected and labelled, and then transfected cells were allowed to migrate away from the surface of the lateral ventricles during four days. The result was that transfection of shRNAs against Dyx1c1 mRNA caused significant reductions in the distance that neurons migrated away from the ventricular zone surface in embryonic rat neocortex relative to controls (figure 10 in discussion).

Cells were transfected with two controls, one of which targets beta-3-tubulin (Yu et al., 2002) without an effect on migration (Bai et al. 2003), and another contains a 3-base mismatch relative to pU6-DyxHPB. The former migrated 519 ± 82 µm (n=6) away from the ventricular zone surface in four days and the latter 509 ± 74 µm (n=6). In contrast, cells transfected with either of two shRNA constructs that decrease Dyx1c1 expression, pU6-DyxHPB and pU6-DyxHPE, migrated 229 µm ± 59 and 212 ± 90 µm (n=6) respectively from the VZ surface of the embryonic neocortex. The cells transfected with the shRNAs targeting Dyx1c1 expression did not migrate into the cortical plate within 4 days like controls, but instead remained within the intermediate zone (IZ), subventricular zone (SVZ), and ventricular zone (VZ).

3.2. Overexpression of DYX1C1 rescues migration

To test that the effect of *Dyx1c1* knockdown using shRNAs on migration is specific, rescue experiments were performed. The Dyx1c1 expression vector with a strong promoter driving expression of Dyx1c1-eGFP (pCA-Dyx-eGFP) was co-transfected with shRNA vectors targeting Dyx1c1. Co-transfection induced expression of *Dyx1c1* in COS-7 cells and in neurons above background levels in presence of shRNA transfection. The experiments in migrated neurons showed that addition of Dyx-eGFP rescued the migration also in the embryon, despite shRNAs transfected at the same time. The migration distance for these cells was comparable to shRNA controls (532 ± 47 µm). It was tested that overexpression of *Dyx1c1* alone did not impair migration and also that *Dyx1c1* overexpression did not rescue migration impaired by RNAi of *Dcx*, another dyslexia candidate gene, which has an effect on neuronal migration (data not shown). These results indicated that impaired migration following RNAi of *Dyx1c1* is specific to that dyslexia candidate gene.

In order to determine the role of Dyx1c1 in the transition out of the multipolar stage, the percentages of transfected neurons that were in the bipolar and multipolar stages were compared. Neurons within SVZ and IZ and 1, 2 and 4 days following transfection were counted. There was an increase in the number of cells that progress into the multipolar stage from 1 to 2 days after transfection for both control and Dyx1c1 RNAi-treated cells. By 4 days after transfection the majority of control transfected cells had re-established a bipolar morphology. In contrast, most *Dyx1c1* RNAi treated cells remained multipolar in morphology. This result suggests that Dyx1c1 is not necessary for transition into the

multipolar stage of migration, but is required for the normal rate of transition out of the multipolar stage.

3.3. TPR domains of DYX1C1 have significant role in neuronal migration

To study the potential distinct functions of N and C terminal motifs of Dyx1c1 protein, RNAi rescue experiments using truncated mutants were performed. N-terminal construct contained the first 320 amino acids including p23 domain, and C-terminal construct contained the final 108 amino acids including the TPR domains. The third deletion construct used was missing only the 4 C-terminal amino acids, which are not part of the TPR domains. Transfection of the N-terminal construct was not effective at rescuing migration arrest when transfected along with the shRNA construct (pU6-DyxHPB). Instead of that, the C-terminus alone rescued the migration when transfected with shRNA, as well as the construct with C-terminal four amino acid deletion. The result indicates that the C-terminal TPR domains are both necessary and sufficient to Dyx1c1 function in neuronal migration.

4. The axon guidance receptor gene roundabout homologue 1 (*ROBO1*) (III)

4.1. Discovery

The karyotype analysis revealed a translocation t(3;8)(p12;q11) in patient with dyslexia phenotype. The translocation occurred in the same region on chromosome 3 that showed linkage to the dyslexia phenotype in a previous study with large Finnish pedigree (Nopola-Hemmi et al. 2001). Although only the index patient had a translocation in his family, and he and his sister both have the dyslexia phenotype (see materials and methods), the translocation breakpoint was mapped in the hope that a new dyslexia candidate gene might be revealed. Fluorescence *in situ* hybridisation was used to narrow down to the translocation region in bacterial artificial chromosome clone RP11-143B12 and the exact breakpoint was determined by Southern hybridisation. The breakpoint was mapped within the ortholog of the *Drosophila roundabout (robo)* gene, *ROBO1,* which is also known as *DUTT1* (Deleted in U Twenty Twenty). This known axon guidance receptor gene, a plausible candidate gene for dyslexia susceptibility, was disrupted by translocation between exons 1 and 2. Two other positional candidate genes, dopamine D3 receptor gene

(*DRD3*) and 5-hydroxytryptamine receptor 1F (*5HT1F*), were also located in the region (Nopola-Hemmi et al. 2001).

4.2. Genotyping

In order to study whether the *ROBO1* associates with dyslexia in a large linkage family linked to 3p12, genotyping by sequencing of the exons, splice sites, 1 kb of ROBO1 promoter region upstream of exon 1 and the extended 3′ UTR region of *ROBO1* variant 2 from the genomic DNA was performed. A dyslexic individual and his parents (father dyslexic, mother unaffected) from the large linkage family were sequenced as well as all exons from the cDNA of dyslexic individual from the extended family. Comparison of these sequences to *ROBO1* and *ROBO1* variant 2 sequences in NCBI GeneBank revealed altogether seven sequence variations, two of them previously known. Dyslexic individuals had two silent single nucleotide polymorphisms (SNPs) in *ROBO1* exons 12 and 18 (1741G>A, 2794C>A; numbering according to *ROBO1*), an exonic 3-bp deletion and insertion polymorphism (DIP6203–6205; numbering for *ROBO1* variant 2), four SNPs in 3′ UTR (UTR, 6227C > A, 6483T > A, 6651T > A, 6923T > G; numbering for *ROBO1* variant 2), and four intronic SNPs (intron 2: 59567 and intron 7: 1451; numbering for BAC RP11-588D3; intron 25: 16181 and 16198; numbering for BAC RP11-26M20). Observed changes were confirmed in three additional pedigree members (dyslexic father, son, and unaffected mother) by sequencing.

Exonic SNPs of ten additional family members and two non-dyslexic individuals were genotyped and a specific SNP haplotype segregated with dyslexia was revealed. Two other positional candidate genes, *DRD3* and *5HT1F,* were mapped outside the shared haplotype. None of the polymorphisms was uniquely observed only in dyslexic individuals and none of the four observed intronic SNPs produced alternative splice variants. Because ROBO1 gene spans about 990 kb of genomic DNA and contains altogether over 2,200 intronic SNPs (according to the NCBI SNP database), only these four intronic SNPs were investigated in this study.

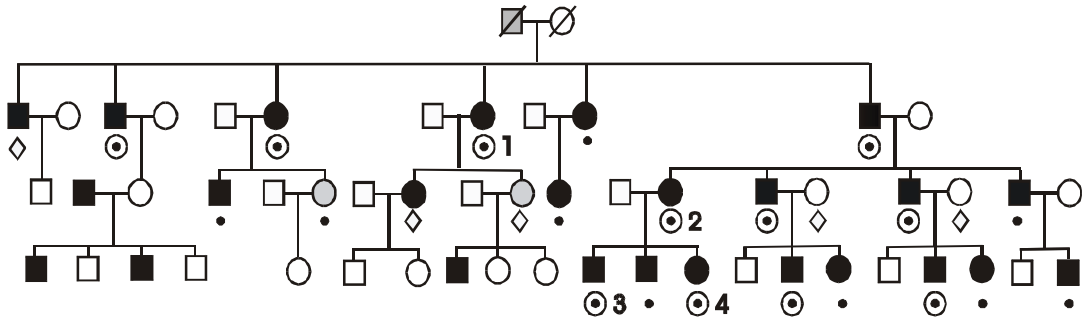

Figure 8. An abridged pedigree of the extended family linked to *DYX5* on chromosome 3 (study III). Affected individuals are shaded black and unverified dyslectics are shaded gray. Numbers refer to samples studied for *ROBO1* expression, a dot indicates carriers of the dyslexia-linked haplotype and circled dots indicate individuals genotyped for all markers (also outside of dyslexia-linked haplotype). Diamonds denote individuals genotyped for all markers, but not sharing the haplotype.

4.3. Splice variants and new exon in *ROBO1*

Because exonic and intronic polymorphisms, which seem to be silent could induce disease-related splice variants (Pagani and Baralle 2004), alternative splicing of ROBO1 was studied. All *ROBO1* and *DUTT1* exons from a dyslexic individual in the linkage family, an unrelated healthy control, and adult human brain cDNA were screened by RT-PCR and seven novel splice variants were detected. Their cloning and sequencing revealed the exclusion of exons 2 (88 bp, 89–169 of *ROBO1*), 19 (27 bp, 2813–2829 of *ROBO1*), and 29 (196 bp 4745–4939 of *ROBO1*) entirely and exclusively of *DUTT1* exon 2 (346 bp 1019–1345 of *DUTT1*); the initial 165 bp of exon 22 (3037–3201 of *ROBO1*); 905 bp ranging from exons 24 to 28 (3603–4508 of *ROBO1*); and 878 bp ranging from exons 25 to 28 (3641–4528 of *ROBO1*).

No splice variants were uniquely different between dyslexic and control individuals; however, quantitative differences could not be reliably assessed. Comparison of the genomic and cDNA sequences for *DUTT1* in several individuals suggested that exon 7 of *DUTT1* is not co-linear with the genomic sequence. Instead, *DUTT1* bases 1891–1900 (gttgggtct: valine, glycine, and serine), in the beginning of *DUTT1* exon 7 (*ROBO1* exon 8) belong to a new short exon, marked exon 7b corresponding to bases 5987–5995 of BAC RP11-588D3. These bases have previously been reported as part of the *DUTT1* gene but they are not included in the *ROBO1* cDNA sequence. In all individuals sequenced, the cDNA sequence included the new exon 7b, indicating that it is included in the major splice form in at least brain and lymphoblast RNA.

The BLAST search for expressed sequence tags (ESTs) homologous for the 5′ ROBO1 region and 5'RACE revealed a new exon. This exon, referred to as ROBO1 exon a, located upstream of exon 1, includes 455 bp and spans bases 28919–28466 in the BAC clone AC125624

4.4. Haploinsufficiency

To measure whether both alleles of *ROBO1* were equally transcribed in dyslexic individuals segregating the dominant susceptibility haplotype, comparison of genomic and cDNA sequences was performed. DNA and cDNA from lymphocyte RNA were investigated from four patients and four useful SNPs were found: SNP 2794C>A was heterozygous in one, 6483T>A in four, 6651T>A in four, and 6923T>G in one patients. The normal readers' lymphocyte RNA and commercially available brain RNA were used as controls. The result was that the expression of the dyslexia-linked allele was significantly attenuated as measured by allelic peak heights ($p = 0.017$ by two-tailed t test, figure 9). In control samples, biallelic expression was consistently observed. By the repeated measurements, the mean expression level of the dyslexia-associated allele in dyslexic participants was 66% of the same allele in controls ($p < 0.0004$ by two-tailed t test).

To verify the result of haploinsufficiency studies of SNPs 6483T>A and 6651T>A, the SNP 6483T>A was sequenced again in the whole sample set. The result of these additional SNP assays was that the allelic imbalance in cases versus controls was highly significant ($p < 0.00005$ by t test). In order to study that the attenuated expression of dyslexia-associated allele involved only *ROBO1*, SNPs from genes *GBE1* and *HTR1F* in the four dyslexic individuals of the large family were genotyped. Heterozygosity was detected for the *GBE1* SNPs 2363A>G and 646A>G in three patients. For these polymorphisms, normal biallelic expression was observed in all three patient samples in contrast to the finding with *ROBO1,* suggesting that transcription of *ROBO1* was specifically silenced.

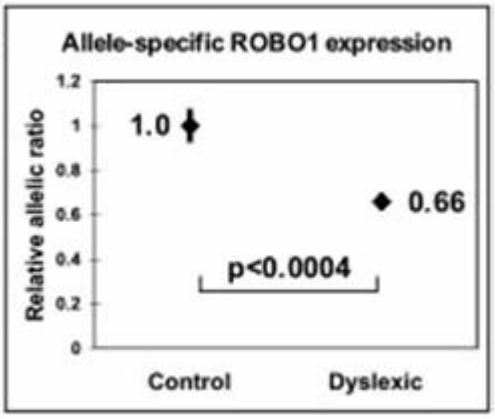

Figure 9. Attenuation of *ROBO1* mRNA expression from the dyslexia-associated allele (SNP 6483 A/T, study III). Allelic ratios were assessed by five to six replicated sequencing tracings in four controls and four dyslexic individuals. Data are shown as mean ± 1 standard error of the mean (bars). Significance was assessed by two-tailed t test.

Because there was no specific SNP for the *ROBO1* or *DUTT1* transcripts, the isoform-specific down-regulation could not be detected. In the translocation patient, two SNPs were heterozygous, in both regions corresponding to exons common to both *ROBO1* and *DUTT1* transcripts (6651T>A and 6923T>G). These SNPs revealed two alleles present in cDNA in the translocation patient, suggesting that *DUTT1* might be biallelically expressed even though the genomic structure of *ROBO1* was disrupted by translocation in one chromosome.

5. Evolution (II, III)

The mouse *Dyx1c1* was predicted by connecting overlapping mouse EST clones. It encodes a protein of 421 amino acids and it is 78% identical with human *DYX1C1*. The non-human primates, chimpanzee, pygmy chimpanzee, gorilla and orangutan were sequenced for the genomic segments corresponding to human exons. The predicted proteins differed by 3, 2, 5 and 6 amino acids (0.7%, 0.5%, 1.2% and 1.4% of residues), respectively. *ROBO1* in four non-human primates was also sequenced and rat was used as an out-group in sequence comparisons. Seven amino acid changes were detected between human and chimpanzee, and 20 between human and orangutan, which indicated a high level of variation between the related species and humans.

Non-synonymous and synonymous (dN/dS) ratio in *ROBO1* was smaller than 1 in all lineages, implicating purifying Darwinian selection. The likelihood ratio test rejected the null hypothesis of fixed dN/dS ratio in all lineages. A model in which the omega value is higher in lineages leading to humans, chimpanzees and gorillas was significantly better than a free-ratio model (p <0.001). Although ratios higher than one are considered a sign of positive selection, it has been observed that genes that are expressed in the brain are under stronger selective pressure than many other genes (Duret et al. 2000).

DISCUSSION

1. The field of dyslexia research in 2007

Since 2001, research on dyslexia has been advancing at a revolutionary speed. The number of candidate loci has increased from five to nine, and four candidate genes have been revealed (table II). In addition to the support for the linkage between dyslexia and locus *DYX1* on chromosome 15q22 (Marino et al. 2002, Chapman et al. 2004, Bates et al. 2006), *DYX2* and *DYX4* on chromosome 6p22 and 6q11 (Kaplan et al. 2002, Turic et al. 2003, Deffenbacher et al. 2004, Francs et al. 2004, Cope et al. 2005a, Meng et al. 2005, Schumacher et al. 2006, Harold et al. 2006, Bates et al. 2006), *DYX3* on chromosome 2p11-p16 (Fisher et al. 2002, Francs et al. 2002, study I) and *DYX5* on chromosome 3p12-q13 (Stein et al. 2004, Bates et al. 2006), three additional loci has been found: locus *DYX6* on chromosome 18 (Fisher et al. 2002, Bates et al. 2006), *DYX7* on chromosome 11p15.5 (Hsiung et al. 2004), and *DYX9* on chromosome Xq26-q27 (Fisher et al. 2002, deKovel et al. 2004, Bates et al. 2006). Locus *DYX8* was confirmed to be a dyslexia locus on chromosome 1p34 (Grigorenko et al. 2001) and chromosome 7q31 has been linked to dyslexia in two independent studies so far (study I, Bates et al. 2006). Furthermore, two novel linkages have shown suggestive support for new dyslexia loci on chromosomes 4p15.33-p16.1 and 17p13.3 (Bates et al. 2006). Dominant inheritance has been proposed for the dyslexia loci on chromosomes 2 and 3 (Fagerheim et al. 1999, Nopola-Hemmi et al. 2001). For other dyslexia loci, the genes are merely expected to increase the risk for dyslexia, compatible with the common disease/common variant hypothesis. However, in a complex disorder even a modest increase in the genetic risk is interesting.

Table II Dyslexia loci. The following abbreviations have been used: TLNG: targeted linkage, GWS: genome-wide scan, HE: Haseman –Elston regression analysis, DF: DeFries-Fulger regression analysis and VC: variance-component analysis. QTL indicates that phenotype has been treated as quantitative trait.

LOCUS	AUTHORS	METHOD	PHENOTYPE	MATERIAL families,country	FINDINGS
DYX1 Chrom. 15	Smith et al. 1983	TLNG, param	global	9, USA	LINKAGE:LOD 3.24 chrom.15p11.1-q11.1
	Smith et al. 1991	TLNG,HE	global, QTL	19, USA	LINKAGE: 15q15-15qter, p=0.009 / 15q15-15qter, p=0.03
	Grigorenko et al. 1997	TLNG, param/NPL	components	6, USA	LINKAGE:LOD 3.15 in 15q21 for single word reading/significant in 15q11
	Schulte-Körne et al. 1998	TLNG, param/NPL	global	7, Germany	LINKAGE: 15q21multipoint p=0.0042/15q21 multipoint p=0.03
	Morris et al. 2000	association	global	178 trios, UK	ASSOCIATION: 15q15, DD-three marker haplotype p<0.001
	Nopola-Hemmi et al. 2000	translocations	global	2, Finland	TRANSLOCATIONS: t(2;15)(q11;q21), t(2;15)(p13;q22)
	Marino et al. 2002	association , TDT	global	121 families	ASSOCIATION: 15q15-15qter chromosomal region
	Study II	association	global	109/195.	ASSOCIATION: DYX1C1 locus
	Wigg et al. 2004	association	components	148, Canada	ASSOCIATION: DYX1C1 locus
	Chapman et al. 2004	TLNG, param/VC	components, QTL	111, USA	LINKAGE: 15q (word identification)
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE: 15q21.1, regular-word spelling
	Marino et al. 2007	association	components		ASSOCIATION: DYX1C1, short-term memory
DYX2&DYX4	Smith et al. 1991	TLNG,HE	global, QTL	19, USA	LINKAGE: 6p21.3, p<0.02 / 6p21.3, p<0.0001
Chrom. 6	Cardon et al. 1994,1995	TLNG,DF	global, QTL	19,46 DZ pairs, USA	LINKAGE: 6p21.3, p=0.04 / 6p21.3, p=0.009
	Grigorenko et al. 1997	TLNG, param/NPL	components	6, USA	LINKAGE: no significant / 6p22.3-21.3, multipoint p=<0.005 strongest for phoneme awarenessp<0.000001, weakest for single-word reading p<0.005
	Fisher et al. 1999	TLNG,HE/VC	components, QTL	82, UK	LINKAGE: 6p21.3,multipoint, phonological decoding p=0.007, orthographic coding p=0.006 / 6p21.3,multipoint, phonological decoding p=0.004, orthographic coding p=0.007
	Gayan et al. 1999	TLNG,DF	components, QTL	79 twin-based fam,USA	LINKAGE: 6p21, LOD for phoneme awareness 1.46, phonological decoding 2.42 and orthographic coding 3.1.
	Petryshen et al. 2000	TLNG,HE/VC	components, QTL	79, Canada	no significant linkage / weak linkage 6p23-21.3, phon. decoding, rapid autom.naming, spelling
	Grigorenko et al. 2000	TLNG,NPL	components	8, USA	LINKAGE: 6p21.3 for a variety of phenotypes
	Fisher et al. 2002	GWS,VC, DF	components, QTL	173, UK;119,USA	LINKAGE 6p21.3, phonological&ortographic processing
	Kaplan et al. 2002	TLNG,HE,DF,TDT	components, QTL	104, USA	LINKAGE 6p21.3
	Turic et al. 2003	association , TDT	components	101+ 77 trios, UK	ASSOCIATION 6p21.3, no vocabulary or ADHD

	Deffenbacher et al. 2004	sib-pair lng, HE,DF,TDT	components, QTL	349, USA	ASSOCIATION 6p21.3, severe RD, orthographic and phonological skills
	Francks et al. 2004	association	components	264,UK;159,USA	ASSOCIATION 6p22.2:*TTRAP* and *KIAA0319*
	Meng et al. 2005	association	global	156, USA	ASSOCIATION 6p22: DCDC2, deletion
	Cope et al. 2005	association	global	143 trios;223/ 273,UK	ASSOCIATION 6p22: *KIAA0319*
	Schumacher et al. 2006	association	global	376 trios	ASSOCIATION 6p22: *VMP/DCDC2/KAAG1* locus
	Harold et al. 2006	association	global	264; 350/273co, UK	association with 5' flanking region of *KIAA0319* (p=0.0004)
DYX4	Petryshen et al. 2001	TLNG,NPL,VC	components, QTL	96 affected sib-pairs	LINKAGE: 6q11.2-q12, phonological decoding
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE 6q11.2, non-regular word spelling
DYX3	Fagerheim et al. 1999	GWS, NPL	global	1, Norway	LINKAGE 2p15-p16
Chrom. 2	Petryshen et al. 2002	TLNG,NPL,VC	components, QTL	96, Canada	LINKAGE 2p15-p16, the highest LOD for spelling
	Fisher et al. 2002	GWS,VC, DF	components, QTL	173, UK;119,USA	LINKAGE 2p15-p16
	Francks et al. 2002	fine map,association	global	119, USA	SUPPORT 2p12-p16
	Study I	GWS,param/NPL	global	11, Finland	LINKAGE 2p11
	Raskind et al. 2005	GWS,VC, MCMC	components, QTL	108,USA	LINKAGE 2q22, speed of phonological decoding
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE 2q22.3, regular-word spelling
DYX5	Nopola-Hemmi et al. 2001	GWS, param	global	1, Finland	LINKAGE 3p12-q13
Chrom. 3	Study III	translocation	global	1, Finland	t(3;8)(p12;q11)
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE 3p12-q13
DYX6 Chrom.18	Fisher et al. 2002	GWS,VC, DF	components, QTL	173, UK;119,USA	LINKAGE 18p11.2, phonological&ortographic processing
DYX7 Chrom. 11	Hsiung et al. 2004	TLNG, association	global	100, Canada	LINKAGE 11p15.5
DYX8	Rabin et al. 1993	TLNG	global	9,USA	LINKAGE 1p34-p36
Chrom. 1	Froster et al. 1993	Translocation	global	1, Germany	TRANSLOCATION: t(1;2)(p22;q31)
	Grigorenko et al. 2001	TLNG, param/NPL	components	8,USA	SUPPORT 1p
	Tzenova et al. 2004	TLNG, NPL,VC	global, QTL	100, Canadan	LINKAGE 1p34-p36, spelling
DYX9	Fisher et al. 2002	GWS,VC, DF	components, QTL	173, UK;119,USA	LINKAGE Xq26
Chrom. X	de Kovel et al. 2004	GWS	global	1, Holland	LINKAGE Xq27.3
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE Xq27
Chrom.7	Study I	GWS,param/NPL	global	11, Finland	LINKAGE 7q32
	Bates et al. 2006	GWS, VC	components, QTL	403 twin fam., Australia	LINKAGE 7q32-34, non-word spelling

Altogether four candidate genes for dyslexia have been found. The first was *DYX1C1* on chromosome 15q21 and the second was *ROBO1* on chromosome 3. In addition to them, two other candidate genes, *DCDC2* and *KIAA0319* have been found in the most replicated dyslexia linkage region on chromosome 6p22 (Meng et al. 2005, Schumacher et al. 2006, Cope et al. 2005a, Harold et al. 2006). These findings can explain the observed QTL effects on chromosome 6. Except the axon guidance receptor gene *ROBO1*, all of the candidate genes had unknown function.

2. Genome-wide scan in Finnish families

The genome-wide scan in 11 Finnish families revealed two linkages of dyslexia, one novel locus on chromosome 7q32, which has been replicated once (Bates et al. 2006) and a previously confirmed linkage to 2p11 (Fagerheim et al. 1999, Petryshen et al. 2002). Interestingly, the locus on chromosome 7 had previously been linked to autism and the first gene implicated in a speech and language development, *FOXP2,* located in the same region (IMGSAC 1998, Lai et al. 2001). Although *FOXP2* appears to have no role in autism or SLI, it could be a candidate gene for a more specific language disorder such as dyslexia. It has been connected to several facets of language processing and grammatical skills, which are also deficient in dyslexic subjects (Newbury 2001). The results did not support the role of candidate gene for *FOXP2*, but a functional polymorphism might hide in the promoter or intronic regions of the gene, which we did not screen. In addition to *FOXP2* there are several other interesting genes in the linkage region: the G protein-coupled receptor 37 *GPR37* (Marazziti 1997), the actin regulation protein *WASL* (Fukuoka 1997), the protein tyrosine phosphatase receptor type zeta-1 *PTPRZ1*, which is expressed only in CNS (Levy et al. 1993), a putative signalling molecule *WNT2* involved in CNS development (Wassink 2001) and *NRCAM*, a neuronal cell adhesion molecule (Lane et al. 1996). These all are expressed in brain, and the *WASL* protein is involved in the Slit-Robo pathway and has an influence on actin polymerisation and neuronal migration (Wong et al. 2001). In the absence of a more exact genetic localisation of our linkage peak and latter information of *ROBO1*, none of these genes were sequenced.

The linkage peak on chromosome 2p11 maps approximately 34 cM from the peak reported earlier (Fagerheim et al. 1999). This locus might be the same and the difference in the result may be caused by dissimilar sample sets, diagnostic criteria or markers used.

Alternatively, there might be two dyslexia loci in the same chromosomal region. Fine-mapping was performed in this linkage region and the same set of Finnish families was used (Peyrard-Janvid et al. 2004). These studies suggested a candidate gene *TACR1* (tachykinin receptor 1), which is involved in the modulation of neuronal activity, inflammation and mood (De Felipe et al. 1998, Derocq et al. 1996, Kramer et al. 1998). However, this gene was excluded based on sequencing of the coding regions and the lack of haplotype conservation between dyslexic individuals (Peyrard-Janvid et al. 2004).

3. *DYX1C1*, the dyslexia gene in cortical migration

A new gene, *DYX1C1* that associates strongly with dyslexia, was found. Altogether four distinct pieces of evidence support our finding. Firstly, a translocation breakpoint disrupting *DYX1C1* is transmitted with dyslexia in one family. This breakpoint is located within a TPR-domain coding region of the gene, and thus is likely to disrupt protein function. Secondly, two SNPs show association with dyslexia in a Finnish population independently and as a haplotype with a significant TDT result. A new SNP in the promoter region of the gene has been found and it shows association with dyslexia also in other populations (Dahdouh et al. submitted). Thirdly, inhibition of *Dyx1c1* expression disrupts the neuronal migration in the developing brain neocortex of rat embryo, leading to anatomical changes similar to those found earlier in dyslexics, and also to changes in auditory processing, learning and motor skills (Frenkel et al. 2000).

3.1. Translocation on *DYX1* locus

The location of DYX1 locus on chromosome 15 is not completely clear (Grigorenko et al. 1997, Schulte-Körne 1998, Morris et al. 2000, Nopola-Hemmi et al. 2000). Due to the imprecision of genetic linkage in multifactorial phenotypes, *DYX1C1* might correspond to *DYX1*, but there might also be more than one locus for dyslexia on chromosome 15. The peaks of two previous linkage studies map about 7 megabases or 2.2 centimorgans and 16 Mb (Marino et al. 2004) proximally from the breakpoint defined in our study. According to the previous results, the SNPs that have been found in the Finnish population do not associate with dyslexia in other populations (Scerri et al. 2004, Meng 2005, Marino 2005, Cope et al. 2005b), although the DYX1C1 locus has been confirmed (Wigg et al. 2004). In addition to that, the SNP1249, which causes a premature truncation of the protein by four amino acids, does not affect the localisation of DYX1C1 protein in the cell or its function in neuronal migration. These results are consistent with a recently found novel SNP in the

promoter of DYX1C1. This SNP affects transcription factor binding and is suggested to be a new dyslexia risk haplotype (Dahdouh et al. submitted).

3.2. DYX1C1 in the cell

We have detected the localisation of DYX1C1 protein in cells and we observed both nuclear and cytoplasmic expressions. In COS-1 cells it localises more to the nucleus whereas in COS-7 cells it has been detected predominantly in the cytoplasm. *In vivo* studies with human brain tissue indicated expression in the nucleus in a subset of human glial and neuronal cells and in rat brain tissue it is detected in addition to nuclei in the cytoplasms in a minority of neurons. Protein products of sizes 24 kD and 48 kD were detected: short C-terminal fragment with TPRs and full length protein were expressed mainly in the cytoplasms and short N-terminal fragment with p23 domain were detected mainly in the nucleus. This result expanded our earlier results with DYX1C1 construct with C-terminal epitope in COS-1 cells, where DYX1C1 localises mainly in the nucleus. These results indicate further that there might be cell specific regulation factors, which affect the localisation of the DYX1C1 protein.

The protein structure of DYX1C1 offers little information about its cellular function. TPR domains in the C-terminus of the protein are common protein-protein interaction domains, which are found in a wide variety of proteins and are thought to be of ancient origin. The p23 domain in the N-terminal part of the protein is homologous to the binding site of *p23* co-chaperone, which binds to Hsp90 with it (Sullivan et al. 1997, Fang et al. 1998). More information about the interactions of DYX1C1 was published by Hatakeyama et al. (2004). They used a yeast two hybrid system and *in vivo* binding assays where DYX1C1 interacts with CHIP (C-terminus of Hsp70-interacting protein) and Hsp70. CHIP (official gene nomenclature STUB1) is a ubiquitin-protein ligase in the ubiquitin pathway, which plays an important role in the rapid degradation of short-lived regulatory proteins. These proteins are involved in response to stress or to extracellular signals, morphogenesis and organelle biogenesis (Weissman 2001). Hsp70 and Hsp90 are well-known stress proteins that protect cells from death and assist protein folding (Morimoto 1993, Freeman and Morimoto 1996). They are also chaperones for CHIP in chaperone-dependent mechanisms for substrate recognition, and they are a part of the protein quality control system of the cell (Connell et al. 2000). A deficit in this system is connected to a number of neurogenerative disorders, such as Parkinson's disease, Huntington disease and Alzheimer's disease (Meriin and Sherman 2005). Hatakeyama et al. (2004) have suggested that together DYX1C1 and Hsp70 might be a chaperone-dependent mechanism

for substrate recognition. Due to its structural similarity with XAP2, a component of dioxin receptor chaperone complex (Kuzhandaivelu et al. 1996), DYX1C1 also might inhibit the activity of CHIP (Hatakeyama et al. 2004). Whether DYX1C1 has a role as a protector of some receptor or as a co-chaperone in the degradation pathway, remains to be seen. Interestingly, it really co-localises with CHIP *in vitro*, especially right after heat shock (unpublished data).

3.3. *DYX1C1* in stress reaction

The altered localisation of DYX1C1 in ischaemic human brain tissue, the finding of the p23 domain in the structure and new information about interactions led us to perform stress reactions *in vivo* and *in vitro*, and to investigate the function and potential interactions. In a stress reaction *in vivo,* the density of immunoreactive cells elevated in rat brain tissue and both in *in vitro* and *in vivo* studies, DYX1C1 localised more in the cytoplasm. The TaqMan result indicated that the expression of *DYX1C1* did not elevate in heat-shocked 293 cells. This might indicate translational regulation of *DYX1C1*, which is an appropriate mechanism for stress proteins to be active even if the gene expression at transcriptional and post-transcriptional level is inhibited by stress, at least in the *Drosophila* (Ahmed and Duncan 2004). Alternatively, the regulation of *DYX1C1* expression in stress might function in a cell-specific manner. Co-localisation experiments *in vitro* with heat-shocked stress proteins Hsp70, Hsp90 and DYX1C1 indicated that interactions are possible in small punctate staining signals in cytoplasm: after heat shock with Hsp90 and right after heat shock, before distinct localisations after recovery period, with Hsp70. Regardless, our *in vivo* study showed segregated topographic expression of *Dyx1c1* and *Hsp70* in rat brain, suggesting that Dyx1c1 is not involved in the molecular network of Hsp70. Due to the widespread expression of *Hsp90* no specific interactions with *Dyx1c1 in vivo* could be detected.

We suggest that *DYX1C1* is not a housekeeping gene because of its presence only in a small number of neurons by immunostaining. Examination of *DYX1C1* expression in ischaemic brain tissue indicated that it is involved dynamically in the functional cell state, changing in the face of a metabolic challenge. Immunohistochemical data from ischaemic rat brain indicated that *Dyx1c1* expression was enhanced in a distant region but not in the border region right after infarct core. This suggests that *DYX1C1* might be involved, in addition to stress reaction, also in messages that are transported by axonal or transsynaptic mechanisms.

3.4. *DYX1C1* in neuronal migration

We have found out that *DYX1C1* has a role in the migration of neocortical neurons in the developing brain of rat embryo (figure 10). The result indicates that it is required for the transition out of the multipolar stage of migration. This stage occurs when bipolar progenitor cells from the ventricular zone become radially migrating neurons and the stage is particularly vulnerable to disruptions causing neuronal migration disorders (LoTurco and Bai 2006). The same effect has also been seen with genes *Dcx, Lis1* and *FlnA* (Bai et al. 2003, Nagano et al. 2004, Tsai et al. 2005) in which *Dcx* and *Lis1* have been connected to type 1 lissenchepaly and *FlnA* mutations cause nodular peri-ventricular heterotropia (Chang et al. 2005). *DCX* and the new dyslexia candidate gene *DCDC2* are members of the same protein family and individuals suffering from nodular peri-ventricular heterotropia also have language and reading difficulties (Chang et al. 2005, Sokol et al. 2006). However, unpublished research results indicate that Dyx1c1 does not interact with Dcx or Lis1 (Wang et al. 2006).

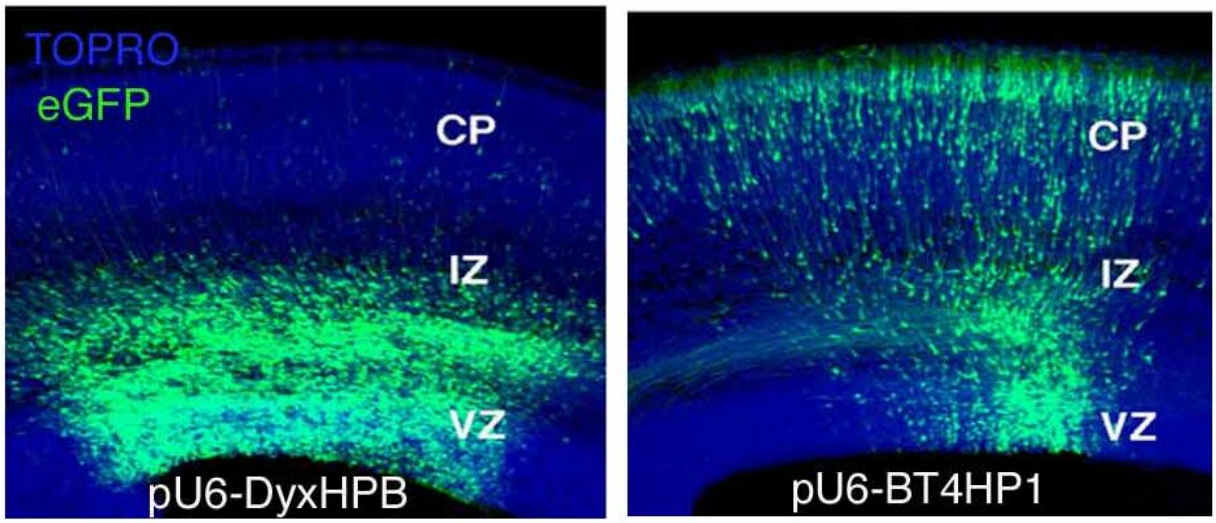

Figure 10. RNAi of Dyx1c1 disrupts migration in developing neocortex. Image of embryonic neocortex 4 days following electroporation of shRNA vectors (*in utero* RNAi), pU6-DyxHPB RNAi plasmids on the left, pU6-Bt4HP1 control plasmids on the right. eGFP-transfected cells are green, blue is TOPRO (molecular probes) counterstain for the nuclei of the cells.

RNAi studies and a translocation breakpoint in TPR domains indicated that these domains are important in migration. The SNP that associates more commonly in dyslexia is on the promoter region of *DYX1C1*, which indicates that instead of structural changes there are alterations in the gene expression that may influence the risk for dyslexia. (Dahdouh et al.

submitted). Recently, there has been a similar RNAi study with rats that has been used in our work, but the rats were allowed to mature. Interestingly, the inhibition of Dyx1c1 causes the same kind of alterations in adult rats that has been seen in the brain of dyslexic adults: pockets of unmigrated cells in the white matter, cortical laminar displacement of neurons and ectopias in molecular layer in cortex (Rosen et al. 2007). Furthermore, these rats have shown impairments in auditory processing and spatial learning, which is new evidence that strengthen the role of *DYX1C1* in dyslexia (Threlkeld et al. 2007).

3.5. *DCDC2* and *KIAA0139* in neuronal migration

In addition to *DYX1C1,* the dyslexia candidate genes *DCDC2* and *KIAA0319* are also involved in radial migration in the developing rat neocortex (Meng et al. 2005, Paracchini et al. 2006). *DCDC2* contains two doublecortin peptide motives similar to *DCX*. These domains are suggested to be crucial in neuronal migration due to mutations in this motif in double cortex syndrome. Comparing the RNAi studies of *DCX* (Bai et al. 2003) and *DCDC2,* it has been suggested that the *DCX* is necessary for neuronal migration and *DCDC2* could have just a modular effect on it (Meng et al. 2005). DCDC2 is expressed in the entorhinal cortex, inferior temporal cortex, medial temporal cortex, hypothalamus, amygdale and hippocampus (Meng et al. 2005). These particular regions are associated with the region of fluent reading and they are mostly activated in both normal and dyslexic readers during reading tasks. Due to only small alterations in the dyslexia phenotype, the explanation could be an altered regulation of expression rather than a total disruption of the protein products.

KIAA0319 is a putative glycosylated membrane protein, and it has been proposed to have a role in changing the relationship between radial glia and migrating neurons. The expression of its risk haplotype is reduced in dyslexic individuals (Paraccini et al. 2006). It has a very specific spatial-temporal expression pattern during brain development (Paraccini et al. 2006) and expression was detected in the superior parietal cortex, primary visual cortex and occipital cortex (Meng et al. 2005). This evidence suggests its role in brain development and developmental dyslexia.

4. *ROBO1* in dyslexia

Our results indicate that two functional copies of *ROBO1* gene are required to acquire normal reading ability. The expression of another ROBO1 allele was disrupted by translocation in one dyslexic individual and attenuated in dyslexics in a family with linkage in the same region on chromosome 3. *ROBO1* encodes a transmembrane receptor, which is a member of the immunoglobulin superfamily. It has immunoglobulin domains, three fibronectin domains, a transmembrane domain and a long intracellular region with no recognizable motifs but four proline-rich repeats that are suggested to act with downstream signalling molecules (Kidd et al. 1998, Bashaw et al. 2000). ROBO1 is spliced alternatively in a complex manner and the cell or tissue specificity or function of these splice variants is unknown. There was considerable variation of ROBO1 mRNA in lymphoblasts between individuals tested in our study, which suggests that the regulation of expression is complex.

The human orthologue of *robo*, *ROBO1* is also named *DUTT1* and was previously identified as a tumour suppressor gene in a small-cell lung cancer cell line (Sundaresan et al. 1998). These two genes are suggested to be alternative splice variants with different initiation exons and initiation codons and therefore probably with distinct functions. Instead of lymphomas and lung adenocarcinomas, which have been found in heterozygous *Robo1* knockout mice at high frequency (Xian et al. 2004), the deleted human *ROBO1/DUTT1* has been found in a child with a developmental delay and congenital anomalies, but without cancer (Petek et al. 2003). This can indicate the diverse roles of ROBO1 gene, or the same mechanism concerning its function in the guidance and growth. It is worth remembering that there is also another receptor involved in midline signalling called *DCC*, *Deleted in Colon Cancer* (Culotti and Merz 1998).

4.1. *ROBO1* in brain development

In *Drosophila,* Robo is a receptor for Slit, which is a repellent signal molecule. The Robo-Slit-signalling system controls the decision by axons to cross the midline of the nervous system (Seeger et al. 1993, Kidd et al. 1998). Studies with mice have revealed the role of Robo1 in axon growth and neuronal migration. Homozygous knockout mice died after birth and have prenatally shown major defects in axon pathfinding and in cortical interneuron migration. The defects in axon pathfinding include dysgenesis of corpus callosum and hippocampal comissure. Based on the mouse model, it was suggested that callosal and hippocampal comissure axons, instead of maintaining distinct fibre tracts as

normal, were mixed together in the midline (Andrews et al. 2006). This is consistent with the known function of the Robo-Slit signalling system that controls the decision of crossing the midline of nervous system. Inadequate signalling might disrupt the structure of axon comissures, which link two sides of the nervous system together.

Defects in tangential interneuron migration were also detected in Robo1 knockout mice. In addition to the midline of the nervous system, Slit is secreted also from the ventricular zone and extracortical proliferation zone, lateral ganglionic eminence (LGE). It is needed so that interneurons migrate along their normal pathway to the neocortex (Zhu et al. 1999). Interestingly, the dyslexia locus *DYX9* on chromosome Xq27.3 (de Kovel 2004) includes the *SLITRK2* and *SLITRK4* genes, which are members of the SLIT and NTRK-like families of genes. These proteins show high homology to the Slit proteins and are also involved in mouse neurite outgrowth (Aruga et al. 2003a, 2003b).

5. Neurodevelopmental mechanisms of developmental dyslexia

Two distinct neurodevelopmental mechanisms have been revealed. One is a deficit in neocortical migration, where *Dyx1c1*, *Dcdc2* and *Kiaa0139* are involved. They arrest or delay the radial migration of cortical neurons. The effect of RNAi targeted to *Dcdc2* during cell migration in the developing neocortex is weaker than the effect of RNAi targeted to *Dyx1c1* or *Kiaa0319*. Inhibition of these two proteins arrested the migration instead of the cortical plate into the IZ, SVZ and VZ. Similar structural anomalies have also been seen in morphological and brain imaging studies, which support these findings. The other mechanism involving *ROBO1* affects the tangential migration of cortical neurons. The lower expression of *ROBO1* might alter the migration and could impair connections between distinct brain regions in the cortex.

The second mechanism alters neuronal pathfinding, which means impairments in axonal navigation and thus in connections between the two hemispheres and malformation of the corpus callosum. The corpus callosum is formed by neurons from each of six cortical layers and the major projection across it is derived from neurons in layers 2/3 and 5 (Richards et al. 2004). Axons of the corpus callosum have to grow a long way to reach their final target and so even a slight alteration in the expression of the critical gene such as guidance molecules or receptors, could negatively affect pathfinding in migration. *Robo1* and *Dcx* knockout mice have shown alterations in both, corpus callosum (Kappeler

et al. 2007) and neocortical migration, Robo1 in tangential migration and Dcx in radial migration, respectively.

Robo1 and *Dcx* knockout mice also have alterations in hippocampal comissures (Andrews et al. 2006) and the hippocampus (Corbo et al. 2002, Kappeler et al. 2007), which may play a part in memory and learning (Bear et al. 2007). Interestingly, rats with inhibited *Dyx1c1* expression also have alterations there (Rosen et al. 2007).

6. The molecular mechanisms of dyslexia

The dyslexia candidate genes can be divided into two groups according to their function. ROBO1 and KIAA0139 are transmembrane proteins, of which ROBO1 has a known function in axon guidance and KIAA0139 has also been proposed to have a role in axon guidance as an adhesion protein. They receive signals from the extracellular matrix and forward messages inside the cell and finally to the cytoskeleton that produces the movements. DCDC2 has been associated with the microtubule cytoskeleton of the cell, due to the doublecortin peptide domain. Therefore it most probably has a role downstream in the cascade that is guided by extracellular signal molecules. The function of DYX1C1 is still very obscure, but it localises inside the cell and could have a role in intracellular signalling. No links or common pathways have yet been found between these genes.

Candidate gene studies have shown that the alterations in dyslexic individuals are linked to the regulation of gene expression. Complex mechanisms and cascades in brain development suggest that there is conscientious temporo-spatial regulation of the genes involved. The alterations in knockout mice and RNAi-exposed rat embryos are dramatic, but due to this data it is more comprehensible that even a slight alteration in the expression of a certain gene or genes, at a certain time and place, may have an effect on the fine adjustment of brain development. This slight shift in gene regulation might cause subtle alterations in some vulnerable stage and, together with the environmental factors, bring out a specific phenotype of dyslexia. Heritable variation in allelic expression levels has been documented for several genes, and might conceivably arise by a number of different mechanisms, like variation in enhancer and suppressor elements, splicing efficiency, transcript stability, or epigenetic modifications (Yan et al. 2002). Based on this variability of gene expressions it is easy to understand the unique brain structure and function of all individuals.

7. The theory of dyslexia

Although our studies have revealed two neurodevelopmental mechanisms that are associated with dyslexia, the exact molecular mechanisms are still unknown. At the moment we have two susceptibility genes that are associated with anomalies in more than one distinct brain region: *Dyx1c1* in the neocortex and hippocampus and *Robo1* in the neocortex, corpus callosum and hippocampal comissures. Furthermore, inhibition of *Dcdc2* expression has disrupted neuronal migration in the neocortex and it is also expressed in the hippocampus (Meng et al. 2005), and thus might have a role in its normal development. There are also several guidance molecules like Netrins, Semaphorins and Slits that are involved in both axonal navigation and neuronal migration (Tessier-Lavigne and Goodman, 1996). These results strongly suggest that there is a common and extremely complex molecular pathway, or alternatively distinct pathways, that are involved in the migration in the neocortex and pathfinding between the hemispheres and hippocampus. However, due to the scarce information of dyslexia genes and the complexity of brain development, such as spatial and temporal regulation, it is too early to make further conclusions.

Deficits in neuronal migration cause alterations in the connections between language regions in the brain and in the future the exact dyslexia phenotype might be determined by the localisation of these alterations. This could explain the strong support for the phonological theory. The region for phonological processing localises in the parietotemporal region on the brain and occipitotemporal region is considered the interface between visual information and language domains (Tarkiainen et al. 1999, Devlin et al. 2006). The anomalies in both the occipitotemporal and parietotemporal regions or in the connections between them most probably have effects on phonological processing. Structural alterations in the inferior frontal gyrus that are also associated with dyslexia, likely cause a more serious phenotype than dyslexia. The inferior frontal gyrus is proposed to be connected with higher levels of the linguistic system and it is used for compensation (Brunswick et al. 1999, Shaywitz et al. 2002). Animal studies support the scenario that anomalies in migration induce thalamic alterations, which could be interpreted in such a way that phonological deficits are primary and sensory impairments are secondary alterations (Herman et al. 1997, Peiffer et al. 2002). This also supports the primary role of phonological processing in dyslexia and partly explains the variety of symptoms in the dyslexia phenotype.

There is a growing consensus that reading and language disorders are heterogeneous and that many children or adults may not fit into the diagnostic categories. Previous genetic studies have shown that most probably there are common aetiological factors in neurodevelopmental disorders like SLI, ADHD, autism, SSCH and dyslexia, which have come out also in this thesis. Overlapping genes, the same neurodevelopmental mechanisms and the same molecular pathways might hide in the background of all these disorders and therefore dyslexia research will shed light also on the aetiology of them. How close these disorders really are to each other remains to be seen.

CONCLUSIONS AND FUTURE PROSPECTS

Developmental dyslexia is a neurodevelopmental disorder with a complex genetic background. Altogether nine loci in the genome have been linked to dyslexia and two of them have been proposed to inherit as dominant traits. The other loci have rather a quantitative effect on the dyslexia phenotype. Four susceptibility genes, *DYX1C1, ROBO1, DCDC2* and *KIAA0139*, have been revealed so far. The discovery of the two first genes, *DYX1C1* and *ROBO1*, has been described in this thesis. These two genes have offered an opportunity to study the development of the brain and they have revealed two distinct neurodevelopmental mechanisms behind the aetiology of developmental dyslexia.

All these genes are involved in the development of the neocortex in the developing brain. *DYX1C1* has a role in the radial migration from the proliferative region to the cortex. The inhibition of gene expression leads to ectopias and other structural anomalies, which are also detected in post-mortem dyslexic brain and brain imaging studies. *ROBO1* is involved in the tangential migration of interneurons from intracortical or extracortical proliferation regions to the cortex. Furthermore, previous studies have suggested that the deficit occurs in the regulation of the gene expression of these susceptibility genes.

Another neurodevelopmental mechanism is involved in axonal pathfinding. A considerable number of signalling molecules and receptors are needed to guide the growing axon to its final and exact target. The pathway is long for axons, which connect the cortexes of the two hemispheres together via the corpus callosum, in which alterations have been detected in dyslexic individuals. The corpus callosum locates in the midline of the central nervous system and axonal crossing there is tightly regulated. Unorganized crossing of the midline causes anomalies in the function of the nervous system and therefore the *SLIT-ROBO1*-signalling system, which participates in this controlling is required for normal brain development.

DYX1C1 and ROBO1 represent two kinds of molecules in neuronal migration. ROBO1 is a receptor and a transmembrane protein. It receives signals from the extracellular matrix and forwards messages inside the cell and finally to the cytoskeleton which produces the movements. *DYX1C1* has a role inside the cell and might be involved in the rapid regulation of cellular signalling. None of these four known susceptibility genes are functionally linked together so far. This can be caused by too many missing parts of the complex network or alternatively they are involved in separate pathways. However, there

are still at least five loci associated with dyslexia left in the genome and thus the hunting for genes will continue.

The total number of genes behind dyslexia remains to be seen, as well as the reason for the variation in the phenotype. The work to find out the function of the candidate genes will continue intensively. In the future, efforts should be focused on revealing the spatial and temporal regulation of the expression of dyslexia candidate genes during brain development. That might enlighten the variation in the dyslexia phenotype, as well as other related learning and language disorders.

The genetic analyses of dyslexia suffer from inconsistent phenotype testing. Universal tests and thresholds should be determined and they should be taken into use worldwide. Larger sample sets would also be of great advantage and they would increase the statistical power of the analysis and therefore co-operation is needed. The NEURODYS project that was started in 2006 could be a solution for these problems. It is a European Union-funded dyslexia research project in which 13 research groups from 10 European countries will investigate the biological basis of dyslexia. The aim of the project is to understand the aetiology of dyslexia by integrating the results of three fields: genetics, the environment and neuroscience (www.neurodys.com).

Hopefully our study and dyslexia research in general will prove useful for dyslexic individuals and relieve their everyday lives. By understanding the neurodevelopmental mechanisms of dyslexia, as well as of the other learning disorders, the diagnosis could be made earlier and adequate support and teaching methods could be outlined.

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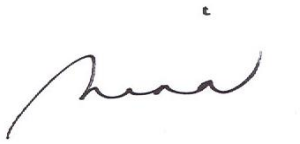

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