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The Axon Guidance Receptor Gene ROBO1 Is a Candidate Gene for Developmental Dyslexia

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Dyslexia, or specific reading disability, is the most common learning disorder with a complex, partially genetic basis, but its biochemical mechanisms remain poorly understood. A locus on Chromosome 3, DYX5, has been linked to dyslexia in one large family and speech-sound disorder in a subset of small families. We found that the axon guidance receptor gene ROBO1, orthologous to the Drosophila roundabout gene, is disrupted by a chromosome translocation in a dyslexic individual. In a large pedigree with 21 dyslexic individuals genetically linked to a specific haplotype of ROBO1 (not found in any other chromosomes in our samples), the expression of ROBO1 from this haplotype was absent or attenuated in affected individuals. Sequencing of ROBO1 in apes revealed multiple coding differences, and the selection pressure was significantly different between the human, chimpanzee, and gorilla branch as compared to orangutan. We also identified novel exons and splice variants of ROBO1 that may explain the apparent phenotypic differences between human and mouse in heterozygous loss of ROBO1. We conclude that dyslexia may be caused by partial haplo-insufficiency for ROBO1 in rare families. Thus, our data suggest that a slight disturbance in neuronal axon crossing across the midline between brain hemispheres, dendrite guidance, or another function of ROBO1 may manifest as a specific reading disability in humans.


Introduction

Dyslexia refers to a difficulty in reading and writing despite normal intelligence, senses, and adequate education. The primary difficulty lies in phonological processing, rapid naming, and the recognition of phonemes [1]. Dyslexia is a common disorder, affecting 3% to 10% of the population [2]. Familial occurrence has been reported and twin and family studies indicate a strong genetic component in its etiology [3]. So far, genome-wide screens have linked dyslexia to loci on Chromosomes 1p34–36 (DYX8), 2p16-p15 (DYX3), 3p12-q13 (DYX5), 6p21.3 (DYX2), 11p15.5 (DYX7), 15q21 (DYX1), 18p11.2 (DYX6), Xq27.3 (DYX9), and 7q32 (http://www.ncbi.nlm.nih.gov/omim)[4–12]. The first dyslexia candidate susceptibility gene, DYX1C1 at 15q21, was recently identified, and a region corresponding to DYX2 has been narrowed down to a small number of candidate genes [12–16].

We ascertained earlier a large four-generation family including 21 dyslexic individuals available to the study with severe dyslexia segregating in a dominant fashion, and mapped the susceptibility gene by a genome-wide scan to a region on Chromosome 3 that was named the DYX5 locus [17]. A 20-cM long haplotype was shared between 19 to 21 dyslexic individuals, giving statistically significant support for the mapping of the gene, but insufficient resolution to identify the gene [17]. Recently, speech-sound disorder was studied in a large set of 77 families from the United States, with results supporting association of this disorder to the DYX5 locus in the majority of families [18]. Phonological processing was the common phenotypic component found deficient in both studies [17–19].

We report here the localization of a translocation in an individual with dyslexia and a translocation t(3;8)(p12;q11) to the DYX5 region and specifically to the first intron of ROBO1. ROBO1 is a neuronal axon guidance receptor gene involved in brain development, and thus an attractive candidate gene for dyslexia susceptibility [20–22]. Furthermore, in the large pedigree with 19 dyslexic individuals genetically linked to DYX5 and more specifically, a specific and rare haplotype of ROBO1, the expression of ROBO1 from this haplotype was absent or attenuated in affected individuals. We conclude that dyslexia may be caused by partial haplo-insufficiency for ROBO1. In addition, we searched for novel exons and splice variants of ROBO1 that may help in understanding the
Dyslexia, or specific reading disability, is a common learning disorder with a complex, partially genetic basis. A number of chromosomal regions harboring genes involved in dyslexia have been identified, and in this study the authors describe a candidate gene from one such locus, called ROBO1, on Chromosome 3. The authors show that an axon guidance receptor gene, ROBO1, is disrupted by a chromosomal translocation in one dyslexic individual; furthermore, this study shows that the expression of ROBO1 is reduced on chromosomes from dyslexics in a large pedigree in which dyslexia has been linked to DYX5. ROBO1 has a role in regulating axon crossing across the midline between brain hemispheres and guidance of neuronal dendrites. As suggested by these findings, dyslexia may be caused in rare families by a small change in the expression of ROBO1, such as loss of one functional copy. Thus, ROBO1 is a candidate for a dyslexia susceptibility gene.

Because of its known function in neuronal axon guidance in the developing brain, ROBO1 was a plausible candidate gene for dyslexia susceptibility [20–22]. We sequenced its 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 of initially one dyslexic individual and his parents (father dyslexic, mother unaffected) from the large linkage family (Figure 2A). All exons were also sequenced from the cDNA of another dyslexic individual from the extended family. Comparison of the sequences to ROBO1and ROBO1 variant 2 sequences revealed altogether seven sequence variations, two of them previously known [23]. All of the observed changes were confirmed in three additional pedigree members (dyslexic father, son, and unaffected mother) by sequencing. 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) (Figure 2B).

Genotyping of the exonic SNPs in ten additional family members and two unrelated non-dyslexic individuals confirmed that a specific SNP haplotype segregated with dyslexia, consistent with the previously observed linkage, but revealed that none of the polymorphisms was uniquely observed only in dyslexic individuals. For example, the DIP6203–6205GAT+ allele had an allele frequency of 22% (94/434 among healthy control participants' chromosomes) and did not show significant association to dyslexia in our replication sample set of 96 dyslexic individuals from other families.

None of the four observed intronic SNPs produced alternative splice variants. Because the ROBO1 gene spans about 990 kb of genomic DNA and contains altogether over 2,200 intronic SNPs (according to the NCBH SNP database), their exhaustive listing in our family members was impractical. In accordance with our previous genome scan on dyslexia in Finland, suggesting that DYX5 locus is not involved in most families [9], the same haplotype as in this large family was not observed in other, unrelated families (unpublished data).

The silent and 3' UTR SNPs provided assays to study the transcription of ROBO1. Our rationale was to measure whether both alleles of ROBO1 were equally transcribed in dyslexic individuals segregating the dominant susceptibility haplotype. Comparison of genomic and cDNA samples from four dyslexic individuals showed that ROBO1 mRNA was only weakly or not at all transcribed from the allele that segregated with dyslexia (Figure 2C), whereas in non-readers' lymphocytes, as well as control brain RNA, biallelic expression was consistently observed. Of note, there was considerable variation between individuals, suggesting that the regulation of expression is complex. The SNP 2794C > A was heterozygous in one patient, 6483T > A in four, 6651T > A in one, and 6923T > G in one, and combining all results, the expression was significantly attenuated for the dyslexia-linked allele as measured by allelic peak heights (p = 0.017 by two-tailed t test). To verify this initial analysis, we repeated the assay for all four dyslexic and four control participants by sequencing the SNP 6483T > A again. The results from five to
six replicated sequencing assays for each participant are shown in Figure 2D. 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 exclude the possibility that an individual SNP behaved aberrantly in the analysis, we also sequenced the SNP 6651T $\rightarrow$ A, yielding similar findings, and both SNP assays combined, the observation of allelic imbalance in cases versus controls was highly significant ($p = 0.00005$ by $t$ test). As no SNP was specific for the $ROBO1$ or $DUTT1$ transcript only, we cannot assess isoform-specific down-regulation in the large family. In the translocation
patient, two SNPs were heterozygous, both in the region 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.

To study the possibility that the suppression of expression involved other genes than ROBO1 in the dyslexia susceptibility haplotype, we genotyped known SNPs in the nearby genes GBE1 (341C/G, 646A/G, 1597A/G, 1794C/T, 2349T/G, 2363A/G, 2761A/T) and HTR1F (528C/T, 783T/A) in the four dyslexic individuals of the large family (Figure 1C). 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. Heritable variation in allelic expression levels has previously been documented for several genes, and might conceivably arise by a number of different mechanisms, such as variation in enhancer and suppressor elements, splicing efficiency, transcript stability, or epigenetic modifications [24]. Two other positional candidate genes, DRD3 and 5HT1F, mapped outside the shared haplotype [17].

Seemingly silent exonic and intronic polymorphisms may induce disease related splice variants [25]. Thus, we studied the possibility of ROBO1 alternative splicing by RT-PCR of all
ROBO1 and DUTT1 exons from a dyslexic individual in the linkage family, an unrelated healthy control, and adult human brain cDNA. 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) (Figure 1D). 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 colinear with 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 (Figure 1D) 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 [23]. 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 lymphoblastoid RNA.

As the known ROBO1 sequence AF040990 starts from the transcription initiation site, we sought to determine the ROBO1 5' UTR sequence by a BLAST search for expressed sequence tags homologous for the 5' ROBO1 region. The expressed sequence tag AW450262, homologous to ROBO1 exons 1 and 2, indicated an additional site (referred to as ROBO1 exon a) upstream on BAC AC125624 (bases 28508–28470) (Figure 1D). RT-PCR with an initial primer in the exon a sequence and primers in ROBO1 exons 1 and 4 revealed an additional 52 bp (bases 34119–34168 of BAC AC125815) 5' of the transcription initiation site on ROBO1 exon 1 and also confirmed the presence of the novel exon a on the BAC AC125624. Additional primers were designed 5' to the novel exon a, and RT-PCR performed similarly as above showed the a exon to span at least 129 bp (bases 28593–28466 of BAC AC125624). 5' rapid amplification of cDNA ends (RACE) revealed additional 326 bps, stretching the exon to 28919–28466 on the BAC AC125624.

A significant fraction of human genes has been under positive Darwinian selection since the common ancestor of humans and chimpanzees [26]. For example, FOXP2, the gene implicated in speech and language, has undergone a selective sweep during human evolution [27]. Therefore, we sequenced ROBO1 from chimpanzee, pygmy chimpanzee, gorilla, and orangutan, and used the rat ROBO1 sequence as the out-group. We used likelihood ratio test to analyze variation in the selective pressure in ROBO1 sequence in the different lineages. Non-synonymous and synonymous (dN and dS) ratio was smaller than 1 in all lineages, implicating purifying Darwinian selection. However, the likelihood ratio test rejected the null hypothesis of fixed dNdS ratio in all lineages. A model in which omega value was higher in lineages leading to humans, chimpanzees, and gorillas was significantly better than a free-ratio model (p < 0.001) (Figure 3 and Tables S1 and S2). This suggests that the selective pressure for ROBO1 gene has changed 12 to 16 million years ago, after the divergence of the orangutan branch.

![Figure 3. Coding Changes of ROBO1 during Primate Evolution](https://www.plosgenetics.org/doi/10.1371/journal.pgen.0010050.g003)

Phylogenetic tree of ROBO1 protein evolution in hominoids. Rat was used as the out-group in sequence comparisons. dN/dS ratios of the branches were calculated with the Codeml program, assuming a freely varying ratio. A model in which omega value was higher in lineages leading to humans, chimpanzees, and gorillas was significantly better than a free-ratio model (p < 0.001).

**Discussion**

Our data suggested that two functional copies of ROBO1 are required in brain development to acquire normal reading ability, and partial haplo-insufficiency for ROBO1 may predispose humans to specific dyslexia. robo was originally identified in *Drosophila* in a search for genes controlling the midline crossing of axons; in mutant robo embryos, axons cross and recross across the midline too many times [20–22]. The human ortholog of robo, ROBO1 (also named DUTT1), was identified as a potential tumor-suppressor gene in a small-cell lung cancer cell line [28]. ROBO1 and DUTT1 are presumed to be alternative splice variants with different initial exons and initiation codons and may thus have in part distinct functions [29]. Homozygous Robo1/Dutt1 knockout mice are embryonically lethal, but heterozygous mice were found to develop lymphomas and lung adenocarcinomas at high frequency [29]. The human ROBO1/DUTT1 locus has been found deleted in a child with developmental delay and congenital anomalies but without cancers [30]. These observations seem to pose a dilemma for understanding the functions of ROBO1 in different species.

robo encodes a transmembrane receptor that belongs to the immunoglobulin superfamily. It consists of immunoglobulin domains, three fibronectin domains, a transmembrane...
In the fruit fly, robo is a receptor for secreted repellent slit proteins and acts as a gatekeeper of axonal crossing on the left-right axis. Activation of robo makes axons indifferent to the chemoattractant netrin, a ligand of the DCC receptor [32]. Netrin-dependent activation of the DCC receptor increases transient phosphorylation of Erk1, Erk2, and the transcription factor Elk1. The activation of the Erk pathway has been suggested as necessary for experience-dependent plasticity and for long-term potentiation of synaptic transmission in visual cortex development in the rat [33]. Interestingly, a possible Elk1 binding site was altered and associated with dyslexia in DUX1C1, a candidate gene for dyslexia susceptibility on Chromosome 15q21, and Elk1 has been implicated in learning in the rat [13,34–36]. A suggested functional role for DUX1C1 remains unconfirmed, because its associations have not been unambiguously replicated, leaving the relevance of the Elk1 binding site open [37–42]. Slit/Robo/DCC signaling has also been implicated in cortical dendritic guidance and development [43,44]. Furthermore, a recently identified dyslexia susceptibility locus on Xq27.3 includes the SLITRK2 and SLITRK4 genes, which belong to the SLIT and NTRK-like family of genes involved in mouse neurite outgrowth and show high homology to Slit proteins [10,45,46]. Thus, the identification of ROBO1 as a susceptibility gene in dyslexia may implicate a key developmental pathway in which slight disturbances may lead to specific reading disability.

Genomic sequences and predicted transcripts for ROBO1 in four apes revealed a high level of variation between the related species and humans (Figure 3). We detected seven amino acid changes between human and chimpanzee and 20 between humans and orangutan. An analysis of dN/dS substitutions revealed that the selective pressure on ROBO1 has changed after the divergence of the orangutan branch. Although according to stringent criteria, only dN/dS ratios higher than 1 are regarded as signs of positive selection; it has been shown that genes expressed in the brain are under stronger selective pressure than genes expressed, for example, in the liver [47]. In addition, in primates many brain-expressed genes show significantly higher dN/dS ratios than housekeeping genes when compared to the rodent counterparts, even though the dN/dS ratios in primates are still well below 1. Interestingly, it was recently shown that the evolution of SLIT1, a ligand for ROBO1, has been significantly faster in primates than in rodents [48]. Also other proteins involved in axonal path-finding, such as SEMA4F and EPHA6, were shown to have undergone adaptive evolution [48]. We propose that ROBO1 might have undergone rapid changes in the recent primate evolution that may be related to its largely uncharacterized functions in the human brain.

Taken together, these results implicate a well-known pathway of neuronal development in a highly specific cognitive function in humans. This function attributed here to the ROBO1 transcript variant including newly discovered exons is distinct from the role that has been suggested in lung tumorigenesis for the alternatively spliced transcript variant DUTT1. This insight may open new visions for understanding complex brain processes and provide a framework for building testable hypotheses for the biology of reading.

Materials and Methods

Patients. A multiplex four-generation family with severe dyslexia segregating in a dominant fashion (Figure 2A) has been previously studied [49]. Genetic linkage and phenotypic analysis was diagnosed in 27 out of 74 family members by thorough testing including an intelligence test, a Finnish reading and writing test for adults and for children according to their school grade, and a neuropsychological test battery [49–52]. Detailed psychological evaluation of this family has been reported elsewhere [19].

A dyslexic individual with a balanced reciprocal translocation t(3;8) (p12;q11) came to our attention because of infertility and was diagnosed with oligospermia. He has three siblings, and all four children have been neuropsychologically evaluated at a specialist hospital. However, the family members were not available for retesting and thus our data are based on the clinical records. The translocation carrier and his sister were diagnosed with severe dyslexia while 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 a normal karyotype, whereas the parents were not available for karyotyping. Because the index case presented with infertility, but no such history or miscarriages were recorded for his mother, it is likely that the translocation had arisen de novo. Thus, the translocation and dyslexia did not apparently cosegregate in two siblings, but as dyslexia is a complex phenotype, no inference is conclusive to either reject or confirm a possible causal association of the translocation with dyslexia in the index case.

For association studies, dyslexic and non-dyslexic individuals were recruited from 23 unrelated families and 35 non-dyslexic and non-dyslexic couples from the Department of Pediatric Neurology at the Hospital for Children and Adolescents, University of Helsinki, and the Child Research Centre, Jyväskylä, Finland. Additional population controls consisted of 100 anonymous blood donors. The diagnosis and degree of dyslexia were determined by Finnish reading and spelling tests designed for children and adults [49,50]. Intelligence was estimated by Wechsler tests for adults (WAIS-R) or for children (WISC-R) [51,52]. The diagnostic criteria for dyslexia included normal performance intelligence quotient (PIQ > 85) and remarkable deviation (depending on age, at least two years) in reading skills. This study has been approved by the ethical review board of the Helsinki University Central Hospital, and informed consent was obtained from the participants.

Fluorescence in situ and Southern hybridization. YAC clones A130E9 (Washington University, St. Louis, Missouri, United States), 34FC9, 15AC10, 39F13, 2DG9, 25DH8, 35AH8 (ICII/Zeneca), 912A11, 934E8, 422A6, 950F5, 650C2, 938D4, and BAC RP11-143B12 were used as probes in fluorescence in situ hybridization experiments. The genomic sequence of clone RP11-143B12 was obtained by BLAST search with the BAC 5’ and 3’ ends (AQ373182, T7, and AQ373179 (specifically) to a Chromosome 3 scaffold sequence on the Celera public database (http://public.celera.com/cds/login.cfm) and to BACs in the National Center for Biotechnology CBI database (http://www.ncbi.nlm.nih.gov/).

Southern hybridization probes were PCR-amplified from genomic fragments from non-repetitive regions on the BAC clones RP11-143B12. The repeats in the clone sequence were detected by RepeatMasker (http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker) and PCR primers were designed by Primer3 (http://frodo.wi.mit.edu/).
were identified and 2 kb of genomic DNA and the cDNA of selected individuals from the large
homolog of the Drosophila roundabout gene, ROBO1 (AF040990), the first intron of ROBO1 variant 2 (NM_002941), ROBO1 variant 2 (NM_133631), Rat ROBO1 (NM_022188), Homo sapiens clone sequences for BACs RP11-588D3 (AC055751) and BAC RP11-20M20 (AC106720).

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Author contributions. KHJ, NKA, MT, and JK conceived and designed the experiments. KHJ, NKA, MT, and RE performed the experiments. KHJ, NKA, MT, and JK analyzed the data. [NH and HK contributed reagents/materials/analysis tools. KHJ, NKA, and JK wrote the paper.

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