An Evaluation of the Reading Disabilities Candidate Genes $DYX1C1$ and $ROBO1$

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Science
University of Toronto

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2012

Abstract

Reading disabilities (RD) have a significant genetic basis and chromosomes 3p12-q13 and 15q15-21 have shown replicated linkage to RD or reading measures. This study evaluated two RD candidate genes within these regions: DYX1C1 on chromosome 15q21 and ROBO1 on chromosome 3p12. DYX1C1 was tested for association using a family-based analysis of two independent samples. No statistically significant association was observed between the 10 tested DYX1C1 single nucleotide polymorphisms (SNPs) and RD or any of the quantitative traits. A review and meta-analysis of the potentially functional SNPs at the -3G/A and 1249G/T positions did not find strong support for these alleles as risk alleles for RD. ROBO1 was also evaluated in this study using SNPs that previously showed association with memory and reading measures in a population-based sample. None of the SNPs showed significant association with RD or any of the quantitative traits after correction for multiple testing.
Acknowledgments

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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>BAS</td>
<td>British Abilities Scale</td>
</tr>
<tr>
<td>catmap</td>
<td>Case-control and TDT meta-analysis package</td>
</tr>
<tr>
<td>ChIPS</td>
<td>Children’s Interview for Psychiatric Syndromes</td>
</tr>
<tr>
<td>CMS</td>
<td>Children’s Memory Scale</td>
</tr>
<tr>
<td>CELF-3</td>
<td>Clinical Evaluation of Language Fundaments- 3rd Edition</td>
</tr>
<tr>
<td>CTOPP</td>
<td>Comprehensive Test of Phonological Processing</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>DYXIC1</td>
<td>Dyslexia susceptibility 1 candidate gene 1</td>
</tr>
<tr>
<td>DYX</td>
<td>Dyslexia susceptibility region</td>
</tr>
<tr>
<td>FBAT</td>
<td>Family-based association test</td>
</tr>
<tr>
<td>GxE</td>
<td>Gene-environment interaction</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HBAT</td>
<td>Haplotype-based association test</td>
</tr>
<tr>
<td>HuGENet™</td>
<td>Human Genome Network</td>
</tr>
<tr>
<td>IBD</td>
<td>Identical by descent</td>
</tr>
<tr>
<td>IBS</td>
<td>Identical by state</td>
</tr>
<tr>
<td>IG-like</td>
<td>Immunoglobulin-like</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of the odds</td>
</tr>
<tr>
<td>MEG</td>
<td>Magnetoencephalography</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense mediated decay</td>
</tr>
<tr>
<td>PLA2G4B</td>
<td>Phospholipase A2, group IVB</td>
</tr>
<tr>
<td>PLCB2</td>
<td>Phospholipase C β 2</td>
</tr>
<tr>
<td>P-Chips</td>
<td>Children’s Interview for Psychiatric Syndromes</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRTG</td>
<td>Protogenin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>RD</td>
<td>Reading disabilities</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rGE</td>
<td>Gene-environment correlation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROBO1</td>
<td>Roundabout homolog 1</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hair RNA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSD</td>
<td>Speech and sound disorder</td>
</tr>
<tr>
<td>TTI</td>
<td>Telephone Teacher Interview</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeat domains</td>
</tr>
<tr>
<td>TDT</td>
<td>Transmission disequilibrium test</td>
</tr>
<tr>
<td>WISC-III</td>
<td>Wechsler Intelligence Scale for Children-III</td>
</tr>
<tr>
<td>WISC-IV</td>
<td>Wechsler Intelligence Scale for Children-IV</td>
</tr>
<tr>
<td>WISC-R</td>
<td>Wechsler Intelligence Scale for Children-Revised</td>
</tr>
<tr>
<td>WJ-R</td>
<td>Woodcock Johnson Psychoeducation Battery - Revised</td>
</tr>
<tr>
<td>WRAT-III</td>
<td>Wide Range Achievement Test-III</td>
</tr>
</tbody>
</table>
Chapter 1– Introduction

1.1 Introduction to Reading Disabilities (RD)

Reading is an important skill that provides numerous advantages including greater educational and socioeconomic opportunities (Batiz-Rivera, 1990; Maughan, 1995; McIntosh and Vignoles, 2001). It is not surprising then, that promoting literacy is a priority around the world (Robinson, 2005; Ainscow and Miles, 2008). Even with adequate instruction, however, there are individuals who do not develop into fluent readers. Reading disabilities (RD), or developmental dyslexia, refer to an unexpected difficulty in learning to read despite normal intelligence, education, and socioeconomic opportunity (Habib, 2000; Demonet et al., 2004; Williams and O'Donovan, 2006). With an estimated prevalence ranging from 5-17.5% in school-aged children, RD is the most common learning disability and affects four out of five individuals with learning disabilities (Shaywitz et al., 1990; Shaywitz, 1998; Katusic et al., 2001).

Individuals with RD are most commonly identified in childhood when significant reading difficulties are observed in the classroom. RD is not a developmental delay; children diagnosed with RD will continue to show poor reading skills in adolescence and adulthood relative to normal readers (Bruck, 1992; Shaywitz et al., 1999). However, remediation programs can be used to improve reading skills in individuals with RD (Lovett et al., 2000; Temple et al., 2003).
1.2 Phonological Deficit Hypothesis and Alternative Theories of RD

Fluent reading involves decoding and comprehending text with accuracy and efficiency. This is a complex task, requiring a number of overlapping cognitive components. Children suspected of having RD are often tested for these different components through the administration of psychometric tests. Common tests used to evaluate children with reading difficulties have been summarized by Paracchini et al. (2007), and are listed in Table 1-1. RD was originally referred to as ‘congenital word blindness’, implying that visual problems were the underlying cause of the observed reading difficulties (Morgan, 1896; Hinshelwood, 1911). Today, extensive evidence suggests that RD is a language-based disorder and that it is caused by a deficit in mapping written text to their phonemes, the most basic unit of speech sounds (Shaywitz, 1998). Thus, the reading problems observed in individuals with RD are due to decoding processes rather than comprehension. Although RD cannot be “cured”, remediation programs that include training and developing phonological processing skills can be used to significantly improve reading abilities (Lundberg et al., 1988; Hurford et al., 1994; Bus and van IJzendoorn, 1999; Schneider et al., 2000).

Three cognitive skills that require phonological processing have been shown to be impaired in individuals with RD: phonological awareness, phonological memory, and rapid automatized naming (Denckla and Rudel, 1976; Mann and Liberman, 1984; Bruck, 1992; Meyer et al., 1998; Wilson and Lesaux, 2001). Phonological awareness involves the recognition and manipulation of phonemes, and is assessed by asking subjects to segment words into their constituent phonemes (e.g. breaking down the word *dog* into the sounds /d/ /o/ /g/) and manipulate phonemes within words (e.g. switching the /g/ and the /b/ sounds in the word *bog* to get *gob*). Remediation programs that focus on phonological skills often aim to improve
phonological awareness. Phonological short-term memory refers to the temporary storage of verbal information. Tests that involve memorizing strings of digits (Digit Span Tests) assess short-term memory, while non-word repetition tasks specifically evaluate phonological short-term memory. Finally, rapid automatized naming is the rapid access and retrieval of phonological representations from long-term memory. It is measured by assessing the subject’s ability to rapidly name a sequence of objects, colours, digits, or shapes. The use of alphanumeric sequences in rapid automatized naming tests has been shown to be a stronger predictor of reading ability compared to sequences of colours and objects (Semrud-Clikeman et al., 2000; van den Bos et al., 2002; Schatschneider et al., 2004; Bowey et al., 2005).

The phonological deficit hypothesis is currently the most robust in explaining the difficulties that individuals with RD face with reading. Another related theory is the ‘double deficit hypothesis’, which considers both phonological awareness and rapid automatized naming as two sources of reading dysfunction in RD (Wolf and Bowers, 1999). Individuals with one of the two deficits show limited reading impairment while individuals with both deficits are more severely affected. Support for the double deficit hypothesis has been provided by multiple studies where individuals with a ‘double deficit’ performed significantly poorer on reading tests compared to individuals with a single deficit and unaffected readers (Lovett et al., 2000; Cirino et al., 2005; Escribano, 2007).

In addition to the phonological-based hypotheses, a number of other causes for RD have been proposed. The cerebellar theory suggests that reading problems stem from a disorder of the cerebellum, which is involved in the automatization of articulation and motor skills (Nicolson et al., 1995; Nicolson et al., 2001). The magnocellular theory implicates a dysfunction in the magnocellular pathways involved in the visual perception of motion (Stein and Walsh,
If images or words are moved off target from the fovea, the magnocells bring the eyes back on target, stabilizing the visual image. The magnocells are also thought to be involved in the visual “jumps” from word to word, allowing the individual to have a stable fixation on each word. No theory can explain all of the observed deficits in individuals with RD but the phonological deficit theory is currently the most robust in explaining the majority of reading problems. This may also suggest that RD is a heterogeneous syndrome and that dysfunctions in different systems may lead to reading impairments.
### Table 1-1. Common tests used to assess reading-related cognitive skills

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single word reading and spelling</td>
<td>Assesses the individual's ability to read and spell real words of increasing difficulty. Single-word reading is often used in the diagnosis of RD.</td>
</tr>
<tr>
<td>Nonword reading</td>
<td>Test involves the reading of pseudowords (e.g. rop, potwid), which assesses the individual's ability to decode unfamiliar words. Along with single-word reading, this task is also often used in the diagnosis of RD.</td>
</tr>
<tr>
<td>Phoneme segmenting and spoonerism tasks</td>
<td>Individuals are asked to break down words into their constituent phonemes and manipulate phonemes within or between words. Both tests evaluate the individual's phonological awareness abilities.</td>
</tr>
<tr>
<td>Orthographic word choice</td>
<td>Individuals are given pseudohomonyms (e.g. height and hite), and are asked to identify the real word. This test assesses orthographic coding, or the ability to recognize words as a whole unit rather than through decoding.</td>
</tr>
<tr>
<td>Rapid naming tasks</td>
<td>Test involves measuring the speed in which an individual can name presented stimuli (e.g. objects, digits, or colours). This task measures the individual's rapid automatized naming abilities.</td>
</tr>
</tbody>
</table>


1.3 RD Across Languages

The development of reading skills varies across languages. In a cross-national study of fifth grade children, the prevalence of RD was found to be significantly higher in the US compared to Italy, with the differences depending on the severity of RD (Lindgren et al., 1985). The underlying factor that affects the disparity in reading ability is the level of orthographic consistency between languages (Goswami, 2008). Greek, Finnish and Italian are considered to have transparent orthographies where each phoneme is mapped onto one grapheme, the smallest unit in written language. In contrast, English is considered to have an opaque orthography as many different combinations of letters can be used to represent the same phoneme. For example, the /f/ sound can be represented by f (in farm), ff (in staff) ph (in photo), or gh (in laugh). The complex mapping between graphemes and phonemes makes the acquisition of English reading skills relatively more challenging compared to languages with more transparent orthographies. A study comparing 13 different European orthographies found that children reading in languages with opaque orthographies (English and Danish) performed significantly poorer on real word and nonword reading tasks compared to children reading transparent orthographies (Greek, Finnish and German) (Seymour et al., 2003).

Regardless of the orthographic differences between languages, there is a common phonological deficit that is observed in individuals with RD. In a cross-language comparison of English, French and Italian dyslexics, it was found that the Italian dyslexics performed better on reading tasks (Paulesu et al., 2001). However, all three dyslexic groups performed significantly poorer relative to their controls on reading and phonological processing tasks. Another study comparing English and German readers also confirmed that dyslexics in both languages show common deficits: slower reading speeds, deficits in nonword reading that is more pronounced than deficits in real word reading, and significantly lower phonological decoding skills (Ziegler
et al., 2003). In non-alphabetic languages including Chinese, phonological processing skills have also been shown to have an effect on reading acquisition and are significantly poorer in individuals with low reading ability (Hu and Catts, 1998; Ho et al., 2000). Thus, although orthographic differences affect the ease in which children acquire reading skills, dyslexics across languages share common underlying deficits.

1.4 RD Comorbidity with Speech Sound Disorder (SSD)

Speech and sound disorder (SSD), also known as articulation disorder, is a developmental disorder which results in a difficulty in the production and use of speech sounds. Children with histories of SSD show phonological awareness impairments and are at significantly higher risk for RD (Raitano et al., 2004; Rvachew and Grawburg, 2006; Peterson et al., 2009). In addition, SSD linkage studies have identified susceptibility regions that are shared with RD including chromosome 1p34-36 (Smith et al., 2005; Miscimarra et al., 2007), 3p12-q13 (Stein et al., 2004), 6p22 (Smith et al., 2005), and 15q15-21 (Smith et al., 2005; Stein et al., 2006). The overlapping linkage results suggest that there may be shared genetic influences between both disorders.

1.5 Study Designs and Important Concepts in Establishing Gene-Disease Relationships

1.5.1 Twin Studies

Twin studies can be used to investigate whether a trait or phenotype has a genetic influence. If a disease is found to aggregate in families, it is possible that this may be due to genetic factors. However, shared environmental influences may also account for the higher rates among family
members. Comparing the concordance rates between monozygotic (MZ) and dizygotic (DZ) twins can help support whether the trait of interest has a genetic basis. MZ twins share the same genetic information while DZ twins, on average, share only half. Assuming similar environmental exposures among twin members, significantly higher concordance rates between MZ twins relative to DZ twins suggest that there may be genetic factors underlying the trait.

Twin studies have also been used to estimate the heritability of reading-related traits (DeFries and Fulker, 1985). Heritability refers to the proportion of phenotypic variance in a population that is due to genetic variance. Broad-sense heritability \( (H^2) \) represents the proportion of phenotypic variation due to additive, dominant and epistatic genetic effects, while narrow-sense heritability \( (h^2) \) represents the phenotypic variation solely due to the variance in additive genetic effects. Narrow-sense heritability is important as natural and artificial selection act through additive genetic effects (Mousseau and Roff, 1987). Furthermore, the resemblance of complex traits within families is mainly due to additive genetic effects (Hill et al., 2008).

In the method of DeFries and Fulker (1985), individuals in a set of MZ or DZ twins were recruited if they fell at the lower extreme of a quantitative trait. Then, the scores of the co-twins were compared to determine the proportion of genetic and the environmental influences. For the co-twin in a MZ pair, the trait should on average be as extreme as the original proband if genetics was the sole influence acting on the trait. In contrast, the co-twin in a DZ pair should on average, regress half-way between the original proband and the population mean because they share half of their genetic information. If a trait is influenced entirely by the shared environment, both the MZ and DZ pairs would be positioned in the lower extremes. Finally, if a trait is influenced entirely by unshared environmental influences, the co-twin should regress towards the population mean in both MZ and DZ pairs. Most traits, however, will fall in between the three
extravagant, and the group heritability \((h_g^2)\), shared environment \((c_g^2)\) and unshared environment \((e_g^2)\) can all be estimated.

1.5.2 Linkage Studies

Linkage studies are performed to locate regions of the genome that may contain genes affecting disease susceptibility (Dawn Teare and Barrett, 2005). This is achieved by genotyping families for polymorphic markers including microsatellites or single nucleotide polymorphisms (SNPs), and identifying chromosomal regions that co-segregate with a trait within families. There are two main classes of linkage analyses, including parametric and non-parametric, which can both be used to study binary (categorical) and quantitative (continuous) traits. In parametric linkage analyses, also known as model-based linkage, families are genotyped for polymorphic markers spaced across a region or the whole genome, and a genetic model is applied to test for linkage between the genotyped markers and an unknown disease causing loci. Parameters that are required for the model include the disease penetrance, inheritance pattern, location of the trait-causing locus, allele frequencies at the genotyped loci and disease locus and transmission frequencies between the parents and offspring (Lander and Schork, 1994). The likelihood of linkage between a marker and trait is expressed by the logarithm of the odds (LOD) score, in which a greater positive score suggests a higher probability of linkage, and a greater negative score suggests an absence of linkage. Traditionally, in the context of a candidate gene approach, LOD scores of >3 were considered to be significant evidence for linkage (indicating a 1000 to 1 odds that the observed linkage was not due to chance), and scores of <2 were considered to be strong evidence against linkage. However, for genome-wide studies, a greater cutoff of 3.3 is considered to be significant at the 0.05 level (Lander and Kruglyak, 1995). Parametric linkage
methods are effective when studying Mendelian traits but have less power for complex traits that may be influenced by multiple genes. Furthermore, specifying an incorrect model may reduce the power of the analysis (Clerget-Darpoux et al., 1986; Lander and Schork, 1994).

For traits with unknown mode of transmission, which is often the case with common complex traits, non-parametric linkage analyses may be more effective. They do not require parameters and assumptions of a specific transmission model, and are therefore more robust. In non-parametric analyses, alleles or haplotypes that are identical by descent (IBD) are identified in families. IBD refers to alleles that are shared among relatives and originate from the same ancestor. This differs from an allele that is identical by state (IBS) which is also shared among relatives, but can originate from the same or different ancestors. Both IBD and IBS are distinct, but are not mutually exclusive as an allele IBD is also IBS. In non-parametric linkage analyses, if affected members in a family share alleles or haplotypes IBD that are greater than expected by chance, it suggests that a gene influencing the trait may be located in the region. Non-parametric methods, although more robust when the mode of inheritance is unknown, require a large number of families to achieve sufficient statistical power (Lander and Schork, 1994).

1.5.3 Association Studies

Genetic association studies test whether specific genetic polymorphisms are associated with a categorical or quantitative phenotype in populations (Cordell and Clayton, 2005). Unlike linkage methods, which can identify large chromosomal susceptibility regions housing hundreds of genes, association studies can be used to narrow genes or even specific polymorphisms influencing a trait. For this reason, association studies are considered to be a higher resolution technique compared to linkage methods.
There are two main types of genetic association studies: case-control and family-based. The case-control analysis is the traditional method of testing for association (Cardon and Bell, 2001). A sample of cases and controls are genotyped for polymorphic markers and the genotype or allele frequencies between both groups are compared. If a specific allele is found in the cases at a statistically significant higher or lower proportion compared to the control group, the allele is considered to be associated with the trait. The selection of the cases and controls is a critical consideration in case-control studies. If a study uses different ethnic groups between the cases and controls, a significant result may be due to differences in allele frequencies and linkage disequilibrium (LD) between the groups, rather than reflecting a true association with the disease. Therefore, stratification must be accounted for to reduce the risk of spurious associations (Freedman et al., 2004). One method of correcting for population stratification in case-control studies includes genotyping markers that are unlinked with any candidate markers that showed nominally significant association (Devlin et al., 2000; Reich and Goldstein, 2001). The association statistic is calculated and averaged for the unlinked markers, and is then compared to the candidate marker. If the significance of the candidate marker is dramatically different from the unlinked markers, it may suggest that the candidate marker is causal or is near the causal locus. However, if the significance of the candidate marker is similar to the unlinked markers, the association may be due to population stratification. Another correction method has been developed specifically for genome-wide association studies (GWAS) (Price et al., 2006). A principal components analysis is performed to model ancestry differences between cases and controls, allowing for the identification of population substructure within the sample. The correction is then applied to a candidate marker, based on its variable frequency across the ancestral populations.
The family-based design is robust to population stratification and focuses on the transmission of alleles within families rather than allele or genotype frequencies (Spielman et al., 1993). In the transmission disequilibrium test (TDT), the transmission of alleles from heterozygous parents to affected offspring is compared at a particular locus. Under the null hypothesis, the transmission of alleles from heterozygous parents to affected offspring should be found in a 1:1 ratio. If one allele shows statistically significant biased transmission, it suggests an association between that allele and the trait. Since the TDT measures the transferring of alleles among families and not the allele frequency differences between cases and controls, population stratification is not an issue. The disadvantages of the TDT are that some of the collected genetic information is discarded (e.g. homozygous parents), and that it may be more difficult to ascertain families. Extensions of the TDT have been developed to test for association using multiple siblings (Curtis, 1997), haplotypes (Clayton and Jones, 1999), and quantitative traits (Laird et al., 2000).

1.5.4 Linkage Disequilibrium and Haplotype Analyses

Linkage disequilibrium (LD) measures the extent to which multiple alleles are found on the same haplotype across a population (Dawn Teare and Barrett, 2005). If two biallelic markers A/a and B/b are in Hardy-Weinberg equilibrium and segregate independently (with the frequency of each allele being 0.5), the four possible haplotypes that can result (AB, Ab, aB, ab) should be found in equal proportions. However, if it is found that certain alleles are always inherited together (e.g. only Ab and aB are found in a sample), then the markers are considered to be in LD. The main forces behind the decay of LD include recombination and to a lesser extent, recurrent mutations (Ardlie et al., 2002). Markers that are located close to each other tend to be in LD because there
is a lower probability of recombination events occurring to break down the LD. Because of this, regions of markers across the genome, called LD blocks, are found to be in LD.

LD between markers is often measured using $D$ (Lewontin and Kojima, 1960), $D'$ (Lewontin, 1964) and $r^2$ (Hill and Robertson, 1968). When the gametic phase is known, $D$ is the difference between the observed frequency of the haplotype and the expected probabilities if the alleles segregated independently. $D'$ measures the degree to which two markers segregate independently, with a value of 1 corresponding to the markers being in complete LD. The $r^2$ is the correlation coefficient between a pair of markers, based on the major and minor allele frequencies. A value of 1 indicates that two markers are highly correlated and are considered to be in perfect LD.

The presence of LD between surrounding markers have important consequences in the design and analysis of a genetic association study. Polymorphisms can be selectively genotyped so that they are more informative and represent larger regions. For example, genotyping one marker in an LD block, referred to as a tag SNP, can be informative for the surrounding markers in the same LD block.

Correction for multiple testing in association studies should take into account the LD between genotyped markers. When multiple polymorphisms and phenotypes are tested for association, the threshold for significance should be modified to prevent the incorrect rejection of the null hypothesis, referred to as Type I error. The most common method is Bonferroni correction, or modified versions of the procedure, which takes into account the number of independent tests that are performed. The correction is calculated by $\alpha/n$, where $\alpha$ is the desired significance level and $n$ is the number of hypothesis tests. However, SNPs that are in LD would not be considered independent, and Bonferroni correction would be too conservative. The LD
between the tested SNPs should be considered to ensure that the appropriate threshold for statistical significance is determined for a study.

1.6 RD Twin Studies

Since the earliest stages of RD research, it has been established that RD tends to run in families and that family history was a significant factor in the development of RD (Hallgren, 1950; Finucci et al., 1976; DeFries et al., 1978; Volger et al., 1984). These studies suggested that RD may have genetic influences, although shared environmental factors between family members may have also contributed to the development of RD. Using MZ and DZ twins that shared similar environmental backgrounds, a study of US twins found a concordance of RD in 84% of the MZ twins, significantly greater than the 29% concordance found in DZ twins (Bakwin, 1973). A subsequent study which tested an independent sample of twins from the US, known as the Colorado Twin Study sample, also found a significant difference with 68% of MZ pairs and 38% of DZ pairs concordant for RD (DeFries and Alarcon, 1996).

In addition to suggesting a genetic basis for RD, twin studies have shown that the reading skills in individuals with RD have a heritability ranging from 0.30-0.72 (DeFries et al., 1987; Gayan et al., 1999; Gayan and Olson, 2001; Harlaar et al., 2005). Using the Colorado Twin Study sample, moderate to high group heritability was found for measures of word recognition ($h_g^2 = 0.54$), phonological decoding ($h_g^2 = 0.71$), orthographic coding ($h_g^2 = 0.67$), phoneme deletion ($h_g^2 = 0.72$) and phoneme transposition ($h_g^2 = 0.69$) (Gayan and Olson, 2001). In the same study, bivariate heritability estimates were calculated to determine if there was genetic variance contributing to the overlap between the traits. A high correlation was found between phonological decoding and phoneme awareness ($h_g^2 = 0.67$), suggesting that there may
be shared genetic influences on both traits. In contrast, the genetic correlation between orthographic coding and phoneme awareness was considerably lower ($h_g^2 = 0.28$), suggesting mostly separate genetic influences underlying both traits. An independent study in the UK using twins in the bottom 10th percentile of a normally distributed sample also found moderate to high group heritability for word recognition ($h_g^2 = 0.68$ in males, and $h_g^2 = 0.50$ in females) (Harlaar et al., 2005).

1.7 RD Linkage Regions

Linkage studies have identified at least 14 chromosomal regions that may affect RD susceptibility. Nine of these regions are designated as DYX1-9 but five additional regions have also shown significant linkage. The susceptibility regions DYX1 and DYX5 and their corresponding candidate genes are discussed below.

1.8 Review of Linkage and Candidate Gene Studies in DYX1

1.8.1 Linkage Studies

The first reported RD susceptibility region was DYX1, which encompasses a large region on chromosome 15q15-21. In the initial study, a parametric analysis of 9 multi-generational families found linkage to the centromeric region of chromosome 15 using an autosomal dominant model (Smith et al., 1983). Subsequent replication studies did not find linkage to this region (Bisgaard et al., 1987; Rabin et al., 1993; Fisher et al., 2002). Instead two studies found linkage between 15q15 and 15qter, one of which used a non-parametric analysis of an expanded sample from
Smith et al. (1983) (Fulker et al., 1991; Smith et al., 1991). Another study using categorically
developed traits for components of reading found significant linkage with single-word reading at
D15S143, located on chromosome 15q21 (Grigorenko et al., 1997). Three studies were able to
replicate linkage with RD to 15q21, prompting further investigation into possible RD candidate
genes in this region (Chapman et al., 2004; Bates et al., 2007; Schumacher et al., 2008).

1.8.2 Association Studies
The first RD association studies in DYX1 tested for the transmission of microsatellite markers
across the susceptibility region. In a two-stage study using UK families, a three-marker
haplotype D15S994/D15S214/D15S146 was found to be significantly associated with RD
(Morris et al., 2000). A later study using Italian families found a different three-marker
haplotype to be associated with RD D15S214/D15S508/D15S182 (Marino et al., 2004). The
D15S994 marker is located in the phospholipase C β 2 (PLCB2) gene and is located 1.6Mb from
phospholipase A2, group IVB (cytosolic; PLA2G4B). It has been suggested that phospholipid
metabolism may be involved in RD, with dyslexic adults showing higher levels of cytosolic
phospholipases A2 (cPla2) (Taylor et al., 2001). Increased cPla2 may result in a deficiency of
unsaturated fatty acids, which has been observed in children with RD (Richardson et al., 2000).
Morris et al. (2004) followed up their original findings by genotyping a case-control and family-
based sample for 4 PLCB2 SNPs and 11 PLA2G4B SNPs. However, none of the SNPs in either
sample showed significant association with RD.

The first reported RD candidate gene was on chromosome 15q21, identified through
a t(2;15)(q11;21) chromosomal translocation that cosegregated with RD in a six member, two
generation Finnish family (Taipale et al., 2003). In that family, the affected father and two
affected offspring carried the translocation. A third offspring also carried the translocation but his affection status was unknown due to low overall performance on tests measuring cognitive ability and intelligence. The identified translocation disrupted *dyslexia susceptibility 1 candidate gene 1 (DYX1C1)*, also known as *EKN1*. The *DYX1C1* gene is located in DYX1 and lies within the linkage regions identified in several studies (Fulker et al., 1991; Smith et al., 1991; Chapman et al., 2004). An association study of *DYX1C1* was subsequently performed using Finnish dyslexics and controls that included both nondyslexics and anonymous blood donors. The sample that was analyzed included 58 dyslexics and 61 nondyslexics from 20 unrelated families, combined with a replication set of 3 families and 33 unrelated dyslexic-nondyslexic couples. Statistically significant association with RD was found at the -3G/A and 1249G/T positions, with both the minor A and T alleles showing greater frequencies in the dyslexic group. In addition, the frequency of the -3A:1249T haplotype was significantly higher in the dyslexic group (14/106 cases) compared to the controls (10/192 nondyslexic and anonymous controls). A TDT analysis of 9 informative family trios found significant overtransmission of the same -3A:1249T haplotype.

The two significant SNPs were of particular interest because they were proposed as having potentially functional effects. The -3G/A SNP, located in the first *DYX1C1* intron, was thought to have a role in transcription as it is in the predicted binding sequence of the Elk-1, HSTF, and TFII-I transcription factors. On the *DYX1C1* mRNA sequence, the -3G/A SNP is located within the Kozak consensus sequence which plays a crucial role in initiating translation. The 1249T variant introduces a premature stop codon, shortening the protein from 420 to 416 amino acids but subsequent functional studies found that the truncated form did not alter the function of *DYX1C1* in an *in vivo* assay (Wang et al., 2006). Nonetheless, *DYX1C1* became a promising candidate for playing a role in the development of RD.
The findings of Taipale et al. (2003) brought considerable attention to *DYX1C1*, but subsequent association studies have yielded mixed results (Table 1-2). A family-based association study by Wigg et al. (2004) did not find association between RD and the two previously reported SNPs, but the major -3G allele was found to be significantly associated with poorer performance on language, reading and memory measures. In addition, a different SNP (rs11629841) showed significantly biased transmission with RD.

Scerri et al. (2004) studied the association between *DYX1C1* and quantitative reading measures in dyslexic families from the UK. Although a test for orthographic coding word choice showed nominal significance, it was with the major 1249G allele, and was not significant after correction for multiple testing. Another study of families in the US found statistically significant association with the same 1249G allele, which showed biased transmission with RD (Brkanac et al., 2007).

A family-based sample from Italy has been analyzed in several *DYX1C1* association studies. In the first study, no significant association was found between individual SNPs and RD, or reading and spelling measures (Marino et al., 2005). However, a later study which used a larger sample and added memory tests found that the -3A allele was significantly associated with lower performance on a single letter backward span test (Marino et al., 2007). Another study testing this sample for language and mathematics abilities found that the -3A variant was associated with mental calculation tasks, but associations with language measures were not significant (Marino et al., 2011).

In addition to the single SNP analyses a number of studies tested for association with *DYX1C1* haplotypes, also with mixed results. Contrasting the initial study, which found overtransmission of the -3A:1249T haplotype, Wigg et al. (2004) found the -3G:1249G
haplotype to be associated with RD. Marino et al. (2007) did not find an association between the -3G/A:1249G/T haplotype and RD, but there was biased transmission of the -3A/1249T haplotype in the working memory task, supporting the initial findings of Taipale et al. (2003). A study by Scerri et al. (2004) did not find any association between a specific -3G/A:1249G/T haplotype and quantitative reading-related measures. In a study of 366 German families, statistically significant association was observed in a gender-stratified analysis (Dahdouh et al., 2009). Overtransmission of several DYX1C1 haplotypes, the most significant being rs3743205G:rs3743204G:rs600753G, was found among the 66 families with female probands. This haplotype was also found to be associated with short term memory in the females.

Association studies of DYX1C1 have been performed using unselected populations to investigate whether DYX1C1 is associated with normal variation in quantitative traits. In the first study, which used twin families, three SNPs were associated with a number of reading, spelling and memory measures: rs685935 (digits span forwards), rs17819126 (irregular reading and spelling, nonword reading), rs3743204 (nonword reading) (Bates et al., 2010). The second study, which analyzed a cohort of 10 year old children for general reading and spelling abilities, found association between DYX1C1 SNPs and reading (rs8040756), and spelling measures (rs8043049, rs8037376, rs7174102 ) (Paracchini et al., 2011).

In contrast to the previous studies, which either found replicated findings of the initial study or association with other SNPS, a number of studies did not find association between DYX1C1 SNPs and RD or quantitative traits (Bellini et al., 2005; Cope et al., 2005; Meng et al., 2005; Saviour et al., 2008; Newbury et al., 2011; Venkatesh et al., 2011). With a wide range of results, the status of DYX1C1 as an RD candidate gene remains unclear.
<table>
<thead>
<tr>
<th>Study</th>
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<th>Significant Results</th>
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<tr>
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<td>Case-control</td>
<td>Finland</td>
<td>RD</td>
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<td>-3A (9 quantitative traits)</td>
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<td>RD</td>
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<tr>
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<td>Marino et al. (2007)</td>
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<td>Quantitative</td>
<td>-3A (Single Letter Backwards Span)</td>
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<td>India</td>
<td>RD</td>
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<tr>
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<td>Design</td>
<td>Location</td>
<td>Condition</td>
<td>Significant SNPs</td>
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<td>Germany</td>
<td>RD</td>
<td>rs3743204G:rs600753G&lt;sup&gt;2&lt;/sup&gt; rs3743205G:rs600753G&lt;sup&gt;2&lt;/sup&gt; rs61761345G&lt;sup&gt;2&lt;/sup&gt; rs16787A:rs3743205G:rs600753G:rs61761345G&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>Quantitative</td>
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</tr>
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<td>Cohort (Population-Based)</td>
<td>Australia</td>
<td>Quantitative</td>
<td>rs8040756 (Reading) rs8043049 (Spelling) rs8037376 (Spelling) rs7174102 (Spelling)</td>
</tr>
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<td>Case-control</td>
<td>UK (2 samples)</td>
<td>RD</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>1</sup> Association was found with measures for Reading, Spelling, Word ID, Word Attack, Receptive Language, Expressive Language, Rapid Digit Naming, Nonword Repetition, and Phonological Awareness

<sup>2</sup> Association was only when the analysis was restricted to female probands
1.8.3 Functional Studies of DYZ1C1

While the association findings generated interest in DYZ1C1, functional studies have made DYZ1C1 a biologically plausible RD candidate gene. Prior to the initial association study, the function of DYZ1C1 was unknown. Taipale et al. (2003) found that DYZ1C1 was abundantly expressed in most tissues including the brain, and that the protein product contained three tetratricopeptide repeat domains (TPR), which suggests a role in mediating protein-protein interactions.

Wang et al. (2006) first studied the function of DYZ1C1 through knockdown and rescue experiments in rat embryos. In the brains of the rats treated with the short hairpin RNA (shRNA) plasmids, decreased neural migration from the subventricular zone to the cerebral plate was observed. The neurons were also noted to be different than the control rats after 4 days, with bipolar morphologies rather than the multipolar found in the controls. The authors then performed individual rescue experiments using different motifs of DYZ1C1. The TPR domains are located on the C-terminal end and a p23 motif is located on the N-terminal end, which may suggest different functional properties. Rescue experiments using only the C-terminus was able to rescue the normal neural migration. Furthermore, a truncated form of the C-terminus end missing 4 amino acids also fully rescued normal migration. The results of this study showed that the truncation caused by the 1249T allele may not drastically alter the function of DYZ1C1, at least as measured by these assays. However, the authors acknowledged that while the truncated protein may not have a functional effect, it may be differential levels of DYZ1C1 expression that affect RD susceptibility.

Similarly, a study by Rosen et al. (2007) found that RNA interference (RNAi) treatment in rats resulted in a number of unique characteristics that were not found in the sham
Among the 21 treated rats, all were found to have abnormal neural migration (n=21), while some had molecular ectopias in the cortex (n=5) and heterotopias in the hippocampus (n=5). Interestingly, these characteristics have been found in postmortem examinations of dyslexic brains (Galaburda et al., 1985). Before histological examination of the brains, the rats were tested for auditory processing and spatial learning abilities (Threlkeld et al., 2007). Differences were observed in the adult RNAi treated rats in auditory processing tasks, measured through the reduction of startling reflexes from auditory stimuli. The rats with hippocampal malformations also performed significantly poorer on the spatial learning tasks compared to the shams and the rats that had only cortical malformations.

In a later study, a different group of $DYX1C1$ RNAi treated rats were tested for spatial working memory abilities (Szalkowski et al., 2011). Consistent with previous studies, cortical and hippocampal abnormalities were only found in a subset of the treated rats. The RNAi treated rats, regardless of the presence or absence of visible malformations, performed significantly poorer compared to the controls, committing more errors throughout the 48-day testing period. There was no difference in performance between the observed malformations.

Studies of the heterotopias found that the neurons were both transfected and non-transfected, suggesting that both cell autonomous and non-cell autonomous processes are involved in the disruption of the neural migration (Currier et al., 2011). A cell-autonomous effect refers to an abnormal phenotype that is only observed in the genetically mutant cells, while non-cell autonomous effects have normal cells involved in the pathology as well. The results of the $DYX1C1$ functional studies were consistent with the theory that RD is caused by a dysfunction in neural migration, and contributed to providing a biologically plausible role for $DYX1C1$ in RD.
1.9 Review of Linkage and Candidate Gene Studies in DYX5

1.9.1 Linkage Studies

Chromosome 3p12-q13 (DYX5) was identified as a RD susceptibility region through a large Finnish family spanning 4 generations and consisting of 74 members, 27 of whom were affected with RD (Nopola-Hemmi et al., 2001). In a genome-wide linkage scan using a subset of this family, 19 of the 21 genotyped affected members shared a 35Mb haplotype IBD that spanned chromosome 3p12-q13. The affected members that were studied performed significantly worse on phonological awareness, rapid naming and verbal short-term memory tests. An independent genome-wide linkage study using sibpair samples from the UK and US found linkage to 3p13 in the UK sibpair sample and 3q13 in the American sibpair sample (Fisher et al., 2002). The quantitative traits that were significant in the study included phonological decoding and orthographic coding. In addition to the two RD studies that found linkage to DYX5, a third study using families affected with SSD found this same region to be significantly linked with a phonological memory task (Stein et al., 2004). SSD has been shown to share common phonological processing deficits with RD, and is also often comorbid with RD. These findings suggest that DYX5 may be involved in the common phonological deficits found in both RD and SSD.

1.9.2 Functional Studies of ROBO1

ROBO1 is a member of the evolutionary conserved ROBO family of receptor proteins, and is orthologous to the Drosophila roundabout gene (ROBO). There are 4 ROBO homologs in mammals, which are all defined by five immunoglobulin-like (IG-like) domains, three
fibronectin type-III repeats, a transmembrane region, and a cytoplasmic tail (Kidd et al., 1998; Wong et al., 2002). ROBO was first identified in a Drosophila study screening for genes that controlled axon midline crossing in the central nervous system (Seeger et al., 1993). In the ROBO mutants, the normally ipsilateral axons crossed the midline, while commissural axons were found to cross the midline multiple times.

The ligand for ROBO receptors were found to be Slit proteins, which function as extracellular chemoattractants (Brose et al., 1999; Kidd et al., 1999). Three Slits have been identified in mammals, each containing 4 leucine rich regions, epidermal growth factor repeats, a laminin G region and a cysteine rich C-terminus (Wong et al., 2002). The leucine rich region of the Slit molecules interacts with the IG-like domains of ROBO receptors, activating the Slit-ROBO signaling pathway. In mammals, the Slit proteins are all expressed in the ventral midline of the neural tube and are expressed from development into adulthood. The Slits act as chemorepellants, preventing the growth of axons by collapsing of growth cones at the tips of extending neurons. The role of Slit and ROBO genes in the development of the olfactory bulb has been extensively studied but both genes are also expressed in the inner ears of rodents and chicks (Nguyen-Ba-Charvet and Chedotal, 2002; Webber and Raz, 2006). Thus, the development of the auditory system may also be affected by Slit and ROBO genes.

1.9.3 Expression and Magnetoencephalographic (MEG) Studies of Roundabout Homolog 1 (ROBO1)

One affected Finnish individual was found to have a t(3;8)(p12;q11) chromosomal translocation in DYX5, although this translocation was not found in any of the three siblings, one of whom was affected with RD (Hannula-Jouppi et al., 2005). This translocation disrupted the roundabout
homolog 1 (ROBO1) gene, and it was hypothesized that this gene may be involved in the development of RD. Using a subset of the large Finnish family in the study by Nopola-Hemmi et al. (2001), the expression of ROBO1 in individuals with RD was investigated (Hannula-Jouppi et al., 2005). Four of the affected family members who carried one copy of the risk haplotype were selected for the study, and the relative allelic expression in lymphocytes was compared to four controls through the sequencing of one SNP within the haplotype. In the affected individuals with the risk haplotype, there was a statistically significant reduction in expression of the risk allele compared to the common allele, whereas the controls with two copies of the common haplotype had similar allelic expression. This led the authors to propose that differential expression of ROBO1 contributes to the development of RD.

During normal development, the majority of axons from each ear will cross the midline to the contralateral auditory cortex, while the rest remain on the ipsilateral side. Magnetoencephalography (MEG) has been used to evaluate the processing of auditory stimuli in the auditory cortices (Fujiki et al., 2002). The individual is first supplied with auditory stimuli in each ear separately. The stimuli are “frequency-tagged”, so that the frequency given to each ear is different. During monaural listening, the response of each auditory cortex is measured for each stimulus. Then, the stimuli are provided simultaneously, with the same frequency-tagged stimulus given to the same ear and the response of the auditory cortices is again measured. The MEG signal for the left and right inputs can be separated from each other and compared to the monaural signal to detect any suppression of the input. In the first studies using MEG to measure auditory processing, the right auditory cortex showed a symmetrical suppression of inputs but in the left hemisphere, the ipsilateral input was significantly suppressed compared to the contralateral inputs (Fujiki et al., 2002). However, a later study found that there was significant
ipsilateral suppression in both sides (Kaneko et al., 2003). Thus, the normal MEG response of the left and right auditory cortices during binaural listening is still unclear.

After the initial linkage findings in the Finnish family, the MEG method was used to examine the levels of binaural suppression and its relationship to ROBO1 expression in lymphocytes (Lamminmaki et al., 2012). It was hypothesized that the lower expression of ROBO1 in the individuals with the risk haplotype weakened the crossing of auditory neurons from the ear to the contralateral auditory cortex, which would result in less ipsilateral suppression. Ten of the members carrying the ROBO1 risk haplotype underwent the MEG experiment using frequency-tagged stimuli and the levels of binaural suppression were compared to 10 non-dyslexic, healthy, age- and sex-matched controls. The expression of ROBO1 was assessed through quantitative real-time PCR (qRT-PCR). Levels of ROBO1 expression in the haplotype-carrying family members were compared to anonymous blood donors, who were different from the controls of the MEG experiment. The study found significantly lower ipsilateral suppression in the ROBO1 subjects compared to the controls. Among the ROBO1 group, there was a dose dependent response, with a correlation between ROBO1 expression and levels of ipsilateral suppression. In the expression analysis, the authors hypothesized that the overall biallelic expression of ROBO1 in the individuals with the risk haplotype would be 83% of the individuals with two copies of the haplotype. This value was derived from the previous study by Hannula-Jouppi et al. (2005), where it was observed that the allelic expression of the risk haplotype was 66% of the normal haplotype. It was predicted that an individual who carried one copy of the normal haplotype (where ROBO1 allelic expression is at 100%) and one copy of the risk haplotype (where ROBO1 allelic expression is at 66%) would have ROBO1 expression levels at 83% of an individual carrying two normal haplotypes ([(100% + 66%)/2]. Although the differences between the affected individuals and the controls did not reach statistical
significance, the authors concluded that the 83% reduction in \textit{ROBO1} expression was consistent with their hypothesis. Thus, the findings of this study suggested that lower \textit{ROBO1} expression levels may weaken the crossing of auditory axons across the midline, resulting in abnormal auditory processing. Deficits in auditory processing have also been observed in other studies, although with rapidly changing auditory stimuli, rather than continuous tones as was tested in the study by Lamminmaki et al. (2012) (Tallal, 1980; De Martino et al., 2001; Rey et al., 2002). Nonetheless, the results of these studies suggested that a dysfunction of the auditory system may lead to reading difficulties in a subset of individuals with RD.

1.9.4 \textit{ROBO1} Association Studies
To date, one \textit{ROBO1} association study has been conducted using a population-based sample from Australia (Bates et al., 2011). A total of 538 families with monozygotic twins, dizygotic twins or triplet offspring were tested for reading measures, and 505 of those families were later tested for language measures. Four quantitative reading measures were tested in the subjects including phonological buffering (nonword repetition task), short-term verbal memory (digits forward memory task), working memory (letter number sequencing task), and reading and spelling principle components (irregular and non-word spelling and reading tasks). Subjects were genotyped for 144 tag SNPs across \textit{ROBO1}. After correcting for the number of tested SNPs, the phonological buffering task remained significantly associated with two SNPs: rs6803202 \((p=8.7 \times 10^{-5})\) and rs4535189 \((p=9.3 \times 10^{-5})\). Several other SNPs also showed nominal significance for the reading measures. Consistent with a previous linkage study using families with SSD, the results of this study suggest that \textit{ROBO1} may be responsible for variation in phonological buffering abilities.
1.10 Introduction to Systematic Reviews and Meta-analyses

With advances in genotyping technologies, which have made testing polymorphisms more efficient and affordable, there has been a rapid increase in the number of published genetic association studies. Although many studies have reported associations, subsequent studies have often failed to replicate the initial findings and tend to show more modest effect sizes (Ioannidis et al., 2001). Thus, it is important to critically evaluate the evidence to determine if there is an association between a trait and polymorphism or gene. This is often achieved through systematic reviews and meta-analyses.

The goal of a systematic review is to investigate a specific research question through an evaluation of the literature. As outlined by Sagoo et al. (2009), there are 5 objectives in a systematic review of genetic association studies: identify the studies that investigated the associations of interest, assess the validity of the cumulative evidence, determine if there is an association, assess whether there is a consistent effect across studies, and quantify the effect size if there is an association. Systematic reviews differ from narrative literature reviews in that they are designed as rigorous research studies and offer a more objective appraisal of the evidence (Garg et al., 2008; Sagoo et al., 2009). Unlike narrative literature reviews, systematic reviews are more transparent in describing the methods for collecting studies, assessing their quality, and synthesizing the collected data to draw objective conclusions. As a result, systematic reviews are less prone to bias and error (Teagarden, 1989). A meta-analysis is often used in a systematic review as a quantitative method to synthesize the data and determine if there is an overall association. In addition, a meta-analysis allows the evaluation of between-study heterogeneity. Guidelines for systematic reviews of genetic association studies have been developed by the
Human Genome Network (HuGENet™), as outlined in The HuGENet™ HuGE Review Handbook version 1.0 (Little and Higgins). A summary of the relevant considerations in systematic reviews and meta-analyses are discussed below.

1.10.1 Collection of Studies and Search Strategy

Since the validity of a systematic review and meta-analysis is dependent on the analysis of the complete body of evidence, it is important to identify all relevant studies that address the research question (Yoshii et al., 2009). To achieve this, a comprehensive search of electronic bibliographic databases should be performed and reported. Multiple databases should be used in the collection of studies (e.g. MEDLINE, EMBASE and BIOSIS) since there may be low overlap between the different indexes, depending on the field of study (Sagoo et al., 2009). The search terms should take the form “(gene OR synonyms) AND (disease OR synonyms)” (Little and Higgins). In addition to identifying studies through the bibliographic databases, there may be studies that are not published in peer-reviewed journals, referred to as ‘grey literature’. The methods of the data collection, including the bibliographic databases used and the search strategies, should be explicitly defined so that they can be readily reproduced. This is often a problem in systematic reviews, as the search strategies are often inadequately described (Yoshii et al., 2009). Two individuals should independently perform the search strategy and data extraction to ensure that the results are similar. If there are discrepancies in the extracted data, a third individual should perform the data extraction or alternatively, the discrepancies should be resolved by consensus.
1.10.2 Assessing Study Validity

Information on the design of each study should be obtained from the article or through direct correspondence with the authors. By assessing the individual studies, potential biases can be identified. These biases may affect the results of the individual studies and should be addressed in the systematic review.

Studies are susceptible to several types of biases including phenotype definitions, confounding, and genotyping errors (Little and Higgins; Ioannidis et al., 2008; Sagoo et al., 2009). The phenotype definition may have important effects on the study results. For diseases that may be defined by quantitative measures, different thresholds may select for different severity of cases. The types of cases analyzed in a study may also be affected by the methods of ascertainment, as different sources may select for a more or less severe phenotype (e.g. schools for children with learning disabilities may select for more extreme cases of RD than regular schools). The definition of the controls is also important since unselected, population controls may yield different results than selected controls (e.g. individuals without the disease). For case-control studies, there is a risk of confounding due to population stratification (Cardon and Bell, 2001). Different ethnic groups may have different allele frequencies at a specific locus. Thus, the control and cases should ideally be composed of the same ethnic group to prevent any false-positive associations. The genotyping methods may also be a source of bias in a genetic association study. Misclassification of genotypes can affect the allele-disease associations and can be caused by a number of sources including the variation in DNA sequence, quality of DNA and human error (Pompanon et al., 2005). Quality-control measures should be performed to identify and prevent genotyping errors. Some examples of quality-control measures include genotyping samples for replication, testing for Hardy-Weinberg equilibrium, and checking for...
Mendelian errors in family-based studies. However, bias can never be ruled out completely, even in a high quality study (Ioannidis et al., 2008).

1.10.3 Identification and Assessment of Publication Bias in the Systematic Review

Publication bias is an important consideration in systematic reviews. It has been well established in the medical literature that studies with positive or interesting results are more likely to get published (Easterbrook et al., 1991; Dwan et al., 2008). In contrast, studies with negative results may experience a “file drawer” effect, where they may be set aside rather than be submitted for publication (Rosenthal, 1979). Although the evidence of publication bias has extensively been illustrated in clinical or drug trials, similar risks apply to the reporting of genetic association studies (Ioannidis et al., 2001; Salanti et al., 2005).

In addition to reporting biases, significant studies are more likely to be published sooner, receive more citations, and be published in an English language journal (Egger et al., 1997; Stern and Simes, 1997; Egger and Smith, 1998). These factors may affect how accessible a study may be, and how easily a study can be identified for a systematic review. It is important to ensure that a thorough search of the literature, and unpublished studies if possible, is performed to identify all relevant studies.

A common method of assessing publication bias in meta-analyses is to interpret funnel plot asymmetry (Egger et al., 1997). In a funnel plot, the effect size is plotted against the study size. Normally, studies with larger samples tend to show modest effect sizes while smaller studies tend to show more extreme effects in both directions, creating an inverted funnel. A
regression analysis can be used to test for significant asymmetry from the funnel shape, which would suggest publication bias.

1.10.4 Meta-analysis and Assessment of Between-Study Heterogeneity

Meta-analyses are used to combine the effect size estimates from each individual study and obtain a pooled result. Methods have been developed to combine the results from case-control and TDT designs, accommodating the inclusion of both tests (Kazeem and Farrall, 2005). Two different models can be used in the meta-analysis: a fixed-effects model or a random-effects model. There are important distinctions between both models, and the appropriate model should be selected depending on the included studies and the research question (Munafo and Flint, 2004). The fixed-effects model assumes that the study samples are derived from the same population, and that the effect size of the genetic variant in question is the same in all samples. Any variance that is observed in a fixed-effects model is attributed to sampling error. On the other hand, the random-effects model assumes that the study samples are derived from different populations and that the effect size of the genetic variant may differ between samples. The sources of variance in a random-effects model are from both sampling error and between-study heterogeneity. This heterogeneity can be due to the use of different populations, study designs or phenotype definitions. Random-effects model meta-analyses give a more conservative estimate than fixed-effects models and are generally preferred over fixed-effects model if there is any anticipation of between-study heterogeneity (Kavvoura and Ioannidis, 2008). Furthermore, the random-effects model is more robust, as the results are similar to fixed-effects meta-analyses when there is no heterogeneity.
Assessing between-study heterogeneity, or the consistency of effect sizes across the samples, can be performed using statistical methods. A common method is the Cochran’s $Q$ statistic, which tests for statistically significant evidence of between-study heterogeneity (Cochran, 1950). However, Cochran’s $Q$ has low power when there are few studies, and has excessive power when many samples are included (Hardy and Thompson, 1998). Furthermore, a non-significant p-value in Cochran’s $Q$ statistic does not necessarily confirm homogeneity among the studies. Another measure, called the $I^2$ statistic, was developed to quantify the degree of heterogeneity, and is derived from the $Q$-statistic (Higgins and Thompson, 2002). $I^2$ values are reported as percentages and describe the proportion of total variation that is due to heterogeneity. Both the $Q$ and $I^2$ statistics have been suggested to be used together as complementary methods in a meta-analysis (Huedo-Medina et al., 2006).

1.10.5 Venice Criteria

In a systematic review, it is important to evaluate the credibility of the evidence and the likelihood that an association exists with the collected evidence. The Venice Criteria was developed as a semi-quantitative measure to assess the cumulative evidence of genetic association studies (Ioannidis et al., 2008). The cumulative evidence is evaluated using three criteria including the amount of evidence, replication, and protection from bias, with each criterion given a categorical rating (A-C, with A being the strongest grade). The amount of evidence refers to the sample size with the risk allele. Replication refers to the extent of between-study heterogeneity among the samples, measured by the $I^2$ statistic. Protection from bias is determined by the presence of the biases discussed in Chapters 1.10.2-3. Combining the ratings from all three categories, a grade of ‘strong’, ‘moderate’ or ‘weak’ can be assigned to the
evidence. For example, a ‘strong’ grade requires that all three categories are given an A rating. The Venice Criteria can be used in a systematic review to describe the overall credibility of the collected studies.
1.11 Research Aims and Hypotheses

1.11.1 Study I: Evaluation of the RD Candidate Gene \textit{DYX1C1}

\textbf{Rationale}

\textit{DYX1C1} is located on chromosome 15q21, within the DYX1 region that has shown linkage to RD in multiple independent samples. The two \textit{DYX1C1} SNPs -3G/A and 1249G/T previously showed statistically significant association with RD. In a previous study using a subset of the Toronto sample collected at that time (n=148), significant association was also found between \textit{DYX1C1} SNPs and RD, as well as with quantitative reading-related measures. Although \textit{DYX1C1} has been extensively studied, subsequent association studies have yielded mixed results and the association between \textit{DYX1C1} SNPs and RD remains unclear.

\textbf{Aims}

I. To test whether \textit{DYX1C1} SNPs are associated with RD using two independently ascertained, family-based samples.

II. To test whether \textit{DYX1C1} SNPs are associated with reading-related quantitative measures using the Toronto sample

III. To evaluate the overall association between the -3G/A and 1249G/T SNPs and RD through a thorough review and meta-analysis, and to assess the credibility of the cumulative evidence.
Hypothesis

I. Through the cumulative evidence and an increased sample size, the common \textit{DYX1C1} SNPs will be associated with RD and standardized quantitative reading measures.

1.11.2 Study II: Family-based association analysis of \textit{ROBO1}

Rationale

Chromosome 3q (DYX5) has shown linkage to phonological processing and memory measures in two independent studies. A subsequent study using a normal population found statistically significant association between two \textit{ROBO1} SNPs and a phonological memory task. Despite the designation of \textit{ROBO1} as a RD candidate gene, there has not yet been a study to directly investigate the association of \textit{ROBO1} with RD.

Aim

II. To validate the association between \textit{ROBO1} SNPs and RD, and measures of reading, phonological memory and working memory, with a specific focus on the most significant SNPs in a previous study by Bates et al. (2011).

Hypothesis

I. \textit{ROBO1} is associated with RD and with phonological memory.
Chapter 2 – General Materials and Methods

2.1 Subjects

2.1.1 Toronto Sample

The sample included families that were recruited primarily from the Greater Toronto Area and surrounding regions. The sample consisted of 1416 individuals from 421 families, each with at least one child who exhibited difficulties in reading. Probands ranged from 6-16 years of age, and siblings within the same age range were also invited to participate in the study regardless of their reading ability. Families were self-referred through information provided to a number of sources including websites, schools, regional organizations for learning disabilities, and referrals from general education teachers, special education teachers, speech/language pathologists, and psychologists.

To be included in the study, the proband must have learned English as their first language or attended an English speaking school for at least five years. The ethnicity of the sample was primarily Caucasian, with 68.1% of individuals reporting European descent and 26% reporting “Caucasian Canadian” background. The remaining subjects reported South American (1.8%), non-European (2.9%), or non-European mixed background. The protocol for the Toronto sample was approved by The Hospital for Sick Children Research Ethics Board and written informed consent was obtained from all participants.
2.1.1.1 Psychometric Testing

Probands and siblings underwent an intensive and comprehensive battery of tests measuring cognitive, reading and language abilities. A subset of the administered tests was used in the analysis of the quantitative traits. The subtests analyzed in this study were administered to all 588 children except for the Digit Span Forwards and Backwards subtests of the Wechsler Intelligence Scale for Children-III (WISC-III), which were administered to 418 of the 588 children. The decreased sample for the WISC-III Digit Span measures was due to the use of an updated version (WISC-IV) once it became available (Wechsler, 2003). All tests were standardized and corrected for age and sex. Descriptions of the analyzed subtests are provided below. The pairwise correlation coefficients for all of the analyzed subtests are provided in Table 2-1 and the summary statistics are provided in Table 2-2.

2.1.1.2 Wechsler Intelligence Scale for Children – 3rd and 4th Editions (WISC-III and WISC-IV)

The WISC measures intelligence in children 6-16 years of age, without the use of reading or writing tasks. The first 418 of the 588 subjects were tested using the WISC-III, with the remaining 170 tested with the WISC-IV once it became available. In the WISC-III, two composite scores were used for this study: Verbal and Performance IQ. Verbal IQ was calculated from the scores of five subtests including Information (answering general knowledge questions), Similarities (explaining how two objects or concepts are similar), Arithmetic (answering oral math problems), Vocabulary (defining words), and Comprehension (answering questions that test social judgment and verbal reasoning). Performance IQ was calculated from five subtests including Picture Completion (identifying the missing parts of a picture), Coding (copying symbols that are paired with shapes or numbers), Picture Arrangement (placing pictures in a
sequential order to tell a logical story), Block Design (copying designs using blocks) and Object Assembly (constructing a picture or object from its fragmented pieces).

For the children who were administered the WISC-IV, the Verbal Comprehension and Perceptual Reasoning Indices were used to assess IQ, which are analogous to the Verbal and Performance IQ of the WISC-III. The Verbal Comprehension Index consists of 4 of the subtests used in WISC-III (Similarities, Vocabulary, Comprehension and Information) and includes an additional subtest measuring Word Reasoning (identifying an object using a series of clues). The Perceptual Reasoning Index consists of the Block Design and Picture Completion subtests from the WISC-III, and includes 2 new subtests measuring Picture Concepts (selecting pictures that have a similar characteristic) and Matrix Reasoning (viewing an incomplete picture or pattern, and selecting the missing piece).

The Digit Span Forwards and Backwards subtests of the WISC-III were used to assess verbal short-term memory and working memory abilities in the subjects (Wechsler, 1991). In the Digit Span Forwards subtest, the subject is orally presented with a sequence of numbers that progressively increases in length and is asked to repeat the sequence in the correct order. In the Digit Span Backwards subtest, the subject is also presented with a sequence of numbers, but the task involves repeating the sequence in reverse order. Scores on both tests were standardized to a mean value of 10.

2.1.1.3 Children’s Memory Scale (CMS)

The CMS is a test for children aged 5-16 that measures a number of memory dimensions including attentional and working memory; verbal and visual memory; short- and long-delay
memory; recall and recognition; and learning characteristics (Cohen, 1997). For this study, two measures of verbal short-term and working memory were used: Numbers Forwards and Numbers Backwards. The Numbers Forwards subtest is analogous to the Digit Span Forwards subtest in the WISC-III, where the child is asked to orally repeat a sequence of digits. The Numbers Backwards subtest is analogous to Digit Span Backwards, where the sequence of digits is repeated in reverse order. Scores for both tests were age standardized to a mean value of 10.

2.1.1.4 Wide Range Achievement Test-III (WRAT-III)

The WRAT-III is an achievement test measuring word recognition, spelling and arithmetic (Wilkinson, 1993). The test can be administered to individuals from 5-75 years old. Data from the Spelling and Reading subtests were used for this study. The Spelling subtest assesses the subject’s ability to write their name and single dictated words. The Reading subtest measures the ability to read aloud single words. All scores were age standardized with a US population mean of 100.

2.1.1.5 Woodcock Reading Mastery Test-Revised (WRMT-R)

The WRMT-R is a test measuring reading ability in individuals aged 5-75 (Woodcock, 1987). The Word Attack and Word Identification subtests in the WRMT-R were used to assess real-word and non-word reading abilities. In the Word Identification subtest, subjects are required to read single real words that increase in difficulty. The Word Attack subtest is a phonological decoding task which involves the reading of non-words that test 67 different letter-sound categories. Scores were standardized to a mean of 100.
2.1.1.6 Comprehensive Test of Phonological Processing (CTOPP)

The CTOPP was used to measure the subjects’ phonological awareness, phonological memory and rapid naming skills. Two different versions of the test are administered, one for children 5-6 years of age and another version for children 7-24 which included extended items for the Phonological Awareness tests (Wagner et al., 1999). For this study, the composite score for Phonological Awareness was used, in addition to the scores for the Phonological Memory and Rapid Digit Naming subtests. The Phonological Awareness composite score comprises subtests for 5-6 year olds that measure the ability to segment words into their phonemes, form words using individually presented segments, and match words based on their initial or last sound. For children 7 years and older, the first two Phonological Awareness subtests are used in the calculation of the composite score. The Phonological Memory composite score is based on a nonword repetition subtest, where the subject is tested on their ability to repeat nonwords ranging from 3-15 sounds. In the Rapid Digit Naming subtest, the individual is asked to recite a series of 72-digits as quickly and accurately as possible. The standardized score for the Phonological Awareness composite score was 100, and both the Rapid Digit Naming and Nonword Repetition subtests were standardized to a mean score of 10.

2.1.1.7 Clinical Evaluation of Language Fundamentals- 3rd Edition (CELF-3)

The CELF-3 is a test for language ability designed for children 6-21 years of age (Semel et al., 1995). Two composite scores are produced, Receptive and Expressive Language, both of which were standardized to a mean score of 100. The Receptive Language composite score is derived
from tasks involving concepts and directions; word classes; and sentence structure or semantic relationships. The concepts and directions subtest evaluates the subject’s ability to interpret, recall and execute oral commands by identifying objects in response to oral directions. In the word classes subtest, the subject is given 3 or 4 words and is asked to choose a pair of words based on semantic relationships. The sentence structure subtest was given to subjects 7-8 years of age, and consists of choosing a picture that corresponds to an orally presented sentence. For individuals ≥9 years of age the semantic relationship subtest was given. Like the sentence structure subtest, the subject is also presented with one or multiple sentences but instead of choosing a picture, the task involves selecting 2 correct choices from 4 options.

Three expressive language subtests were administered that involved formulating sentences; recalling sentences; and word structure or sentence assembly. In the formulated sentences subtest, the subject is given a picture and asked to generate a sentence that includes a target word or phrase. The sentence recall subtest measures the subject’s ability to repeat sentences of varying length and complexity. Individuals 7-8 years of age were given a word structure task, which evaluates their ability to complete a partial sentence based on a picture. Subjects ≥9 years of age were instead given the sentence assembly subtest, where they are asked to rearrange words and phrases to create a complete sentence.

2.1.1.8 Behavioural and Medical Assessment

To screen for symptoms of possible neurological, medical and psychiatric disorders, parents were interviewed using the structured Children’s Interview for Psychiatric Syndromes (ChIPS) (Weller et al., 2000), and teachers were interviewed using the semi-structured Teacher Telephone Interview (Tannock et al., 2002). This information was supplemented using two
standardized questionnaires: the Conners Parent and Teacher Rating Scales- Revised (Conners, 1997) and the Ontario Child Health Survey Scales- Revised (Boyle et al., 1993).

2.1.1.9 RD Phenotype Definition and Exclusionary Criteria

Subjects were excluded if they scored <80 on the Performance and Verbal Scales of the WISC-III, or <80 on the Perceptual Reasoning and Verbal Comprehension Indices of the WISC-IV. Additional exclusionary criteria include evidence of neurological or chronic medical illness, bipolar affective disorder, psychotic symptoms, Tourette syndrome or chronic multiple tics.

To determine the affection status of the probands and siblings for the categorical analyses, scores from the three reading tests were used: WRAT-III Reading, WRMT-R Word Attack and WRMT-R Word Identification. Subjects with a score 1.5 standard deviations below the population mean on two tests, or 1 standard deviation below the mean on the average of all three tests, were considered to have RD. Of the 588 children in the sample, a total of 236 probands and 36 siblings met the categorical cutoff. The subjects that did not make the categorical cutoff for RD were included in the quantitative trait analyses.
Table 2-1. Pairwise correlation coefficients for the analyzed reading measures

<table>
<thead>
<tr>
<th></th>
<th>CLF-REC</th>
<th>CLF-EXP</th>
<th>CTP-RDN</th>
<th>CTP-NWR</th>
<th>CTP-PA</th>
<th>CMS-FS</th>
<th>CMS-BS</th>
<th>WSC-FS</th>
<th>WSC-BS</th>
<th>WRA-RD</th>
<th>WRA-SP</th>
<th>WRM-ID</th>
<th>WRM-AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLF-REC</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLF-EXP</td>
<td>0.699</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP-RDN</td>
<td>0.325</td>
<td>0.281</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP-NWR</td>
<td>0.376</td>
<td>0.407</td>
<td>0.182</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP-PA</td>
<td>0.420</td>
<td>0.439</td>
<td>0.326</td>
<td>0.342</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>CMS-FS</td>
<td>0.439</td>
<td>0.504</td>
<td>0.194</td>
<td>0.435</td>
<td>0.356</td>
<td>1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CMS-BS</td>
<td>0.286</td>
<td>0.255</td>
<td>0.302</td>
<td>0.178</td>
<td>0.315</td>
<td>0.263</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>WSC-FS</td>
<td>0.364</td>
<td>0.493</td>
<td>0.239</td>
<td>0.330</td>
<td>0.328</td>
<td>0.604</td>
<td>0.304</td>
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<tr>
<td>WSC-BS</td>
<td>0.341</td>
<td>0.323</td>
<td>0.270</td>
<td>0.188</td>
<td>0.251</td>
<td>0.205</td>
<td>0.346</td>
<td>0.230</td>
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<tr>
<td>WRA-RD</td>
<td>0.471</td>
<td>0.515</td>
<td>0.475</td>
<td>0.350</td>
<td>0.566</td>
<td>0.300</td>
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<td>0.314</td>
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<td>WRA-SP</td>
<td>0.344</td>
<td>0.370</td>
<td>0.445</td>
<td>0.257</td>
<td>0.462</td>
<td>0.241</td>
<td>0.344</td>
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<td>0.319</td>
<td>0.751</td>
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<td>WRM-ID</td>
<td>0.439</td>
<td>0.472</td>
<td>0.468</td>
<td>0.281</td>
<td>0.543</td>
<td>0.273</td>
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<td>0.309</td>
<td>0.872</td>
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<td>WRM-AT</td>
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<td>0.403</td>
<td>0.416</td>
<td>0.355</td>
<td>0.576</td>
<td>0.312</td>
<td>0.369</td>
<td>0.292</td>
<td>0.292</td>
<td>0.796</td>
<td>0.726</td>
<td>0.795</td>
<td>1</td>
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</tbody>
</table>

(All correlation coefficients were significant with p<0.001)

CLF-REC and CELF-EXP: CELF-III Receptive and Expressive Language

CTP-RDN, CTP-NWR and CTP-PA: CTOPP Rapid Digit Naming, CTOPP Non-word Repetition, and Phonological Awareness

CMS-FS and CMS-BS: CMS Numbers Forwards and Backwards

WSC-FS and WSC-BS: WISC-III Digits Span Forwards and Backwards

WRA-RD and WRA-SP: WRAT-III Reading and Spelling

WRM-ID and WRM-AT: WRMT-R Word ID and Word Attack
Table 2-2. Summary statistics for the analyzed reading measures

<table>
<thead>
<tr>
<th>Reading Measure</th>
<th>Full Sample (n=588) Mean</th>
<th>Std. Dev.</th>
<th>Individuals that met affection criteria (n=272) Mean</th>
<th>Std. Dev.</th>
<th>Individuals did not meet affection criteria (n=316) Mean</th>
<th>Std. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRAT-III Reading</td>
<td>88.19</td>
<td>13.85</td>
<td>77.00</td>
<td>8.20</td>
<td>97.82</td>
<td>9.92</td>
</tr>
<tr>
<td>WRAT-III Spelling</td>
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<td>79.70</td>
<td>7.89</td>
<td>93.53</td>
<td>10.25</td>
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<td>WRMT-R Word ID</td>
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<td>74.31</td>
<td>10.41</td>
<td>96.22</td>
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<td>WRMT-R Word Attack</td>
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<td>13.84</td>
<td>75.27</td>
<td>9.99</td>
<td>94.69</td>
<td>9.79</td>
</tr>
<tr>
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<td>8.12</td>
<td>2.21</td>
<td>7.24</td>
<td>2.08</td>
<td>8.90</td>
<td>2.03</td>
</tr>
<tr>
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<td>8.32</td>
<td>2.30</td>
<td>7.73</td>
<td>2.08</td>
<td>8.83</td>
<td>2.37</td>
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</table>
2.1.2 Calgary Sample

The sample consisted of nuclear families with affected siblings from Calgary, Alberta (Field and Kaplan, 1998; Petryshen et al., 2001; Hsiung et al., 2004; Tzenova et al., 2004). In contrast to previous published studies, which included members of the extended families, only members of the nuclear family were used in the study. Most of the affected sibpair families in the sample were ascertained through a proband (>8 years of age) who was enrolled in a special school for children with learning disabilities. Although a few affected sibpair families were derived from the extended family and were non-sibling relatives of the proband, only one affected sibpair family from each extended family was analyzed.

The sample was also primarily white Caucasian, with 4 of the original 100 multi-generational families containing one unaffected non-European parent. The protocol for the Calgary sample was approved by the University of Calgary Ethics Review Board and written informed consent was obtained from all participants.

2.1.2.1 Phenotype Definition and Exclusionary Criteria

The RD status of the subjects was determined using the Word Attack subtests from the Woodcock Reading Mastery Test and the Revised Woodcock-Johnson Psychoeducation Test, both of which comprise phonological coding tasks. Subjects <18 years of age who performed ≥2 years below the age norm were considered to be affected with RD. Full-scale IQ was estimated using a short form of the Wechsler Intelligence Scale for children for subjects 8-16 years of age (Wechsler, 1974) and a short form of the Wechsler Adult Intelligence Scale for adult subjects (Wechsler, 1981). Since verbal IQ relies in information acquired through reading, and since the
short form of the WISC was used to estimate IQ, low IQ was not used as an exclusionary criterion. For the 95 families in which DNA was available, 86 families included at least two children who were diagnosed with RD, with a total of 207 affected children.

2.2 DNA Extraction

In both samples, DNA was extracted from white blood cells using a high-salt extraction method (Miller et al., 1988).

2.3 SNP Genotyping

2.3.1 TaqMan® Genotyping Assay

The TaqMan® 5’ nuclease assay (Applied Biosystems, Foster City, CA) was the primary method of genotyping in this study. Polymerase chain reactions (PCR) were performed using forward and reverse primers, along with two probes that each bind to a specific SNP variant. On each probe, there is a fluorescent tag on the 5’ end and a quencher on the 3’ end. The fluorescence of the tag is absorbed by the quencher when the probe is intact. During the PCR, the AmpliTaq Gold® polymerase with 5’ nuclease activity will cleave the probe that binds to its specific DNA site, releasing the fluorescent tag from the quencher. By detecting the relative level of fluorescence for each probe, the genotype of the individual can be determined.

The primers and probes used to genotype the SNPs were available through Assay-on-Demand® or Assay-by-Design® formats (Applied Biosystems). The Assay-on-Demand®
assays are predesigned and tested by Applied Biosystems, while Assay-by-Design® assays are designed by Applied Biosystems based on a submission of the DNA sequence flanking the SNP of interest. The University of California, Santa Cruz (UCSC) Genome Browser was used to retrieve the flanking sequence for the SNPs genotyped using the Assay-by-Design® (Fujita et al., 2011).

Genotyping was carried out in 96-well plates consisting of two negative controls. Each PCR consisted of 10µL volume reactions with the following reagents: 30ng of genomic DNA, 10µmol of TaqMan® Universal PCR Master Mix (Applied Biosystems) and 0.25µL of allelic discrimination mix (Applied Biosystems) with 36µM of each primer and 8µM of each probe. The reaction conditions consisted of the following steps: 50°C for 2min, 95°C for 10min, and 40 cycles of 94°C for 15s and an annealing temperature of 59 °C for 1min. Plates were then read with the ABI 7900-HT Sequence Detection System using the allelic discrimination endpoint analysis mode of the SDS software package, v2.0 (Applied Biosystems).

2.3.2 Restriction Fragment Length Polymorphism (RFLP) Assay
The RFLP assay takes advantage of the specificity of restriction enzymes to genotype SNPs (Botstein et al., 1980). If a SNP is within a restriction site, it is possible to amplify a PCR fragment containing the site and digest the product with the corresponding restriction enzyme. By running the product on a gel, and determining whether the PCR product was cleaved, the allele at that site can be determined.

For the RFLP assay, a 20µL PCR was performed with 100ng of each primer, 0.2mM dNTP, 1.5mM magnesium chloride and 0.5U Taq polymerase. All of the PCR conditions
consisted of a denaturation step at 94ºC for 3min, 35 cycles at 94ºC for 30s, 60ºC for 30s and 72ºC for 30s, and ending with a 10min extension at 72ºC. The PCR product (3µL) was digested with 6 U of the restriction enzyme 2h at 37ºC. Digested products were run in 1.5% agarose gels to determine the alleles.

2.4 Statistical Analysis Tools

2.4.1 Categorical Association Analyses with Single SNPs
The transmission disequilibrium test (TDT) was used to test for association between single SNPs and RD. The TDT is a modified $\chi^2$ test that tests for biased transmission of alleles to affected offspring (Spielman et al., 1993). Under the null hypothesis, a heterozygous individual for a SNP would have an equal probability of transmitting each allele to an affected offspring. If there is a significant deviation, where one allele is transmitted more often than would be expected by chance, it suggests an association between the allele and the trait. The $\chi^2$ value can be calculated with the following equation and tested for significance using one degree of freedom:

$$\chi^2 = \frac{(b - c)^2}{(b + c)}$$

The variables $b$ and $c$ represent the number of transmitted alleles for a SNP. A deviation from the expected 1:1 ratio of transmitted alleles results in a greater $\chi^2$ value. The program Haploview v4.2 was used to calculate the TDT for the association between single SNPs and RD (Barrett et al., 2005).
2.4.2 Categorical Association Analyses with Haplotypes
Markers in LD can be used to map disease genes and localize the susceptibility region (Zhao et al., 2003). The program Transmit v2.5.4 was used to test for association between specific haplotypes and RD (Clayton, 1999). The robust estimator option was selected which accounts for the inclusion of families with multiple affected siblings, even in the presence of linkage. Transmit calculates a modified TDT to test for the biased transmission of multi-locus haplotypes within families. For unknown transmissions in families, the transmission is averaged over all possible haplotype assignments to parents and offspring. Unaffected siblings, although not used in the TDT, are used to narrow down the possible genotypes in the unknown or ambiguous phase of genotypes in the parents. The biased transmission of each haplotype is calculated separately using a 1 degree of freedom (df) $\chi^2$ test. In addition, a global $\chi^2$ test is performed with H-1 df, where H represents the number of haplotypes. For the global test, rare haplotypes with frequencies <0.1 are pooled.

2.4.3 Single SNP and Haplotype Association with Quantitative Traits
The Family Based Association Test (FBAT) program v2.0.3 was used to test for single SNP and haplotype association with the quantitative measures (Laird et al., 2000). Offsets were provided for all tests, which represented the population mean score. The imputed offsets for each quantitative trait are provided in Chapter 2.1.1. The empirical variance option was selected, as previous evidence showed linkage to the studied chromosomal regions. An additive model was assumed for the analysis because it is robust to model misspecification. The HBAT package in FBAT was used to test for association between haplotypes and quantitative measures.
2.4.4  Correction for Multiple Testing

For the single SNP association tests, Bonferroni correction was performed to account for multiple testing. However, this method is conservative when the variables are correlated, for example, in the presence of LD between SNPs. To determine the number of independent SNPs, the SNPSpD tool was used (Nyholt, 2004). In this method, a correlation matrix is created from the imputed pedigree file. A principle components (factor) analysis generates eigenvalues, with the number of values equaling the number of variables (Cheverud, 2001). Conceptually, eigenvalues represent the amount of variance that is accounted for by the factor. The first eigenvalue accounts for as much of the variability as possible, and each subsequent eigenvalue accounts for as much of the remaining variability (ie. the first eigenvalue will always be the largest, and subsequent eigenvalues will be equal to or less than the first). If all of the variables are perfectly correlated, then the first eigenvalue will equal the number of variables and all subsequent eigenvalues will have a value of 0 (ie. the first eigenvalue can account for all of the variance between the variables). Conversely, if all of the variables are uncorrelated, then all of the eigenvalues will have a value of 1. The variance of the eigenvalues is then used to calculate the effective number of independent tests ($M_{eff}$), with a higher variance resulting in less number of effective independent tests. An alternative method to calculate $M_{eff}$ using eigenvalues by Li and Ji (2005), is also given by SNPSpD. This method is less conservative, and more accurate, for variables that show moderate correlation. Following the author’s suggestions, the $M_{eff}$ calculated using the method of Li and Ji (2005) was used if it was less than the value $M_{eff}$ calculated by the method of Nyholt (2004).
2.4.5 Visualization of LD Blocks

Haploview v4.2 was used to visualize pair-wise LD between SNPs. The method to determine LD blocks was based on the algorithm of Gabriel et al. (2002). The $D'$ 95% confidence intervals were calculated, and each comparison was classified as either “strong LD”, “inconclusive”, or “strong recombination”. For two SNPs to be in “strong LD”, the minimum lower and upper confidence interval values were set to 0.7 and 0.98, respectively.

2.4.6 Genotype Quality Control

Haploview v4.2 and Merlin v1.1.2 were used to check for Mendelian errors, crossovers between markers, and Hardy-Weinberg equilibrium for all genotyped SNPs (Abecasis et al., 2002; Barrett et al., 2005). Subjects with unclear genotype calls were genotyped a second time.

2.4.7 Meta-analysis

The catmap package was used to synthesize the data and evaluate the overall association among the pooled studies (Nicodemus, 2008). This package utilizes the method of Kazeem and Farrall (2005) to combine both case-control and TDT studies. For each study, an odds ratio and standard error are calculated. Each study is also given a weight based on the inverse of its standard error. Since larger studies tend to have smaller standard errors, their results are given greater weight than smaller studies. The catmap package allows the use of either fixed-effects (Kazeem and Farrall, 2005) or random-effects models (DerSimonian and Laird, 1986) to obtain a pooled effect size. For the meta-analysis, the random-effects model was used to synthesize the studies. The statistical program R v2.8.1 was used to run the catmap package.
2.4.8 Assessment of Heterogeneity

Two methods were used to quantify heterogeneity for the meta-analysis. The Cochran’s \( Q \) statistic is a \( \chi^2 \) test that is calculated by summing the squared deviation of each study’s effect estimate from the pooled estimate, with each study given a weight based on its inverse variance (Cochran, 1954). The \( Q \) statistic measures if there is statistically significant evidence for heterogeneity but only tests for the presence or absence of heterogeneity, rather than the extent of heterogeneity. As has been previously suggested, the level of significance was loosely set to \( p=0.1 \) because of the low power of the test, particularly when there are few studies (Fleiss, 1986). The second measure of heterogeneity that was used was the \( I^2 \) statistic (Higgins and Thompson, 2002). The \( I^2 \) value is derived from the \( Q \) statistic, and is given as a percentage. It is interpreted as the proportion of total variation that is due to heterogeneity. An \( I^2 \) value of 0 indicates that all observed differences between studies are due to general sampling error, whereas an \( I^2 \) value of 100 suggests that all observed differences are due to heterogeneity between studies. Using the \( I^2 \) statistic, low heterogeneity is considered between 0-24% \( (I^2=0-24) \), medium between 25-49% \( (I^2=25-49) \), and high above 50% \( (I^2>50) \). The calculation of the \( Q \) statistic was performed in catmap, while the \( I^2 \) values and their 95% uncertainty levels were calculated using the method of Higgins and Thompson (2002).

2.4.9 Assessment of Publication Bias

One challenge in the evaluation of association studies is that some studies are more likely to be published than others, as reviewed in Chapter 1.10.3. To assess publication bias, the Egger test was used (Egger and Smith, 1998). This is a statistical test for funnel plot asymmetry, with the
normalized effect estimate plotted against their precision (the reciprocal of the standard error of the effect estimate). A linear regression is tested to determine whether the y-intercept significantly deviates from 0, suggesting publication bias. The Egger test was performed using the metabias package in STATA v11.1 (StataCorp LP, College Station, Texas).

2.4.10 Statistical Power

An important consideration in genetic association studies is the level of statistical power, which refers to the ability of a sample to detect a true association between a genetic variant and a specific phenotype. Specifically, statistical power is the probability of correctly rejecting the null hypothesis. By setting a number of parameters including the sample size, risk and marker allele frequencies (and the LD between the risk and marker alleles) and the estimated disease/trait frequency, the effect size that can be detected using the sample can be determined. Power analyses were performed for all of the analyzed samples, with the type-I error rate and acceptable power defined at 0.05 and 80%, respectively.

The Genetic Power Calculator was used to calculate the statistical power of the samples analyzed in this study (Purcell et al., 2003). For the Toronto and Calgary samples, the statistical power of each sample to detect an association with RD was calculated. The effect size that could be detected with the sample was then calculated, with a range of allele frequencies and \( D' \) values.

The power of the sample analyzed in the meta-analysis was also calculated. The imputed prevalence of RD was 0.1. The observed allele frequencies were used as the marker and risk allele frequencies. The SNPs that were analyzed were proposed as being causal alleles, and
the $D'$ was set to 1. For the meta-analysis, data from both case-control and TDT analyses were included but there was no software option that allows the combination of samples using both study designs. Instead, the statistical power of the sample was calculated using both the case-control and TDT options, with the combined number of affected subjects used as the number of cases and number of family trios. We were able to confirm that similar power calculations were given for both options.

The statistical power of the Toronto sample was calculated for the quantitative trait analyses using the Genetic Power Calculator (Purcell et al., 2003). A dominant model was assumed, the case threshold was set to 1, and the recombination fraction was set to 0. The marker and risk allele frequencies were set equal. The minimum QTL variance that could be detected with the sample was calculated, with a range of allele frequencies and $D'$ values.
Chapter 3 – A Family-Based Association Analysis and Meta-Analysis of the Reading Disabilities Candidate Gene

DYX1C1

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3.1 Abstract

Reading disabilities (RD) have a significant genetic basis and has shown linkage to multiple regions including chromosome 15q. *Dyslexia susceptibility 1 candidate gene 1* (*DYX1C1*) on chromosome 15q21 was originally proposed as a candidate gene with two potentially functional polymorphisms at the -3G/A and 1249G/T positions showing association with RD. However, subsequent studies have yielded mixed results. We performed a systematic review and meta-analysis of the -3G/A and 1249G/T polymorphisms, including new unpublished data from two family-based samples. Ten markers in *DYX1C1* were genotyped in the two independently ascertained samples. Single marker and -3G/A:1249G/T haplotype analyses were performed for RD in both samples, and quantitative trait analyses using standardized reading-related measures was performed in one of the samples. For the meta-analysis, we used a random-effects model to summarize studies that tested for association between -3G/A or 1249G/T and RD. No significant association was found between the *DYX1C1* SNPs and RD or any of the reading-related measures tested after correction for the number of tests performed. The previously reported risk haplotype (-3A:1249T) was not biased in transmission. A total of 9 and 10 study samples were included in the meta-analysis of the -3G/A and 1249G/T polymorphisms, respectively. Neither polymorphism reached statistical significance, but the heterogeneity for the 1249G/T polymorphism was high. The results of this study do not provide evidence for association between the putatively functional SNPs -3G/A and 1249G/T and RD.
3.2 Introduction

Specific reading disabilities (RD), or developmental dyslexia, refer to an unexpected and specific difficulty in learning to read despite normal or above average intelligence, education, and socioeconomic opportunity. RD is likely caused by a dysfunction of neural systems involved in cognitive skills required for reading (Habib, 2000). A core deficit observed in dyslexics involves the processing of phonemes, the most basic unit of speech sounds, which persist into adulthood (Bruck, 1992; Shaywitz et al., 1999; Ramus et al., 2003). Individuals with RD have difficulty segmenting words into their phonological elements, impairing their ability in learning to read. Several other reading components have been shown to be impaired in individuals with RD including orthographic coding, single-word reading, rapid automatic naming, and spelling (Denckla and Rudel, 1976; Paulesu et al., 2001; Francks et al., 2002). With an estimated prevalence ranging from 5-17.5% in school-aged children, RD is the most common learning disability and affects four out of five individuals with learning disabilities (Katusic et al., 2001; Shaywitz, 1998; Shaywitz et al., 1990). There is substantial evidence to suggest that RD is both familial and heritable. RD has been shown to cluster in families (Finucci et al., 1976; Hallgren, 1950; Volger et al., 1984) and twin studies estimate the heritability of reading components in the range of 0.30 to 0.72 (DeFries et al., 1987; Wadsworth et al., 2000).

Linkage studies have identified at least nine susceptibility regions (designated as DYX1-9), including DYX1 on chromosome 15q15.2-21.2 which has been replicated in multiple linkage studies (Chapman et al., 2004; Grigorenko et al., 1997; Morris et al., 2000; Schulte-Korne et al., 1998; Schumacher et al., 2008). One gene in this region, dyslexia susceptibility 1 candidate gene 1 (DYX1C1) on chromosome 15q21 (also known as EKNI) is among the most well-studied RD candidate genes. DYX1C1 was originally proposed as a candidate gene based on
a t(2;15)(q11;q21) chromosomal translocation that cosegregated with RD in three affected members of a six-member, two generation Finnish family (Taipale et al., 2003). One additional offspring in the family also carried the translocation, but his RD affection status was unknown due to low verbal and non-verbal performance on cognitive achievement tests. In the same study, a case-control association analysis was performed using a sample of Finnish dyslexics and a control group comprising nondyslexics and anonymous blood donors. Two single nucleotide polymorphisms (SNPs) at the -3G/A (rs3743205) and 1249G/T (rs57809907) positions of the transcript showed significant association, with the minor -3A and 1249T alleles increasing risk for RD. The frequency of the -3A:1249T haplotype was significantly greater in the dyslexic group (14/106 cases) compared to the controls (10/192 nondyslexic and anonymous controls). In addition, an analysis of 9 informative family trios found significant overtransmission of the -3A:1249T haplotype to affected offspring.

The results of the Finnish study were particularly promising because of the proposed functionality of the two significant SNPs. The -3G/A position is located in the predicted binding sequence of the Elk-1, HSTF, and TFII-I transcription factors, and was also predicted to affect the Kozak sequence which plays a role in initiating translation. The 1249T variant introduces a premature stop codon, shortening the protein from 420 to 416 amino acids although a latter study indicated that the truncation may not affect function (Wang et al., 2006). However, there were considerable study design issues that may have affected the results of the initial study (Taipale et al., 2003). The association analysis included 58 dyslexics and 61 nondyslexics from 20 unrelated families, and a replication set of 3 families and 33 unrelated dyslexic-nondyslexic couples. The $\chi^2$ or Fisher’s exact test was used to test for association between the genotyped $DYX1C1$ SNPs and RD, and Bonferroni correction was applied to account for multiple testing. The transmission disequilibrium test (TDT) was used to assess the transmission of the risk haplotype -3A:1249T in
9 informative trios. Because the analytic strategy used in the study did not take into account the greater allele sharing in the related individuals taken from the same families, the observed results may be biased toward positive spurious associations. In addition, the significant overtransmission of the -3A:1249T haplotype was based on an analysis of only 9 informative trios, and these significant findings may be due to the small sample size.

In addition to the significant associations, functional studies have supported a potential role for \textit{DYX1C1} in RD, as \textit{DYX1C1} has been implicated in neural migration. RNA interference (RNAi) of \textit{DYX1C1} in rats disrupted the migration of neurons, causing malformations in the neocortex and hippocampus (Rosen et al., 2007; Wang et al., 2006). This is consistent with studies of post-mortem dyslexic brains which have reported ectopias and dysplasias, signs of abnormal neural migration (Galaburda et al., 1985; Humphreys et al., 1990). \textit{DYX1C1} RNAi-treated rats also performed significantly poorer in spatial working memory and auditory processing tasks (Szalkowski et al., 2011; Threlkeld et al., 2007). Wang et al. (2006) showed that RNAi rescue experiments using the shortened 416 amino acid version of \textit{DYX1C1} predicted by the 1249T variant restored normal neural migration, indicating that this polymorphism was not sufficient to alter the function of \textit{DYX1C1}, at least as measured by this specific assay in rats. Thus the reported role of \textit{DYX1C1} in neural migration is consistent with the neurobiology of RD, although the 1249T allele may not be detrimental to gene function. The initial association findings, in combination with the functional evidence, generated strong interest in \textit{DYX1C1} as a candidate gene.

Previous association studies have illustrated that although \textit{DYX1C1} has been widely studied, the results have been inconsistent and often conflicting, and the status of this candidate gene remains unclear. In a family-based association study by Wigg et al. (2004), a different
marker rs11629841 was found to be significantly associated with RD which was in high LD with both -3G/A and 1249G/T. Brkanac et al. (2007) found association with the 1249G/T polymorphism, but it was the major G allele that was overtransmitted rather than the T allele which showed association in Taipale et al. (2003). A number of other studies did not find association between DYX1C1 SNPs and RD (Bellini et al., 2005; Cope et al., 2005; Marino et al., 2005; Newbury et al., 2011; Saviour et al., 2008; Venkatesh et al., 2011).

In addition, previous studies have tested for association between DYX1C1 SNPs and quantitative reading and language measures. Wigg et al. (2004) found that the major -3G variant was significantly associated with nine measures of reading skills including phonological awareness, word identification, decoding, rapid automatized naming, language ability, and verbal short-term memory. Marino et al. (2007) found the opposite allele -3A was significantly associated with lower performance in a short-term memory test. Other studies did not find association between DYX1C1 SNPs and quantitative reading tasks (Meng et al., 2005; Scerri et al., 2004). Two independent Australian cohort studies also performed quantitative analyses of reading and reading related measures in population-based samples (Bates et al., 2010; Paracchini et al., 2011). Bates et al. (2010) found three SNPs that were significantly associated with different measures: rs685935 with short-term memory; rs17819126 with irregular reading, nonword reading, and irregular spelling; and rs3743204 with nonword reading. Paracchini et al. (2011) found that spelling was associated with the three markers rs7174102, rs8043049 and rs8037376, and reading associated with rs8040756.

The purpose of this study was to evaluate the cumulative epidemiological evidence of DYX1C1 as a RD candidate gene. First, we performed a family-based association analysis using two independent samples, one of which was an extension of the sample used in Wigg et al.
Second, we summarized and evaluated the -3G/A and 1249G/T SNPs through a literature review and meta-analysis of genetic association studies. Our aim was to determine whether specific SNPs in DXY1C1 were associated with RD, or with reading-related quantitative measures.

3.3 Materials and Methods

Subjects and Assessment – Toronto Sample

The first sample included families recruited primarily from the Greater Toronto Area and surrounding regions, each with at least one child exhibiting reading difficulties. Proband ranged from 6-16 years old, and siblings within the same range were invited to participate in the study regardless of reading ability. The total sample consisted of 1416 individuals from 421 nuclear families, consisting of 421 probands and 167 siblings. Of the 421 nuclear families, 148 were previously analyzed in an association analysis of DXY1C1 (Wigg et al., 2004).

Proband and siblings in the Toronto sample were tested for IQ, reading and language measures, which have all been described previously (Elbert et al., 2011; Wigg et al., 2004). The Wechsler Intelligence Scale for Children (WISC-III or WISC-IV) was used to assess intelligence and cognitive ability, and children were excluded from the study if they scored <80 on the Verbal and Performance Scales of the WISC-III, or on the Verbal Comprehension and Perceptual Reasoning Indices of the WISC-IV (Wechsler, 2003; Wechsler, 1981; Wechsler, 1991). The Wide Range Achievement Test-III (WRAT-III) (Wilkinson, 1993), Woodcock Reading Mastery Test-Revised (WRMT-R) (Woodcock, 1987), and Comprehensive Test of Phonological Processing (CTOPP) (Wagner et al., 1999) were used to measure reading, spelling,
phonological awareness and rapid naming skills. Receptive and expressive language ability was assessed using the Clinical Evaluation of Language Fundamentals-3rd edition (CELF-3) (Semel et al., 1995). Three subtests that measured single word and non-word reading; WRAT-III Reading, WRMT-R Word ID and WRMT-R Word Attack, were used to determine RD affection status for the categorical analysis. Probands and siblings were considered to have RD if they scored 1.5 standard deviations below the mean on 2 of the 3 measures or 1 standard deviation below the mean on the average of all 3. For the analysis of the quantitative traits, scores from the WRAT-III, WRMT-R, CTOPP, and CELF-3 subtests were used as the quantitative phenotypes and information from all of the 588 subjects in the sample was included.

To screen for symptoms of possible neurological, medical and psychiatric disorders, parents were interviewed using the structured Children’s Interview for Psychiatric Syndromes (ChIPS) (Weller et al., 2000), and teachers were interviewed using the semi-structured Teacher Telephone Interview (Tannock et al., 2002). This information was supplemented with two standardized questionnaires: the Conners Parent and Teacher Rating Scales- Revised (Conners, 1997) and Ontario Child Health Survey Scales- Revised (Boyle et al., 1993). Subjects were excluded if there was evidence of neurological or chronic medical illness, bipolar affective disorder, psychotic symptoms, Tourette syndrome or chronic multiple tics.

Subjects were either native English speakers or were educated for at least five years in an English-speaking school. The ethnicity of the sample was primarily Caucasian, with 68.1% of individuals reporting European descent and 26% reporting “Caucasian Canadian” background. The remaining subjects reported South American (1.8%), non-European (2.9%), or non-European mixed background. The protocol for the Toronto sample was approved by The
Hospital for Sick Children Research Ethics Board, and written informed consent was obtained from all participants.

Subjects and Assessment – Calgary Sample

The second sample genotyped for this study consisted of nuclear families with affected siblings from Calgary, Alberta. Full descriptions of the recruitment and assessment of the 95 families have previously been described (Field and Kaplan, 1998; Hsiung et al., 2004; Petryshen et al., 2001; Tzenova et al., 2004). In contrast to previous studies, which included members of extended families, only members of the nuclear family were used in the study. Most of the affected sibpair families were ascertained through a proband >8 years of age who attended a special school for children with learning disabilities. A few affected sibpair families derived from extended families were close (non-sibling) relatives of the proband but only one affected sibpair family per extended family was utilized. For the 95 families in which DNA was available, 86 families included at least two children who were diagnosed with RD, with a total of 207 affected children. In the Calgary sample, RD was diagnosed based on the word attack subtests from the Woodcock Reading Mastery Test and the Revised Woodcock-Johnson Psychoeducation Test, both which comprise phonological coding tasks. Subjects <18 years of age who performed ≥2 years below the age norm were considered affected. Full-scale IQ was estimated using a short form of the Wechsler Intelligence Scale for children for subjects 8-16 years of age (Wechsler, 1974) and a short form of the Wechsler Adult Intelligence Scale for adult subjects (Wechsler, 1981). Since these IQ tests only estimate IQ, and since they include reading components (which probably depresses the scores of dyslexic individuals), low IQ was not used as an exclusionary criterion.
The sample was also primarily white Caucasian, with 4 of the original 100 multigenerational families containing one unaffected non-European parent. The protocol for the Calgary sample was approved by the University of Calgary Ethics Review Board and written informed consent was obtained from all participants.

**SNP Genotyping for the Toronto and Calgary Samples**

For both the Toronto and Calgary samples, genomic DNA was extracted from whole blood using a high-salt extraction method (Miller et al., 1988). A total of 10 markers were genotyped in the Toronto and Calgary samples. Six of the markers, including rs2007494, rs57809907 (1249G/T), rs3743205 (-3G/A), rs3743204, rs11629841, and rs692691, were previously genotyped in a subset of the Toronto sample which included 148 families (Wigg et al., 2004). For this study, the remaining 273 families of the Toronto sample were genotyped for the 6 SNPs. In the Calgary sample, the 6 SNPs were genotyped in all 95 families.

Four additional markers, rs600753, rs16787, rs17819126, rs12899331 were also selected for genotyping in the entire Toronto and Calgary samples. The markers rs600753 and rs16787 were previously found to be significant in several combinations of haplotypes (Dahdouh et al., 2009). The rs1789126 marker was selected because of a positive association in an Australian sample (Bates et al., 2010), and the rs1289331 was selected as it was suggested to be the binding site of the SP1 transcription factor (Tapia-Paez et al., 2008).

All 10 markers were genotyped using the ABI 7900-HT Sequence Detection System (Applied Biosystems) using the TaqMan® 5’ nuclease assay for allelic discrimination. The 1249G/T, rs600753, rs3743204, rs2007494, rs16787 and rs12899331 were genotyped using
Assay-by-Design probes while rs692691, rs11629841 and rs17819126 were genotyped using Assay-on-Demand probes. The -3G/A polymorphism was previously genotyped using a restriction fragment length polymorphism assay (Wigg et al., 2004). Later, Assay-by-Design probes were available and were used to genotype the remaining sample.

Genotyping was carried out in 96-well plates consisting of two negative controls. 10µL PCR reactions were performed with the following reagents: 30ng of genomic DNA, 10µmol of TaqMan® Universal PCR Master Mix (Applied Biosystems) and 0.25µL of allelic discrimination mix (Applied Biosystems) with 36µM of each primer and 8µM of each probe. The reaction conditions consisted of the following steps: 50°C for 2min, 95°C for 10min, and 40 cycles of 94°C for 15s and an annealing temperature of 59 ºC for 1min. Plates were then read with the ABI 7900-HT Sequence Detection System using the allelic discrimination end-point analysis mode of the SDS software package, v2.0 (Applied Biosystems). Samples with undetermined or unclear calls were genotyped for a second time.

**Statistical Analysis**

The genotyped samples were checked for genotyping and family structure errors using Haploview v4.2 and Merlin v1.1.2, which determines unlikely crossover events suggesting erroneous genotypes (Abecasis et al., 2002; Barrett et al., 2005). Non-Mendelian inheritance was resolved before analysis by genotyping families a second time and all markers were tested for Hardy-Weinberg Equilibrium. Haploview v4.2 was also used to perform the TDT for association between single SNPs and categorical RD (Barrett et al., 2005). Bonferroni correction was used to account for multiple testing, with a type I error rate of 0.05. To account for SNPs in LD for the
Bonferroni correction, we used the SNP SpD tool to determine the number of independent tests (Nyholt, 2004). For the single SNP association analysis with RD, the threshold for statistical significance was corrected for the number of independent SNPs (p=0.05/8=0.006), while the quantitative trait analysis was corrected for both the number of independent SNPs (p=0.05/8=0.006) and number of independent quantitative measures (p=0.006/7=0.0009).

The transmission of the -3G/A:1249G/T haplotypes was analyzed using Transmit v2.5.4 with the robust estimator option (Clayton and Jones, 1999). In the Toronto sample, association with quantitative measures was assessed using scores from nine reading and language tests: WRAT-III reading and spelling; WRMT-3 word ID and word attack; CTOPP non-word repetition, phonological awareness and rapid digit naming; and CELF-3 receptive and expressive language. The program FBAT v.2.0.3 was used to carry out the quantitative trait analyses (Laird et al., 2000). For all results, the two-tailed p-values are reported.

Systematic review and meta-analysis

Search Strategy

For the systematic review and meta-analysis, we included the -3G/A and 1249G/T SNPs because they were the most commonly genotyped across DYX1C1 association studies and were proposed as having a functional role. We searched MEDLINE, EMBASE, Scopus, BIOSIS, and HuGE Navigator with the keywords “DYX1C1”, “EKN1”, “dyslexia” and “reading disabilities” for studies examining an association between categorical RD and the -3G/A or 1249G/T variants. The HuGE navigator was used to search for genome wide association studies (GWAS) using the search terms “dyslexia” and “reading disabilities”, and we filtered the results to only GWAS.
Genetic linkage studies, conference proceedings, review articles, functional studies were excluded from the meta-analysis. The reference lists from all *DYX1C1* association studies and review articles were examined for additional studies.

Studies that met the inclusion criteria used either a case-control or family-based association design. The following information was collected from each study: country and language, sample size, ethnicity of sample, definition of cases and controls, and whether SNPs were in Hardy-Weinberg Equilibrium. For case-control studies, allele frequencies for the two SNPs were obtained for the cases and controls. For family-based studies, the allele frequencies and the number of transmissions for each allele were obtained for both SNPs. Information that was not provided in the articles was requested from the corresponding authors. If a study used both case-control and TDT analyses, only the TDT data were used because it is robust to population stratification, whereas the case-control design is not.

*Meta-analysis*

Each study was summarized by constructing two-by-two tables from case-control studies and one-by-two tables from TDT studies. To synthesize the case-control and TDT studies, the method of Kazeem and Farrall (2005) was used. The odds ratios and 95% confidence intervals for individual studies were calculated, with each study given a weight based on the inverse of its variance. Studies using larger samples would carry greater weight as their variances would be smaller. Heterogeneity between studies was assessed using the Cochran’s *Q* statistic and the *I*\(^2\) statistic (Higgins and Thompson, 2002). For the pooled estimate of the odds ratio and its 95% confidence interval, a fixed- (Kazeem and Farrall, 2005) or random-effects (DerSimonian and
Laird, 1986) model could be applied. As the 1249G/T SNPs showed evidence of between-study heterogeneity (p=0.037), we used the random-effects model. Although the -3G/A SNP did not show statistically significant evidence of heterogeneity, the random-effects model was still applied. Since there is evidence for genetic heterogeneity in RD (Fagerheim et al., 1999; Pennington et al., 1991), which may suggest different effect sizes between populations, the random-effects model was more appropriate than the fixed-effects model, which assumes a common genetic effect (Cohn and Becker, 2003). In addition, the random-effects model is more robust and generally preferred because it accounts for between-study heterogeneity but also gives similar results to fixed-effects models in the absence of heterogeneity (Kavvoura and Ioannidis, 2008).

Subgroup analyses were subsequently performed within the meta-analysis to explore potential sources of heterogeneity. In the first, studies were restricted to white Caucasian populations. In the second, studies that included phonological awareness or decoding tasks in the diagnosis of RD were analyzed together. The third subgroup analysis was restricted to English speaking subjects and English language reading tests.

Publication bias was assessed with the Egger test which uses a linear regression approach to test for funnel plot asymmetry (Egger et al., 1997). In one study there was an absence of the 1249T allele in the control group (Saviour et al., 2008). To calculate a non-zero odds ratio, we increased the allele count to 1. However, for the Egger test, an allele count of 0 was used to prevent substantial inflation of the standard error. The catmap package was used to assess between-study heterogeneity, calculate the pooled ORs, and carry out the sensitivity analysis (Nicodemus, 2008). The open-source statistical program R v2.8.1 was used to run the
catmap package. For the calculation of the Egger test, we used the metabias package in STATA v11.1 (StataCorp LP, College Station, Texas).

A power analysis of the pooled sample was performed using the Genetic Power Calculator (Purcell et al., 2003). Although there was no option that combined case-control and TDT samples, we estimated and compared the power of the individual modules. The total sample size was pooled using the cases from the case-control and TDT studies, as the power between the same number of family trios and cases (with 1:1 ratio with controls) is virtually equal (McGinnis et al., 2002). Both the -3G/A and 1249G/T SNPs were hypothesized as being the causal alleles, and the $D'$ value was set to 1, with the marker allele frequency set equal to the risk allele frequency. A dominant model was used as this model of inheritance reflected previous association studies. The RD prevalence risk used was 0.1, based on the estimates provided in the literature (Shaywitz et al., 1990). For the case-control module, a case:control ratio of 1 was used, and the unselected controls option was selected because more than 50% of the pooled control group were unscreened controls. The type I error rate was defined at 0.05 and the threshold for acceptable power was 80%.

The Venice criteria were used to evaluate the credibility of the evidence for association between RD and the -3G/A or 1249G/T variants (Ioannidis et al., 2008). Three criteria are taken into consideration including the amount of evidence (e.g. sample size with the risk allele, power, false-discovery rate), extent of replication (e.g. results from meta-analysis, between-study heterogeneity) and protection from bias (e.g. genotyping errors, population stratification). Each criterion is categorized and using a semi-quantitative index, a final grade of “strong”, “medium”, or “weak” is given to the strength of the evidence.
3.4 Results

Association Analysis

Of the 10 markers that were genotyped, 6 were located within introns and 4 within exons (Figure 3-1). The parental minor allele frequencies were similar in both the Toronto and Calgary samples for all 10 markers (Table 3-1). The LD between the SNPs for both samples was also comparable (Figure 3-2). For the single SNP analysis, no marker showed significantly biased transmission with RD in either sample. For the haplotype analysis, there was significant undertransmission of the rare -3A/1249G haplotype in the Toronto sample (frequency=0.013, p=0.002), but this rare haplotype was not observed in the Calgary sample (Table 3-2). For the quantitative trait analysis, three measures showed association at a p-value of <0.05 in the Toronto sample: CELF-III Receptive language and rs17819126 (p=0.034), CTOPP Phonological awareness and rs17819126 (p=0.044), and CELF-III expressive language and rs11629841 (p=0.034). However, these results were not significant after Bonferroni correction.

Literature Review and Meta-Analysis

Including the unpublished data from the Toronto and Calgary samples, 11 studies met inclusionary criteria for the literature review and meta-analysis. Later, one study was excluded because we were unable to contact the corresponding authors to obtain the number of transmitted and untransmitted alleles for the -3G/A and 1249G/T polymorphisms (Cope et al., 2005), and two studies were excluded because subsets of the samples were duplicated in a subsequent study included in this meta-analysis (Marino et al., 2005; Wigg et al., 2004). A total of 8 studies, which analyzed 10 independently ascertained samples, were included in the meta-analysis. Three
GWAS were identified using RD samples but were not included. Two of the studies tested for association with non-reading phenotypes. The third study performed a quantitative trait analysis, comparing readers of high and low ability selected from a representative UK sample (Meaburn et al., 2008).

Characteristics of the 10 samples are summarized in Table 3-3. All 10 samples were genotyped for the 1249G/T SNP, and 9 of the 10 samples were genotyped for the -3G/A SNP. The study design distribution included 5 case-control and 4 family-based for the -3G/A, and 6 case-control and 4 family-based for the 1249G/T. In one study, one of the groups of cases was nested within a case-cohort study (Newbury et al., 2011). Cases and probands were recruited from different sources including hospitals, research centers and institutions (Bellini et al., 2005; Newbury et al., 2011; Taipale et al., 2003); regular schools (Brkanac et al., 2007; Saviour et al., 2008; Venkatesh et al., 2011) (Toronto sample, this study); schools for the learning disabled (Saviour et al., 2008; Venkatesh et al., 2011) (Calgary sample, this study); as a part of a case-cohort study (Newbury et al., 2011); or a combination of referrals from teachers and paediatricians (Marino et al., 2007). For the case-control studies, controls were selected from the proband’s partner and/or family (Taipale et al., 2003), anonymous blood donors (Taipale et al., 2003), unselected European controls from the European Collection of Cell Cultures (Newbury et al., 2011) unmatched healthy individuals (Bellini et al., 2005), unmatched individuals with no history of reading, spelling or other academic difficulties (Saviour et al., 2008), or group-age-matched individuals without RD (Venkatesh et al., 2011). All of the case-control studies tested for association by comparing the allele frequencies between groups, and no genotype analyses were performed. The ethnicity of the samples included white Caucasians only (Bellini et al., 2005; Marino et al., 2007; Newbury et al., 2011; Taipale et al., 2003), primarily white Caucasian
The RD diagnosis in all studies was based on significantly low reading ability, with 8 of the 10 studies reporting the specific tests that were administered to the subjects. The psychometric tests that were used to measure reading ability and the cutoffs used for diagnosis varied between and among languages. All of the samples, with the exception of the Calgary sample, used low IQ as an exclusion criterion with the minimum IQ ranging from 80-90. The most common test that was used to assess intelligence was the Wechsler Intelligence Scale for Children. Five of the samples also reported the use of neurological, medical or sensorial disorders as exclusionary criteria, although the specific disorders that were screened varied across the samples (Bellini et al., 2005; Brkanac et al., 2007; Marino et al., 2007; Newbury et al., 2011) (Toronto sample, this study).

Within the pooled case-control studies, a total of 786 cases and 756 controls were included. For one study, the allele frequencies in two independent samples were compared to the same group of controls but for the meta-analysis, the controls were only counted once (Newbury et al., 2011). Within the family-based studies, a total of 940 affected subjects were included. Of the studies that were included in the analysis, we were able to contact all corresponding authors and confirm that both SNPs were in Hardy-Weinberg equilibrium except for Taipale et al. (2003).

In the 9 samples that were analyzed for the -3G/A polymorphism only the study by Taipale et al. (2003) showed a statistically significant result. For the 1249G/T studies, three studies showed significant association. Two studies showed significant association with the T allele including Taipale et al. (2003) and Marino et al. (2007). In contrast, the study by Brkanac
et al. (2007) found that the T allele carried a decreased risk for RD. Using the random-effects model, there was no statistically significant evidence of an association between RD and the -3G/A (OR=1.16, 95% CI: 0.900, 1.490) or 1249G/T polymorphisms (OR=1.26, 95% CI: 0.981, 1.624), with significance at p<0.05 (Figures 3-3 and 3-4). The level of heterogeneity for both SNPs was medium to high, with an $I^2$ value of 34% (95% CI: 1, 55) for the -3G/A and 50% (95% CI: 28, 64) for the 1249G/T. There was no evidence of publication bias for either -3G/A (p=0.445) or 1249G/T (p=0.313). The four additional subgroup analyses did not yield considerably different results.

Using the Venice criteria to evaluate the credibility of the evidence, a “weak” grade was given for both the association between -3G/A and 1249G/T polymorphisms and RD. There was a potential for information and misclassification bias which could have affected the results of the individual studies, while heterogeneity was moderate for the -3G/A and high for the 1249G/T polymorphism.

3.5 Discussion

DYX1C1 is a prominent RD candidate gene and has been extensively studied. Although two potentially functional SNPs previously showed significant association with RD in the initial study, subsequent studies yielded contradictory results. We performed a family-based association analysis, expanding on the sample that was used by Wigg et al. (2004). With 273 additional families and an increase from 101 to 272 subjects meeting the categorical cut off, we tested whether the previous reported associations remained significant, in addition to testing 4 new additional SNPs. A second independent sample from Calgary was also used to test for categorical association with single SNPs and the -3G/A:1249G/T haplotypes. The rs11629841
marker, which previously showed significantly biased transmission, was not significant in the two samples. For the quantitative trait analysis, the previous associations between the -3G variant and all nine reading measures also did not remain statistically significant in this study. Although we found three reading/language measures with p<0.05, they were not statistically significant when corrected for the multiple tests performed.

In the Toronto sample, there was a statistically significant undertransmission of the -3A/1249G haplotype, differing from the -3G/1249G haplotype that was overtransmitted in the previous study by Wigg et al. (2004) and the -3A/1249T haplotype that was associated with RD in the initial study by Taipale et al. (2003). However, this haplotype was rare in both the samples, found in only 0.013 of parental chromosomes in the Toronto sample and absent in the Calgary sample. Although it is possible that this may suggest a protective effect, it is more likely that these results were due to the relative frequencies as the -3A/1249G, -3A/1249T, -3G/1249T haplotypes were all rare in both samples (all <0.06).

We also conducted the first meta-analysis of the two significant DYX1C1 SNPs reported by Taipale et al. (2003). Similar to other meta-analyses of genetic association studies, the initial study showed strong association, while subsequent studies tended to find marginal to no significance (Ioannidis et al., 2001). With 786 cases, 756 controls, and 940 family trios, the meta-analysis did not find significant association between -3G/A or 1249G/T and RD. Combining the cases and family trios, the sample had >80% power to detect an odds ratio as small as 1.3 for both the -3G/A and 1249G/T SNPs, if such an effect existed. The case-control and TDT modules calculated virtually the same power estimate when the cases and the family-trios were equal. However, the between-study heterogeneity likely reduced the true statistical power.
The level of between-study heterogeneity was moderate to high for both SNPs, which may reflect differences in study design, phenotype definition, quality control methods for genotyping, and population stratification. Most studies defined RD as having significantly poor reading skills with normal IQ except for the Calgary sample which did not use IQ to define RD. However, there were differences in the tests administered and the cut-offs used to determine diagnosis. For example, in the studies that used the WISC-III, the cutoff for normal IQ ranged from 80-90. Some studies indicated that they excluded neurological, medical or psychiatric disorders. The sources that were used to recruit subjects differed between studies. In addition, among the case-control studies, different types of controls were used including unselected, unmatched and matched individuals. These differences in the cases and controls may have affected the results of the individual studies and contributed to the observed between-study heterogeneity.

The quality control measures for the included studies were largely unknown which could create a potential for bias. Misclassification of both genotypes and phenotypes may have affected the results but the approximate effect is difficult to predict. Population stratification was less likely because the cases and controls were of the same ethnicity, and family-based designs are robust to such problems. Narrowing the studies to only white Caucasian subjects and narrowing the RD phenotype in the subgroup analyses did not yield a significantly different result.

Apart from the Toronto and Calgary samples, we included only published studies, and there was a possibility that unpublished data were not considered in this meta-analysis. If there were unpublished results, however, these would have likely contributed a negative result due to publication biases (Thornton and Lee, 2000). Of the studies that met inclusionary criteria,
one independent study was excluded from the analysis because the specific data were unavailable. This study did not find an association between the two SNPs and RD, and would have also likely contributed a negative result.

Applying the Venice criteria, the strength of the cumulative evidence for an association between -3G/A or 1249G/T and RD was “weak”. With a risk allele count of 207 for -3G/A and 306 for 1249G/T, the sample size was considered to be of moderate size (“B” rating). However, there were considerable differences in RD phenotype definition, study populations, and study findings, and a null overall effect was found in the meta-analysis (“C” rating). There was also a potential for bias which could have affected the results (“B” rating).

Overall, we did not find strong evidence for an association between DYX1C1 SNPs and RD. In the Toronto sample, previous associations were no longer significant when a larger sample was tested, and an analysis of an independent sample from Calgary did not yield any significant results. The meta-analysis did not find an association between -3G/A or 1249G/T and RD, and there was weak credibility of evidence for both SNPs due to the high between-study heterogeneity.

3.6 Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research number MOP-89855. C.T. was supported by the National Sciences and Engineering Council of Canada-Alexander Graham Bell Canada Graduate Scholarship and University of Toronto Open Fellowship. F.G. holds a Canada Research Chair.
3.7 Tables and Figures

Figure 3-1. Relative positions of the genotyped DYS1C1 markers
Figure 3-2. Inter-marker LD across the genotyped $DYX1C1$ SNPs in the Toronto (A) and Calgary (B) samples. The red unnumbered boxes represent inter-marker $D'$=1 and are indicative of high LD. The LD blocks in each sample are outlined in bold.
Table 3-1. Single SNP analysis for the Toronto and Calgary samples

<table>
<thead>
<tr>
<th>Marker</th>
<th>Transmission Ratio (Allele)</th>
<th>Minor Allele Frequency (Allele)</th>
<th>$\chi^2$</th>
<th>P-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Toronto Sample)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12899331</td>
<td>86(A):76(G)</td>
<td>0.229 (G)</td>
<td>0.617</td>
<td>0.432</td>
</tr>
<tr>
<td>rs16787</td>
<td>62(A):55(C)</td>
<td>0.164 (C)</td>
<td>0.419</td>
<td>0.518</td>
</tr>
<tr>
<td>rs2007494</td>
<td>90(A):79(T)</td>
<td>0.247 (A)</td>
<td>0.716</td>
<td>0.398</td>
</tr>
<tr>
<td>-3G/A</td>
<td>32(A):30(G)</td>
<td>0.070 (A)</td>
<td>0.158</td>
<td>0.691</td>
</tr>
<tr>
<td>rs3743204</td>
<td>90(T):82(G)</td>
<td>0.235 (T)</td>
<td>0.372</td>
<td>0.542</td>
</tr>
<tr>
<td>rs17819126</td>
<td>33(T):23(C)</td>
<td>0.067 (T)</td>
<td>1.786</td>
<td>0.181</td>
</tr>
<tr>
<td>rs11629841</td>
<td>108(G):94(T)</td>
<td>0.344 (G)</td>
<td>0.970</td>
<td>0.325</td>
</tr>
<tr>
<td>rs692691</td>
<td>131(T):102(C)</td>
<td>0.404 (T)</td>
<td>3.609</td>
<td>0.058</td>
</tr>
<tr>
<td>rs600753</td>
<td>119(T):97(C)</td>
<td>0.494 (C)</td>
<td>2.241</td>
<td>0.134</td>
</tr>
<tr>
<td>1249G/T</td>
<td>50(T):35(G)</td>
<td>0.093 (T)</td>
<td>2.722</td>
<td>0.099</td>
</tr>
<tr>
<td>(Calgary Sample)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rs12899331</td>
<td>80(A):64(G)</td>
<td>0.261 (G)</td>
<td>1.778</td>
<td>0.182</td>
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<tr>
<td>rs16787</td>
<td>45(C):39(A)</td>
<td>0.174 (C)</td>
<td>0.429</td>
<td>0.513</td>
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<tr>
<td>rs2007494</td>
<td>59(A):53(T)</td>
<td>0.203 (A)</td>
<td>0.321</td>
<td>0.571</td>
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<td>-3G/A</td>
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<td>0.049 (A)</td>
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<tr>
<td>rs3743204</td>
<td>66(T):62(G)</td>
<td>0.203 (T)</td>
<td>0.125</td>
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</tr>
<tr>
<td>rs17819126</td>
<td>26(T):23(C)</td>
<td>0.075 (T)</td>
<td>0.184</td>
<td>0.668</td>
</tr>
<tr>
<td>rs11629841</td>
<td>80 (G):80 (T)</td>
<td>0.337 (G)</td>
<td>0.000</td>
<td>1.000</td>
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<tr>
<td>rs692691</td>
<td>83(T):68(C)</td>
<td>0.388 (T)</td>
<td>1.490</td>
<td>0.222</td>
</tr>
<tr>
<td>rs600753</td>
<td>75(T):65(C)</td>
<td>0.488 (C)</td>
<td>0.714</td>
<td>0.398</td>
</tr>
<tr>
<td>1249G/T</td>
<td>25(T):22(G)</td>
<td>0.076 (T)</td>
<td>0.191</td>
<td>0.662</td>
</tr>
</tbody>
</table>

$^1$Alleles refer to the plus-strand, except for the -3G/A and 1249G/T polymorphisms which refer to the transcript

$^2$Threshold for significance set at 0.006
Table 3-2. Haplotype analysis for the Toronto and Calgary samples

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Observed</th>
<th>Expected</th>
<th>Var(O-E)</th>
<th>Chisq (1df)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toronto Sample&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3G 1249G</td>
<td>0.895</td>
<td>479.97</td>
<td>480.30</td>
<td>25.64</td>
<td>0.004</td>
<td>0.949</td>
</tr>
<tr>
<td>-3A 1249G</td>
<td>0.013</td>
<td>0.03</td>
<td>4.38</td>
<td>1.99</td>
<td>9.540</td>
<td>0.002</td>
</tr>
<tr>
<td>-3G 1249T</td>
<td>0.039</td>
<td>20.87</td>
<td>20.55</td>
<td>9.74</td>
<td>0.011</td>
<td>0.918</td>
</tr>
<tr>
<td>-3A 1249T</td>
<td>0.052</td>
<td>39.13</td>
<td>34.77</td>
<td>15.16</td>
<td>1.254</td>
<td>0.263</td>
</tr>
<tr>
<td>Calgary Sample&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>-3G 1249G</td>
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<td>375.58</td>
<td>14.21</td>
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<td>14.00</td>
<td>18.77</td>
<td>9.11</td>
<td>2.500</td>
<td>0.114</td>
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</table>

<sup>1</sup>Global chisquared test, on 3 degrees of freedom = 10.907 (p=0.012)

<sup>2</sup>Global chisquared test, on 2 degrees of freedom = 5.0643 (p=0.079)
<table>
<thead>
<tr>
<th>Study (Location)</th>
<th>Language</th>
<th>Study design</th>
<th>Ethnicity</th>
<th>Tests used to assess reading ability and intelligence</th>
<th>Scores for inclusionary criteria</th>
<th>Other Exclusionary Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taipale 2003 (Finland)</td>
<td>Finnish</td>
<td>Case-Control</td>
<td>Caucasian</td>
<td>Finnish reading and spelling tests for children and adults, and neuropsychological tests for phonological awareness, rapid automatized naming, and verbal short-term memory. Tests include A Developmental Neuropsychological Assessment, Luria’s Neuropsychological Test, -Wechsler Adult Intelligence Scale Revised (WAIS-R) and Wechsler Adult Intelligence Scale for Children Revised (WISC-R)</td>
<td>≥2 years deviation in reading skills</td>
<td>- Performance IQ&gt;85</td>
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<tr>
<td>Bellini 2005 (Italy)</td>
<td>Italian</td>
<td>Case-Control</td>
<td>Caucasian</td>
<td>Standard text reading from the &quot;test for speed and accuracy in reading, developed by the MT group&quot; -Single word and non-word reading from the &quot;battery for the assessment of developmental reading and spelling disorders&quot; -WISC-R</td>
<td>Reading performance ≥2 standard deviations (SD) below the mean for the same age and/or school degree</td>
<td>- IQ ≥85</td>
</tr>
<tr>
<td>Marino 2007 (Italy)</td>
<td>Italian</td>
<td>Family-based</td>
<td>Caucasian</td>
<td>Accuracy and speed of text reading from the &quot;test for speed and accuracy in reading, developed by the MT group&quot; -Timed single word and single non-word reading from the &quot;battery for the assessment of developmental reading and spelling disorders&quot; -WISC-R</td>
<td>Either accuracy or speed ≤1 SD on the timed text, single word or single non-word reading tasks</td>
<td>-Full scale IQ&gt;80</td>
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<tr>
<td>Study</td>
<td>Language(s)</td>
<td>Design</td>
<td>Ethnicity</td>
<td>Measures</td>
<td>Criteria</td>
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<td>Brkanac 2007 (US)</td>
<td>English</td>
<td>Family-based</td>
<td>Predominantly Caucasian</td>
<td>Woodcock Reading Mastery Test - Revised (WRMT-R) word ID and word attack subtests&lt;br&gt;Test of Word Reading Efficiency real word reading efficiency and phonemic decoding efficiency subtests&lt;br&gt;Gray Oral Reading Test (3rd edition) rate and accuracy scores&lt;br&gt;Spelling subtests from the Wide Range Achievement Test - Revised, Wechsler Individual Achievement Test, and Woodcock Johnson Psychoeducation Battery-Revised (WJ-R)&lt;br&gt;Timed Alphabet Task&lt;br&gt;Wechsler Intelligence Scale for Children-3rd Edition (WISC-III)</td>
<td>Prorated verbal IQ ≥90&lt;br&gt;Score below the population mean and ≥1 SD below their verbal IQ on at least one of the 10 measures&lt;br&gt;Absence of mental retardation, developmental delay, primary language disorder, neurological disorder including traumatic brain injury or seizure disorder, and psychiatric disorder (except for attention-deficit hyperactivity disorder).</td>
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<td>Saviour 2008 (India)</td>
<td>Kannada/English</td>
<td>Case-Control</td>
<td>Ethnic Indian</td>
<td>Graded reading and spelling tests&lt;br&gt;Raven’s (Coloured) Progressive Matrices&lt;br&gt;School examination marks</td>
<td>Performance on reading and spelling tests ≥2 grades deviation&lt;br&gt;Individuals with behavioural or emotional problems that would cause poor academic achievement, and individuals who are below normal on intelligence/reasoning function</td>
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<td>Newbury 2011 (UK)</td>
<td>English</td>
<td>Case-Control</td>
<td>European Caucasian</td>
<td>British Abilities Scales (BAS) single-word reading&lt;br&gt;BAS similarities or BAS matrices subtests&lt;br&gt;WAIS-R for siblings &gt;17.5 years</td>
<td>BAS single-word reading score ≥1 SD below the population mean for their age - IQ 90&lt;br&gt;Diagnosed with co-occurring developmental disorders such as SLI, autism or attention deficit-hyperactivity disorder.</td>
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<tr>
<td>Newbury 2011 independent sample (UK)</td>
<td>English</td>
<td>Case-Control (cases selected as a part of case-cohort)</td>
<td>European Caucasian</td>
<td>British Abilities Scales 2 (BAS2) single-word reading&lt;br&gt;BAS similarities or BAS matrices subtests</td>
<td>BAS2 single-word reading score ≤100 for their age, and &gt;1.5 SD below their predicted score based on IQ&lt;br&gt;Not specified</td>
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<td>Venkatesh 2011 (India)</td>
<td>Kannada/English</td>
<td>Case-Control</td>
<td>Ethnic Indian</td>
<td>General intellectual ability and reading/dictation school tests used in India</td>
<td>Not specified&lt;br&gt;Not specified</td>
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<tr>
<td>Study</td>
<td>Year</td>
<td>Country</td>
<td>Setting</td>
<td>Methods</td>
<td>Definition of Dyslexia</td>
<td>Exclusion Criteria</td>
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</tbody>
</table>
| Tran 2011 | 2011 | Canada | Family-based | Primarily Caucasian | - Performance and verbal IQ > 80  
| | | | | | - Score of ≥ 1.5 SD below the mean on two of the three tests, or if the mean of the average of the three scores is ≥ 1 SD below the mean | Absence of neurological or chronic medical illness, bipolar affective disorder, psychotic symptoms, Tourette syndrome or chronic multiple tics |

| Tran 2011 | 2011 | Canada | Family-based | Primarily Caucasian | WJ-R and WRMT-R word attack subtests  
| | | | | | - IQ not used to define dyslexia phenotype | ≥ 2 years deviation in reading skills using the word attack subtests |

| | | | | | Not specified |
The relative weights of the samples are represented by the boxes, and the 95% confidence intervals are represented by the lines.
The relative weights of the samples are represented by the boxes, and the 95% confidence intervals are represented by the lines.
Chapter 4 – A Family-Based Association Analysis of the Reading Disabilities Candidate Gene ROBO1

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4.1 Abstract

Linkage studies have identified chromosome 3 as a RD susceptibility region (DYX5). In addition, this region has shown linkage with speech and sound disorder (SSD), which is often comorbid with RD and shares similar phonological processing and phonological memory difficulties. One gene in this region, roundabout homolog 1 (ROBO1), has been proposed as a RD candidate gene, and has showed significant association with a measure of phonological memory. In this study, we conducted a family-based association analysis, testing for association between ROBO1 SNPs and RD in two independent samples. We also tested for association with quantitative reading, phonological memory and working memory traits in one of the samples. Haplotype analyses were performed using the SNPs in LD blocks for both samples. We did not find significant association between any of the ROBO1 SNPs and RD in either sample. For the digit span backwards measure, there were several SNPs with p<0.05, but these results were not statistically significant after correction for multiple testing. In contrast to the previous study, we did not find significant evidence for association between ROBO1 and RD or any of the reading measures.
4.2 Introduction

Specific reading disabilities (RD), or developmental dyslexia, refer to an unexpected and specific difficulty in learning to read despite normal intelligence, education, and socioeconomic opportunity. RD is the most common learning disability with estimated prevalence rates ranging from 5-17.5% (Shaywitz, 1998; Shaywitz et al., 1999; Katusic et al., 2001). A core deficit observed in individuals with RD involves the processing of phonemes, the basic unit of speech sounds. Three cognitive skills that require phonological processing include phonological awareness, phonological memory, and rapid automatized naming, all of which have been shown to be impaired in individuals with RD (Denckla and Rudel, 1976; Mann and Liberman, 1984; Bruck, 1992; Meyer et al., 1998; Wilson and Lesaux, 2001). Short-term phonological memory refers to the temporary storage of verbal information, while working memory is conceptualized as including both the short-term storage and manipulation of verbal information. The short-term storage of unfamiliar phonological forms, measured through the administration of nonword repetition tests, has been implicated in the development of vocabulary and reading skills (Gathercole et al., 1992; Passenger et al., 2000). Individuals with reading difficulties have been shown to perform poorer on nonword repetition tasks (Snowling et al., 1986; Brady et al., 1987; Kamhi et al., 1988).

Significant evidence suggests that RD is influenced by genetic factors. Studies comparing RD rates in monozygotic and dizygotic twins have found significantly higher concordance rates in monozygotic twins (Bakwin, 1973; DeFries and Alarcon, 1996). The heritability of reading component skills range from 0.30-0.72, and moderate heritability has been reported for short-term memory, as measured through digit span tests \( h_g^2=0.27-0.66 \) (Finkel et al., 1995) and phonological memory, as measured through non-word repetition tasks \( h_g^2=0.61 \).
(Bishop et al., 2006). Two independent RD linkage studies have identified a pericentromeric region on chromosome 3 as a RD susceptibility region, designated as DYX5. This region was originally identified in a study of a large Finnish family, with 19 of 21 tested affected members sharing a 35Mb region on chromosome 3p12-q13 (Nopola-Hemmi et al., 2001). The affected individuals in this family showed significantly lower performance in tasks for phonological awareness, rapid automatized naming and verbal short-term memory. A subsequent study found linkage to 3p13 in a British sample, and 3q13 in an American sample, both within the linkage region found in the initial study (Fisher et al., 2002). In that study, measures of phonological memory and reading showed significant linkage. Furthermore, a linkage study using affected families with speech and sound disorder (SSD) found significant linkage between this region and a phonological memory task (Stein et al., 2004). Both RD and SSD are often comorbid and share similar difficulties in phonological awareness (Raitano et al., 2004; Rvachew and Grawburg, 2006; Peterson et al., 2009). Thus, this region may influence phonological processing that results in the deficits observed in RD and SSD.

The roundabout homolog 1 (ROBO1) gene on chromosome 3p12.3 was proposed as a candidate gene when one affected Finnish individual carried a t(3;8)(p12;q11) chromosomal translocation, disrupting the gene (Hannula-Jouppi et al., 2005). In that study, the ROBO1 expression was found to be reduced in four members of the original Finnish linkage family carrying the risk haplotype, compared to four controls. It was hypothesized that lower expression of ROBO1 may contribute to the development of RD. A follow up study using 10 of the affected family members found that ROBO1 expression levels were correlated with levels of auditory cortex suppression of ipsilateral inputs (Lamminmaki et al., 2012). Since ROBO1 is known to control axon guidance across the midline (Seeger et al., 1993; Kidd et al., 1998; Nguyen-Ba-Charvet and Chedotal, 2002; Wong et al., 2002), it was proposed that the lower expression of
ROBO1 in the family members affected the crossing of auditory pathways across the midline, ultimately affecting their processing of the auditory stimuli.

To date, one RD association study of reading traits has been performed with ROBO1 in a population-based sample (Bates et al., 2011). Using families with twins or triplets, two ROBO1 SNPs were found to be significantly associated with a phonological memory task after correcting for the number of markers tested: rs6803202 (p=8.7 x 10^{-5}) and rs4535189 (p=9.3 x 10^{-5}). Several other SNPs also showed nominal significance with nonword repetition, short-term verbal memory, working memory, and reading and spelling principle components.

Despite the status of ROBO1 as a RD candidate gene, no study has yet to examine the association between ROBO1 SNPs and RD or reading measures using a RD sample. The purpose of this study was to evaluate ROBO1 as a RD candidate gene through a family-based association analysis. Using two independently ascertained samples, we tested for association between ROBO1 SNPs and RD. A quantitative trait analysis was also performed in one of the samples, where similar measures were available for the phenotypes that were analyzed in Bates et al. (2011).

4.3 Materials and Methods

Toronto Sample

Subjects and Assessment

A family-based sample from the Greater Toronto Area and surrounding regions was used for the analysis. Descriptions of the sample and assessment methods have been described previously
Briefly, the sample consisted of 1416 individuals from 421 families, each with at least one child who exhibited difficulties in reading. Proband s ranged from 6-16 years of age, and siblings within the same age range were also invited to participate in the study regardless of their reading ability. Families were self-referred through information from a number of sources including websites (Learning Disabilities Association of Ontario), schools, regional organizations for learning disabilities, and referrals from general education teachers, special education teachers, speech/language pathologists, and psychologists. Subjects were either native English speakers or were educated for at least five years in an English-speaking school. The ethnicity of the sample was primarily Caucasian, with 68.1% of individuals reporting European descent and 26% reporting “Caucasian Canadian” background. The remaining subjects reported South American (1.8%), non-European (2.9%), or non-European mixed background. Written informed consent was obtained from all participants, and protocols were approved by The Hospital for Sick Children Research Ethics Board.

Proband s and siblings underwent a comprehensive battery of tests for language and reading abilities, as well as IQ. The performance on three single word and non-word reading subtests were used to determine the RD affection status of the probands and siblings for the categorical analysis: Wide Range Achievement III (WRAT-III) Single Word Reading (Wilkinson, 1993), Woodcock Reading Mastery Test-Revised (WRMT-R) Word Identification (Woodcock, 1987) and WRMT-R Word Attack. Subjects were considered to be affected with RD if they scored 1.5 standard deviations below the mean on 2 of the 3 measures, or 1 standard deviation below the mean on all 3. The Wechsler Intelligence Scale for Children (WISC-III or WISC-IV) was used to assess intelligence and cognitive ability, and children were excluded from the study if they scored <80 on the Verbal and Performance Scales of the WISC-III, or Verbal
Comprehension and Perceptual Reasoning Indices of the WISC-IV (Wechsler, 1991; Wechsler, 2003). Of the 588 probands and siblings, 272 met the categorical cutoff.

Isolation of DNA and SNP Genotyping

DNA was extracted directly from white blood cells using a high-salt extraction method (Miller et al., 1988). A total of 16 SNPs were selected for genotyping, based on the findings of Bates et al. (2011). These include the 2 SNPs that were significantly associated with the phonological memory measure, and 14 SNPs among the next strongest findings for the phonological memory, short-term verbal memory, and working memory measures. All 16 SNPs were genotyped using the TaqMan® 5’ nuclease assay for allelic discrimination (Applied Biosystems, Foster City, CA). Genotyping was carried out in 96-well plates consisting of two negative controls. 10µL PCR reactions were performed with the following reagents: 30ng of genomic DNA, 10µmol of TaqMan® Universal PCR Master Mix (Applied Biosystems) and 0.25µL of allelic discrimination mix (Applied Biosystems) with 36µM of each primer and 8µM of each probe. The reaction conditions consisted of the following steps: 50ºC for 2min, 95ºC for 10min, and 40 cycles of 94ºC for 15s and an annealing temperature of 59 ºC for 1min. Plates were then read with the ABI 7900-HT Sequence Detection System using the allelic discrimination end-point analysis mode of the SDS software package, v2.0 (Applied Biosystems).

To check for genotyping and family structure errors, Haploview v4.2 and Merlin v.1.1.2 were used (Abecasis et al., 2002; Barrett et al., 2005). Hardy-Weinberg Equilibrium was checked for each marker using Haploview v4.2. None of the 16 SNP showed significant deviation from Hardy-Weinberg equilibrium. Samples with undetermined calls were genotyped a
second time. After retyping samples, there were no identified Mendelian errors or unlikely crossovers between markers.

**Selection of Quantitative Traits for Analysis**

The selection of quantitative traits for analysis was based on the measures tested by Bates et al. (2011). In the previous study, a single measure was used for reading and spelling principle components. There were no equivalent measures in the Toronto sample. Instead, the WRAT-III reading and spelling subtests were used to assess single word reading and spelling separately (Wilkinson, 1993). Bates et al. (2011) assessed phonological memory using a combined score from two nonword repetition tests (Gathercole et al., 1994; Dollaghan and Campbell, 1998). For this study, the Comprehensive Test of Phonological Processing (CTOPP) non-word repetition task was used for the analysis (Wagner et al., 1999). Short-term and working memory were previously assessed using the Wechsler Adult Intelligence Scale-III (WAIS-III) Digits Forward and Letter Number Sequencing tasks, respectively (Wechsler, 1997). For this study, the Digit Span Forwards and Backwards measures from the WISC-III and Children’s Memory Scale (CMS) were analyzed (Wechsler, 1991; Cohen, 1997).

For the reading, spelling, and non-word repetition measures, data were available for all 588 probands and siblings. Due to the switch from the WISC-III to the WISC-IV, data for the WISC-III version of the Digit Span Forwards and Backwards subtests were available for the first 418 of the 588 subjects, with the remainder of the subjects being tested with the WISC-IV version of the subtests (Wechsler, 2003). However, all 588 subjects were tested for the CMS Numbers Forwards and Backwards measures.
**Statistical Analysis**

To test for association between single SNPs and RD, the TDT statistic was calculated using Haploview v4.2 (Barrett et al., 2005). Pairwise LD between SNPs was also visualized using Haploview v4.2, and LD blocks were determined using the method of Gabriel et al. (2002). The minimum lower and upper confidence limits for the $D'$ were set to 0.7 and 0.98.

The quantitative trait analysis was performed using FBAT v2.0.3 (Laird et al., 2000). The empirical variance option was used since linkage has been established to the studied chromosomal region. An additive model was also assumed as it is robust, even if the true model is not additive. Offsets were used in the analysis of all quantitative traits, based on population norms. To test for association between the haplotypes, determined by the LD blocks, the HBAT command in FBAT was used.

Bonferroni correction was used to account for testing multiple SNPs and quantitative traits. Because there was a correlation among the SNPs and phenotypes, correcting for all genotyped SNPs and tested traits would have been over conservative. We corrected for the number of independent SNPs as calculated by SNPSpD ($p=0.05/8=0.006$) and the number of calculated independent quantitative measures as calculated by matSpD ($p=0.006/6=0.001$) (Nyholt, 2004).

A statistical power analysis of the sample was calculated using the Genetic Power Calculator (Purcell et al., 2003). The TDT module for discrete traits was used under a dominant model. A total of 272 family trios were used for the analysis as 272 probands and siblings met the categorical cutoff for RD in the sample. The RD prevalence was estimated at 0.1, based on
the literature. The type-I error rate was defined at 0.05 and the threshold for acceptable power was 80%. Assuming a dominant model, the sample had adequate power to detect a genotype relative risk of 1.8 for a causative marker, with a risk allele frequency ranging from 0.11-0.32. At a risk allele frequency of 0.2, the sample had adequate power to detect a genotype relative risk of 1.8 for a marker with a $D'$ ranging from 0.9-1.0.

The power of the sample for the quantitative trait analysis was also calculated using the Quantitative TDT module in the Genetic Power Calculator (Purcell et al., 2003). The number of subjects used in the analysis was 588, and the case threshold was set to 1 SD. The marker allele frequency was set equal to the quantitative trait loci (QTL) allele frequency, and the recombination fraction was set to 0. Using the observed marker allele frequencies 0.04-0.49, the sample had adequate power to detect a QTL attributing 0.02 of the variance of a trait. For a risk allele frequency of 0.04-0.19, the sample had adequate power to detect a QTL attributing 0.02 of the variance of a trait with $D'$ ranging from 0.8-1.0.

**Calgary Sample**

**Subjects and Assessment**

The sample consisted of nuclear families with affected siblings from Calgary, Alberta, which have been previously described (Field and Kaplan, 1998; Hsiung et al., 2004; Petryshen et al., 2001; Tzenova et al., 2004). The affected sibpair families were mostly ascertained through probands aged >8 who attended a specialized school for children with learning disabilities. Although previous studies included extended family members in the analysis, this study only included the nuclear sibpair family recruited from the special education schools.
RD affection status was determined by performance on two phonological coding tasks: the Woodcock Reading Mastery Test and the Revised Woodcock-Johnson Psychoeducation Test. Subjects <18 years of age with scores ≥2 years below the age norm were considered to be affected with RD. A total of 207 affected children from 95 families were included in the sample. Full-scale IQ was estimated using a short form of the WISC-R for subjects aged 8-16 years, and a short form of the Wechsler Adult Intelligence Scale for adults. A total of 95 families including 207 affected children were included in the analysis.

**Selection of SNPs and Statistical Analysis for the Calgary Sample**

A genome-wide scan was previously performed using the Calgary sample, and all genotyped SNPs within ROBO1 were selected for analysis. Haploview v4.2 was used to test for association between ROBO1 SNPs and RD (Barrett et al., 2005).

The TDT module of the Genetic Power Calculator (Purcell et al. 2003) for discrete traits was used to calculate the power of the sample, using the same prevalence, type-I error rate, and power threshold as the Toronto sample. The number of trios included in the analysis was 207. Assuming a dominant model, the sample had adequate power to detect a genotype relative risk of 1.9 for a causative marker, with a risk allele frequency ranging from 0.14-0.27. At a risk allele frequency of 0.2, the sample had adequate power to detect a genotype relative risk of 1.8 for a marker with a $D'$ ranging from 0.96-1.0.
4.4 Results

A total of 16 SNPs were genotyped in the Toronto sample, with the relative positions shown in Figure 4-1. Three LD blocks were identified among the SNPs (Figure 4-2). The first block encompassed 8 markers: rs4680960, rs4130991, rs7653197, rs4535189, rs6803202, rs7628757, rs4564923, and rs4264688. The second block included rs6548621 and rs7622444, and the third block consisted of rs9853895 and rs9857859. In the Calgary sample, a total of 34 SNPs were genotyped in ROBO1 and were included in the analysis.

We did not find significant association between the ROBO1 SNPs (Table 4-1 and 4-2) or haplotypes (not shown) and RD in either sample. In the quantitative trait analysis of the Toronto sample, none of the genotyped SNPs were significant for the tested reading measures, and we could not replicate the findings of Bates et al. (2011) (Table 4-3). The results from the WISC-III Digit Span Backwards was nominally significant, with the strongest findings at rs9853895 (p=0.0028), rs6548621 (p=0.0068) and rs9857859 (p=0.0090). However, these results were not considered to be statistically significant after Bonferroni correction, as the adjusted threshold for significance was p=0.001. In addition, several haplotypes containing these markers showed nominal significance for the WISC-III Digit Span Backwards measure (Tables 4-4 to 4-6). The major haplotype in Block 2 rs6548621A:rs7622444A haplotype was biased in transmission (p=0.0068). In Block 3, rs9853895C:rs98557859C was a protective haplotype (p=0.0057), while rs9853895C:rs98557859C was preferentially not transmitted (p=0.0054). The CMS Numbers Backwards measure, which also tests for working memory, did not show statistically significant association with any of the single SNPs or haplotypes. Although one SNP rs333491 showed nominal significance (p=0.0194), this was different than the nominally significant SNPs in the WISC-III Digit Span.
4.5 Discussion

*ROBO1* was tested as a RD candidate gene because of its role in axon guidance and because individuals from a Finnish family carried a rare haplotype that was associated with lower *ROBO1* expression. This was the first study testing for association between *ROBO1* SNPs and RD using RD samples. However, we did not find significant association between *ROBO1* SNPs and RD, and were not able to replicate the findings of Bates et al. (2011) which found significant association with a measure of short-term phonological memory. Several SNPs in that study showed a trend towards association with the WISC-III Digit Span Backwards measure but this was not significant after correction for multiple testing. These results were not replicated in a related test, the CMS Numbers Backwards. Because only a subset of the full sample was tested for the WISC-III Digit Span measures, the observed results may be due to the reduced sample size.

The haplotype analysis was performed as it may be informative to evaluate which ancestral chromosomes may carry the risk allele. Analyses of haplotypes with categorical RD did not show statistically significant association. Although several haplotypes showed nominal significance in the Toronto sample, these included the SNPs that showed the strongest results in the single SNP analysis. Since the transmission of haplotypes is related to the transmission of the SNPs individually, the nominally significant results should be interpreted with caution.

There were a number of factors that may account for the lack of replication between the study used in Bates et al. (2011) and the Toronto sample. First, the samples analyzed in both studies differed considerably. The previous study used a population-based twin sample (mean age=17.9 years, SD=2.9 years, range 12.3-25.1 years), whereas this study used a selected sample
(mean age=10.4 years, SD=2.5 years, range 6-16 years). In addition to the differences between the samples, the use of different quantitative measures may have also contributed to the different results. Although related measures were selected to analyze similar reading-related skills, the specific tests in this study differed from the study by Bates et al. (2011). Thus, differences in study design may have accounted for the discrepancies between the studies.

An important consideration was the method of correcting for multiple testing in both studies. Whereas the previous study corrected for the number of independent SNPs, this study corrected for both the number of independent SNPs and quantitative traits. Although this may have led to a more stringent threshold for statistical significance, we chose to additionally correct for the number of quantitative traits because they also contributed to the number of hypothesis tests and should be taken into account to prevent Type-I error. Nonetheless, this factor may have affected the interpretation of the results in both studies.

*ROBO1* was first identified as a possible RD candidate gene based on a chromosomal translocation. Using a subset of the family that originally showed linkage to DYX5, it was suggested that *ROBO1* expression levels may be lower in individuals with RD and that this may affect the processing of auditory stimuli (Hannula-Jouppi et al., 2005; Lamminmaki et al., 2012). In those studies, affected members in a large Finnish family carrying a rare *ROBO1* haplotype showed lower expression of the gene. Reduced *ROBO1* expression levels were also correlated with the lower suppression of ipsilateral auditory inputs. It was proposed that because *ROBO1* is involved in axonal guidance across the midline, lower suppression of ipsilateral auditory inputs is the result of less auditory neurons crossing the midline due to reduced expression of *ROBO1*. However, several questions remained from these studies. First, the translocation that was identified in an affected individual was not found in any other family members, including an
affected sibling. Thus, the disruption of *ROBO1* being causal in the individual remained questionable. Second, the findings of the expression studies may not be generalizable to individuals with RD. In the initial expression analysis, four affected family members were compared to four controls. With a small sample size, the expression levels may not be representative of other family members with the risk *ROBO1* haplotype and the normal population. The follow-up study compared 10 affected family members to 10 anonymous controls and found that the average expression level in the affected group was 83% of the control group. Although this reduced expression was expected by the authors (the normal haplotype showed 100% *ROBO1* expression and the risk haplotype expresses *ROBO1* at 66% of the normal haplotype, the overall *ROBO1* expression in an individual carrying a normal haplotype and a risk haplotype would be predicted to be 83% of an individual with two normal haplotypes) this difference may be due to chance as it was not statistically significant. Third, the relationship between the *ROBO1* expression levels and the ipsilateral suppression of auditory stimuli may not be generalizable to the greater population. Although a correlation was found in the affected family members, a comparison could not be made with the control group since different controls were used for the auditory processing and expression experiments. Furthermore, the unaffected family members were not included in any of the experiments. It is unclear whether the observed relationship was specific only to individuals with the *ROBO1* risk haplotype or if it is specific to the family. It has not yet been shown if this correlation applies to population-based and RD samples.

Since brain biopsies are an unrealistic option in living subjects, blood lymphocytes are often used as a probe for brain expression, particularly for psychiatric disorders (Gladkevich et al., 2004). However, it has not yet been established whether *ROBO1* expression in lymphocytes can be used to accurately represent *ROBO1* expression in the brain. This is particularly important
as a study comparing gene expression between human peripheral blood mononuclear cells (lymphocytes, monocytes and macrophages) and brain samples found that only ~23% of 17 859 genes were found to have similar expression levels (Rollins et al., 2010). It is therefore important to determine whether ROBO1 expression levels in both lymphocytes and the brain are correlated, so that we can confirm that the previous ROBO1 expression results may be representative of the brain.

The results of this study did not support an association between ROBO1 SNPs and RD or quantitative measures of phonological memory. Although the genome-wide data from the Calgary samples covered 34 SNPs across ROBO1, the possibility of SNPs within ROBO1 affecting RD susceptibility cannot be completely ruled out, as the ROBO1 gene spans over 1Mb. In the quantitative trait analysis, between-study heterogeneity may account for the different findings in this study compared to the study by Bates et al. (2011). However, this study showed that the SNPs that were previously associated with phonological short-term memory could not be replicated in a RD sample.

4.6 Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research number MOP-89855. C.T. was supported by the National Sciences and Engineering Council of Canada-Alexander Graham Bell Canada Graduate Scholarship and University of Toronto Open Fellowship.
4.7 Tables and Figures

Figure 4-1. Relative positions of the genotyped ROBO1 SNPs in the Toronto sample.
Figure 4-2. Inter-marker LD across the genotyped ROBO1 SNPs in the Toronto sample. The red unnumbered boxes represent inter-marker $D^{'}=1$ and are indicative of high LD. The three haplotype blocks (Blocks 1-3) are outlined in bold.
Figure 4-3. Relative positions of the genotyped ROBO1 SNPs in the Calgary sample.
Figure 4-4. Inter-marker LD across the genotyped ROBO1 SNPs in the Calgary. The red unnumbered boxes represent inter-marker $D'=1$ and are indicative of high LD. The two haplotype blocks (Blocks 1-2) are outlined in bold.
Table 4-1. Single SNP categorical analysis for 16 genotyped ROBO1 SNPs in the Toronto sample

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position</th>
<th>Minor Allele Frequency (Allele)</th>
<th>HW P-value</th>
<th>Transmission Ratio (Allele)</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs333491</td>
<td>78808849</td>
<td>0.448 (C)</td>
<td>0.289</td>
<td>111:103 (T:C)</td>
<td>0.299</td>
<td>0.585</td>
</tr>
<tr>
<td>rs4680960</td>
<td>79449565</td>
<td>0.391 (T)</td>
<td>0.872</td>
<td>107:102 (C:T)</td>
<td>0.120</td>
<td>0.730</td>
</tr>
<tr>
<td>rs4130991</td>
<td>79469021</td>
<td>0.393 (G)</td>
<td>0.975</td>
<td>109:105 (A:G)</td>
<td>0.075</td>
<td>0.785</td>
</tr>
<tr>
<td>rs7653197</td>
<td>79470783</td>
<td>0.393 (T)</td>
<td>0.875</td>
<td>111:105 (G:T)</td>
<td>0.167</td>
<td>0.683</td>
</tr>
<tr>
<td>rs4535189</td>
<td>79489970</td>
<td>0.494 (A)</td>
<td>0.655</td>
<td>119:118 (A:G)</td>
<td>0.004</td>
<td>0.948</td>
</tr>
<tr>
<td>rs6803202</td>
<td>79499152</td>
<td>0.491 (C)</td>
<td>0.839</td>
<td>115:114 (C:T)</td>
<td>0.004</td>
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<tr>
<td>rs9853895</td>
<td>79585157</td>
<td>0.408 (T)</td>
<td>0.612</td>
<td>117:102 (C:T)</td>
<td>1.027</td>
<td>0.311</td>
</tr>
<tr>
<td>rs7644521</td>
<td>79784533</td>
<td>0.201 (C)</td>
<td>0.929</td>
<td>66:65 (C:T)</td>
<td>0.008</td>
<td>0.930</td>
</tr>
<tr>
<td>rs7629503</td>
<td>79813291</td>
<td>0.303 (A)</td>
<td>0.883</td>
<td>96:91 (C:A)</td>
<td>0.134</td>
<td>0.715</td>
</tr>
</tbody>
</table>
Table 4-2. Single SNP categorical analysis for the genotyped ROBO1 SNPs in the Calgary sample

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position</th>
<th>Minor Allele Frequency (Allele)</th>
<th>HW P-value</th>
<th>Transmission ratio (Allele)</th>
<th>( \chi^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6807667</td>
<td>78723181</td>
<td>0.455 (C)</td>
<td>1.000</td>
<td>99:91 (G:C)</td>
<td>0.337</td>
<td>0.562</td>
</tr>
<tr>
<td>rs6786778</td>
<td>78733249</td>
<td>0.063 (C)</td>
<td>0.918</td>
<td>27:19 (C:T)</td>
<td>1.391</td>
<td>0.238</td>
</tr>
<tr>
<td>rs1507417</td>
<td>78733334</td>
<td>0.228 (T)</td>
<td>0.448</td>
<td>47:43 (T:C)</td>
<td>0.178</td>
<td>0.673</td>
</tr>
<tr>
<td>rs4681006</td>
<td>78760885</td>
<td>0.042 (T)</td>
<td>1.000</td>
<td>16:16 (G:T)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>rs3773216</td>
<td>78778741</td>
<td>0.265 (A)</td>
<td>0.373</td>
<td>59:53 (A:G)</td>
<td>0.321</td>
<td>0.571</td>
</tr>
<tr>
<td>rs3773217</td>
<td>78778831</td>
<td>0.451 (G)</td>
<td>0.071</td>
<td>95:85 (T:G)</td>
<td>0.556</td>
<td>0.456</td>
</tr>
<tr>
<td>rs10865570</td>
<td>78798290</td>
<td>0.275 (C)</td>
<td>0.905</td>
<td>69:61 (C:T)</td>
<td>0.492</td>
<td>0.483</td>
</tr>
<tr>
<td>rs6788434</td>
<td>78816376</td>
<td>0.349 (C)</td>
<td>1.391</td>
<td>91:79 (T:C)</td>
<td>0.847</td>
<td>0.357</td>
</tr>
<tr>
<td>rs6788511</td>
<td>78816649</td>
<td>0.174 (C)</td>
<td>0.967</td>
<td>48:45 (G:A)</td>
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<td>0.756</td>
</tr>
<tr>
<td>rs17016466</td>
<td>78816649</td>
<td>0.174 (G)</td>
<td>0.967</td>
<td>79:63 (C:T)</td>
<td>1.803</td>
<td>0.179</td>
</tr>
<tr>
<td>rs1457659</td>
<td>78837167</td>
<td>0.235 (T)</td>
<td>0.360</td>
<td>93:76 (C:T)</td>
<td>1.710</td>
<td>0.191</td>
</tr>
<tr>
<td>rs333472</td>
<td>78866958</td>
<td>0.497 (T)</td>
<td>0.536</td>
<td>28:23 (T:C)</td>
<td>0.490</td>
<td>0.484</td>
</tr>
<tr>
<td>rs452705</td>
<td>78907601</td>
<td>0.070 (C)</td>
<td>1.000</td>
<td>73:65 (C:A)</td>
<td>0.464</td>
<td>0.496</td>
</tr>
<tr>
<td>rs17311169</td>
<td>78912442</td>
<td>0.312 (C)</td>
<td>0.942</td>
<td>79:63 (C:T)</td>
<td>1.803</td>
<td>0.179</td>
</tr>
<tr>
<td>rs451056</td>
<td>78929186</td>
<td>0.320 (G)</td>
<td>0.451</td>
<td>77:68 (G:C)</td>
<td>0.559</td>
<td>0.455</td>
</tr>
<tr>
<td>rs7629522</td>
<td>78929347</td>
<td>0.339 (T)</td>
<td>1.000</td>
<td>95:94 (C:T)</td>
<td>0.005</td>
<td>0.942</td>
</tr>
<tr>
<td>rs1031932</td>
<td>78945847</td>
<td>0.423 (T)</td>
<td>0.944</td>
<td>70:62 (T:C)</td>
<td>0.485</td>
<td>0.486</td>
</tr>
<tr>
<td>rs162429</td>
<td>78995892</td>
<td>0.255 (C)</td>
<td>1.000</td>
<td>47:43 (C:T)</td>
<td>0.178</td>
<td>0.673</td>
</tr>
<tr>
<td>rs162263</td>
<td>79019090</td>
<td>0.228 (A)</td>
<td>0.429</td>
<td>51:47 (A:C)</td>
<td>0.163</td>
<td>0.686</td>
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<tr>
<td>rs189022</td>
<td>79038893</td>
<td>0.245 (T)</td>
<td>0.588</td>
<td>59:53 (T:A)</td>
<td>0.321</td>
<td>0.571</td>
</tr>
<tr>
<td>rs4680919</td>
<td>79065986</td>
<td>0.270 (G)</td>
<td>0.159</td>
<td>53:47 (G:A)</td>
<td>0.360</td>
<td>0.549</td>
</tr>
<tr>
<td>rs11925923</td>
<td>79108258</td>
<td>0.186 (A)</td>
<td>1.000</td>
<td>92:78 (G:A)</td>
<td>1.153</td>
<td>0.283</td>
</tr>
<tr>
<td>rs11920038</td>
<td>79310241</td>
<td>0.489 (C)</td>
<td>0.530</td>
<td>71:64 (C:T)</td>
<td>0.363</td>
<td>0.547</td>
</tr>
<tr>
<td>rs17395825</td>
<td>79324469</td>
<td>0.243 (T)</td>
<td>0.824</td>
<td>26:25 (C:T)</td>
<td>0.020</td>
<td>0.889</td>
</tr>
<tr>
<td>rs6769328</td>
<td>79440078</td>
<td>0.074 (G)</td>
<td>1.000</td>
<td>22:18 (G:C)</td>
<td>0.400</td>
<td>0.527</td>
</tr>
<tr>
<td>rs12629799</td>
<td>79455525</td>
<td>0.059 (G)</td>
<td>1.000</td>
<td>19:18 (A:G)</td>
<td>0.027</td>
<td>0.869</td>
</tr>
<tr>
<td>rs7617893</td>
<td>79459699</td>
<td>0.061 (C)</td>
<td>1.000</td>
<td>93:90 (T:C)</td>
<td>0.049</td>
<td>0.825</td>
</tr>
<tr>
<td>rs4550794</td>
<td>79501790</td>
<td>0.409 (A)</td>
<td>0.665</td>
<td>86:85 (T:A)</td>
<td>0.006</td>
<td>0.939</td>
</tr>
<tr>
<td>rs9853895</td>
<td>79526435</td>
<td>0.487 (T)</td>
<td>0.791</td>
<td>47:41 (T:C)</td>
<td>0.409</td>
<td>0.522</td>
</tr>
<tr>
<td>rs9815393</td>
<td>79667847</td>
<td>0.136 (G)</td>
<td>0.216</td>
<td>95:94 (C:G)</td>
<td>0.005</td>
<td>0.942</td>
</tr>
<tr>
<td>rs9876243</td>
<td>79669081</td>
<td>0.423 (C)</td>
<td>0.944</td>
<td>49:41 (C:T)</td>
<td>0.711</td>
<td>0.399</td>
</tr>
<tr>
<td>rs9882958</td>
<td>79682156</td>
<td>0.135 (C)</td>
<td>0.224</td>
<td>27:24 (C:G)</td>
<td>0.176</td>
<td>0.674</td>
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<tr>
<td>rs9828140</td>
<td>79687183</td>
<td>0.071 (C)</td>
<td>0.736</td>
<td>49:41 (C:T)</td>
<td>0.711</td>
<td>0.399</td>
</tr>
<tr>
<td>rs12714482</td>
<td>79687531</td>
<td>0.135 (G)</td>
<td>0.224</td>
<td>18:18 (A:G)</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4-3. Quantitative Trait Analysis for the Genotyped ROBO1 SNPS

<table>
<thead>
<tr>
<th>SNP</th>
<th>WISC-III Digit Span Backwards (n=418)</th>
<th>CMS Numbers Backwards (n=588)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs333491</td>
<td></td>
<td>0.0194</td>
</tr>
<tr>
<td>rs4680960</td>
<td>0.0199</td>
<td></td>
</tr>
<tr>
<td>rs4130991</td>
<td>0.0194</td>
<td></td>
</tr>
<tr>
<td>rs7653197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4535189</td>
<td>0.0130</td>
<td></td>
</tr>
<tr>
<td>rs6803202</td>
<td>0.0116</td>
<td></td>
</tr>
<tr>
<td>rs7628757</td>
<td>0.0173</td>
<td></td>
</tr>
<tr>
<td>rs4564923</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4264688</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6548621</td>
<td>0.0068</td>
<td></td>
</tr>
<tr>
<td>rs7622444</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9853895</td>
<td>0.0028</td>
<td></td>
</tr>
<tr>
<td>rs9857859</td>
<td>0.0090</td>
<td></td>
</tr>
<tr>
<td>rs7429525</td>
<td></td>
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</tr>
<tr>
<td>rs7644521</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7629503</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only p-values <0.01 were reported. The threshold for significance was p=0.001. The other subtests did not yield any results with p<0.01 (CTOPP Non-Word Repetition, WISC-III Digits Span Forwards, CMS Numbers Forwards, WRAT-III Reading and Spelling).
### Table 4-4. Haplotype Analysis (Block 1) for the WISC Digit Span Backwards Measure

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Freq</th>
<th>Fam#</th>
<th>S-E(S)</th>
<th>Var(S)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4680960</td>
<td>rs4130991</td>
<td>rs7653197</td>
<td>rs4535189</td>
<td>rs6803202</td>
<td>rs7628757</td>
<td>rs4564923</td>
</tr>
<tr>
<td>G</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

### Table 4-5. Haplotype Analysis (Block 2) for the WISC-III Digit Span Backwards Measure

<table>
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<tr>
<th>Haplotype</th>
<th>Freq</th>
<th>Fam#</th>
<th>S-E(S)</th>
<th>Var(S)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6548621</td>
<td>rs7622444</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>0.410</td>
<td>144</td>
<td>71.993</td>
<td>706.973</td>
<td>2.708</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>0.397</td>
<td>147</td>
<td>-39.327</td>
<td>577.750</td>
<td>1.636</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>0.193</td>
<td>94</td>
<td>-35.173</td>
<td>412.028</td>
<td>-1.733</td>
</tr>
</tbody>
</table>

### Table 4-6. Haplotype Analysis (Block 3) for the WISC-III Digit Span Backwards Measure

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Freq</th>
<th>Fam#</th>
<th>S-E(S)</th>
<th>Var(S)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9853895</td>
<td>rs9857859</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>0.593</td>
<td>147</td>
<td>-76.5</td>
<td>767.25</td>
<td>2.762</td>
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<tr>
<td>T</td>
<td>T</td>
<td>0.404</td>
<td>146</td>
<td>77.0</td>
<td>767.00</td>
<td>2.780</td>
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</tbody>
</table>
Chapter 5 – Summary of Findings, Overall Discussion and Future Directions

5.1 Overall Discussion

*DYX1C1* and *ROBO1* are two prominent RD candidate genes that have a biologically plausible role in the development of RD. Both genes are located within regions that have shown replicated linkage with RD and have also previously shown association with RD or quantitative reading measures. However, the findings of this study did not support the hypothesis that *DYX1C1* and *ROBO1* SNPs would be associated with RD and quantitative reading traits.

In the *DYX1C1* study, we focused on the -3G/A and 1249G/T SNPs because of their potential effect on *DYX1C1* expression levels and function. The -3G/A polymorphism was thought to affect gene translation because it is located in the Kozak consensus sequence (Taipale et al., 2003). This sequence, which occurs on eukaryotic mRNA, lies upstream of the start codon and is important for the initiation of translation. In a study of 699 vertebrate mRNAs, the consensus sequence was elucidated to be (GCC)CC(G/A)CCAUGG, with AUG serving as the translation start site (Kozak, 1987). The most highly conserved position within the Kozak sequence is at -3G/A (3 positions upstream of the start codon), with 97% of vertebrates carrying a purine at this position (61% of transcripts carried an adenine and 36% carried a guanine). While this position is conserved, it is not specific to guanine, and it is only crucial to carry a purine for efficient translation (Kozak, 1989). Thus, individuals who carry the -3A allele in *DYX1C1* may not have a drastically altered protein expression levels. However, further studies would need to be performed to confirm whether this specific position has an effect on *DYX1C1* protein expression levels.

The 1249T allele in *DYXIC1* introduces a premature stop codon which was thought to have a potentially functional effect on *DYX1C1* (Taipale et al., 2003). Knockdown and rescue
experiments showed that the shortened *DYX1C1* protein resulting from the 1249T allele was able to recover the normal phenotype, but this ectopic expression assay may not be representative of normal endogenous conditions (Wang et al., 2006). Premature stop codons can be produced by nonsense mutations in the coding region or by errors in mRNA splicing. In addition to altering the protein product, premature stop codons may induce nonsense mediated decay (NMD), a mRNA surveillance mechanism that triggers the degradation of mRNAs carrying premature stop codons (Chang et al., 2007). However, NMD applies to nonsense mutations occurring >50-55 nucleotides upstream of an exon-exon junction, referred to as the '50 nucleotide rule' (Nagy and Maquat, 1998). It is unlikely that the 1249T allele triggers NMD as it is located in the last exon. Nonetheless, there are other effects that may be triggered with the 1249T allele in addition to changes in protein structure.

In the Toronto sample we genotyped *DYX1C1* and *ROBO1* SNPs that showed nominal significance in previous studies. The Calgary sample was also genotyped for the same SNPs in *DYX1C1* but for the *ROBO1* study, we analyzed SNPs that were genotyped across the gene from a previous genome-wide scan. Although we did not find statistically significant association in the analyses, other SNPs in *ROBO1* may potentially affect RD susceptibility, and the observed between-study heterogeneity may be due to the differences in LD between samples. Future studies with a more thorough coverage of tag SNPS across *ROBO1* may help to determine whether polymorphisms in *ROBO1* are associated with RD. In the field of RD, large-scale GWAS have not yet been performed to test for association with RD and reading-related traits. Future GWAS that analyze markers across the genome may be informative in confirming previous associations, or identifying new candidate genes.
Alternatively, an association was not observed in the *DYX1C1* and *ROBO1* SNPs because the effect size of the causal loci was too small to be detected with the utilized samples. With complex traits and diseases, it is likely that many genes contributing small effect sizes underlie susceptibility (Yang et al., 2005). The purpose of the meta-analysis was to increase the level of statistical power by combining results from multiple samples. Compared to the individual Toronto and Calgary samples, the meta-analysis sample had adequate power to detect a smaller effect size for a SNP conferring susceptibility to RD (odds ratio of 1.3 compared to 1.8 and 1.9 for the Toronto and Calgary samples). The importance of statistical power highlights the potential effectiveness of consortiums, which would facilitate the collection of large sample sizes and allow the detection of variants with modest effects.

An important limitation in this study was the issue of between-study heterogeneity. In the meta-analysis of *DYX1C1*, there was considerable heterogeneity in the individual effect-size estimates which may be due to the differences in study design. This may also be due to genetic or allelic heterogeneity, where different risk genes or alleles influence RD in different subjects. Consistent effects need to be observed in order to generalize the findings of a meta-analysis and thus, the pooled effect may not be applicable to the studied populations (Higgins et al., 2003).

Although we did not find an association between the -3G/A and 1249G/T in the meta-analysis, the presence of between-study heterogeneity likely reduced the power of the combined sample. In the *ROBO1* analysis, we tested whether previous associations in a population-based twin sample could be replicated in a RD sample. However, the differences between the sample characteristics and the reading tests that were administered may have accounted for the lack of replication.
One possible reason for the observed between-study heterogeneity is that there is no current ‘gold standard’ test to diagnose and define RD. Although there is a general agreement that phonological processing deficits contribute to reading difficulties, the methods to test for reading problems can differ between studies. For example, the samples that were included in the meta-analysis diagnosed RD using combinations of tests for text reading (measuring reading accuracy and speed), single word and non-word reading, and spelling. In the English-language studies, there was considerable variation in the reading tests that were administered. Using different reading tests and assessing different reading skills, which involve different subcomponents, may result in different RD phenotypes between studies. Greater consistency in the measurement of reading ability can reduce the levels of between-study heterogeneity. However, the practical and financial considerations to reach a consensus definition and implement the tests in existing samples would remain a great challenge.

Correction for multiple testing was also a relevant issue in the analyses of both genes. For example, several *DYX1C1* and *ROBO1* SNPs reported p-values of <0.05 in their association with different quantitative reading measures but these were not statistically significant after correction for multiple testing. Since multiple SNPs and phenotypes were tested in both studies, it was important to adjust the threshold for statistical significance to prevent the inflation of type-I error. In this study, Bonferroni correction was applied with careful consideration to only correct for the number of independent tests. However, the *ROBO1* study by Bates et al. (2011), which found statistically significant association of two SNPs with a measure of phonological memory, only corrected for number of analyzed SNPs. This may have affected the threshold for significance and may have also accounted for the positive findings in the previous study. It is not known whether correcting for the multiple phenotypes in the study by Bates et al. (2011) would have changed the statistical significance of the positive SNPs. Nonetheless, the different methods
of correcting for multiple testing in both studies may have influenced the interpretation of the observed results.

The analysis of *DYX1C1* and *ROBO1* illustrates the importance of critically analyzing the literature, particularly in genetic association studies. In a previous study that performed metanalyses of 36 different genetic associations for various diseases, it was commonly observed that the initial findings suggested a stronger effect than subsequent studies (Ioannidis et al., 2001). Another study that examined 166 positive genetic associations found only 6 were consistently replicated in subsequent studies (Hirschhorn et al., 2002). In the case of *DYX1C1*, it may have been the bias and small sample size in the initial study that resulted in the stronger effect size of -3G/A and 1249G/T, compared to subsequent studies. The *ROBO1* analysis and its comparison to a previous association study highlighted how different analytical methods may have contributed to different results. In addition, questions raised from the *ROBO1* expression studies suggest that further studies are required to clarify whether *ROBO1* expression levels are associated with RD. Additional studies testing for association between *ROBO1* and RD, as well as reading-related measures, are required to determine if the non-significant findings of this study are consistent with other RD samples. By assessing the literature and understanding the cumulative evidence, we may be able to better distinguish spurious findings from true associations.

In this study, we tested for association with both discrete (RD) and continuous (quantitative reading-related measures) traits. RD can be considered the endpoint, where the individual shows significant difficulty in learning to reading. However, as discussed in Chapter 1.2, deficits in different cognitive skills may lead to RD. The identification of multiple RD linkage regions also suggests that different genes may contribute to the reading problems in different individuals. Studies of RD have increasingly used quantitative association methods to
evaluate the association of SNPs with cognitive components of reading (Fisher and DeFries, 2002). RD is a complex trait and there may be multiple pathways that ultimately cause reading difficulties. Quantitative trait analyses take into account the variation in different cognitive skills and allow us to identify genes that may affect specific deficits that are observed in individuals with RD.

Unlike oral language, which is a naturally acquired skill, the development of reading only occurs with instruction. Therefore, the environment in which a child is raised in is an important determinant of reading ability. Print exposure, for example, has been linked to orthographic processing skills (Stanovich and West, 1989; Chateau and Jared, 2000). To better understand variation of reading ability in a population, the relationship between environmental and genetic factors contributing to the development of reading must be considered. Gene-environment correlation (rGE) refers to the effects of genetics on the environment or behaviours, which have been well documented in complex traits (Plomin and Bergeman, 1991; Kendler and Baker, 2007). In reading, it is possible that genetic risk factors may affect the level of print material in the household environment, which would in turn affect a child’s print exposure. Gene-environment interactions (GxE) occur when the influence of genetic factors is affected by environmental exposures (Plomin et al., 1977). In twin studies, reading achievement was found to have a greater genetic influence when children received higher quality of teacher instruction, and have a greater genetic influence with greater levels of parental education (Friend et al., 2008; Taylor et al., 2010). By understanding the role of rGE and GxE on reading development, we can better understand the combinations of genetic and environmental risk factors that may lead to RD. While genetic association studies have been performed to identify genetic risk factors in RD, rGE and GxE studies may help in identifying which individuals carrying the risk variants are at highest risk for reading difficulties, depending on their environmental exposures. This
would allow a more efficient allocation of educational resources, and children who are at risk can be targeted and remediated sooner.

### 5.2 Future Directions

This study did not find association between *DYX1C1* SNPs and RD. However, this does not exclude chromosome 15q15-21 (DYX1) as a susceptibility region, and it is possible that other genes within DYX1 may be associated with RD or quantitative reading measures. The *protogenin* (*PRTG*) gene, also located on chromosome 15q21, was previously found to be associated with attention deficit hyperactivity disorder (ADHD) in a family-based sample (Wigg et al., 2008). ADHD and RD are often comorbid, with 20-40% of individuals ascertained for ADHD being affected with RD (August and Garfinkel, 1990; Dykman and Ackerman, 1991; Del'Homme et al., 2007). Twin studies have suggested shared genetic influences between the inattention symptom of ADHD and reading difficulties (Willcutt et al., 2000; Willcutt et al., 2007), and a whole-genome linkage scan of sibpair families with ADHD found linkage to chromosome 15q (Bakker et al., 2003). Since *PRTG* has been implicated in ADHD, it may also have pleiotropic effects and future studies may include testing for association with RD.

Previous studies of *ROBO1* have suggested association with RD, and that individuals with RD have lower expression of *ROBO1* in lymphocytes. For complex diseases it is likely that differences in gene expression, rather than changes in the coding region, may contribute to disease risk (Dermitzakis, 2008). These differences in gene expression may be generated from SNPs that are located within cis-regulatory elements. In the study by Hannula-Jouppi et al. (2005), affected individuals carrying a rare haplotype spanning *ROBO1* showed lower allelic expression relative to individuals carrying the normal haplotype. Although we were not able to
replicate the previous association findings, a future study may test for allelic ROBO1 expression in individuals with RD. One method of measuring allele-specific expression is through pyrosequencing, which compares allele-specific expression levels in heterozygous individuals at a specific SNP (Wang and Elbein, 2007). Using the RD sample, we can test whether affected individuals show imbalanced allelic expression at specific ROBO1 loci, which would suggest that a SNP may be involved in the regulation of expression or is in LD with the regulatory region. Similar to the methods of Hannula-Jouppi et al. (2005), lymphocyte samples are available for a subset of the probands in the Toronto sample, and we will investigate whether the previous findings can be replicated.
References


Kidd, T., K. Brose, et al. (1998). "Roundabout controls axon crossing of the CNS midline and


Laird, N. M., S. Horvath, et al. (2000). "Implementing a unified approach to family-based tests of
association." Genet Epidemiol 19 Suppl 1: S36-42.

Lamminmaki, S., S. Massinen, et al. (2012). "Human ROBO1 regulates interaural interaction in


Lander, E. S. and N. J. Schork (1994). "Genetic dissection of complex traits [published erratum


Li, J. and L. Ji (2005). "Adjusting multiple testing in multilocus analyses using the eigenvalues of a
correlation matrix." Heredity (Edinb) 95(3): 221-227.

dyslexia in Italy and the United States." Child Dev 56(6): 1404-1417.


Lovett, M. W., K. A. Steinbach, et al. (2000). "Remediating the core deficits of developmental


memory." J Learn Disabil 17(10): 592-599.

DYX1C1 in developmental dyslexia." Genes Brain Behav.


