**DRD2 and PPP1R1B (DARPP-32) polymorphisms independently confer increased risk for autism spectrum disorders and additively predict affected status in male-only affected sib-pair families**

Joe A Hettinger1, Xudong Liu2,3, Melissa L Hudson2,3,4, Alana Lee2,3,4, Ira L Cohen4,5, Ron C Michaelis6, Charles E Schwartz7, Suzanne ME Lewis4,8,9 and Jeanette JA Holden1,2,3,4,10,11*

**Abstract**

**Background:** The neurotransmitter dopamine (DA) modulates executive functions, learning, and emotional processing, all of which are impaired in individuals with autism spectrum disorders (ASDs). Our previous findings suggest a role for dopamine-related genes in families with only affected males.

**Methods:** We examined two additional genes which affect DA function, the DRD2 and PPP1R1B (DARPP-32) genes, in a cohort of 112 male-only affected sib-pair families. Selected polymorphisms spanning these genes were genotyped and both family-based and population-based tests were carried out for association analysis. General discriminant analysis was used to examine the gene-gene interactions in predicting autism susceptibility.

**Results:** There was a significantly increased frequency of the DRD2 rs1800498TT genotype ($P = 0.007$) in affected males compared to the comparison group, apparently due to over-transmission of the T allele ($P = 0.0003$). The frequency of the PPP1R1B rs1495099CC genotype in affected males was also higher than that in the comparison group ($P = 0.0002$) due to preferential transmission of the C allele from parents to affected children ($P = 0.0009$). Alleles rs1800498T and rs1495099C were associated with more severe problems in social interaction ($P = 0.0002$ and $P = 0.0016$, respectively) and communication ($P = 0.0004$ and $P = 0.0046$), and increased stereotypic behaviours ($P = 0.0021$ and $P = 0.00072$). General discriminant analysis found that the DRD2 and PPP1R1B genes additively predicted ASDs ($P = 0.00011$; Canonical R = 0.26) and explain ~7% of the variance in our families. All findings remained significant following corrections for multiple testing.

**Conclusion:** Our findings support a role for the DRD2 and PPP1R1B genes in conferring risk for autism in families with only affected males and show an additive effect of these genes towards prediction of affected status in our families.

**Keywords:** Autism spectrum disorders, Dopamine receptors, DARPP-32, Association study, Candidate gene
Introduction
Autism spectrum disorders (ASDs) are characterized by repetitive behaviours and interests, as well as deficiencies in communication and social interaction. They are believed to be complex, polygenic disorders predominantly characterized by multifactorial inheritance [1], although Zhao et al. [2] (2007) suggested that Mendelian inheritance may apply to autism risk in a subgroup of families with affected males. To address the significant genetic heterogeneity and phenotypic variation seen among affected individuals, which has confounded the conclusive identification of candidate genes for the majority of cases, we have been testing genes for evidence of association with specific ASD endophenotypes in an effort to identify a subgroup within the ASD population whose members share an underlying pathophysiology.

Abnormalities in neurotransmitter pathways can account for the deficits seen in persons with ASDs. In contrast to the attention which has been directed to the study of genes involved in the glutamate [3], GABA [3] and serotonin pathways [1], genes related to the synthesis, function and metabolism of dopamine (DA) have received little attention [4].

We have argued [5] that genes in the dopaminergic (DAergic) pathway are excellent candidates based on their affect on ASD behaviours. DA modulates motor functions [6], cognitive processes (including executive functions [7] and learning [8]), and emotional regulation [9] - all of which are abnormal in individuals with autism [10-13]. DA also plays a role in social interactions [14] and the pathophysiology of stereotypies [15]; impairments in social interaction and the presence of increased stereotypies are core features of autism. Furthermore, there is decreased DAergic activity in the medial prefrontal cortex (PFC) in children with autism [16], and increased levels of the major metabolite of DA, homovanillic acid (HVA), in cerebrospinal fluid from affected children compared to controls [17], indicating altered DAergic function in these individuals.

Based on our earlier findings on the dopamine β-hydroxylase (DBH) gene, which encodes the enzyme that converts DA to norepinephrine, in mothers from male-only affected sib-pair families [18], we have pursued a comprehensive study of DA-related genes in mothers and sons with ASDs. Since our initial study with DBH, in which we found an increased frequency of the 19-bp deletion in mothers from male-only affected sib-pair families [18], we identified a 3-marker risk haplotype in the dopamine D1 receptor (DRD1) gene [5] in our family cohort having only affected sons. Here we report our findings on two other genes affecting DA levels and function, the dopamine D2 receptor (DRD2) and protein phosphatase 1, regulatory subunit 1B (PPP1R1B) genes, and results of tests for gene-gene interactions.

The DRD2 gene comprises eight exons [19] and maps to 11q22-q23 [20]. It encodes the dopamine D2 receptor which, in addition to its role in postsynaptic neurons, acts as an autoreceptor mediating DA synthesis [21] and neurotransmission [22] in DAergic neurons. The dopamine D2 receptor is involved in the DAergic modulation of executive functions [23], reversal learning [24] and emotional processing [25]. Drd2−/− mice have abnormal gait similar to that of individuals with Parkinson disease [26], and the administration of antipsychotic medications (e.g., risperidone, a dopamine D2 receptor antagonist) has proven efficacious in treating symptoms associated with ASDs [27].

The PPP1R1B gene, located at chromosome 17q12 and comprising 7 exons (http://www.ncbi.nlm.nih.gov; Gen-eID 84152), encodes DARPP-32, which is expressed in dopaminergic (DAptive) neurons [28] and mediates the effects of both D1 and D2 dopamine receptor classes [29]. For example, dopamine D2 receptor antagonist-induced catalepsy in rats is attenuated in Ppp1r1b−/− mice [30] and knockout mice are impaired in reversal learning [31]. Genetic [32,33] and immunoblot [34] studies showed an association of PPP1R1B with altered PFC DARPP-32 protein levels in schizophrenia and bipolar disorder, two conditions for which DA dysfunction is evident and which exhibit comorbidity with autism [35].

There are two previous studies which examined the DRD2 gene as a candidate gene for autism. The first [36] reported an increased frequency of the TaqI A1 allele in persons with autism (N = 33) compared to controls (N = 314), whereas the second [37] found no evidence for transmission disequilibrium of an intragenic microsatellite in 39 affected sib-pair families. No association studies have examined the role of PPP1R1B as a candidate gene for ASDs.

Based on our hypothesis that DA-related genes are important in male-only affected sib-pair families [18], we examined four markers at the DRD2 locus that are commonly used to investigate possible associations between DAergic function and behavioural abnormalities [38], and three polymorphisms at the PPP1R1B locus to determine whether there was an association of these DA-related genes with autism.

Materials and methods
Subjects
The 112 affected sib-pair families and the comparison group (N = 443) were previously described [5]. Briefly, the study group included 28 families from Canada [18], 5 from the South Carolina Autism Project [39], and 79 families obtained through the Autism Genetic Resource Exchange (AGRE) in the United States [40]. All families have two or more children with either autism or an ASD, including Asperger syndrome and pervasive
developmental disorder (PDD) variants. This study was approved by the Queen’s University Research Ethics Board and written informed consent was obtained from parents of all participating families from Canada and South Carolina, and through AGRE [40].

All 443 samples from the comparison group (blood-spots from anonymous newborns taken for the purpose of PKU testing and made available from the Ontario Ministry of Health) were available for the study of the PPP1R1B locus. Two hundred and fifty-three comparison group samples were used for the DRD2 gene studies. There were no significant differences in the allele frequencies for DRD2 \((P = 0.57–0.93)\) and PPP1R1B \((P = 0.28–0.91)\) markers in males and females from the respective comparison cohorts and thus our comparison cohorts included both males and females. Although comprehensive information regarding psychiatric and behavioural disorders is not available for the comparison group, we do not expect the prevalence of ASDs in this comparison group to be greater than that in the general population, or approximately 1/110 [41].

Marker amplification and genotyping

**DRD2**

Four polymorphisms (rs1799732 Ins/Del, rs1079597 G/A, rs1800498 T/C and rs1800497 C/T) were studied at the DRD2 locus (Figure 1). The DRD2 gene contains four haplotype blocks [Haploview 4.1; available at http://hapmap.ncbi.nlm.nih.gov] [42]. Three of the blocks are small (<4 kb) and one block is 20 kb in size. One of four polymorphisms used in this study, rs1079597, is part of the HapMap dataset. Both this SNP and rs1800498 are located within the larger haplotype block and were examined in previous studies of other neuropsychiatric disorders. Primer sequences, PCR and digestion conditions are shown in Table 1. All PCR reactions were carried out using 5 ng of template DNA; digestion products were separated on either 2% (rs1079597, rs1800498 and rs1800497) or 2.5% (rs1799732) agarose gels and visualized using ethidium bromide and UV illumination. In order to minimize genotyping errors, DNAs from affected individuals and family members were randomly arranged on 96-well plates, and all results were independently scored and tabulated by two persons.

**PPP1R1B**

Three polymorphisms (rs1495099 G/C, rs907094 T/C and rs3764352 A/G) were examined in the PPP1R1B gene (Figure 2). These variants were chosen from the NCBI dbSNP Build 121 database from the Human Genome Project (available at http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) based on the following criteria: the markers span the PPP1R1B locus, they have minor allele frequencies (MAFs) of approximately 20%, and alleles at rs907094 and rs3764352 are associated with changes in DARPP-32 mRNA expression and measures of cognitive performance [32]. The PPP1R1B locus has a single haplotype block which includes rs907094 and rs3764352 as haplotype-tagged SNPs (htSNPs). PCR reactions were carried out using 5 ng of template DNA and amplicons were digested using conditions shown in Table 1. All digestion products were separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. In order to minimize genotyping errors, DNAs from affected individuals and family members were randomly arranged on 96-well plates, and all results were independently scored and tabulated by two persons.
Statistical analyses

Prior to carrying out analyses, Mendelian errors were checked in the family cohort using the FBAT program, v1.5.5 [44]. DRD2 marker data on five families and PPP1R1B marker data on two families were excluded from the analyses due to identified Mendelian errors. Single gene analyses were performed as previously described [5]. To avoid allele and genotype frequency distortion from using related individuals in case–control comparisons, one affected individual was selected at random from each family using SPSS v14.0 (SPSS, Chicago, IL) with the same cohort of randomly chosen individuals used for single marker allele and genotype frequency comparisons for all polymorphisms as well as general discriminant analyses. Family-based association tests (FBAT), including quantitative disequilibrium tests (QTDT), were done using FBAT v1.5.5. Because FBAT v1.5.5 can accommodate multiple affected individuals from each family, all affected individuals including those used for case–control comparisons, were included for FBAT and QTDT analyses. The domains, ‘reciprocal social interaction,’ ‘communication’ and ‘repetitive stereotyped behaviours’ used for QTDT analyses were derived from the total scores from the ‘Qualitative Abnormalities

Table 1 PCR and digestion conditions for DRD2 and PPP1R1B markers used in this study

| DRD2 Marker (5′ → 3′) | Primers | [MgCl₂] | Annealing temperature | # of cycles | Restriction enzyme (U)² |
|----------------------|---------|---------|-----------------------|-------------|-----------------------|
| rs1799732 Ins/Del    | F 5′-GAGAAGACTGGGAGCAGAC-3′ R 5′-CCACCAAGGGCGCTGACCT-3′ | 1.5 mM | 63°C | 35 | BstNI (0.05) |
| rs1079597 G/A³       | F 5′-GATACCCACTCAGGAGA-3′ R 5′-CATGAAAGAAGCTGACGTACAG-3′ | 1.0 mM | 55°C | 34 | TaqI (0.4) |
| rs1800498 T/C³       | F 5′-CCCAGCAGGAGGAGGAAGTA-3′ R 5′-GACAATGACTTGTGAAGCATG-3′ | 1.0 mM | 55°C | 34 | TaqI (0.4) |
| rs1800497 C/T³       | F 5′-CCGTTCGACCGCTGGCGCAATGTGTC-3′ R 5′-CCGTCGACCTCCTGAGTGCATCA-3′ | 1.0 mM | 58°C | 34 | TaqI (0.4) |

| PPP1R1B Marker (5′ → 3′) | Primers | [MgCl₂] | Annealing temperature | # of cycles | Restriction enzyme (U)² |
|-------------------------|---------|---------|-----------------------|-------------|-----------------------|
| rs1495099 G/C          | F 5′-TTGTTGCTGAGCTGAGATGC-3′ R 5′-CTCCAGGGAATGCAAAAG-3′ | 1.0 mM | 60°C | 35 | PvuII (0.3) |
| rs907094 T/C           | F 5′-ACCAGAAGGCCGAGAGAGCTG-3′ R 5′-GAAGTCGAGGGCGCTGACGTACAG-3′ | 1.0 mM | 60°C | 34 | MseI (0.3) |
| rs3764352 A/G          | F 5′-CTGTTTTGGAGGGGCTGTC-3′ R 5′-TGGAATCTGAGAAGATGC-3′ | 1.0 mM | 60°C | 35 | BccI (0.3) |

¹Rs1799732 Ins/Del, rs1079597 G/A, rs1800498 T/C, and rs1800497 C/T previously known as –141 C Ins/Del, TaqI B, TaqI D, and TaqI A, respectively.
²Restriction enzymes obtained from New England Biolabs, Pickering, ON, Canada.
³Primer sequences have been reported previously [43].

Figure 2 Illustration of the PPP1R1B locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs1495099, rs907094 and rs3764352 listing r² of the comparison group (N=435) followed by r² of parents from families (N=216). Legend: ■ exon; □ intron; □ untranslated region.

0.67 0.72
0.68 0.73
0.94 0.97
0.94 0.97
9.7kb

rs1495099
rs907094
rs3764352
in Reciprocal Social Interaction’ (A1 to A4), ‘Qualitative Abnormalities in Communication’ (B1, B2(V), B3(V) and B4) and ‘Restricted, Repetitive, and Stereotyped Patterns of Behaviour’ (C1 to C4) subdomains in the ADI-R diagnostic algorithm [45].

General Discriminant Analysis (GDA) was used to evaluate the predictive value of our single gene findings in discriminating between individuals with and without autism, as well as to test for evidence of interaction effects between genes. Genotypes were coded as categorical variables and GDA was performed using Statistica 9.1 [Statsoft, Tulsa, OK, USA].

Corrections for multiple comparisons
The contribution of a single gene to autism susceptibility is predicted to be relatively small and thus difficult to detect statistically. Although corrections for multiple testing must be made in genetic association studies, Bonferroni correction is thought to be too stringent, with a high risk for rejecting true significant findings. The false discovery rate (FDR) approach [46] is a compromise between not correcting for multiple comparisons, which is too lax, and Bonferroni adjustments, which are too strict. FDR has two methods, the Benjamini and Hochberg (BH) method and the Benjamini and Liu method (BL) [46]. The BH method is appropriate for correcting for both independent and positively-dependent comparisons [47], and thus is appropriate for genetic studies using polymorphisms.

FDR corrections were performed separately for single gene case–control and family-based comparisons, as well as GDA findings, using an initial FDR threshold of 0.050.

Results
Linkage disequilibrium of polymorphisms at the DRD2 and PPP1R1B loci
High \( r^2 \) measures of linkage disequilibrium (LD) were observed between DRD2 polymorphisms rs1079597 and rs1800497, with no LD between rs1799732 and rs1079597 and rs1800497 (Figure 1). R-squared measures between PPP1R1B markers showed high LD (\( r^2 > 0.9 \)) between rs907094 and rs3764352, and lower LD between rs1495099 and rs907094 and rs3764352 (Figure 2).

Case-control comparisons
All four markers of DRD2 were in HWE in the comparison and family cohorts with the exception of rs1799732 and rs1800498 in affected males (\( P = 0.009 \) and \( P = 0.012 \), respectively); all markers were in HWE in the parents from these families. As shown in Table 2, there was an increased frequency of the rs1800498 TT genotype (\( P = 0.007 \)) in affected males (43.4% versus 28.7% in the comparison group), which remained significant following FDR correction. No significant differences in genotype frequencies of the other three markers were seen between cases and the comparison group (\( P = 0.32–0.51 \)) (Table 2). Separate analyses using the extended-transmission disequilibrium test (ETDT) showed that significant over-transmission of the rs1800498 T allele was not from mothers (21 transmitted, 11 untransmitted; \( \chi^2 = 3.125, df = 1, P = 0.077 \)) but was from fathers (26 transmitted, 12 untransmitted; \( \chi^2 = 5.158, df = 1, P = 0.023 \)) to affected sons. No differences in rs1800498 T allele or rs1800498 TT genotype frequencies were found between mothers (\( P = 0.69 \) and \( P = 0.26 \), respectively) or fathers (\( P = 0.90 \) and \( P = 0.65 \), respectively) and the comparison group (data not shown).

All three PPP1R1B markers were in HWE in the comparison group; none were in HWE in the cohort of affected individuals (\( P = 0.008, P = 0.033 \) and \( P = 0.033 \), respectively), although all markers were in HWE in the parents (data not shown). As shown in Table 2, the rs1495099 CC (\( P = 0.002 \)), rs907094 CC (\( P = 0.028 \)) and rs3764352 GG (\( P = 0.025 \)) genotype frequencies were increased in affected males (22.0%, 14.5% and 14.5%, respectively) relative to the comparison group (9.9%, 6.9% and 6.7%, respectively). Findings on alleles of these polymorphisms were similar, with the minor allele frequencies of all three markers, rs1495099 C, rs907094 C and rs3764352 G, being increased in the affected males relative to the comparison group (\( P = 0.001, P = 0.014 \) and \( P = 0.021 \), respectively, all remained significant following FDR correction; data not shown).

Because we hypothesize that maternal effects including genetic factors may contribute to autism susceptibility in some autism families [18], we compared frequencies of rs1495099 C alleles and rs1495099 CC genotypes between mothers (33.2% and 12.7%, respectively) and the comparison group (28.7% and 9.9%, respectively) but found no significant differences (\( P = 0.19 \) and \( P = 0.39 \), respectively; data not shown).

Family-based association tests
FBAT showed that the DRD2 rs1800498 T allele was over-transmitted to affected males (\( P = 0.0003 \); significant following FDR correction), while no evidence of preferential allele transmission was found for the other three markers (\( P = 0.16–0.94 \)) (Table 3). The rs1799732 Ins - rs1079597 G - rs1800498 T - rs1800497 C (Ins-G-T-C) haplotype, consisting of the major alleles for all four markers, was over-transmitted to affected males but with a P-value (\( P = 0.0009 \); data not shown) slightly higher than that observed with rs1800498 T alone (\( P = 0.0003 \)). It should be noted that the additive model was used in FBAT for polymorphisms at the DRD2 locus because of the increased CT and TT genotype frequencies found for rs1800498.
For PPP1R1B, a recessive model was used in FBAT because of the increased frequency of rs1495099 CC, rs907094 CC and rs3764352 GG genotypes found in affected individuals compared to the comparison cohort. Family-based association analyses and FDR-based corrections showed significant over-transmission of rs1495099 C (P = 0.00092) but not of rs907094 C (P = 0.11) or rs3764352 G (P = 0.09) (Table 3). The rs1495099 C - rs907094 C - rs3764352 G (C-C-G) haplotype was not significantly over-transmitted to affected males (P = 0.031; not significant following FDR correction; data not shown) compared to that of rs1495099 C alone (P = 0.00092).

**Genotype-phenotype associations**

We next used quantitative transmission disequilibrium tests (QTDT) to determine whether the extent of impairment in the core behaviours was more pronounced in affected males with the risk allele. The DRD2 rs1800498 T allele was associated with more severe impairments in reciprocal social interaction (P = 0.0002), verbal communication (P = 0.0004), and repetitive and stereotyped behaviours (P = 0.0021); these findings remained significant following corrections for multiple comparisons.

The PPP1R1B rs1495099 C allele was associated with higher ADI-R domain scores (more severe problems) in affected males for social interaction (P = 0.0016), nonverbal communication (P = 0.0046), and stereotyped behaviours (P = 0.00072) (Table 4), with strong evidence for association shown by multivariate QTDT between rs1495099 C and the combined effect of all three ADI-R subdomains (P = 0.00042; data not shown). All findings were significant following FDR-based corrections.

**General discriminant analyses**

We used GDA to determine whether DA-related genes predict ASD susceptibility in affected males compared to the comparison group and to test for gene-gene interactions. Tests were performed based on our single gene findings for DRD2 and PPP1R1B from this study, and
our previous findings with DRD1 [5]. DRD2 rs1800498
genotypes and PPP1R1B rs1495099 genotypes each sig-
ificantly contributed to prediction of ASDs in our fam-
ilies (P = 0.0063 and P = 0.00086, respectively), as well as
when weighted and tested together (P = 0.00011; Can-
onical R = 0.26) (Table 5). We generated a classification
matrix to determine the percent correct classification of
individuals with and without autism based on
DRD2 rs1800498 and PPP1R1B rs1495099 genotypes, and
found that 97% of individuals from our comparison co-
hort and 13% of individuals with autism were correctly
classified (the analysis was based on the a priori baseline
frequency of 70% of individuals without autism and 30%
percent of individuals with autism). Using the weighted scores to
determine group membership, we found that 72% of control
individuals and 64% of affected individuals were pre-
dicted correctly using DRD2 and PPP1R1B genotypes.
However, while ~7% of the variance was explained with
DRD2 rs1800498 and PPP1R1B rs1495099 genotypes, addition of our previously identified DRD1 rs265981–
rs4532–rs686 haplotypes to the analyses did not result in
a significant improvement in the overall canonical cor-
relation (Canonical R = 0.27).

Adding all possible two-way interactions between
DRD2 rs1800498 and PPP1R1B rs1495099 genotypes, as
well as comparisons between DRD1 rs265981–rs4532–
rs686 haplotypes and DRD2 rs1800498 genotypes, and
DRD1 rs265981–rs4532–rs686 haplotypes and PPP1R1B
rs1495099 genotypes, we found no evidence for gene-
genre interactions (P = 0.35–0.75; data not shown).

Discussion
Our model for the involvement of the DA pathway in de-
termining some of the core deficits of ASDs is based on
earlier results implicating the DBH gene as a maternal
effect locus, and on our hypothesis that autism suscepti-
bility is determined by a combination of fetal susceptibil-
genre genes and fetal gender as well as maternal effects
including maternal genetic factors [18]. Following our
findings with the DRD1 gene [5], and as part of our
investigation to determine whether other DA-related genes are significant factors in the etiology of ASDs, we found evidence for association of the \textit{DRD2} and \textit{PPP1R1B} genes with autism in affected males from multiple-incidence families.

We found an increased frequency of the \textit{DRD2} rs1800498 TT genotype ($P = 0.007$) in affected males (43.4%) compared to the comparison group (28.7%) (Table 2), and the rs1800498 T allele was over-transmitted to affected children ($P = 0.0003$) (Table 3). The rs1800498 risk allele was associated with more severe impairments in social interaction ($P = 0.0002$), verbal communication ($P = 0.0004$) and stereotyped behaviours ($P = 0.0021$) in affected males (Table 4), and the rs1800498 TT genotype was associated with an increased risk for ASD with an OR of 1.9 [$95\% \text{ CI: } 1.5–2.5$]. In addition, we examined three polymorphisms at the \textit{PPP1R1B} locus and identified the rs1495099 C allele as a recessive risk allele for susceptibility to ASDs in male-only affected sib-pair families. The CC genotype frequency was increased in affected males (22.0%) relative to the comparison group (9.9%, $P = 0.002$), and family-based association tests using FBAT with a recessive model showed distorted allele transmission with over-transmission of this allele in families ($P = 0.00092$) (Table 3). This allele was associated with greater impairments in social interaction ($P = 0.0016$) and nonverbal communication ($P = 0.0046$), and more severe stereotyped behaviours ($P = 0.00072$), core features of ASDs. Finally, the rs1495099 CC genotype was associated with an increased risk for ASD with an OR of 2.6 [$95\% \text{ CI: } 1.9–3.6$]. All findings were significant following FDR-based corrections for multiple comparisons.

### Functional effects of \textit{DRD2} and \textit{PPP1R1B} risk alleles on gene expression

Our findings at the \textit{DRD2} and \textit{PPP1R1B} loci may reflect the functional effects of unidentified risk variants in LD with rs1800498 at \textit{DRD2} and rs1495099 at \textit{PPP1R1B}. Functional analyses of these markers have not been reported but \textit{in silico} analyses performed using PupaSuite [available at http://pupasuite.bioinfo.cipf.es/] [48] did not identify any putative functional role for these polymorphisms while analyses using FASTSNP [available at http://fastsnp.ibms.sinica.edu.tw/pages/input_CandidateGeneSearch.jsp] [49] predicted a ‘very low-to-low’ effect for rs1800498 at \textit{DRD2} as an intronic enhancer and a ‘very low-to-medium’ effect for rs1495099 at \textit{PPP1R1B} as a regulatory region/intronic enhancer. Meyer-Lindenberg et al. [32] (2007) identified a common 7-marker \textit{PPP1R1B} haplotype that was associated with increased DARPP-32 mRNA expression and improved performance on measures of working memory and cognitive flexibility. This haplotype included the T and A alleles of rs907094 and rs3754352 respectively, while a haplotype containing the minor alleles at these loci (i.e. rs907094 C and rs3754352 G) was associated with decreased mRNA expression in post-mortem brain. Houlihan et al. [50] (2009) screened this 7-marker haplotype to test \textit{PPP1R1B} as a genetic determinant of cognitive ageing and found that rs907094 C

| ADI-R Subdomain | \textit{DRD2} rs1800498 |
|-----------------|----------------------|
| Social          | T                    |
| rs1800498       | 56                   |
| Observed        | 2909.0               |
| Expected        | 2452.5               |
| Z               | 3.7                  |
| $P$             | 0.0002               |
| FDR threshold   | 0.017                |
| Interaction     | C                    |
| rs1800498       | 56                   |
| Observed        | 1557.0               |
| Expected        | 2013.5               |
| Z               | −3.7                 |
| $P$             | 0.0002               |
| Verbal          | T                    |
| rs1800498       | 46                   |
| Observed        | 1364.0               |
| Expected        | 1103.5               |
| Z               | 3.6                  |
| $P$             | 0.0004               |
| Communication   | C                    |
| rs1800498       | 46                   |
| Observed        | 666.0                |
| Expected        | 926.5                |
| Z               | −3.6                 |
| $P$             | 0.0004               |
| Stereotyped     | T                    |
| rs1800498       | 56                   |
| Observed        | 876.0                |
| Expected        | 754.5                |
| Z               | 3.1                  |
| $P$             | 0.0021               |
| Behaviours      | C                    |
| rs1800498       | 56                   |
| Observed        | 502.0                |
| Expected        | 623.5                |
| Z               | −3.1                 |
| $P$             | 0.0021               |

| ADI-R Subdomain | \textit{PPP1R1B} rs1495099 |
|-----------------|---------------------------|
| Social          | C                         |
| rs1495099       | 19                        |
| Observed        | 480.0                     |
| Expected        | 283.5                     |
| Z               | 3.2                       |
| $P$             | 0.0016                    |
| FDR threshold   | 0.017                     |
| Interaction     | G                         |
| rs1495099       | 36                        |
| Observed        | 535.0                     |
| Expected        | 579.5                     |
| Z               | −0.5                      |
| $P$             | 0.59                      |
| FDR threshold   | 0.042                     |
| Nonverbal       | C                         |
| rs1495099       | 10                        |
| Observed        | 108.0                     |
| Expected        | 52.3                      |
| Z               | 2.8                       |
| $P$             | 0.0046                    |
| FDR threshold   | 0.025                     |
| Communication   | G                         |
| rs1495099       | 20                        |
| Observed        | 130.0                     |
| Expected        | 123.3                     |
| Z               | 0.2                       |
| $P$             | 0.82                      |
| FDR threshold   | 0.050                     |
| Stereotyped     | C                         |
| rs1495099       | 19                        |
| Observed        | 142.0                     |
| Expected        | 79.8                      |
| Z               | 3.4                       |
| $P$             | 0.00072                   |
| FDR threshold   | 0.0083                    |
| Behaviours      | G                         |
| rs1495099       | 36                        |
| Observed        | 176.0                     |
| Expected        | 200.3                     |
| Z               | −0.9                      |
| $P$             | 0.38                      |
| FDR threshold   | 0.033                     |

\(^{1}\text{All affected males were included for QTDT analyses.}\)

\(^{2}\text{P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined.}\)

\(^{3}\text{P-value} \leq \text{FDR threshold is significant.}\)
and rs3754352 G alleles were associated with decreased cognitive ability. However, our findings do not support an association of either rs907094 C or rs3764352 G with autism in our family cohort. Unfortunately, because rs1495099 was not included in these studies, its functional role is not known. With respect to the DRD2 locus, the rs1800498 polymorphism was found to be in low LD with rs1799732, a functional variant in the DRD2 promoter [51], in our comparison group and parents from families (Figure 1). To investigate whether alleles from rs1799732 are contributing as a risk factor for autism susceptibility in our families, comparisons between family-based tests of rs1800498 and rs1799732 alleles considered separately (P = 0.0003 and P = 0.16, respectively), and haplotypes containing alleles from both rs1800498 and rs1799732, showed that the observed over-transmission in families is derived from rs1800498, and not because of the rs1799732 polymorphism (data not shown). However, only 31 families were informative for rs1799732 compared to 73 families for rs1800498, so we cannot determine from these findings whether alleles from the functional variant rs1799732 are contributing to autism susceptibility in our families. Another genetic variant at the DRD2 locus, rs1076560GT, has been associated with altered mRNA isoform expression [52,53] and differences in striatal post-synaptic D2 receptor abundance [54]. Bertolini et al. [53] (2009) found in control and schizophrenia cohorts (N = 114 and N = 91, respectively) that individuals heterozygous for the minor “T” allele perform worse in the N-back test of working memory but only at a high level of difficulty (2-back) compared to individuals homozygous for the major “G” allele while Zhang et al. [52] (2007) found using healthy subjects (N = 117) that heterozygous individuals performed worse at higher attentional loads in the variable attentional control (VAC) task, and had increased activity as measured using BOLD fMRI in PFC and striatum compared to individuals homozygous for the major allele. However, the true contribution of this variant to DAergic function and cognition is unclear as no genotype effects of this polymorphism to overall working memory performance and fMRI activity were also reported [52,54]. Nevertheless, both rs1076560 and rs1079597 are in high LD in the HapMap CEU panel (r^2 > 0.9) and are found in the same 20 kb haplotype block as rs1800498. However, no information is available regarding LD between rs1076560 and rs1800498. It is of interest that low LD (r^2 < 0.3) was found between rs1800498 and rs1079597 in our comparison group and parents from families (Figure 1). Additional families informative at these DRD2 loci are needed to determine whether these functional variants or another functional polymorphism in LD with rs1800498 is responsible for the increased risk for autism. In addition, functional analyses and resequencing of both genes in affected individuals with this risk haplotype at DRD2 or the risk allele at PPP1R1B are required.

### Pathophysiological contributions of DRD2 and PPP1R1B to risk for autism

The QTDT results support an association of the DRD2 and PPP1R1B loci with autism (Table 4). A role for the DRD2 gene in autism susceptibility is suggested by the fact that antipsychotic medications, which prevent dopamine D2 receptor activation, improve the core symptoms of ASDs [55]. Postsynaptic D2 receptors and presynaptic D2 autoreceptors are involved in the DAergic modulation of cognitive and emotional processes that are impaired in individuals with autism [56,57]. Thus, functional polymorphisms which affect receptor availability (e.g. altered gene expression), either postsynaptically on DAceptive neurons or presynaptically on DAergic neurons, may contribute to the impairments found in individuals with autism.

DARPP-32 mediates the downstream effects of dopamine receptor activation, and thus plays an important role in the modulation of DA-related processes which are abnormal in individuals with autism. Unlike dopamine receptors, which can be studied using systemic or local administration of ligands, DARPP-32 is found in the cytoplasm of DAceptive neurons and there are few studies which have examined its role in DA-modulated processes and behaviours. One such study by Hotte et al. [58] (2006) found that administration of D1 receptor antagonists in mice caused deficits in working memory which coincided with decreased levels of phosphorylated-DARPP-32 in the PFC. Deficiencies in working

| Table 5 General discriminant analysis of DRD2 rs1800498 and PPP1R1B rs1495099 genotypes towards prediction of ASDs in affected males |
|------------------|------------------|------------------|------------------|------------------|------------------|
| **Eigenvalue**   | **Canonical R**  | **Wilk’s Lambda** | **χ^2** | **df** | **P** | **FDR threshold** |
| DRD2 - PPP1R1B   | 0.07             | 0.26             | 0.94   | 23.27 | 4    | **0.00011** | 0.017 |
| DRD2             | 0.97             | 5.14             | 343    | 0.063 | 0.050 | **0.00086** | 0.033 |
| PPP1R1B         | 0.06             | 7.20             | 343    |        |      |            |      |

1One affected individual was randomly chosen from each family.
2Chi-statistic reported for additive test of DRD2 and PPP1R1B and F statistic reported for single gene tests.
3P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined.
4P-value ≤ FDR threshold is significant.
memory [59] and impairments in reversal learning [10] are found in individuals with autism. Both Drd2 −/− mice and Ppp1r1b −/− mice exhibit impairments in reversal learning compared to wild-type mice [31,60].

The role of DARPP-32 in mediating the DA-related changes to neuronal excitability necessary for memory and learning was shown in a study by Calabresi et al. [61] (2000). These authors were unable to induce long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity, in the striatum of Ppp1r1b −/− mice. DA has a role in synaptic plasticity in both the striatum [62] and amygdala [63], subcortical structures that are important for regulating emotional behaviours [64] and social interactions [65], and are implicated in the pathophysiology of repetitive behaviours [66].

Effects between dopamine-related genes and risk for ASD

Based on our findings in this study and our previous findings [5], single-gene analyses showed evidence for association of DRD1, DRD2 and PPP1R1B with autism in male-only affected sib-pair families. We performed general discriminant analysis using Statistica v9.1 to predict ASD susceptibility in affected males, and to test for evidence of gene-gene interactions of DA-related genes and ASDs. We found that DRD2 rs1800498 genotypes and PPP1R1B rs1495099 genotypes significantly contributed to prediction of ASDs in our families when tested separately and together (P = 0.0063–0.00011), which accounted for ~7% of the variance. These results were significant following corrections for multiple comparisons (Table 5). We also found that 97% of individuals from our comparison cohort and 13% of individuals with autism were correctly classified while 72% of control individuals and 64% of affected individuals were predicted correctly using a weighted additive combination of DRD2 and PPP1R1B genotypes. The inclusion of DRD1 rs265981 C −rs4532 A −rs686 T (C-A-T) haplotype from mothers (P = 0.029) [5], and of the DRD2 rs1800498 T allele from fathers (P = 0.023), to affected sons suggests that imprinting effects of these genes are also important risk factors for ASDs. A role for imprinting has been proposed for several brain-related disorders, including autism [67]. There is evidence of imprinting of genes from neurotransmitter pathways implicated in ASDs such as the 5-hydroxytryptamine receptor 2A (HTR2A) gene in the serotoninergic pathway [68].

The dopamine D1 and D2 receptors are expressed in human placenta [69,70] and fetal brains [71,72]. Placental D1 and D2 receptors are involved in DA-mediated release of opioids [73] and lactogen [74] respectively, which are required for fetal development and growth. Both dopamine D1 and D2 receptors are involved in brain development. Dopamine D2 receptors expressed in fetal brain induce neurite outgrowth and axon elongation while dopamine D1 receptors inhibit neurite outgrowth in cortical neuron differentiation [75], with the opposite effects found in striatal neuron differentiation [76]. There is no evidence for the kind of parent-of-origin-specific DNA methylation at the DRD1 or DRD2 loci in either human placenta or fetal brains that is suggestive of imprinting [77]. Further, a review of the ‘imprinted gene and parent-of-origin effect’ database [available at http://igc.otago.ac.nz/home.html] [78] did not yield any evidence supporting imprinting at either locus. The possibility remains, however, that imprinting of these genes may occur during a very narrow developmental period or in a specific subpopulation of brain cells, as has been demonstrated for the Ube3a gene in mice [79]. It should be noted that the evidence presented for parent-of-origin effects in ASDs is based on three markers in the DRD1 gene and one marker in the DRD2 gene and thus, more polymorphisms need to be studied to confirm our findings.

Conclusions

Our results strongly support a role for the DRD2 and PPP1R1B genes in susceptibility to autism spectrum behaviours in males from affected sib-pair families in which there are only affected males, and especially those males where there are severe impairments in social interaction (DRD2 and PPP1R1B), verbal communication (DRD2), nonverbal communication (PPP1R1B) and stereotyped behaviours (DRD2 and PPP1R1B). Our results also support an additive effect of the DRD2 and PPP1R1B genes in predicting ASDs in our families. However, we recognize that large genome-wide association studies have not implicated these genes in autism susceptibility, but those studies did not analyse male-only affected sib-pair families separately.
from other families, and there is no information about the level of impairments in the core characteristics of ASDs. Further studies are needed using additional similar family cohorts and sequencing of the DRD2 and PPP1R1B genes in individuals with the risk alleles to identify functional polymorphisms that could be associated with the clinical findings in order to further develop our model for an association of DA-related gene function and susceptibility to ASD behaviours.

Competing interests
The authors declare they have no competing interests.

Acknowledgments
This work was supported by research grants from the Ontario Mental Health Foundation (OMHF; JJAH, PI); the Canadian Institutes for Health Research (CIHR; #43820, JAH, PI) and the Canada Foundation for Innovation (A8739) to the Autism Spectrum Disorders Canadian-American Research Consortium (ASD-CARC) (JAH, PI; www.asdcarc.com; www.AutismResearch.ca); and funded in part by a grant from the South Carolina Department of Disabilities and Special Needs (SCDDSN)(CES). This work was also supported by a research studentship from the OMHF to JAH. JAH was a trainee with the CIHR/Autism Speaks STHR Interdisciplinary Inter-Institute Autism Spectrum Disorders Training Program (PI: JAH) (www.AutismTraining.ca). MEL is a Career Scholar supported by the Michael Smith Foundation for Health Research. The authors are very grateful to all the families who participated in this research and we gratefully acknowledge the resources provided by the Autism Association of DA-related gene function and susceptibility to inherited autism. Am J Med Genet B Neuropsychiatr Genet 2008, 45:1024–1026.

Rinaldi A, Mandillo S, Oliveira A, Mele A, D1 and D2 receptor antagonist injections in the prefrontal cortex selectively impair spatial learning in mice. Neuropsychopharmacology 2007, 32:309–319.

Pezze MA, Feldon J. Mesolimbic dopaminergic pathways in fear conditioning. Prog Neurobiol 2004, 74:301–320.

Coldren JT, Halloran C. Spatial reversal as a measure of executive functioning in children with autism. J Genet Psychol 2003, 164:29–41.

Gaigg SB, Bowler DM. Differential fear conditioning in Asperger’s syndrome: implications for an amygdala theory of autism. Neuropsychology 2007, 45:2125–2134. 23. Hill EL. Executive dysfunction in autism. Trends Cogn Sci 2004, 8:26–32.

Wensky JA, Damasio AR, Maurer RG. Gait disturbances in patients with autistic behavior: a preliminary study. Arch Neurol 1981, 38:646–649.

Wang Z, Yu G, Cascio C, Liu Y, Gingrich B, Insel TR. Dopamine D2 receptor-mediated regulation of partner preferences in female prairie voles (Microtus ochrogaster): a mechanism for pair bonding? Behav Neurosci 1999, 113:602–611.

Karler R, Calder LD, Thai LH, Bedingfield J. A dopaminergic-glutamatergic basis for the action of amphetamine and cocaine. Brain Res 1994, 658:1–14.

Ernst M, Zamekkin AJ, Matokich J, Pascualvaca D, Cohen RM. Low medial prefrontal dopaminergic activity in autistic children. Lancet 1997, 350:638.

Gillberg C, Svernholm L. CSF monoamines in autistic syndromes and other pervasive developmental disorders of early childhood. Br J Psychiatry 1987, 151:89–94.

Robinson PD, Schutz CK, Maccarri F, White BN, Holden JJ. Genetically determined low maternal serum dopamine beta-hydroxylation levels and the etiology of autism spectrum disorders. Am J Med Genet 2001, 100:30–36.

Lubanik JH, Dabjal M, Sileri L, Grandy DK, Civelli O, McElligott DL, Evans GA. Structure and linkage of the D2 dopamine receptor and neural cell adhesion molecule genes on human chromosome 11q23. Genomics 1992, 14:1010–1018.

Grandy DK, Litt M, Allen L, Buzow JR, Marchionni M, Makam H, Reed L, Magenis RE, Civelli O. The human dopamine D2 receptor gene is located on chromosome 11 at q22-q32 and identifies a TaqI RFLP. Am J Hum Genet 1989, 45:778–785.

Onali P, Ollanas MC. Involvement of adrenergic cyclc inhibition in dopamine autoreceptor regulation of tyrosine hydroxylase in rat nucleus accumbens. Neurosci Lett 1989, 101:261–29.

Mercut NB, Saiardi A, Bondi A, Picietti R, Calabresi P, Bernardi G, Borrelli E. Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. Neuroscience 1997, 79:323–327.

Mehta MA, Sahakian BJ, McKenna PJ, Robbins TW. Systemic sulpiride in young adult volunteers simulates the profile of cognitive deficits in Parkinson’s disease. Psychopharmacology (Berl) 1999, 146:162–174.

Meintzschel F, Zieman U. Modification of practice-dependent plasticity in human motor cortex by neuremodulators. Cereb Cortex 2005, 16:1005–1115.

Greba Q, Gilkins A, Kokkinds L. Inhibition of amygdaloid dopamine D2 receptors impairs emotional learning measured with fear-conditioned startle. Brain Res 2001, 899:218–226.

Bak IH, Picietti R, Saiardi A, Thien T, Derich A, Depaulis A, Le Meur M, Borrelli E. Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. Nature 1995, 377:424–428.

McDougle CJ, Holmes JP, Carlson DC, Pelton GH, Cohen DJ, Price LH. A double-blind, placebo-controlled study of risperidone in adults with autistic disorder and other pervasive developmental disorders. Arch Gen Psychiatry 1998, 55:633–641.

Quinnet CC, Miller PE, Hemminger HC Jr, Walaas SL, Greengard P. DARPP-32, a dopamine- and adenosine 3’5’-monophosphate-regulated phosphoprotein enriched in dopamine- innervated brain regions. III. Immunocytochemical localization. J Neurosci 1984, 4:111–124.

References
1. Bacchelli F, Maestrini E. Autism spectrum disorders: molecular genetic advances. Am J Med Genet C Semin Med Genet 2006, 142C:13–23.
2. Zhao X, Leotta A, Kustanovich V, Lajonchere C, Geschwind DH, Law K, Law P, Qiu S, Lord C, Sebat J, et al. A unified genetic theory for sporadic and inherited autism. Proc Natl Acad Sci U S A 2007, 104:12831–12836.
The control of the false discovery rate in multiple hypothesis testing. Science 1998, 281:652–656.

Hetering C, Blangero J, Ewens WJ: The 3′ region of the DRD2 gene is involved in genetic susceptibility to schizophrenia. Schizophr Res 2004, 67:327–336.

Wang Y, Afifi A, Khan AN, Cooper MS, Sanders SJ, Weiss ST, Eaves LJ: Replication study of candidate genes for cognitive abilities: the Lothian Birth Cohort 1936. Genes Brain Behav 2009, 8:238–247.

uA receptor activity during normal and abnormal pregnancies. J Comp Neurol 2003, 460:559–695.

Amar Texting et al. Behavioral and Brain Functions 2012, 8:1

http://www.behavioralandbrainfunctions.com/8/1/1

Page 12 of 13
73. Stratakis CA, Mitsiades NS, Chrousos GP, Margioris AN: Dopamine affects the in vitro basal secretion of rat placenta opioids in an opioid and dopamine receptor type-specific manner. Eur J Pharmacol 1996, 315:53–58.

74. Petit A, Gallo-Payet N, Vaillancourt C, Bellabarba D, Lehoux JG, Belisle S: A role for extracellular calcium in the regulation of placental lactogen release by angiotensin-II and dopamine in human term trophoblastic cells. J Clin Endocrinol Metab 1993, 77:670–676.

75. Reinoso BS, Undie AS, Levitt P: Dopamine receptors mediate differential morphological effects on cerebral cortical neurons in vitro. J Neurosci 1996, 43:439–453.

76. Schmidt U, Beyer C, Oestreicher AB, Reisert I, Schilling K, Pilgrim C: Activation of dopaminergic D1 receptors promotes morphogenesis of developing striatal neurons. Neuroscience 1996, 74:433–460.

77. Shen HM, Nakamura A, Sugimoto J, Sakamoto N, Oda T, Jinno Y, Okazaki Y: Tissue specificity of methylation and expression of human genes coding for neuropeptides and their receptors, and of a human endogenous retrovirus K family. J Hum Genet 2006, 51:440–450.

78. Glaeser BL, Ramsay JP, Morrison IM: The imprinted gene and parent-of-origin effect database now includes parental origin of de novo mutations. Nucleic Acids Res 2006, 34:D29–D31.

79. Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, Nikawa N, Ogawa M, Wagstaff J, Kishino T: Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. Hum Mol Genet 2003, 12:837–847.

doi:10.1186/s1744-9081-0-1
Cite this article as: Hettinger et al.: DRD2 and PPP1R1B (DARPP-32) polymorphisms independently confer increased risk for autism spectrum disorders and additively predict affected status in male-only affected sib-pair families. Behavioral and Brain Functions 2012 8:1.

Submit your next manuscript to BioMed Central and take full advantage of:

• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit