MUTATION SCREENING OF DOPAMINE AND SEROTONIN CANDIDATE GENES IN TOURETTE’S SYNDROME AND ALCOHOL DEPENDENT PATIENTS

by

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A Thesis submitted in conformity with the requirements for the Degree of Master of Science
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ABSTRACT

Mutation screening of dopamine and serotonin candidate genes in Tourette’s syndrome and alcohol dependent patients

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Pharmacological evidence suggests that both dopamine and serotonin system genes may be involved in the inheritance of Tourette’s syndrome (TS) and alcohol dependence. Dopamine transporter antagonists used to treat attention deficit-hyperactivity disorder (AD-HD) worsen comorbid with TS, emphasizing possible TS dopamine dysregulation. Serotonergic antidepressants treating obsessive compulsive disorder (OCD) comorbid with TS and alcohol dependence withdrawal also implicating serotonin dysregulation in these disorders. Therefore, we conducted a candidate gene study of the dopamine D1 (DRD1), dopamine transporter (DAT1) and serotonin transporter (5HTT) genes in these disorders. The DRD1 gene was found to be non-polymorphic in patient and control groups. Common conserved sequence variants of the DAT1 gene were identified but were not associated with TS or alcohol dependence. The short promoter 5HTT gene was significantly more common in the TS group compared with controls ($\chi^2$P=0.04), suggesting that the 5HTT gene may be a susceptibility locus in TS.
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INTRODUCTION

1.1 Neurotransmitter G protein coupled-receptors and transporters in disease

A number of diseases known to be caused by sequence variants in G protein coupled receptors (GPCRs) have been described (Zastawny et al, 1997). Mutations causing both loss and gain of receptor function have been reported. Retinitis pigmentosa, one of the earliest characterized GPCR pathologies, was found to be due to a gain of function mutation in the rhodopsin gene (Dryja et al, 1990; Dryja et al, 1995). A loss of function mutation in the rhodopsin receptor gene was recently found to cause a form of stationary night blindness (Yamamoto et al, 1997). Other GPCR mutations have been identified. These include a mutation in the V2 vasopressin receptor gene which causes nephrogenic diabetes insipidus type 11 (van den Ouweland et al, 1992) and a mutation in the thyroid stimulating hormone (TSH) receptor gene which causes hyperthyroidism (Kopp et al, 1995). Most recently, a mutation in the CCR5 gene, a coreceptor for the human immunodeficiency virus (HIV) that is expressed on T helper cells, has been found to confer resistance to HIV infection (Michael et al, 1997). The identification of simian immunodeficiency virus (SIV) G protein-coupled coreceptors (Farzan et al, 1997), GPR1 and GPR15 (Heiber et al, 1996; Marchese et al, 1994), suggests that future studies of GPCR genes in disease states may be relevant and fruitful.

Despite the rapidity with which the GPCR genes expressed in the central nervous system (CNS) have been cloned (Marchese et al, 1997; O'Dowd, 1993; Seeman and Van Tol, 1993), no mutations in these genes have yet been discovered to contribute to the inheritance of neuropsychiatric disorders (Petronis and Kennedy, 1995). Pharmacogenetic studies have met with some success in identifying dopamine D4
(DRD4) and serotonin 2A (5HT2A) receptor variants that confer different responses to atypical neuroleptic drug treatment (Seeman et al, 1997; Rao et al, 1997). However, the numerous attempts to utilize genetic markers in order to conduct linkage and association studies of these disorders have not succeeded in convincingly associating GPCRs expressed in the CNS with a neuropsychiatric disorder (Petronis and Kennedy, 1995).

In the work presented here, therefore, we set out to screen candidate genes implicated in Tourette’s syndrome (TS) and alcohol dependence for sequence variants that might provide new insight into the inheritance of these disorders. The emphasis of the work was to screen DNA from patients with TS and alcohol dependence for mutations in genes that had not been subjected to mutation screening previously. Therefore, we screened the GPR19 gene which, while having no known ligand, shows high sequence homology and similar tissue distribution to dopamine system genes including the dopamine DRD2 gene (O'Dowd et al, 1996). From among the GPCRs, we also screened the DRD1 gene for sequence variants in two disorders that may have dopaminergic etiologies: TS and alcohol dependence.

We next elected to screen the dopamine transporter (DAT1) gene for sequence variants in Tourette’s syndrome (TS) and alcohol dependence. The final component of the work presented was based on the results of a similar mutation screening project which identified a sequence variant in the 5’ regulatory region of the serotonin transporter (5HTT) gene that has been reported to alter 5HTT gene expression in vitro (Heils et al, 1996).
1.2 Clinical definition of TS, associated disorders and alcohol dependence

(a) TS and comorbid neuropsychiatric disorders

TS is a relatively rare psychiatric disorder affecting between 0.1 and 0.5 per thousand people throughout the world. The cluster of symptoms that underlie TS have their onset between the ages of 2 and 15. The diagnostic criteria include recurrent, involuntary, rapid motor movements with no purpose (tics) that affect multiple muscle groups. Symptoms may vary over weeks or months or may be suppressed in the short term but must be otherwise unremitting for at least a year in order to diagnose TS. The disorder is usually lifelong.

The tics are the essential feature of TS. Motor tics commonly involve the head, torso and upper limbs. Vocal tics include sounds such as clicks, grunts, yelps, barks, sniffs, coughs and words. An irresistible urge to utter obscenities, coprolalia, is present in 60% of cases. Symptoms are exacerbated by stress but disappear during sleep and absorbing tasks.

Associated features of TS include echokinesis, imitating the movements of someone being observed, and palilalia, repetition of one's own phrases. Obsessive-compulsive or ritual behaviors may also be present. These include obsessive thoughts of doubting, compulsive rituals including complicated movements, impulses to touch objects, retrace steps, squat and twirl when walking (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, 1990).

TS is often diagnosed comorbid with obsessive compulsive disorder (OCD) (Rapoport and Inoff-Germain, 1997; Swedo and Leonard, 1996) or attention deficit-
hyperactivity disorder (AH-HD) (Castellanos et al., 1997; Schuerholz et al., 1996). Up to 50% of TS patients have been reported to be diagnosed comorbid with OCD. Comorbid OCD or AD-HD in TS patients presents a challenge to treatment protocols because of the need to simultaneously treat TS tics and the comorbid OCD or AD-HD. Treatment modalities for OCD and AD-HD are distinctly different from each other and TS treatments (Peterson, 1996). Major depression (Comings and Comings, 1993; Comings, 1994) and substance abuse disorders, such as alcohol dependence (Muller-Vahl et al., 1997; Comings, 1994), have also been diagnosed comorbid with TS. The contrasting pharmacological interventions used to treat TS and associated comorbidities may provide insight into the neuropathies that may underlie TS (Singer et al. 1993) and alcohol dependence (Tiihonen et al., 1995).

(b) Alcohol dependent disorder and associated withdrawal symptoms

Alcohol dependence involves a pattern of pathological alcohol use or impairments in social or occupational functions due to alcohol use. The disorder is characterized by the need for daily alcohol consumption and an inability to stop drinking that is associated with the need for markedly increased amounts of alcohol in order to satisfy alcohol craving (tolerance) (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, 1990). The consequence of a cessation of alcohol consumption for an alcohol dependent patient is alcohol withdrawal, which involves severe anxiety, depression, ataxia and muscle tremors (Lejoyeux, 1996; Anton, 1996; Diagnostic and statistical manual of mental disorders, Fourth Edition, 1990).
1.3 The pharmacology of TS, associated disorders and alcohol dependence

The neurotransmitters dopamine and serotonin have both been implicated in the etiology of TS, associated comorbid disorders and alcohol dependence based on pharmacological evidence (Lejoyeux et al, 1996; Anton et al, 1996; George et al, 1995, Samson et al, 1993; Dyr et al, 1993). Treatment of these disorders is complex but only those aspects salient to understanding the dopaminergic and serotonergic hypotheses of TS and associated disorders will be reviewed here. However, the range of therapeutic interventions used to treat these disorders provides strong evidence for their dysregulated neurotransmitter etiology (Wolf et al, 1996; Castellanos et al, 1997; Uhlenhuth et al, 1995; Narango and Bremner, 1994).

(a) Typical and atypical neuroleptics implicate dopamine pathways in TS

Dopamine pathways are considered to be involved in TS pathology due to the efficacy of typical neuroleptics, such as haloperidol and primozide, in the treatment of the motor and vocal tics that are central to the disease (Peterson, 1996). Typical neuroleptics are dopamine D2 receptor antagonists, although haloperidol is reported to have some $\alpha_1$-adrenergic effects, while primozole is reported to have some calcium channel blocking properties. While side-effects often limit the use of haloperidol (Silva et al, 1996), the fact that it remains a treatment of choice for managing TS motor and vocal tics (Silva et al, 1996; Bruun and Budman, 1996; Iancu et al, 1995) provides the best clinical rationale for the hyperdopaminergic hypothesis of TS (Wolf et al, 1996).
The involvement of serotonin pathways in TS has less pharmacological evidence in its support (Peterson, 1996). There is evidence that the atypical neuroleptic risperidone has some efficacy in treating the tic symptoms of TS (Bruun and Budman, 1996). Atypical neuroleptics, including risperidone and clozapine, antagonize both dopamine and serotonin receptors. Risperidone, a 5HT2 receptor antagonist and a weak dopamine D2 receptor antagonist (Peterson, 1996; Bruun and Budman, 1996), relieves the severity of tics for some patients (Bruun and Budman, 1996), however, in 20% of TS patients treated with risperidone, treatment is curtailed due to weight gain and sedating side effects (Peterson, 1996).

Clozapine, the atypical neurolepic which antagonizes both 5HT2A and dopamine D4 receptors (Seeman et al, 1997), has not been shown to effectively treat tics (Caine et al, 1979) even though it is often effective in treating schizophrenia (Seeman et al, 1997; Rao et al, 1996; Meltzer, 1995; Van Tol et al, 1992). In fact, exacerbation of tic symptoms have been reported in some TS patients undergoing clozapine treatment (Caine et al, 1979). It is unclear whether the side effects of clozapine in TS result from serotonin or dopamine receptor antagonism (Stein et al, 1997; Peterson, 1996). Taken together, there is clinical and pharmacological evidence of a role for hyperserotonergic dysregulation in the motor and vocal tics integral to TS.

(b) **SRIs implicate serotonin pathways in TS-OCD and TS-anxiety**

OCD comorbid with TS is often treated successfully with the combination of a neuroleptic to control the tics and a serotonin antidepressant to relieve OCD symptoms.
Serotonin antidepressants all act, at least in part, to correct a hyposerotonergic state that is hypothesized to underlie depression (Ogilvie et al., 1996; Heils et al., 1996). By extension, the efficacy of serotonergic antidepressants in treating OCD suggests that a hyposerotonergic state may also underlie OCD (Uhlenhuth et al., 1995).

Both tricyclic antidepressants (TCAs) and serotonin reuptake inhibitors (SRIs) are used to treat OCD comorbid with TS (Stein et al., 1997). The tricyclic antidepressant (TCA) clomipramine (Stein et al., 1997; Peterson, 1996; Iancu et al., 1995), which sensitizes serotonin receptors and releases norepinephrine in adrenergic pathways, has been reported to be effective in treating OCD (Iancu et al., 1995). The autonomic side effects of TCA drugs, however, limit the use of clomipramine treatment. Recently, SRI inhibitors such as fluoxetine and fluoxamine (Stein et al., 1997; Peterson, 1996), which antagonize the serotonin transporter, have been reported effective in treating OCD. In order to treat TS-OCD, patients generally require an SRI or TCA for the relief of OCD symptoms and risperidone augmentation for the relief of the motor and vocal tic symptoms of TS.

(c) Methylphenidate implicates dopamine pathways in TS-AD-HD

Treatment of AD-HD commonly involves the use of dopamine psychotropic agents, such as the dopamine transporter antagonist methylphenidate (MP) (Volkow et al., 1996; Volkow et al., 1995; Geogiou et al., 1995). AD-HD treatment, however, often complicates the treatment of comorbid TS symptoms (Peterson, 1996). TS patients with comorbid
AD-HD require two contrasting dopaminergic interventions. Often the sedating properties of typical neuroleptics are used to treat the tics associated with TS and a dopamine psychotropic agent, such as MP, is used to treat AD-HD (Castellanos et al, 1997; Hetchman et al, 1993).

The tic symptoms in patients with TS-AD-HD are commonly reported to worsen immediately after MP treatment commences (Daniels et al, 1996). However, once the MP becomes effective in treating the AD-HD symptoms, tic exacerbation tends to disappear. This makes MP the drug of choice for TS-AD-HD. Other dopamine transporter antagonists, such as dextroamphetamine, result in unabated tic exacerbation in TS-AD-HD patients (Castellanos et al, 1997). Cocaine, a dopamine transporter antagonist with a very high affinity for the dopamine transporter by comparison with MP, results in extreme tic exacerbation (Castellanos et al, 1997). The complex pharmacology of TS-AD-HD treatment suggests that the dopaminergic disregulation in TS-AD-HD may involve many aspects of dopaminergic neurotransmission.

(d) SRIs implicate serotonergic pathways in alcohol dependence withdrawal

SRIs are currently indicated not only for the treatment of obsessive compulsive disorder (Peterson, 1996) but also depression (Ogilvie et al, 1996) and anxiety (Lesch et al, 1996). Detoxification following clinical alcohol dependence is often associated with alcohol related anxiety and depression (Lejoyeux, 1996; Anton, 1996). The common alcohol dependence comorbidities, including affective disorders, often obscure whether anxiety and depression cause or are caused by the withdrawal associated with alcohol
dependence (Anton, 1996). Regardless of their etiology, the symptoms of depression and anxiety associated with detoxification have been reported to be improved when patients are treated with SRIs (Lojoyeux, 1996; Anton, 1996; Oliver et al., 1993). This suggests that a hyposerotonergic state underlies alcohol dependence.

Human drug trials and animal experiments also suggest that SRIs may be effective in reducing alcohol consumption and implicate serotonin dysregulation in the pathways involved with alcohol craving. A recent clinical trial of SRIs in non-depressed and moderately depressed alcoholics found that alcohol consumption was decreased 15-20% in the SRI treatment groups compared to placebo control groups. SRI treatment of alcohol preferring rats has also been shown to result in reduced alcohol consumption. However, evidence for long term reduction of alcohol consumption by SRIs is currently lacking (Narango and Bremner, 1994).

The fact that not all serotonergic antidepressants are reported to treat alcohol dependence with the effectiveness of SRIs, however, suggests that much is still to be learned about the significance of serotonergic dysregulation in alcohol dependence (Lejoyeux, 1996). Clinical trials of other serotonergic drugs, including the 5HT1A receptor agonist buspirone, the 5HT3 antagonist ondansetron and the 5HT2 antagonist ritanserin, have met with less success than the SRIs in treating alcohol withdrawal or ongoing alcoholism (Narango and Bremner, 1996). Therefore, although SRI drugs may reverse a hyposerotonergic state that is common in alcoholics, the serotonergic pathology that may underlie alcohol dependence remains unknown (Lejoyeux, 1996; Oliver et al., 1993).
Dopamine is implicated in animal models of alcohol consumption

Alcohol dependence may also involve aberrant dopamine pathways (Ng et al., 1997; Noble, 1996; Thiihonen et al., 1995; Rassnick et al., 1992). Treatment of the high ethanol preferring C57 mouse with the dopamine D1 receptor antagonist SKF 82958 or the dopamine D2 antagonist quinpirole results in markedly decreased ethanol consumption for 24 hours (Ng et al., 1994). The resumption of ethanol consumption by mice treated with these antagonists has been attributed to receptor desensitization (Ng et al., 1994; George al et, 1995). While this animal model has been used to show the involvement of dopaminergic pathways in alcohol consumption, no neuroleptic treatment is currently used to treat the primary symptoms of alcohol consumption or withdrawal in human alcohol dependent patients. A hypodopaminergic hypothesis of alcohol dependence has been inferred from decreased ethanol consumption in the mouse model following dopamine D1 and D2 antagonist treatment (George et al., 1995; Ng et al., 1994).

1.4 Mode of inheritance of TS, comorbid disorders and alcohol dependence

Debate about whether TS and a range of disorders including OCD, AD-HD and alcohol dependence are part of a heritable TS spectrum disorder (Comings and Comings, 1993) remains controversial (Pauls et al., 1991; Pauls et al., 1988; Pauls et al., 1986). The spectrum disorder hypothesis suggests that expression of TS genes results in not only the tics characteristic of TS but a broad range of associated behaviors including OCD, AD-HD and alcohol dependence. Support for this idea comes from a number of sources. First, there is an unusually high incidence with which these disorders are diagnosed
comorbidly (Castellanos et al, 1997; Stanley, 1997; Comings and Comings, 1993). Second, pharmacological evidence suggests that this group of disorders share at least some aspects of dopaminergic and serotonergic dysregulation (Peterson, 1996). Third, evidence exists that relatives of an affected individual are likely to be affected by the same or one of the associated disorders at a rate exceeding that found in pedigrees of nonpsychiatric individuals (Comings and Comings, 1993; Pauls et al, 1991; Pauls et al, 1988). To date, however, studies that find evidence for a wide spectrum of related disorders that share genetic liabilities (Comings et al, 1996) have not been replicated.

(a) **Autosomal dominant model of TS**

Studies of TS in affected pedigrees have resulted in the hypothesis that TS is inherited as a single autosomal dominant gene that is subject to incomplete penetrance (Wolf et al, 1996; Pauls et al, 1986; Comings et al, 1986). The single major locus hypothesis suggests that the intermediate forms of TS, such as mild tic disorders, may result from the inheritance of a single copy of the susceptibility gene (Comings et al, 1986). Thus, one copy of the TS gene may confer some risk, while two copies may confer greater risk of developing the severe tic disorder that characterizes TS (Comings et al, 1986). However, the range of tic severity found in TS patients (Pauls et al, 1996), combined with the fact that tic severity worsens when one or both parents have a tic disorder (Comings et al, 1986), suggests that TS may better fit a more complex model of inheritance (Walkup et al, 1996). Models describing TS inheritance as semidominant, semirecessive or as being an example of incomplete dominance have also been proposed (Patel, 1996)
(b) Polygenic models of TS, AD-HD, OCD and alcohol dependence

Linkage studies on Tourette's syndrome have recently introduced evidence that tends to exclude the possibility that a single major locus is responsible for the inheritance of TS (Heutink et al., 1995). Complex segregation analysis suggests that susceptibility for TS is conveyed by a major locus in concert with a multifactorial background, including polygenetic and environmental contributions (Walkup et al., 1996). A revised model proposes that the major locus is responsible for half of the observed phenotypic variation described in the clinical literature of TS (Patel, 1996; Walkup et al., 1996). Models of TS inheritance must be interpreted with caution, however, because none have yet met with wide acceptance. The greatest complication to examining disorders such as TS is that its heterogeneity may confound studies of its inheritance (Patel, 1996; Alsobrook and Pauls, 1996; Santengelo et al., 1996; Pauls et al., 1991).

Similar problems must be accounted for when studying the genetic component of alcohol dependence (George et al., 1993; Cloninger et al., 1985; Devor and Cloninger, 1989). While Type II alcoholics have been proposed to have a greater genetic liability, which may include a hyposerotonergic phenotype (Cloninger et al., 1989; Cloninger, 1987), studies of this phenotype have not yet resulted in the identification of novel candidate genes for alcohol dependence (Gordis, 1997). Studies associating alcohol dependence with the dopamine D1 (DRD1), dopamine D2 (DRD2), dopamine D4 (DRD4) and DAT1 genes (to name but a few) all remain controversial (Comings et al., 1997; Muramatsu et al., 1995; Carlos et al., 1993; Uhl et al., 1992). The inheritance of
alcohol dependence, like that of TS (Patel, 1996), is likely to involve complex interaction between many susceptibility loci and environmental factors (Cloninger, 1987; Persico et al, 1993; Muramatsu et al, 1995).

1.5 Candidate gene hypothesis of TS, comorbidities and alcohol dependence

Evidence from monozygotic twin studies suggests that a complex genetic liability for TS, TS associated with AD-HD or OCD and alcohol dependence probably exists (Gordis, 1997; Patel, 1996). Because attempts to isolate candidate genes for TS, AD-HD, OCD and alcohol dependence by linkage analysis have not been successful (Gordis, 1997; Heutink et al, 1995; Gelernter et al, 1995), Mendelian inheritance of these disorders is unlikely. However, failure to detect disease loci by linkage analysis does not exclude polygenic models of the inheritance of TS or alcohol dependence.

In polygenic models, no single gene that contributes to the inheritance of a disorder is sufficient in itself to cause the disorder phenotype. Each contributing gene, or susceptibility loci (Nothen et al, 1992), may contribute additively (Comings et al, 1996) to the inheritance of a sufficient genetic liability to cause a recognizable phenotype. Therefore, much of the recent work on the inheritance of TS, AD-HD, OCD and alcohol dependence has abandoned the method of linkage analysis, the standard means of identifying candidate genes for a genetic disorder (Nothen et al, 1992; Hodge, 1994), in favor of association studies (Owens, 1997; Berrettini, 1997; Hodge, 1994; Nothen et al, 1992).
Association studies involve examining primary candidate genes: genes encoding proteins that are known to be components of a pathway implicated in the pathology of a disease. In their simplest form, association studies involve comparing the frequency of a genetic marker in patient and control groups (Owens, 1997; Hodge, 1994; Nothen et al, 1992). As a result, association studies are based on parametric models. The use of parametric models to test the difference between patient allele and genotype frequencies, however, is correct only if certain assumption are true (Ott, 1991).

Parametric models assume that while parameters cannot be estimated, standard random variables (statistics), such as the $\chi^2$ statistic, can be calculated in order to test whether significant differences exist between patient and control allele and genotype frequency data. The $\chi^2$ statistic, the normalized sum of squares of the differences between patient and control allele or genotype frequencies, is assumed to fit a $\chi^2$ distribution (Ott, 1991). The model also assumes that allele and genotype frequencies in patient and control groups are independent of other variables including the ethnic origin and sex of the members of each group (Hodge, 1994; Nothen et al, 1992). The fact that association studies have often been found to be confounded by variables other than disease (Kennedy et al, 1995; Barr and Kidd, 1993) suggests that the parametric models used in association studies may not always be appropriate.

Linkage analysis, on the other hand, is a non-parametric approach that does not require data to have a close fit to the normal distribution. Non-parametric models accommodate complex systems for which only a few of many variables can be measured. For these reasons, non-parametric linkage analysis is the best approach to isolate
candidate genes for Mendelian traits (Ott, 1991). For complex non-Mendelian traits, however, the non-parametric approach has rarely succeeded in identifying susceptibility loci, probably due to the heterogeneity of these disorders (Heutink et al, 1995). The occasions when linkage analysis has successfully identified susceptibility loci, such as for severe bipolar disorder (Freimer et al, 1996), the affected families studied have been selected for the presence of a founder effect documented to be present by a history of inter-marriage.

Given that linkage analysis of complex traits is only viable under some circumstances, association studies remain a useful if limited tool for the analysis of complex traits. However, care must be taken in sample collection in order to be able to accommodate the assumptions of the parametric model employed in association studies. In addition it should be remembered that unlike linkage studies, association of a marker with a disease, is not evidence that the gene under investigation, or an adjacent gene on the same chromosome, contributes to the inheritance of a disorder (Berrettini, 1997; Hodge, 1994; Nothen et al, 1992).

In psychiatric genetic association studies, candidate genes studied often encode an important site of action for either an endogenous ligand or a drug used to relieve the symptoms of a disorder (Nothen et al, 1992). This is the approach we have used to select the DRD1, DAT1 and 5HTT genes for study in TS, AD-HD, OCD and alcohol dependence. The rationale for selecting each candidate gene will be explained below.

1.6 The dopamine hypotheses of TS, AD-HD and alcohol dependence
Several lines of evidence suggest that TS, AD-HD and alcohol dependence are among the neuropsychiatric disorders for which a dopamine hypothesis implicates the DRD1 gene and the DAT1 gene. The critical role of the DRD1 and DAT1 gene products in sustaining dopaminergic neurotransmission make these genes primary candidates for neuropsychiatric disorders including TS (Gelernter et al. 1993), AD-HD (Cook et al., 1995; Cook et al, 1997) and alcohol dependence (George et al, 1995).

The DRD1 gene, which encodes the dopamine D1 receptor, is a member of the family of genes that encode GPCRs. This receptor, while having 10 fold lower affinity for dopamine than the dopamine D5 receptor (Sunahara et al, 1991), is important to dopamine neurotransmission because of its greater expression in the striatal, cortical and subcortical regions of the human brain (Sunahara et al, 1991). These regions are implicated in emotion, neuropsychiatric disorders and addictions (Weiner et al, 1991).

The DAT1 gene, which encodes the dopamine transporter, is involved in terminating dopaminergic neurotransmission at the synapse. The human dopamine transporter gene (DAT1) belongs to a family of twelve transmembrane domain Na+ and Cl- dependent neurotransmitter transporters (Uhl and Johnson, 1994). The dopamine transporter, like the other members of this family, is a presynaptic re-uptake mechanism (Ding et al, 1994; Nirenbergh et al, 1996) that is integral to the termination of dopaminergic synaptic transmissions by Na+ dependent reuptake (Kitayama et al, 1992, Kilty et al, 1991; Horn, 1990).

The DAT1 gene is implicated in the inheritance of TS (Comings et al, 1996; Vandenergh et al, 1992; Singer et al, 1991; Leckman et al, 1988) and alcohol
dependence (Muramatsu and Higushi, 1995; Thiihonen et al, 1995; Devor and Cloninger, 1989), in addition to attention deficit-hyperactivity disorder (AD-HD) (Gill et al, 1997; Cook et al, 1997; Comings et al, 1996; Cook et al, 1995), Parkinson’s disease (Uhl, 1990) and schizophrenia (Bordea-Pean et al, 1995; Daniels et al, 1995; Li et al, 1994; Persico et al, 1993; Losonczy et al, 1987). Abnormalities of dopamine transporter function have been proposed to contribute to both the hyperdopaminergic (Horn, 1990) and hypodopaminergic hypotheses of the dopamine pathogenesis of these disorders.

(a) The DAT1 and DRD1 genes are candidates for TS

The efficacy of D2-like receptor antagonists, such as haloperidol, in alleviating the motor and vocal tic symptoms of Tourette’s syndrome (Bruun and Budman, 1996; Wong et al, 1996; Wolf et al, 1996) suggests that, as in schizophrenia (Van Tol et al, 1992), a hyperdopaminergic pathology underlies TS (Barr et al, 1996). The possible hyperdopaminergic state that underlies TS may be due to an increased number of dopamine receptors, increased receptor affinity for dopamine (Wolf et al, 1996; Cohen et al, 1978; Butler et al, 1978) or diminished termination of synaptic neurotransmission due to dopamine transporter pathology (Malison et al, 1995; Vandenbergh et al, 1992; Kilty et al, 1991).

The DAT1 gene is a candidate gene in TS because its sequence variants may be responsible for the dopamine transporter dysregulation that is proposed to account for the hyperdopaminergic state that characterizes TS. The potential role of the dopamine transporter in TS is emphasized by the fact that a variety of psychostimulant drugs, such
as cocaine (Kuhar et al, 1990; Wilson et al, 1996) and MP (Volkow et al, 1995) that bind to the dopamine transporter have been reported to worsen the motor and vocal tics of TS patients.

The DRD1 gene is another candidate gene for TS because the hyperdopaminergic state that is proposed to underlie TS may reflect the fact that TS patients have altered dopamine D1 receptor characteristics. Recent evidence from neuroimaging studies has revealed that severely affected TS patients may have increased levels of DRD2 expression or altered dopamine D2 receptor dopamine binding properties (Wong et al, 1997; Robertson, 1996; Wolf et al, 1996). This suggests that dopamine receptors remain valid candidates for TS. However, unlike the dopamine D2 receptor, no evidence of altered DRD1 gene expression in TS has yet been documented. The issue of whether DRD1 function is altered in TS can be investigated by attempting to isolate sequence variants of the DRD1 gene in TS patients as has been done previously in schizophrenic (Ohara et al, 1993) and bipolar patients (Shah et al, 1995).

Dopamine receptor genes have been studied as candidate genes in numerous association studies of sequence variants with TS. As with all association studies, moderate associations of dopamine receptor sequence variants (markers) have been taken as evidence that a functional polymorphic variant, perhaps in the 5' regulatory region, was in linkage disequilibrium with the marker. However, studies finding moderate association of the dopamine D2 (DRD2), D3 (DRD3) and D4 (DRD4) genes with TS (Comings et al, 1996; Grice et al, 1996; Comings et al, 1993; Comings et al, 1991), remain controversial (Nothen et al, 1994; Barr et al, 1996). No evidence for linkage of
the dopamine D1 (DRD1) and D5 (DRD5) genes with TS has yet been found (Gelernter et al, 1991; Barr et al, 1997).

Association studies have not been conducted with DRD1 in TS. However, studies of the 5' flanking region of DRD1 gene, including the Taq1A promoter polymorphism, have failed to show that the DRD1 gene is associated with schizophrenia (Cichon et al, 1996) or bipolar disorder (Coon et al, 1993; Nothen et al, 1992). Point mutation analysis has recently confirmed that the DRD1 gene coding region is non-polymorphic in schizophrenia (Lui Q et al, 1995) and bipolar disorder (Shah et al, 1995). However, studies searching for sequence variants of the DRD1 gene coding region in TS patients have not been reported.

(b) The DAT1 and DRD1 genes are candidates for AD-HD

The DRD1 and DAT1 genes have also been implicated in the dopamine pathology that may underlie AD-HD. The hypodopaminergic hypothesis of AD-HD dopamine pathology (Gill et al, 1997; Cook et al, 1997; Comings et al, 1996; Cook et al, 1995) has been inferred from the pharmacological evidence that psychotropic drugs, such as the dopamine transporter antagonist methylphenidate (MP) (Kuhar et al, 1990; Volkow et al, 1995; Wilson et al, 1996), effectively treat AD-HD (Ding et al, 1994; Hetchman et al, 1994). The hypodopaminergic hypothesis suggests that, by antagonizing the dopamine transporter, MP corrects the low synaptic dopamine levels that characterize AD-HD (Ding et al, 1994), thereby prolonging dopamine receptor stimulation by dopamine. The DRD1 gene is therefore implicated in AD-HD because it encodes the dopamine D1
receptor which has high affinity for dopamine (Sunahara et al, 1990) and is highly expressed in the striatal and cortical brain region implicated in AD-HD (Volkow et al, 1995).

(c) The DAT1 and DRD1 genes are candidates for alcohol dependence

Traits such as alcohol dependence may also involve aberrant dopamine pathways (Ng et al, 1997; Noble, 1996; Tiihonen et al, 1995; Weiss et al, 1990). Dopaminergic neurotransmission in the limbic and mesolimbic pathways of reward located in the midbrain, nucleus accumbens and frontal cortex (Adamson et al, 1995) is implicated in the pathways of drug reinforcement and reward that characterize alcohol dependence (Strange, 1993; Sibley et al, 1992; Wise and Rompre, 1989; Koob and Bloom, 1988; Wise, 1980). A hypodopaminergic hypothesis of alcohol dependence has been advanced (Ng et al, 1997), that suggests that ethanol releases dopamine in some brain regions, thereby contributing to the rewarding and addictive properties of ethanol (Imperato and Di Chiara, 1986; Ng et al, 1997). The identities of the dopamine system genes that may contribute to alcohol dependence, however, remain obscured by complex environmental factors (Cloninger, 1987; Persico et al, 1993; Muramatsu et al, 1995).

The DAT1 and DRD1 genes are candidates for alcohol dependence because of their role in sustaining dopaminergic neurotransmission in brain regions implicated in the pathways of reward. Recent evidence that striatal dopamine transporter densities may be altered in alcohol dependent subjects (Tiihonen et al, 1995) raises the question of whether DAT1 sequence variations contribute to altered dopamine transporter function in alcohol
dependence. It is tempting to suggest that DAT1 or DRD1 sequence variants may contribute to the hypothesized (Ng et al, 1997) effects of dopamine release into the synapse by ethanol. However, no information on the frequency of DAT1 coding region sequence variants is currently available. Information on the frequency of DRD1 sequence variants in alcohol dependent patients is limited to a mutation screening of eight patients which, while revealing no mutations (Lui et al, 1995), cannot be considered to be conclusive given the small sample size.

1.7 The serotonin hypothesis

A number of neuropsychiatric disorders are characterized by serotonergic abnormalities. Severely affected TS patients have been found to excrete 5-hydroxyindoleacetic acid (5HTAA), the major serotonin metabolite, at higher levels than found in controls (Bornstein and Baker, 1991). Post-mortem brain tissue from TS (Anderson, 1991) and depressed patients (Mann et al, 1996; Boyer and Feighner, 1991) have been found to contain altered concentrations of serotonin compared to control post-mortem brain tissue. Evidence for serotonin metabolite dysregulation has also been identified in Type II alcohol dependent patients (Goldman, 1995), those with early onset and high genetic liability (Cloninger et al, 1989; Brown et al, 1985; Cloninger et al, 1985; Linnoila et al, 1983). In addition, serotonergic abnormalities have been noted in obsessive compulsive disorder (OCD) (Heils et al, 1996; Swedo and Leonard, 1994).

The serotonin transporter (5HTT) gene is a candidate gene in the study of the etiology of neuropsychiatric diseases considered to involve serotonergic abnormalities. Due to its
role in clearing serotonin from the synapse, the serotonin transporter is integral to attenuating serotonergic neurotransmission (Heils et al, 1996). The efficacy of SRIs in treating neuropsychiatric disorders, including unipolar depression (Ogilvie et al. 1996; Anderson and Tomenson, 1994), OCD, anxiety and depression comorbid with TS (Riddle et al, 1988), OCD (Swedo and Leonard, 1994) and alcohol dependence (Lejoyeux, 1996; Narango and Bremer, 1994) suggests that the 5HTT gene is a candidate gene in these disorders.

(a) The 5HTT gene is a candidate for TS

The frequency with which TS patients are treated with SRIs in order to control comorbidities (Swedo and Leonard, 1994; Messiha, 1993), provides the pharmacological rational for examining the 5HTT gene as a candidate gene in TS-OCD patients. Mutation screening, however, has shown that coding region variants of the 5HTT gene are very rare (Di Bella et al, 1996) and probably do not contribute to psychiatric disorders. In this context the recently reported 44 base pair deletion/insertion 5HTT promoter polymorphism is of particular interest because it has been shown to reduce 5HTT gene expression in vitro (Heils et al, 1996; Lesch et al, 1996). Therefore, there is in vivo evidence that the sequence variant of the 5HTT promoter may be a mutation of the regulatory region of the 5HTT gene. This discovery raises the question of whether a polymorphism that has functional significance in vitro, is also functional in vivo (Paterson, 1997; Heils et al, 1996).
The hypothesis that the short promoter polymorphism may modulate serotonin transporter expression, thereby influencing the rate of serotonin clearance from the synapse in vivo (Heils et al., 1996), has not been tested. Evidence that 5HTT mRNA expression may be increased in schizophrenics under neuroleptic treatment (Hernandez and Sokolov, 1997) suggests that 5HTT regulatory pathology may underlie some neuropsychiatric disease states. Similar evidence of altered 5HTT mRNA expression or altered binding sites for serotonin antidepressant drugs (Owens and Nemeroff, 1994), however, is not available for TS-OCD patients. However, numerous studies have used the candidate gene approach to conduct association studies of the 5HTT promoter in neuropsychiatric disorder patient groups (Craddock and Owens, 1997).

Associations of the 5HTT gene promoter polymorphism with anxiety traits (Lesch et al., 1996) and depression (Collier et al. 1996) remain controversial (Ebstein et al. 1997) and unsupported. A study of the 5HTT promoter polymorphism in panic disorder found no association (Deckert et al. 1997). The serotonin transporter remains a candidate gene in anxiety disorders and depression because of evidence for the efficacy of SRI treatment (Ogilvie et al., 1996; Lejoyeux, 1996) of the serotonin reuptake pathology (Collier et al., 1996; Iny et al., 1994) that may underlie these disorders. The recent tentative association of the 5HTT with autism (Cook et al., 1997), a disorder frequently characterized by OCD and anxiety (Gordon et al., 1993; McDougle et al., 1996), suggests that other disorders treated with SRIs, including TS and its comorbidities, may also be associated with serotonergic dysregulation (Messiha, 1993) involving the 5HTT gene.
(b) The 5HTT gene is a candidate gene for alcohol dependence

Alcohol dependence withdrawal is characterized by severe anxiety and depression. Both conditions can be relieved by SRIs. While anxiety has been associated with the 5HTT gene by studies of the 5HTT promoter alleles, depression has been associated with the 5HTT gene (Ogilvie et al, 1996) not by genotyping the 5HTT deletion/insertion polymorphism, but by genotyping the 5HTT intron 2 variable number tandem repeat (VNTR). The frequency of comorbid anxiety and depression in alcohol dependent patients (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, 1990) makes the 5HTT gene a candidate for alcohol dependence.

1.8 Screening candidate genes for sequence variants

(a) Mutation screening by SSCP analysis

Single stranded conformational polymorphism (SSCP) analysis is a commonly used method for the detection of gene sequence variants (Lee et al, 1992; Oto et al, 1993; Hongyo et al, 1993). Common variants include single base pair changes, frameshift mutations, microsatellite polymorphisms and nucleotide insertions. Sequence information from mutation screening is valuable because it provides evidence regarding the extent of amino acid sequence conservation in disease and control populations in addition to providing markers useful in linkage and association studies (Weghorst, 1993; Calvert et al, 1995). The method is based on the principle that when DNA is denatured, the single strands containing sequence variants assume a conformation different from those strands containing no sequence variants. The conformational differences in the
single stranded DNA can be resolved on polyacrylamide gel (Glavac and Dean, 1995; Weghorst, 1993) as depicted in Figure 1. When an SSCP band shift is noted, sequence analysis is then performed in order to identify the nature of a sequence variant.

The availability of pre-cast gradient polyacrylamide gels (Novex, San Diego, CA, USA) that are run in the Novex Xcell II minicell system has resulted in improved efficacy of SSCP analysis (Hongyo et al, 1993). Many current protocols for SSCP, however, have not been modified to accommodate these gels (Bardeesy and Pelletier, 1995; Lam et al, 1996). The initial phase of experimental work described here involved optimizing SSCP conditions for pre-cast gels.

1.9 Research objective: to screen candidate genes for sequence variants

(a) Isolate DAT1 gene sequence variants in TS and alcohol dependence

We set out to conduct the first systematic mutation screening of the DAT1 gene in controls, TS and alcohol dependent patients. The DAT1 screening project is the logical sequel to the recent association of the DAT1 gene with TS (Comings et al, 1996), AD-HD (Cook et al, 1997; Gill et al, 1997; Cook et al, 1995) and alcohol dependence (Muramatsu and Higushi, 1995). This study genotyped a variable number tandem repeat located 3' (3' VNTR): the only DAT1 polymorphism reported prior to our effort to screen the DAT1 gene (Vandenbergh et al, 1992). Therefore, the sequence information obtained from screening the DAT1 gene may be a valuable addition to what is known
Figure 1 The principle of PCR-SSCP analysis. Double stranded PCR products are generated for both wild-type (WT) and polymorphic/mutant (M) alleles. Following denaturation, the single strands refold to form specific secondary structures. Polymorphic/mutant single stranded DNA molecules have a different structure than the wild-type molecules. Polyacrylamide gel electrophoresis can resolve the two different wild-type strands and the two different polymorphic/mutant strands (Galvac and Dean, 1995).
about the DAT1 gene conservation in human populations (Donovan et al., 1995; Vandenberghe et al., 1992; Giros et al., 1992). This sequence information will help to evaluate the contribution of sequence variability in dopamine system genes to the etiology of neuropsychiatric disorders.

(b) Isolate DRD1 gene sequence variants in TS and alcohol dependence

We continued our study of the role of GPCR sequence variants in the etiology of TS and alcohol dependence (George et al., 1993; Lam et al., 1996) by screening the coding region of the DRD1 gene for sequence variants. While linkage analysis has excluded the DRD1 gene from having a major gene effect in TS (Heutink et al., 1995; Gelernter et al., 1993), by genotyping the 5'Taq1A polymorphism no mutation screening of the DRD1 coding region in TS has been reported in the literature. Evidence of a role for the DRD1 gene in the inheritance of addictive disorders has recently been reported (Comings et al., 1997) but has not yet been replicated. No study has screened a large sample of alcohol dependent patients for sequence variants in the coding region of the DRD1 gene (Lui et al., 1995).

(c) Genotype 5HTT gene sequence variants in TS and alcohol dependence

Mutation screening of the 5HTT gene in control and neuropsychiatric patient groups, similar to that which we conducted for the DAT1 gene, has shown that its amino acid sequences are conserved in patient and control groups. Di Bella et al., 1996, conducted a
mutation screen in a number of neuropsychiatric patient groups. TS and alcohol dependent patients, however, were not screened.

The evidence for conservation of 5HTT coding region sequences in a number of neuropsychiatric disorders suggested to us that rather than conducting a mutation screening of the 5HTT gene in our patient groups that it would be more fruitful to carry out a candidate gene study of the 5HTT gene in TS and alcohol dependence. For this purpose we genotyped the 5HTT deletion/insertion polymorphism 5HTT and the intron 2 VNTR polymorphisms recently identified (Hiels et al. 1996; Lesch et al. 1994). The 5HTT deletion/insertion promoter polymorphism was of special interest because of evidence of its possible functional relevance. The intron 2 VNTR was genotyped in order to enable us to conduct haplotype analysis of the 5HTT gene in patient and control groups.

1.10 Research strategy for candidate gene screening and association studies

The candidate gene studies presented here were conducted in order to identify gene sequence variants unique to or significantly more common in patients compared to controls. Mutation screening projects inevitably identify a plethora of sequence variants that have no apparent functional significance. We therefore particularly sought to identify sequence variants of the DAT1 and DRD1 genes that would result in alterations to the structure of their respective gene products. The discovery of novel sequence changes of undetermined significance requires pharmacological characterization in vitro by expression in cell lines (discussed by Hiels et al, 1996 and Lam et al, 1996) in order to
establish whether altered protein sequence alters its function. Association studies of TS and alcohol dependence with the novel sequence variants were conducted while we continued to seek sequence variants leading to altered protein structure for characterization in vitro systems.
MATERIALS AND METHODS

2.0 Materials

- Alkaline Phosphatase (Calf intestinal) Pharmacia Biotech (Baie d'Urfe, PQ)
- Agarose Bio-Rad Laboratories
- Ampicillin Sigma (St. Louis, MO)
- Bacto Agar DIFCO Laboratories (Detroit, MI)
- Programmable Power Supply Biorad, Model 3000Xi
- Control DNA (not psychiatric/addicted) Addiction Research Foundation (Toronto, ON)
- Control DNA (not psychiatric/addicted) Psychopharmacology and Dependence Unit.
- Control DNA (addicted, not psychiatric) Psychopharmacology and Dependence Unit, Women's College Hospital (Toronto, ON)
- 7-deaza-deoxy-guanosine tri-phosphate Pharmacia Biotech (Baie d'Urfe, PQ)
- Deionized Formamide Fluka (Ronkonkoma, NY)
- Dimethyl sulfoxide (DMSO) Fluka (Ronkonkoma, NY)
- DIOB Competant Cells Mr. Tuan Nguyen
- DNA Ligase Pharmacia Biotech (Baie d'Urfe, PQ)
- Patient DNA (Tourette's syndrome) City of Hope Medical Centre (Duarte, CA)
- Patient DNA (Alcohol dependent) Addiction Research Foundation (Toronto, ON)
- pBluescript Strategene (la Jolla, CA)
- PCR Geneamp Kit Perkin-Elmer Cetus (Norwalk, CT)
- T7 Sequencing Kit Pharmacia-Biotech (Uppsala, SW)
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<td>Xcell II mini-cell system</td>
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2.1 DNA extraction

Following informed consent, blood samples were obtained from non-psychiatric control individuals, patients with TS, TS-AD-HD, TS-OCD and alcohol dependence with no psychiatric comorbidity. The criteria whereby these diagnoses were made are described below. Genomic DNA was extracted using phenol extraction and ethanol precipitation of DNA from patient blood as previously described (Bardeesy and Pelletier, 1995). Briefly, 5-10 ml of blood was added to an equal volume of lysis buffer (50 mM HEPES, pH 8, 50 mM NaCl, 5 mM MgCl₂, 10% sucrose, 0.5% Triton X-100) and the mixture was kept on ice for 10 minutes. The mixture was then centrifuged at 3000 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in a mixture of TAE buffer and sodium dodecyl sulfate (SDS) before being incubated at 65 °C for 10 minutes. Fifty ul of 10 mg/ml proteinase K and 50 ul of RNase A were then added before incubating the mixture at 65 °C for 90 minutes.

Two extractions with equal volumes of phenol were performed by mixing the phenol/aqueous mixture for 30 minutes on a platform shaker. The aqueous phase from each extraction was saved and extracted twice more with equal volumes of phenol and chloroform before final extraction with an equal volume of chloroform. A one-tenth volume of 3 M sodium acetate in ethanol was then added to the aqueous phase. The DNA was then removed by spooling with a glass rod and rinsing in 70% ethanol before resuspending in TE buffer.
2.2 Clinical samples

(a) Tourette’s syndrome samples

The TS group screened consisted of 109 unrelated patients recruited from the Tourette’s Syndrome Clinic of the City of Hope National Medical Center (COH), Duarte, California (Comings and Comings, 1993; Comings, 1994; Comings et al. 1996). The patients were selected to be Caucasians of Northern and Western European descent. All patients, and available relatives, completed a detailed behavioral questionnaire patterned after the Diagnostic Interview Schedule (DIS) (Robbins et al. 1981). The entire DIS questionnaire, and its use in other studies, is described elsewhere (Comings and Comings, 1993; Comings et al. 1996).

For all patients screened, the final diagnosis of TS, chronic motor and chronic vocal tic disorder, was made by assessing the variables required according to the DSM-III-R (Diagnostic and Statistical Manual of Mental Disorders. Third Edition-Revised. 1987) and DSM-IV (Diagnostic and Statistical Manual of Mental Disorders. Fourth Edition. 1990). In addition to the primary diagnosis of TS, 29 of the patients screened were diagnosed comorbidly with OCD (TS-OCD) and 33 of the patients were diagnosed comorbidly with AD-HD (TS-AD-HD) according to the DSM-III-R (Diagnostic and Statistical Manual of Mental Disorders. Third Edition-Revised. 1987) and DSM-IV (Diagnostic and Statistical Manual of Mental Disorders. Fourth Edition. 1990) criteria. Forty-seven of the TS patients had no comorbid diagnosis. The TS patients screened had not been included in previous studies (David Comings, personal communication).
The TS sample sizes varied for each gene that was screened or genotyped for two reasons. First, DNA samples were still being obtained during the course of this study: allowing sample sizes to increase after the GPR19 and DRD1 studies. Second, the PCR reactions required to complete the 5HTT gene study were less robust than those involved in conducting the DAT1 study. As a result fewer patients were studied at the 5HTT locus by comparison with the DAT1 locus. The DAT1 gene was screened in all patients. The DRD1 gene was screened in 47 patients with a primary diagnosis of TS in addition to three additional TS-OCD patients selected to be the next consecutive patients interviewed (denoted by the COH patient identification number).

(b) Alcohol dependence samples

DNA samples from 72 patients diagnosed with alcohol dependence were obtained from the Addiction Research Foundation (ARF) of Ontario. The patients were diagnosed with alcohol dependence when admitted to the Toronto Hospital between 1989 and 1992 for alcohol-related chronic health problems. The assessment of alcohol dependence was made according to DSM-III-R criteria (Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised, 1987). The alcohol dependent group consisted of unrelated Caucasians of northern and western European descent who resided in the Metropolitan Toronto Area (George et al., 1993).

All 72 samples were screened for mutations in the GPR19 and DRD1 genes. The DAT1 gene was screened in 64 patients. The 5HTT gene was genotyped for the same 64 patients. The Bernoullie multiple testing corrections (discussed below) accounted for the
fact that previous studies by our group have genotyped the DRD4 exon 3 polymorphism (George et al, 1993) and the DRD2 Taq 1A polymorphism in the alcohol dependent group.

(c) Control samples

The GPR19 and DRD1 mutation screening projects involved screening 50 non-psychiatric control samples from the Addiction Research Foundation (ARF) of Ontario, Toronto, Ontario. The subjects were described as being healthy volunteers from the Metropolitan Toronto area with no history of psychiatric treatment at the time blood was drawn.

For the study of the DAT1 and 5HTT genes in TS, groups of 67 and 91 non-psychiatric controls respectively, were selected from the data-base of the Psychopharmacology and Dependence Research Unit, Women’s College Hospital, Toronto, Ontario. Each control was ethnically matched to the TS group. The controls were genotyped for the 3'VNTR and the novel polymorphisms in exons 9 and exon 15 of the DAT1 gene and the deletion/insertion and intron 2 VNTR polymorphisms of the 5HTT genes.

For the study of the DAT1 and 5HTT genes in alcohol dependent patients, 64 controls were genotyped for the 3'VNTR and the novel polymorphisms of exons 9 and 15 of the DAT1 gene. Of the total control group, the 15 exons of the DAT1 gene were screened for 42 controls obtained from the Addiction Research Foundation (ARF) of Ontario, Toronto, Ontario. These controls were healthy volunteers from the Metropolitan Toronto area with
no history of psychiatric treatment at the time blood samples were taken. The remaining 22 controls, selected using the same criteria from the data-base of the Psychopharmacology and Dependence Research Unit, Women’s College Hospital, Toronto, Ontario, were genotyped for the 3'VNTR and the common novel polymorphisms in exons 9 and 15 of the DAT1 gene.

2.3 PCR amplification of gene fragments subjected to SSCP mutation screening

(a) Amplification of GPR19 gene fragments used to refine SSCP method

SSCP analysis of the GPR19 gene was conducted by amplifying the 1245 base pair open reading frame (O’Dowd et al., 1996) into five overlapping fragments using primer pairs designed de novo for this purpose (Table 1). The PCR reactions were conducted using 1 ug of each oligonucleotide primer (Table 1), 2 umol dNTP, 1.5 mmol MgCl$_2$ and 0.5 U Taq DNA polymerase (Life Technologies, Inc. Gaithersburg, MD) in a total reaction volume of 100 ul. 200 ng of template was used per reaction. The PCR was conducted in a Perkin Elmer Therocycler using 40 cycles, at 95 °C for 45 seconds, 55-61 °C for 45 seconds, and 72 °C for 1 minute 30 seconds. The amplified DNA was analyzed by agarose gel electrophoresis prior to mutation screening.

(b) Amplification of DRD1 fragments to be analysed by SSCP

The primers used to amplify the overlapping DRD1 gene fragments were designed de novo for use in this screening project. SSCP analysis of the DRD1 gene encoding the 446 amino acid dopamine D1 receptor (Sunahara et al., 1990) was conducted by amplifying the 1338 base pair DRD1 gene coding region in five overlapping fragments using the
### TABLE 1. Oligonucleotides Used in PCR-SSCP Analysis of Human GPR19 genes

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<tr>
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</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>Primer N</td>
<td>Primer Sequence (5'-3')</td>
<td>Fragment (base pairs)</td>
<td>Anneal Temp. °C</td>
</tr>
<tr>
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<tr>
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<td>GCAAACCCACAGGCAATCTCC</td>
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primer pairs listed in Table 2. The PCR reactions were conducted using 200 ng human genomic DNA, 1 μg of each oligonucleotide primer (Table 2), 2μmol dNTP, 1.5 mmol MgCl₂ and 0.5 U Taq DNA polymerase (Life Technologies, Inc. Gaithersburg, MD). PCR was conducted using 40 cycles, at 95 °C for 30 seconds, 55-60 °C for 40 seconds, and 72 °C for 40 seconds in a Perkin Elmer Gene Amp PCR System 2400 machine. The amplified DNA was analyzed by agarose gel electrophoresis prior to the mutation screening.

(c) Amplification of DAT1 exons analyzed by SSCP

The complete set of PCR primers used to amplify each DAT1 exon and the 3' and 5' flanking regions is listed in Table 3. The primers were designed from unpublished sequence information made available to us through our collaboration with the laboratory of George Uhl, National Institute on Drug Abuse, Johns Hopkins University School of Medicine (David Vandenbergh, personal communication). The PCR reactions were conducted using 200 ng human genomic DNA, 0.5 μg of each oligonucleotide primer (Table 1), 2 μmol dNTP, 1.5 mmol MgCl₂, 10% DMSO and 0.5 U Taq DNA polymerase (Life Technologies, Inc. Gaithersburg, MD) (Lam et al., 1996; Heiber et al., 1996; Marchese et al., 1994). The 15 pairs of PCR primers used to amplify each exon are listed in Table 3. PCR was conducted using 40 cycles, at 95 °C for 30 seconds, 60-68 °C for 40 seconds, and 72 °C for 40 seconds in a Perkin Elmer System 2400 Gene Amp PCR machine. The total reaction volume was 50 μl.
TABLE 3. Oligonucleotide Primers Used in PCR-SSCP Analysis of Human DAT1 gene

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5'-3')</th>
<th>Location</th>
<th>Frag size (bp)</th>
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(d) **Genotyping the DAT1 3'VNTR by PCR amplification**

Amplification of the DAT1 3'VNTR was conducted using primers consisting of: T3-5Long, 5'-TGTGGTGTAAGGAACGCCTGAG-3' and T7-3aLong, 5'-CTTCCTGGAGGTCAACGGCTCAAGG-3. Concentrations of PCR reagents in the final reaction mixture were as described for amplification of DAT1 exons above. The total PCR reaction volume was 50 ul. PCR was conducted using 40 cycles, at 94 °C for 30 seconds, 68 °C for 40 seconds, and 72 °C for 40 seconds in a Perkin Elmer Gene Amp PCR System 2400 machine. DNA was analyzed by 4% agarose gel electrophoresis in order to separate VNTR alleles of different sizes. Amplified DNA was visualized by ethidium bromide staining.

(e) **Genotyping the 5HTT polymorphisms by PCR amplification**

The 5HTT promoter variant was amplified using two sets of oligonucleotide primers. Eighty-five DNA samples from TS patients and 91 TS controls, in addition to 61 of the 64 DNA samples from alcohol dependent patients and 32 controls were genotyped at the 5HTT promoter by amplifying a 484/528-bp fragment using the primers stpr5, 5'-GGCGTTGCGCGCTGGATATGC-3' and stpr 3 5'-GAGGGACTGAGCTGGACAACCCAC-3'. The alcohol dependent DNA samples were genotyped at the 5HTT promoter by amplifying a 406/450-bp fragment using the primers HTtp2A, 5'-TGAATGCCCAGCACCTAACC-3', and HTtp2B, 5'-TTCTGGTGCCACCTAGACGC-3'.
The amplification of the 5HTT promoter was conducted using as little as 100 ng human genomic DNA. One ug of each oligonucleotide primer, 2.0 mmol MgCl$_2$, 0.6 U Taq DNA polymerase and 1X PCR buffer (Life Technologies, Inc. Gaithersburg, MD) were used in each PCR reaction. Each reaction mixture contained 200 umol of each dNTP, with the exception of dGTP which was included at a concentration of 100 umole, plus 100 umole of 7'-deaza-dGTP. The total PCR reaction volume was 25 ul. PCR was conducted using 40 cycles, at 94 °C for 30 seconds, 60 °C for 40 seconds, and 72 °C for 40 seconds in a Perkin Elmer Gene Amp PCR System 2400 machine. The amplified DNA was analyzed by 4% agarose gel electrophoresis and the DNA amplified was visualized by ethidium bromide staining prior to photography over a UV light source.

The 5HTT exon 2 VNTR was amplified for 75 controls matched to TS patients and 32 controls matched to alcohol dependent patients using the primers HTT2X, 5'-TGGATTTCCCTTCCTCACGTGATTGG-3', and HTT2Y, 5'-TCATGTTCTAGTCTTACGCGCGT-3', to amplify the 345-bp (9 copy), 360-bp (10 copy) and 390-bp (12 copy) fragments of DNA encompassing the three alleles of the VNTR. The VNTR of the 85 TS, 64 alcohol dependent, and 16 controls matched to TS patients was amplified using the primers, 5'-CAATGTCTGGCCTCCCTACATAT-3' and 5'-GACATAATCTGTCTTCTGGCCCTCTCA-3'. The PCR products were resolved in 4% acrylamide gel and visualized by silver staining.

Amplification of the VNTR was conducted using 150 ng human genomic DNA, 1 ug of each oligonucleotide primer, 300 umol dNTP, 2.0 mmol MgCl$_2$, 0.6 U Taq DNA polymerase and 10% PCR buffer (Life Technologies, Inc. Gaithersburg, MD) per PCR
reaction. The total PCR reaction volume was 25 ul. PCR was conducted using 40 cycles, at 94 °C for 30 seconds, 57 °C for 40 seconds, and 72 °C for 40 seconds in a Perkin Elmer Gene Amp PCR System 2400 machine. DNA was analyzed by 4% agarose or 4% acrylamide gel electrophoresis and then visualized by ethidium bromide staining or silver staining respectively.

2.7 SSCP mutation screening of GPR19, DRD1 and the DAT1 gene

(a) Overview of the SSCP methodology

Non-denaturing polyacrylamide gel electrophoresis was conducted on pre-cast 4-20% gradient gels (Novex) in the Xcell II mini-cell system (Novex). In some cases the ThermoFlow SSCP Electrophoresis System, consisting of an Xcell II mini-cell system adapted to allow temperature regulated electrophoresis, was used to optimize SSCP conditions. The ThermoFlow re-circulates 1XTBE buffer from the mini-cell through a condenser coil regulated by a temperature controlled water bath (Haake, Karlsruhe, Germany). Mutation screening efficiency was optimized (Oto et al, 1993) by screening each PCR product under at least two temperature conditions by adjusting the TBE buffer temperature. SSCP was routinely conducted at 4 °C in the cold room and at 24 °C (room temperature). Intermediate or higher temperature SSCP (see results section) was conducted in the ThermoFlow.

Initially, electrophoresis was conducted at 200 volts for 2 hours at 24 °C in the ThermoFlow as indicated by recent protocols (Calvert et al, 1995). However, the majority of the SSCP analysis reported here was conducted at between 50-90 volts.
Electrophoresis at 4 °C was conducted at 85 volts for 15-18 hours. At 24 °C the electrophoresis voltage was dropped to 55 volts in order to compensate for the faster running time of SSCP conducted at 24 °C.

b) SSCP protocol adapted for use with Xcell II apparatus (Novex)

Between 3 and 6 ul of the 50 ul PCR reaction was diluted 1:4 with a formamide based SSCP loading buffer in individual 0.2 ul micro-amp PCR tubes (O’Dowd et al, 1996). The denaturing buffer was prepared before SSCP analysis by combining, in a 2:3 ratio, a stock denaturing solution with a stock stop solution. Both stocks were stored at room temperature. The denaturing solution was prepared with 0.1% SDS and 10 mM EDTA. The stop solution was prepared with 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole FF. Samples were denatured at 95 °C for 5 minutes before being soaked at 4 °C in the PCR machine for 5 minutes prior to loading on acrylamide gel (O’Dowd et al, 1996).

The entire 20 ul mixture of denaturing buffer and PCR product was loaded into the separate wells of Novex 4-20 % TBE gels (Novex, La Jolla, California). Most SSCP was run in the cold room or at room temperature, as described. DC Power was supplied by a Biorad, Model 3000Xi Programmable Power Supply (Richmond, CA). A proportion of the total PCR products amplified from patient and control DNA samples were selected for repeated analysis in the ThermoFlow SSCP Electrophoresis System at other temperatures. The Novex SilverXpress Staining kit was used to visualize SSCP band
shifts. Sequencing of the PCR products corresponding to SSCP band shifts was done as described below (O’Dowd et al., 1996).

2.8 Identification of sequence variants by chain-termination DNA sequencing

PCR products identified by SSCP to be potentially polymorphic were subcloned into pBluescript, transformed into *E.Coli* competent cells (strain DIOB), grown overnight in LB broth with ampicillin before miniprepping and sequencing the PCR insert as previously described (Marchese et al., 1994).

2.9 Statistical Methods

The differences between the genotype and allele frequencies of the TS and alcohol dependent groups and their respective control groups were analyzed by two-tailed $\chi^2$ tests using the Statistical Package for the Social Sciences (SPSS), version 7. Hardy-Weinbergh equilibrium for the genotypes at each locus was tested using $\chi^2$ analysis as done previously (Kennedy et al., 1995). The Bonferroni correction for multiple testing was used in order to account for testing associations at three loci of the DAT1 gene and two loci of the 5HTT gene (Aickin and Gensler, 1996). Analysis of sample power was conducted using the Epi Info, Version 5.01a (Public Domain Software for Epidemiology and Disease Surveillance, March 1991). Linkage disequilibrium between haplotypes consisting of the common polymorphisms and the 3'VNTR was analyzed using the maximum likelihood Equilibrium Haplotype (EH) (Terwilliger and Ott, 1994; Ott, 1991) program.
RESULTS

3.1 SSCP mutation screening of the GPR19 gene

The SSCP methodology used to screen candidate genes in this project was refined by screening GPR19 in the 50 caucasian controls and 72 caucasian alcohol dependent patients described in the context of the DRD1 gene mutation screening project. The purpose of this preliminary screening work was two-fold. First, we were interested in establishing the optimal SSCP conditions for screening candidate genes for sequence variants. Second, we were interested in determining whether polymorphic sequence variants of GPR19 existed.

GPR19 is an intronless gene that encodes a 414 amino acid gene product. Our interest in GPR19 stemmed from the fact that the GPR19 gene has many of the characteristics of neurotransmitter G protein-coupled receptors (O'Dowd et al, 1993). Hydrophobic analysis suggests that the deduced amino acid sequence of GPR19 has the seven transmembrane (TM) regions characteristic of GPCRs. The expression of GPR19 in the olfactory tubercle, the islands of Calleja, the caudate-putamen, and the pituitary suggests that GPR19 encodes a neurotransmitter receptor with a pattern of expression similar to the dopamine D2 receptor. The fact that 101 amino acids of the GPR19 gene product were identical to those of the dopamine D2 receptor is further evidence that GPR19 is likely to encode a neurotransmitter receptor. However, no ligand for the GPR19 receptor has yet been identified.

Screening GPR19 revealed a number of single base pair nucleotide changes. The combination of extended SSCP gel running time and reduced voltage was found to result
in more consistent resolution of DNA sequence variants by SSCP conducted on 4-20% pre-cast polyacrilamide gels (Novex). SSCP detected a silent A/G polymorphism at nucleotide 1062 of the GPR19 gene of a control subject when samples were run at 4 °C at 85 volts for 15 hours. This polymorphism was detected by the presence of an additional band (Fig. 2B, lane 2) in the portion of the SSCP gel resolving both single stranded (SS) DNA and double stranded (DS) DNA. The polymorphism was poorly resolved, however, when the samples were electrophoresed at 24 °C at 55 volts for 9 hours (Fig. 2C). The rapid SSCP protocol (300 volts, 2 hours) did not resolve this polymorphism (Fig. 2A, lane 2). These results demonstrate the importance of controlling temperature and voltage conditions in order to ensure sensitive resolution.

The effect of SSCP running temperature on the resolution of sequence variants is also illustrated in Figure 3. SSCP analysis run at 4 °C for 15 hours at 85 Volts resolved band shifts that were revealed by sequencing to be homozygous (Fig 3A, lane 2) and heterozygous (Fig 3B, lane 2) forms of a T/C nucleotide change at base pair 250 located in the portion of GPR19 encoding the putative second transmembrane (TM2) domain. The nucleotide change resulting in the band shifts in Fig 3 was detected by SSCP in less than 2% of the 114 DNA samples screened. The nucleotide change resulted in a conservative Val161le amino acid change. The same heterozygous polymorphism resolved in Fig 3B, lane 2 was also resolved by SSCP analysis run at 24 °C. However, the pattern is very different.

Figure 4 shows the SSCP analysis of the portion of GPR19 encoding the putative third transmembrane (TM3) domain of the receptor. The SSCP band shifts shown here
Figure 2  Optimization of SSCP temperature, running time and voltage to resolve variants. SSCP analysis of GPR19, run at 4 °C at 85 volts for 15 hours, detected a polymorphism (B, lane 2). Note the presence of an additional band in the portion of the SSCP gel resolving both single stranded (SS) and double stranded (DS) DNA. The polymorphism was poorly resolved at 24 °C at 55 volts for 9 hours (C, lane 2). Rapid SSCP (300 volts, 2 hours) did not resolve this polymorphism (A, lane 2).
Figure 3 The effect of SSCP running temperature on the resolution of sequence variants. The SSCP analysis run at 4 °C resolved band shifts revealed to be homozygous (A, lane 2) and heterozygous (B, lane 2) forms of a T/C nucleotide change in GPR19. The heterozygous polymorphism (B, lane 2) resolved into a different pattern when SSCP was conducted at 24 °C (C, lane 2).
Figure 4 Resolution of sequence variants in TM3 of GPR19. Variants were detected by SSCP run at 24 °C but not at 4 °C. The SSCP gel was run at 50 volts for 15 hours using the SSCP conditions discussed.
were detected at 24 °C but not at 4 °C. The band shifts were present in nearly 7% of the DNA samples analyzed. Sequencing showed that this nucleotide change of C/T at base pair 300 resulted in a conservative amino acid change from Ile289Val.

In the course of refining the SSCP protocol, various polyacrylamide gels were compared for their ability to detect a single nucleotide change. Resolution of the common GPR19 polymorphism on pre-cast polyacrylamide non-gradient 10% gels was inferior to resolution on 4-20% gradient gels (Figure 5). The 10% gels were electrophoresed with 60% of the voltage used to run SSCP 4-20% gradient gels, however, high SSCP band shift resolution was not maintained. Therefore, further use of SSCP to screen for sequence variants in candidate genes was undertaken using 4-20% polyacrylamide gels.

The above results represent our first experience with conducting SSCP on pre-cast polyacrylamide gels. The resolution appeared superior compared with that used in our lab by Lam et al, 1996 to screen the 5HT1A receptor for sequence variants in TS patients. The GPR19 gene was found to be highly conserved in patients and controls.

3.2 SSCP mutation screening of the DRD1 gene

The SSCP analysis involved screening the entire coding region of the DRD1 gene for sequence variants using SSCP conditions that were found to be effective in screening the GPR19 gene for sequence variants. No SSCP band shifts were noted in the TS (n=50), the TS-AD-HD (n=35), the TS-OCD (n=30) and the alcohol dependent (n=72) patient groups when SSCP was conducted at 55 volts for 18 hours at 24 °C or at 100
Figure 5 Resolution of TM3 variant on 10% gels was inferior to that on 4-20% gels. When 10% gels were run at 60% of the voltage used to run gradient gels, SSCP resolution was not maintained.
volts for 18 hours at 4 °C. One band shift was observed in the SSCP analysis of the portion of the gene coding for the amino terminus of the DRD1 gene in one of the 50 controls matched to the Tourette’s syndrome population (Figure 6, lane 2). The SSCP analysis was conducted at 100 volts for 18 hours at 4 °C. Sequence analysis of the DRD1 gene in 3 representative patients from each patient and control group confirmed that the SSCP band shift observed for the control subject analysed in figure 6, lane 2 was caused by a base pair substitution of C for T which resulted in the conservation of 49Ile. This sequence was not found among the patients screened.

3.3 SSCP mutation screening of the DAT1 gene

The DAT1 gene mutation screening project was ambitious because of the size and complexity of the gene. The DAT1 cDNA encodes a large 620 amino acid protein, encoded by 15 exons and introns of which all exons but the first are translated. The size of the DAT1 introns remains unknown. However, published (Donovan et al, 1995) and unpublished (Vandenbergh, personal communication) intron-exon junction sequence information enabled us to screen the DAT1 gene by subjecting the PCR products amplified using the primers listed in Table 3 to SSCP analysis. This procedure presented difficulties for those primer pairs that amplified more than one DNA product. For this reason, the primers for exons 4, 10 and 12 were designed more than once before the set presented in Table 3 successfully amplified the correct gene fragment.

The structure of the DAT1 gene was analyzed by SSCP in 225 subjects. Band shifts were noted in all groups when exon 2 (using primer pair EX2ForB and 2C1, Table
Figure 6 Analysis of a fragment (350 bp) of the D1 receptor gene. This silent mutation was only detected by electrophoresis conducted at 85 volts for 24 hours at 1 °C. The arrow indicates the pattern of a polymorphic variant.
3) was analyzed by SSCP conducted at 55 volts for 18 hours at 24 °C (Fig. 7 A, lane 1). Sequence analysis showed that the band shift was due to a sequence variant 171C/T that did not result in an amino acid change. The individual analyzed in lane 1 was revealed, by sequencing, to be heterozygous.

In addition to the silent 171C/T polymorphism, a rare nucleotide change was also discovered in exon 2 (Fig. 7, lane 3) in a single control subject. Sequence analysis showed that this band shift was the result of a 164T/C nucleotide substitution that results in a relatively conserved amino acid change Val164Ala in this control subject.

A second conservative amino acid change was also discovered in another control subject. Sequencing confirmed that the SSCP band shift in exon 8 (Fig 8, lane 1), resolved by 50 volt electrophoresis for 12 hours at 30 °C, resulted from a sequence variant 1445T/C. A Val144Ala amino acid change resulted. The individual analyzed in Fig 8, lane 1 was heterozygous for this sequence variant.

SSCP band shifts were also noted in all groups when exon 9 (Fig 9) and exon 15 (Fig 10) were analyzed by SSCP conducted at 100 volts for 18 hours at 4 °C on 4-20% gradient gels. Sequencing revealed that the exon 9 SSCP band shift (Fig 9, lane 2) resulted from a homozygous sequence variant 1215A/G that did not result in an amino acid change. Sequencing identified the individuals in lanes 1 and 3 to be homozygous non-polymorphic and heterozygous for the polymorphism respectively. The exon 15 SSCP band shift (Fig. 10, lane 1) was confirmed to result from a homozygous sequence variant 1898T/C downstream of the translated portion of exon 15. Sequencing confirmed
Figure 7 SSCP analysis of DAT1 exon 2 in three control subjects. Sequence analysis showed that the SSCP band shift in lane 1 was due to a silent sequence variant 171C/T. This individual was revealed, by sequencing, to be heterozygous. A rare nucleotide change was also discovered in the patient analysed in lane 3. Sequencing showed that this SSCP band shift reflects a 164T/C nucleotide change in exon 2, resulting in a conservative amino acid change Val164Ala in this control subject.
Figure 8  SSCP analysis of DAT1 exon 8 in three control subjects. The band shift in lane 1 was found to reflect a 445T/C sequence variant that results in a Val1144Ala amino acid change. The individual analyzed in lane 1 was heterozygous for this sequence variant. Individuals analyzed in lanes 2 and 3 were shown by sequencing to be non-polymorphic.
Figure 9  SSCP analysis of DAT1 exon 9 in three TS subjects. Sequencing revealed that the exon 9 SSCP band shift in lane 2 resulted from a homozygous 1215A/G sequence variant that did not result in an amino acid change. Sequencing identified the individuals in lanes 1 and 3 to be homozygous non-polymorphic and heterozygous for the polymorphism respectively.
Figure 10 SSCP analysis of DAT1 exon 15 in three control subjects. The band shift in lane 1 was confirmed, by sequencing, to result from a homozygous sequence variant 1898T/C located downstream of the translated portion of exon 15. Sequencing confirmed that the individuals analyzed in lanes 2 and 3 were non-polymorphic and heterozygous for the exon 15 polymorphism respectively.
that the individuals analyzed in lanes 2 and 3 were non-polymorphic and heterozygous for the exon 15 polymorphism respectively (Giros et al, 1992; Vandenbergh et al, 1992).

In order to complete the project, patients and controls were genotyped for a polymorphic 3'VNTR marker at the DAT1 locus (Vandenbergh et al, 1992). Three alleles of the 3'VNTR were identified (Figure 11). Lane 1 shows the 480 base pair band identified to be homozygous for the 10 repeat allele (10/10) of the 3'VNTR. Lane 2 shows the 480 base pair band and the 442 base pair band below it. This individual was heterozygous for the 10 repeat and the 9 repeat alleles (10/9) of the 3'VNTR. Lane 3 shows a 150 base pair band which identifies an individual homozygous for the 3 repeat allele (3/3) of the 3'VNTR.

### 3.4 Association study of DAT1 polymorphisms in TS and alcohol dependence

Allele frequencies of the novel polymorphisms are shown in Table 4. The frequency of the exon 2 polymorphism was between 3.7% and 6% in the TS (n=47), alcohol dependent (n=64) and control groups (n=42) (Table 4). Because of its low frequency, we did not genotype our remaining patient and control samples for the exon 2 polymorphism. The frequencies of the common polymorphisms in exons 9 and 15 were determined in all patient and control groups (Table 4).

The exon 9 A/G frequencies were 19% and 23% in TS and control groups respectively. The frequency of the exon 9 T/C polymorphism was 33% in the alcohol dependent group and 24% in the matched control group. $\chi^2$ analysis (Table 4), however,
Figure 11  PCR genotyping of the DAT1 3'VNTR.  Lane 1 was found to be a 480 base pair band homozygous for the 10 repeat allele (10/10). Lane 2 shows the separation of the 480 base pair band and the 442 base pair band below it. This individual was found to be heterozygous for the 10 repeat and the 9 repeat alleles (10/9). Lane 3 shows the 150 base pair band identifying an individual homozygous for the 3 repeat allele (3/3).
### TABLE 4. DAT1 allele and genotype frequencies: $\chi^2$P values compare population frequencies

<table>
<thead>
<tr>
<th>Population</th>
<th>Exon 2 C/T</th>
<th>N Frequency</th>
<th>Genotype Frequencies</th>
<th>$\chi^2$P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-1</td>
<td>1-2</td>
<td>2-2</td>
</tr>
<tr>
<td>Tourette's</td>
<td>109</td>
<td>0.037</td>
<td>0.26</td>
<td>0.074</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>0.06</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Alcoholics</td>
<td>64</td>
<td>0.047</td>
<td>0.906</td>
<td>0.094</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>-</td>
<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>Exon 9 A/G</th>
<th>Exon 9 Allele 1 A/G</th>
<th>N Frequency</th>
<th>Genotype Frequencies</th>
<th>$\chi^2$P values</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>1-1</td>
<td>1-2</td>
<td>2-2</td>
</tr>
<tr>
<td>Tourette's</td>
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<td>Control</td>
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<tr>
<td>Alcoholics</td>
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<td>Control</td>
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<td>0.76</td>
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<table>
<thead>
<tr>
<th>Exon 15 T/C</th>
<th>Exon 15 Allele 1 T/C</th>
<th>N Frequency</th>
<th>Genotype Frequencies</th>
<th>$\chi^2$P values</th>
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</thead>
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<td></td>
<td></td>
<td>1-1</td>
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<td>2-2</td>
</tr>
<tr>
<td>Tourette's</td>
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<tr>
<td>Control</td>
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<tr>
<td>Alcoholics</td>
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<tr>
<td>Control</td>
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<td>0.76</td>
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<table>
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<th>3'VNTR 48 bp</th>
<th>3'VNTR Genotype Frequencies</th>
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<td>10/10</td>
<td>9/10</td>
</tr>
<tr>
<td>Tourette's</td>
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<td>0.66</td>
</tr>
<tr>
<td>Control</td>
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<td>0.61</td>
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<tr>
<td>Alcoholics</td>
<td>64</td>
<td>0.53</td>
</tr>
<tr>
<td>Control</td>
<td>64</td>
<td>0.56</td>
</tr>
</tbody>
</table>

3'VNTR compared by using 10/10, 9/10 and *Other genotypes to generate $\chi^2$P value.
indicates that the difference between exon 9 A/G allele frequencies and exon 9 A/G genotype frequencies was not significant between patient and control groups.

The exon 15 T/C frequencies were between 21% and 27% for both patient (TS and alcohol dependent) and control groups. $\chi^2$ analysis (Table 4) indicated that the exon 15 T/C frequencies and genotype frequencies were not significantly different between patient and control groups. Genotype and allele frequencies were also found not to be significantly different between the TS group with no comorbidity, the TS-OCD group and the TS-AD-HD ($\chi^2$P values ranging from 0.283 through 0.872). All patient and control groups were found to fit closely to Hardy-Weinbergh equilibrium at each of the three polymorphisms genotyped, including the 3'VNTR. This concurs with the fact that although the DNA was obtained from 4 sources, the patient and control subjects were ethnically matched. The $\chi^2$P values ranged from 0.1 (exon 9 A/G in alcohol dependent patients), through 0.97 (exon 15 T/C in TS).

Linkage disequilibrium between DAT1 haplotypes was also analyzed. The 10/9 repeat polymorphism of the 3'VNTR (VNTR 10/9), found at similar allele and genotype frequencies in each group (Table 4), was in linkage disequilibrium with the exon 15 polymorphism in the TS group ($\chi^2$=60.91, 5df, p<10^-4), the alcohol dependent group ($\chi^2$=19, 5df, p<10^-4) and in their respective control groups ($\chi^2$=27.74, 5df, p<10^-4, $\chi^2$=18.42, 5df, p<10^-4). For the exon 15 T/C-VNTR 10/9 haplotype, linkage disequilibrium coefficients $\delta$ were 0.11 for TS patient and control groups and 0.10 for alcohol dependent patient and control groups. The modest disequilibrium coefficient $\delta$ values, on an absolute value scale from 0-0.25, suggest that, while significant
disequilibrium exists for the exon 15 A/G-VNTR 10/9 haplotype, not all individuals with the exon 15 T/C allele have the VNTR 10/9 allele on the same chromosome.

In the TS group, linkage disequilibrium was also found between the exon 9 polymorphic allele and the 3'VNTR ($\chi^2=18.73$, 5 df, $p<4\times10^{-4}$) and between the exon 9 and exon 15 polymorphic alleles ($\chi^2=12.72$, 3 df, $p<0.04$). The disequilibrium constants $\delta$ were 0.7 and 0.6 for the exon 9 A/G-VNTR 10/9 and exon 9 A/G-exon 15 T/C haplotypes respectively. The more modest $\delta$ values for these TS haplotypes compared to those of the exon 15 T/C-VNTR 10/9 haplotype suggests that, while statistically significant, the exon 9 A/G-VNTR 10/9 and exon 9 A/G-exon 15 T/C haplotypes are less frequent. No evidence for linkage disequilibrium was found between these alleles in either the control groups or the alcohol dependent group.

3.5 Association study of 5HTT polymorphisms in TS and alcohol dependence

The deletion/insertion 5HTT promoter polymorphism and the 5HTT intron 2 VNTR polymorphism were genotyped in all patient and control groups (Figures 12 and 13). The allele and genotype frequencies are summarized in Table 5. $\chi^2$ analysis showed that genotype frequencies at both loci fit closely to Hardy-Weinbergh equilibrium for patient and control groups. For the bi-allelic promoter locus, $\chi^2$P values ranged from 0.26 (for the alcohol dependent group) to 0.92 (for the TS group). For the tri-allelic intron 2 VNTR locus, $\chi^2$P values ranged from 0.11 (for the TS group) to 0.80 (for the alcohol dependent group).
Figure 12 Deletion/insertion 5HTT promoter polymorphism genotyped on 4% agarose. Lane 1 shows the PCR product (~484 bp) of an individual homozygous for the short promoter. Lane 3 shows the PCR product (~528 bp) of an individual homozygous for the long promoter. Lane 2 shows PCR products for the long and short promoter amplified from an individual heterozygous for the long and short promoters.
Figure 13 Intron 2 VNTR polymorphism genotyped on 4% agarose. Lane 1 shows a PCR product (~390 bp) homozygous for the 12 copy (390 bp). Lanes 2 and 3 show two band shifts for a patient heterozygous for the 12 copy and 9 copy (bp) alleles. Lane 4 shows the two band shifts of a patient heterozygous for the 12 copy and 10 copy (bp) alleles.
The SHTT promoter deletion (the short promoter) was significantly more common in the TS group (51%) compared with the control group (36%) ($\chi^2=8.77$, 2 df, $p=0.04$, two tailed, Bonferroni corrected). Genotype frequencies were not significantly different between the TS and control groups ($\chi^2=8.45$, 2 df, $p=0.18$, two tailed) after Bonferroni correction even though the homozygous short genotype was twice as common in the TS group (26%) compared to the control group (13%). Odds Ratio for short promoter allele association with TS was 1.88 [95% CI 1.21-2.93]).

The short promoter allele was not significantly more frequent ($\chi^2=1.52$, 2 df, $p=0.22$, two tailed) in the alcohol dependent group (45%) compared to its matched control group (37.5%). Although the homozygous short promoter allele was more frequent among alcohol dependent patients (28%) compared with the matched control group (16%), the genotype frequencies were also not significantly different between the patient and control groups ($\chi^2=183$, 2 df, $p=0.40$, two tailed).

The allele and genotype frequencies of the intron 2 VNTR were not significantly different between the TS group and matched control group ($\chi^2=4.09$, 2 df, $p=0.129$, two tailed; $\chi^2=6.37$, 3 df, $p=0.1$, two tailed), as reported in Table 5. Between alcohol dependent and control groups, however, the allele frequencies ($\chi^2=20.36$, 1 df, $p=7.2*10^{-5}$, two tailed, Bonferroni corrected) and genotype frequencies ($\chi^2=25.08$, 3df, $p=0.0006$, two tailed, Bonferroni corrected) were significantly different, as reported in Table 5. The Odds Ratio for an alcohol dependent patient having the 10 copy allele was 5.80 [95% CI 2.42-14.37].
<table>
<thead>
<tr>
<th>Group</th>
<th>VNTR</th>
<th>12/12</th>
<th>10/12</th>
<th>10/10</th>
<th>9/12</th>
<th>9/10</th>
<th>9/9</th>
<th>9/All</th>
<th>( \chi^2 ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intron 2</strong></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tourette's</td>
<td>109</td>
<td>0.44</td>
<td>0.37</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>67</td>
<td>0.44</td>
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<td>0.14</td>
</tr>
<tr>
<td>Alcoholics</td>
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<td>0.53</td>
<td>0.18</td>
<td>0.025</td>
<td>0.025</td>
<td>0</td>
<td>0.05</td>
<td>6*10^{-4}</td>
</tr>
<tr>
<td>Control</td>
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<td>0.20</td>
<td>0.03</td>
<td>0.16</td>
<td>0</td>
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</tr>
</tbody>
</table>

\( 5HTT \) intron VNTR genotype frequencies (4X2) compared 12/12, 10/12, 10/10 and 9/All other alleles to generate \( \chi^2 \) value;
\( 5HTT \) allele frequencies (2X2) compared the frequency of the 10 copy allele to the combined frequencies of all other alleles.

<table>
<thead>
<tr>
<th>Group</th>
<th>VNTR</th>
<th>12/12</th>
<th>10/12</th>
<th>10/10</th>
<th>9/12</th>
<th>9/10</th>
<th>9/9</th>
<th>9/All</th>
<th>( \chi^2 ) values</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tourette's</td>
<td>91</td>
<td>0.47</td>
<td>0.24</td>
<td>0.50</td>
<td>0.26</td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.18</td>
</tr>
<tr>
<td>Control</td>
<td>91</td>
<td>0.64</td>
<td>0.42</td>
<td>0.45</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcoholics</td>
<td>64</td>
<td>0.55</td>
<td>0.34</td>
<td>0.38</td>
<td>0.28</td>
<td></td>
<td></td>
<td>0.40</td>
<td>0.22</td>
</tr>
<tr>
<td>Control</td>
<td>32</td>
<td>0.63</td>
<td>0.40</td>
<td>0.44</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Linkage disequilibrium between the deletion/insertion polymorphism and the intron 2 VNTR polymorphism was analyzed in each patient and control group. No evidence of linkage disequilibrium between the polymorphisms was found in the TS group or its control group ($\chi^2=2.51$, 5 df, $p=0.5$; $\chi^2=0.20$, 5 df, $p=0.5$). Linkage disequilibrium was significant between the polymorphisms in the alcohol dependent group ($\chi^2=15.67$, 5 df, $p=0.004$) but not in its control group ($\chi^2=0.19$, 5 df, $p=0.5$). In the alcohol dependent group, the linkage disequilibrium coefficient $\delta$ was 0.1 for the haplotype consisting of the (insertion) long promoter-10 copy VNTR allele. This modest disequilibrium coefficient $\delta$ value suggests that while statistically significant, only partial linkage disequilibrium is present for the short promoter-10 copy haplotype.
DISCUSSION

4.0 Interpreting candidate gene sequence variants in patient and control groups

(a) Conservation of the DRD1 gene in TS and in alcohol dependence

No systematic attempt to associate structural variations in the coding region of the DRD1 gene with TS, alcohol dependence or related disorders has appeared in the literature. The present study has largely ruled out the possibility that mutations in the coding region of the DRD1 gene contribute to the etiology of TS or alcohol dependence in the patients studied. The DRD1 sequence conservation among the 72 alcohol dependent patients reported here confirms the results of a previous study on the DRD1 gene in eight alcohol dependent patients (Lui et al, 1995). The integrity of the DRD1 gene coding region among TS patients confirms the results of a previous study on the role of the DRD1 gene in TS (Gelernter et al, 1993).

The previous work on the DRD1 gene in TS was limited, however, by being confined to analyzing RFLP sites found in the regions flanking the DRD1 gene (Gelernter et al. 1993). The present study, by contrast, effectively excludes the possibility that DRD1 coding region mutations contribute to the etiology of TS or alcohol dependence by systematically screening the DRD1 gene.

(b) DAT1 gene sequence variants in TS and in alcohol dependence

The DAT1 gene mutation screening examined whether sequence variants in the coding region of this gene underlie the polygenic inheritance of TS (Gelernter et al, 1995; Comings et al, 1996), AD-HD (Gill et al, 1997; Cook et al, 1995) and alcohol
dependence (Persico et al. 1993; Muramitsu et al. 1995). This study has succeeded in identifying structural variants in the coding region of the DAT1 gene that may help to clarify association studies of the 3' VNTR of the DAT1 gene with TS (Comings et al. 1996), AD-HD (Cook et al. 1997; Gill et al. 1997; Cook et al. 1995) and alcohol dependence (Muramatsu and Higushi. 1995).

The DAT1 gene variants, however, are not likely to result in altered dopamine transporter function. The two rare amino acid changes, Val54Ala and Val164Ala, are conservative and were found in control subjects. Three common single nucleotide polymorphisms were identified: exon 2 C/T, exon 9 A/G and exon 15 T/C. These polymorphisms were not found at significantly different allele or genotype frequencies in the TS or alcohol dependent groups by $\chi^2$ analysis (Table 4). Therefore, the novel DAT1 polymorphisms do not appear to be associated with TS or alcohol dependence. Power analysis (specifying a type II error rate of $\alpha=0.05$ and power of $1-\beta=0.80$), indicates that patient and control groups of the size used in this study are large enough to detect hypothetical odds ratios of 2.4 and 3.35 for the DAT1 exon 9 A/G and exon 15 T/C alleles to be risk alleles for TS ($n=109$) and alcohol dependence ($n=64$) respectively. Future studies should genotype larger patient and control sample groups in order to verify that these polymorphisms are not associated with TS or alcohol dependence.

Previous studies (Comings et al. 1996; Gelernter et al. 1995) support the evidence presented here that the DAT1 gene is not associated with the vocal and motor tics considered to be the essential features of TS (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. 1990). However, the earlier work by Comings et al. 1996, did
associate the DAT1 VNTR 10/10 genotype with 1-2% of AD-HD diagnosed comorbidly with TS. In the present study, this association was not detected by $\chi^2$ analysis of the frequency of the VNTR 10/10 genotype in TS patients diagnosed comorbid with AD-HD (n=33) compared with TS groups with no AD-HD comorbidity (n=86). Future studies should examine large samples of TS-AH-HD patients in order to confirm the lack of association between DAT1 and TS-AD-HD found here.

Statistically significant, partial linkage disequilibrium was found for the exon 15 T/C-VNTR 10/9 haplotype in all patient and control groups. This suggests that the exon 15 T/C marker may be of use in future haplotype based Haplotype relative risk (HH-RR) studies of TS and alcohol dependent trios. Differences in linkage disequilibrium between patient and control groups were noted. Partial linkage disequilibrium for the exon 9 A/G-VNTR 10/9 and exon 9 A/G-exon 15 T/C haplotypes was statistically significant in the TS group but not in either control group or the alcohol dependent group. However, linkage disequilibrium must be compared with caution between groups of unrelated individuals because a control group unrelated to the patient group cannot control for recombination events that result in different linkage disequilibria in different populations (Terwilliger and Ott, 1994). The linkage disequilibrium between the novel DAT1 polymorphisms, therefore, should be used to examine the family-based association of TS with the DAT1 gene as done previously in studies of AD-HD (Gill et al., 1997; Cook et al., 1997).

The results of the DAT1 candidate gene mutation screening represent a necessary step (Gelman and Gelernter, 1993) toward understanding whether a candidate gene, such as
the DAT1 gene, contributes to the inheritance of a disease state. Our SSCP analysis of the DAT1 gene, like the recent SSCP analysis of the norepinephrine transporter (NET) (Stober et al, 1996), discovered sequence variants common to control and disease states. These polymorphisms may clarify studies of the pharmacology of MP, cocaine (Volkow et al, 1995), antidepressants and endogenous catecholamines (Buck and Amara, 1995) because there is now evidence that amino acid variants, while common in the NET gene, are rare in the DAT1 gene. Research into the selective pharmacology of the dopamine and norepinephrine transporters using transporter chimeras (Buck and Amara, 1995; Buck and Amara, 1994) may be particularly useful in determining the significance of DAT1 sequence conservation.

The DAT1 sequence information, therefore, facilitates further studies of dopamine transporter pharmacology (Volkow et al, 1995), the pharmacogenetics of cocaine addiction (Gelernter et al, 1994; Persico et al, 1993) and future linkage studies of the DAT1 gene in TS, alcohol dependence and other neuropsychiatric disorders including AD-HD. Should evidence of linkage or linkage disequilibrium between disorders and the DAT1 gene be confirmed, a good case could be made for searching for polymorphisms in the 5' regulatory sequences that may contribute to the dopamine transporter pathology (Singer et al, 1991; Tiilhonen et al, 1995) that may underlie these disorders.
(c) **Association studies of 5HTT variants in TS and in alcohol dependence**

We have presented work that provides preliminary evidence that the 5HTT gene may be a susceptibility loci for TS. $\chi^2$ analysis (Table 5) detected a significant difference between allele frequencies ($\chi^2=8.75$, 1 df, $p=0.04$, two tailed, Bonferroni corrected) but not genotype frequencies ($\chi^2=8.45$, 2 df, $p=0.18$, two tailed, Bonferroni corrected) of the 5HTT promoter between TS patient and control groups. The Odds-Ratio of 1.88 for TS patients to have at least one short 5HTT promoter allele, suggests that the 5HTT short promoter may be a risk allele for TS. Ninety-five percent confidence intervals of 1.21-2.73, indicate that the Odds-Ratio is above unity. Power analysis (specifying a type II error rate of $\alpha=0.05$ and power of $1-\beta=0.80$), indicates that patient and control groups of the size used in this study are large enough to detect an odds ration of 2.4: a value toward the upper limit of the 95% confidence interval of 1.21-2.73 of the Odds-Ratio for a TS patient to have at least one short 5HTT promoter risk allele.

The discussion of the results of this study of the 5HTT promoter in TS would not be complete without comparing the frequency of the 5HTT short promoter allele in our control group with that in control groups genotyped for other studies. In the control group used for this study ($n=91$), the short promoter allele frequency was 36%. However, other studies have found the short promoter at higher frequencies. A study of the short allele in 90 German controls and 79 Italian controls found this allele at frequencies of 41% and 42% respectively (Deckert et al, 1997). A study of the short allele in 104 English, 301 Italian and 95 German controls found its frequency to be 45%, 43% and
40% respectively (Collier et al, 1996). Therefore, replication of this work is necessary in order to confirm the control frequency of the 5HTT short promoter allele in the caucasian nonpsychiatric control population. This issue must be considered because a common weakness of association studies in psychiatric genetics has been the fact that statistically significant associations can result from genotyping confounded control groups, even when an attempt to match patients and controls for ethnic origin has been made (Pato et al, 1993; Barr and Kidd, 1993).

The intron 2 VNTR, previously associated with unipolar depression (Evans et al, 1997; Ogilive et al, 1996) but not associated with manic depressive illness (Bellivier et al, 1997), was also genotyped for both of our patient and control groups. VNTR allele and genotype frequencies were not significantly different in the TS group compared to the control group, however, both the alleles and genotypes of this locus were significantly different in the alcohol dependent group (n=64) compared to its control group (n=32) ($\chi^2=22.79$, 2 df, $p=1.2\times10^{-4}$, two tailed, Bonferroni corrected; $\chi^2=25.08$, 4 df, $p=7\times10^{-7}$, two tailed, Bonferroni corrected).

The control group assigned to the alcohol dependence group was small. Therefore, we attempted to verify our analysis by comparing the alcohol dependent VNTR allele and genotype frequencies to those of the control group (n=91) that was originally assigned to the TS patient group. This comparison also found a significant difference between allele and genotype frequencies ($\chi^2=16.64$, 2 df, $p=0.003$, two tailed, Bonferroni corrected; $\chi^2=20.83$, 3 df, $p=0.0012$, two tailed, Bonferroni corrected), although the $\chi^2$P values are larger. This analysis is not strictly valid because the large control group was not
originally assigned to the alcohol dependence group. However, it serves to confirm that the VNTR allele and genotype frequencies are different between the alcohol dependent patient group compared with both of our control groups.

Our VNTR findings for alcohol dependence are different from those reported (Ogilvie et al, 1996) in the study associating the 5HTT VNTR with depression. The depression study found that the 9 copy VNTR allele was associated with risk for unipolar depression. Our study, on the other hand, found that an excess of the 10 copy VNTR allele was significantly more common than the combined 12 and 9 copy VNTR allele group (Table 5) in alcohol dependent patients (n=64) compared to controls (n=32). This analysis was repeated using the larger control group (n=91) that was not originally assigned to the alcohol dependent group. Again, the 10 copy VNTR allele was significantly more common that the combined 12 and 9 copy allele ($\chi^2=17.95$, 1df, $p=0.003$, two tailed, Bonferroni corrected), although the association was less significant ($\chi^2$P values were larger). The Odds Ratios of 5.80 [95% CI 2.42-14.37] and 2.85 [95% CI 1.69-4.81] were calculated for the risk of an alcohol dependent patient having the 10 copy allele, compared with controls from the small and large groups respectively. Therefore, our study suggests that the 10 copy allele may confer risk for alcohol dependence by an unknown mechanism that may involve adjacent gene structures.

The VNTR association should be interpreted with caution, however, because the intron 2 VNTR has no known effect on the 5HTT gene at the transcription level. While alleles of the 5’VNTR of the insulin gene have been shown to correlate with its transcription (Pugliese et al, 1997), there is no evidence as yet that the 5HTT VNTR
alleles have any regulatory function (Ogilvie et al, 1996). The role of Activator Protein-1 (AP-1) motifs adjacent to the 5HTT VNTR remains speculative (Ogilvie et al, 1996; Lesch et al, 1994).

In addition, the 5HTT intron 2 VNTR has not been genotyped in large enough numbers (Evans et al, 1997; Ogilvie et al, 1996) to know the VNTR allele frequency in the caucasian population. Because depression is frequently comorbid with alcohol dependence (Anton, 1996), our result showing an excess of the 10 copy VNTR allele in alcohol dependence may conflict with the previous study showing that an excess of the 9 copy VNTR allele is associated with depression (Ogilvie et al, 1996). Power analysis (specifying a type II error rate of $\alpha=0.05$ and power of $1-\beta=0.80$), however, suggests that our alcohol dependent and control samples are large enough to be able to detect the Odds Ratios for the 10 copy VNTR allele calculated. This work needs to be independently repeated in order confirm these findings because the phenomenon of population stratification (Barr and Kidd, 1993) has been known to confound studies that have attempted to use unrelated controls matched for ethnic origin (Barr and Kidd, 1993; Kennedy et al, 1995).

It has previously been suggested (Ogilvie et al, 1996) that evidence of 5HTT VNTR association with disease might indicate linkage disequilibrium of the VNTR with a functional mutation (Ogilvie et al, 1996). This study found evidence that linkage disequilibrium for the short promoter-VNTR 10 copy haplotype is present in the alcohol dependent group ($\delta=0.1$), but not in TS or control groups. However, the association of the short promoter with alcohol dependence does not reach statistical significance. Power
analysis suggests that alcohol dependent and control groups are large enough to detect an odds ratio of three if it were present.

While this study does present evidence of linkage disequilibrium in the alcohol dependent group that may be disease associated, it ought to be remembered that linkage disequilibrium should be compared with caution between unrelated individuals (Twerwlliger and Ott, 1994). A future study should genotype mother-father-proband trios in order to assess whether the short promoter-VNTR 10 copy haplotype is associated with alcohol dependence. Transmission of the short promoter allele in TS trios may also be informative about the role of the 5HTT gene in TS.

4.1 Conclusions

We have succeeded in confirming the practicality of using SSCP to identify sequence variants of as little as a single base substitution under the conditions developed to screen the GPR19 gene. The fact that SSCP has previously been demonstrated to identify 85-95% of single base pair substitutions (Jordanova et al, 1997; Scheffield et al, 1993; Weghorst et al, 1993) allows us to conclude that we have identified the majority of the sequence variants of the candidate genes we screened in patient and control groups. We have used SSCP to effectively exclude the role of sequence variants in the DAT1 and DRD1 gene coding regions from having a role in the etiology of TS or alcohol dependence. The deletion/insertion polymorphism and the 10 copy VNTR allele of the 5HTT gene, discovered previously (Hiels et al 1996; Lesch et al, 1994), were found to be significantly more common in the TS and alcohol dependent groups, respectively,
compared with the control groups genotyped. The greater frequency of the 5HTT deletion/insertion polymorphism in the TS group is intriguing because there is in vitro evidence that it may alter gene expression.
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