Role of genetic and environmental factors in tic disorders

Netty Bos-Veneman

Publication of this thesis was supported by the
school of behavioural and cognitive neurosciences

Cover design and lay-out: Gaaijo and Netty Bos
Printed by: Ridderprint, Ridderkerk

ISBN 978 90 367 4416 4 (print)
ISBN 978 90 367 4417 1 (digital)

RIJKSUNIVERSITEIT GRONINGEN

Role of genetic and environmental factors in tic disorders

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 1 september 2010
om 13.15 uur

door

Neeltje Gerrita Petronella Bos-Veneman
geboren op 28 mei 1975
te Zutphen

Promotores:	Prof. dr. R.B. Minderaa Prof. dr. C.G.M. Kallenberg
Copromotor:	Dr. P.J. Hoekstra
Beoordelingscommissie:	Prof. dr. J.K. Buitelaar Prof. dr. P.C. Limburg Prof. dr. A.J. Oldehinkel

Voor Gaaijo

Table of contents

Chapter 1

General introduction

General introduction

In 1825, Jean Marc Itard, chief physician at l'Institution Royale des Sourds-muets in Paris, published the story of the twenty-six-year old marquise de Dampierre, who 'in the midst of a conversation that interests her extremely, all of a sudden, without being able to prevent it, interrupts what she is saying or what she is listening to with bizarre shouts and with words that are even more extraordinary and which make a deplorable contrast with her intellect and her distinguished manners'.[1] Sixty years after this publication Georges Gilles de la Tourette, a young neurologist working under the supervision of Jean Martin Charcot at the Salpêtrière Hospital in Paris, borrowed Itard's case of the marquise for his study on the disorder he named 'maladie des tics'.[2] Charcot preferred the eponym of "Gilles de la Tourette" and attached this name to the disorder.

Of the nine patients with bizarre movements and utterances described in Gilles de la Tourette's study, only the marquise would be classified as having Tourette's disorder according to the criteria of the contemporarily used Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR)[3]: presence of multiple motor tics and minimally one vocal tic, lasting at least one year. The DSM-IV-TR also describes criteria for other tic disorders, such as chronic motor and vocal tic disorders, and transient tic disorder, and defines tics as sudden, rapid, recurrent, nonrhythmic movements (i.e., motor tics) and utterances (i.e., vocal tics) that typically mimic some aspect or fragment of normal behavior. Any voluntary movement and vocalization can emerge as a tic and therefore the list of possible tics is limitless. Most people associate Tourette's disorder with coprolalia (i.e., the involuntary utterance of obscene words or socially inappropriate remarks), but only about 10% of patients with Tourette's disorder suffer from this symptom.[4] Although tics can be handicapping, they often cause no hinder. However, the presence of a wide range of comorbid psychiatric conditions and symptoms in patients with a tic disorder is rather the rule than the exception and, in general, these co-occurring symptoms cause more discomfort than the tics itself.[5]

High rates of (symptoms of) attention deficit/hyperactivity disorder (ADHD)[6], obsessive-compulsive disorder (OCD)[6], major depressive disorder (MDD)[7], anxiety disorders[8], and autism spectrum disorder (ASD)[6,9] have been reported in patients with tics. Although clinically and epidemiologically related, the underlying etiological relations between tic disorders and these comorbidities are still under debate. Tics and comorbid disorders may have independent etiologies, as has been suggested especially regarding depressive and anxiety disorders[10], but also with regard to ADHD.[11] OCD and ASD may have shared etiologies with tic disorders[9,12], whereas patients with tics as well as ADHD and OCD, may represent a separate nosological entity.[11] However, most studies have focussed on, but not unravelled, the pathogenesis of tic disorders as such.

Pathogenesis of tic disorders

With the appearance of Freudian psychiatry in the early twentieth century, tic disorders shifted out of neurology largely into psychiatry where tics were thought to be expressions of emotional disturbances. In 1921, the first purely psychoanalytic explanation of tic disorders was proposed by the Hungarian psychoanalyst Sandor Ferenczi who considered tics as a symbolic expression of masturbation.[13] The discovery of the efficacy of neuroleptic drugs in patients with a tic disorder led towards the conceptualization of tics as neurological phenomena in the mid-1960s. Nearly fifty years later the exact etiological mechanisms of tic disorders still remain elusive.

Family and twin studies have provided strong evidence for a genetic basis; however, no independently replicated genes have been identified yet.[12] Serotonergic and dopaminergic genes are likely involved in the phenotypic expression of tic disorders, given that biochemical and pharmacological studies have indicated a substantial contribution of the serotonin and dopamine systems in the etiology of tics.[14-16] A wealth of studies has investigated variable regions of these genes in patients with tic disorders and/or co-occurring conditions, but shown inconclusive results.[17-24]

Susceptibility genes involved in tic disorders may be difficult to identify because of a complex mode of inheritance, comprising several genes that each have only a small effect. Moreover, the clinical heterogeneity of tic disorders, including the presence of co-occurring symptoms and disorders, may hinder the progression of genetic research. Finally, environmental factors, such as perinatal adversities and infections, may modulate or interact with the gene effect, further complicating the search for genes. The involvement of gene-environment interactions in the pathogenesis of tic disorders had been assumed 20 years ago[25], but has yet received little research attention.

Already in 1956 Pasamanick et al.[26] investigated perinatal adverse events in children with tics, and demonstrated that the mothers of children with tics had had more pregnancy and delivery complications than the mothers of healthy children. In general, later studies have confirmed this finding suggesting that perinatal adversities, specifically pregnancy and delivery complications[25], and prenatal smoking exposure[27], may be associated with presence and severity of tics. With regard to comorbid conditions in children with tic disorders, few studies have been performed: delivery complications and prenatal exposure to smoking, coffee, or alcohol may form risk factors for OCD[27,28]; low birth weight, prenatal problems, and exposure to smoking in utero for ADHD.[27,29]

Infections and the immune system have also been suggested to contribute to tic disorders. In 1998 Swedo et al. proposed a putative subgroup of neuropsychiatric disorders, designated as pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) characterized by the sudden onset and/or symptom exacerbations of OCD and/or tic disorder which are temporally related to group A ß-hemolytic streptococcal (GABHS) infections.[30] Introduction of the still controversial concept of PANDAS has greatly fuelled research on the role of immunity

in tic disorders and is roughly based on three possible pathways: involvement of autoimmune responses, altered (cellular) immunity, and/or compromised immune system leading to an increased vulnerability to infections.

Most studies have used Sydenham's chorea (an autoimmune sequel of post streptococcal acute rheumatic fever) as a pathogenetic model: neuronal and streptococcal epitopes show molecular mimicry, and therefore anti-streptococcal antibodies may cross-react with neuronal tissue. Presence of anti-neuronal antibodies[31-33], decreased numbers of regulatory T cells (Treg) that maintain tolerance to self antigens[34], increased single time point serum levels of antistreptococcal antibodies[32,35,36], and identification of candidate auto-antigens[37-39] in patients with a tic disorder hint at autoimmunity being involved in the etiology of tics, although not all of these findings could be confirmed.[40-42]

Few studies have assessed general immune markers in patients with tics and demonstrated higher serum levels of cytokines interleukin (IL)-12 and tumor necrosis factor-α in patients with Tourette's disorder and/or OCD[43], elevated plasma IL-12 levels in patients with Tourette's disorder as well as comorbid OCD[44], and increased serum levels of soluble adhesion molecules E-selectin and vascular cell adhesion molecule-1 in patients with Tourette´s disorder.[45]

Most recently, a new point of view has entered immunological research regarding tic disorders. Patients with tics may exhibit a compromised ability to clear microbial pathogens as has been suggested by the findings that patients with a newly diagnosed tic disorder or OCD were more likely to have had a GABHS infection in the previous year.[46,47] A higher vulnerability to infections may be caused by lower immunoglobulin (Ig) levels. Decreased levels of IgA have indeed been found in patients with Tourette's disorder and/or OCD.[48]

Scope of this thesis

Although a wide spectrum of neurobiological factors that possibly contribute to the development of tics and associated disorders has been studied, the exact pathogenesis is still unknown. This thesis describes studies on possible neurobiological involvement in tic disorders and comorbid symptoms. We have aimed to study possible associations between severity of tics and co-occurring features and perinatal adversities (*chapter 2*), and polymorphisms of serotonergic and dopaminergic genes, both separately as well as in interaction with perinatal adversities (*chapters 3, 4, and 5*), and to clarify the possible role of the immune system (*chapters 6 and 7*) in patients with a tic disorder.

Chapters 2, 3, 4, and 5 report on possible associations between perinatal adversities and severity of tics and co-occurring symptoms in patients with a tic disorder. Presence of pregnancy complications relates to increased tic severity but decreased OCD symptoms, presence of delivery complications to increased severity of tics and ADHD symptoms, and prenatal smoking exposure to more severe comorbid ADHD, ASD, and MDD symptoms but less severe tics. Most interestingly, the study

described in *chapter 2* provides indirect evidence of gene-environment interactions, comprising a positive family history of mental disorders as a proxy for DNA and presence of smoking exposure in utero, contributing to the severity of comorbid ADHD features. Three molecular genetic studies (*chapters 3, 4, and 5*) follow that investigate possible associations between polymorphisms of solute carrier family 6, member 4 (SLC6A4), monoamine oxidase (MAO) A, dopamine receptor (DR) D4, and SLC6A3 genes and severity of tics and comorbid symptoms. We address both main effects of these polymorphisms as well as possible interactions with perinatal adversities. *Chapter 3* describes relations between the SLC6A4 gene and increased severity of tics and comorbid ADHD, OCD, and ASD symptoms in children and adolescents with tics, as well as between the MAOA gene and decreased severity of ASD features. Moreover, the MAOA gene interacts with perinatal adversities to modulate tic severity. In *chapter 4*, we report on a contribution of the DRD4 gene to severity of comorbid OCD and ASD symptoms in children and adolescents with a tic disorder. The DRD4 gene also interacts with delivery complications regarding severity of tics and co-occurring internalizing symptoms. *Chapter 5* demonstrates a relation between the SLC6A3 gene and severity of inattentive symptoms in patients with a tic disorder. Interactions between the SLC6A3 gene and pregnancy complications are associated to severity of obsessions and compulsions. In *Chapter 6* results of a study on general markers of immunity are described. Children and adolescents with tics cannot be distinguished from healthy subjects based on serum levels of several cytokines and soluble adhesion molecules. However, serum IL-2 levels are associated with tic severity, and serum IL-12 levels inversely related to severity of OCD symptoms. In *chapter 7*, we describe Ig profiles of children and adolescents with a tic disorder and healthy volunteers. Serum levels of IgM and IgG3 are decreased in patients compared to healthy children and of IgG1 (trend-level significantly) elevated during tic exacerbations.

In *chapter 8*, our overall results are discussed. Future investigations and concluding remarks are given.

References

1. Itard JMG. Mémoire sur quelques fonctions involontaires des appareils de la locomotion, de la préhension et de la voix. Arch Gén Med 1825;8:385-407
2. Gilles de la Tourette G. Etude sur une affection nerveuse caractérisée par l'incoordination motrice accompagnée d'écholalie et de coprolalie. *Archives de Neurology* 1885;9:19-42, 158-200
3. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
4. Singer HS. Tourette's syndrome: from behaviour to biology. *Lancet Neurol* 2005;4:149-159
5. Cavanna AE, Servo S, Monaco F, et al. The behavioral spectrum of Gilles de la Tourette syndrome. *J Neuropsychiatry Clin Neurosci* 2009;21:13-23
6. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
7. Robertson MM. Mood disorders and Gilles de la Tourette's syndrome: An update on prevalence, etiology, comorbidity, clinical associations, and implications. *J Psychosom Res* 2006;61:349-358
8. Coffey BJ, Biederman J, Smoller JW, et al. Anxiety disorders and tic severity in juveniles with Tourette's disorder. *J Am Acad Child Adolesc Psychiatry* 2000;39:562-568
9. Comings DE, Comings BG. Clinical and genetic relationships between autism-pervasive developmental disorder and Tourette syndrome: a study of 19 cases. *Am J Med Genet* 1991;39:180-191
10. Pauls DL, Leckman JF, Cohen DJ. Evidence against a genetic relationship between Tourette's syndrome and anxiety, depression, panic and phobic disorders. *Br J Psychiatry* 1994;164:215-221
11. Rothenberger A, Roessner V, Banaschewski T, et al. Co-existence of tic disorders and attention-deficit/hyperactivity disorder-recent advances in understanding and treatment. *Eur Child Adolesc Psychiatry* 2007;16 Suppl 1:1-4
12. O'Rourke JA, Scharf JM, Yu D, et al. The genetics of Tourette syndrome: a review. *J Psychosom Res* 2009;67:533-545
13. Goetz CG, Chmura TA, Lanska DJ. History of tic disorders and Gilles de la Tourette syndrome: part 5 of the MDS-sponsored history of movement disorders exhibit, Barcelona, June 2000. *Mov Disord* 2001; 16:346-349
14. Muller-Vahl KR, Meyer GJ, Knapp WH, et al. Serotonin transporter binding in Tourette Syndrome. *Neurosci Lett* 2005;385:120-125
15. Swain JE, Scahill L, Lombroso PJ, et al. Tourette syndrome and tic disorders: a decade of progress. *J Am Acad Child Adolesc Psychiatry* 2007;46:947-968
16. Steeves TD, Fox SH. Neurobiological basis of serotonin-dopamine antagonists in the treatment of Gilles de la Tourette syndrome. *Prog Brain Res* 2008;172:495-513
17. Gizer IR, Ficks C, Waldman ID. Candidate gene studies of ADHD: a meta-analytic review. *Hum Genet* 2009;126:51-90
18. Guo G, Tillman KH. Trajectories of depressive symptoms, dopamine D2 and D4 receptors, family socioeconomic status and social support in adolescence and young adulthood. *Psychiatr Genet* 2009;19:14-26
19. Tarnok Z, Ronai Z, Gervai J, et al. Dopaminergic candidate genes in Tourette syndrome: Association between tic severity and 3' UTR polymorphism of the dopamine transporter gene. *Am J Med Genet B Neuropsychiatr Genet* 2007;144B:900-905
20. Grice DE, Leckman JF, Pauls DL, et al. Linkage disequilibrium between an allele at the dopamine D4 receptor locus and Tourette syndrome, by the transmission-disequilibrium test. *Am J Hum Genet* 1996;59:644-652
21. Wendland JR, Moya PR, Kruse MR, et al. A novel, putative gain-of-function haplotype at SLC6A4 associates with obsessive-compulsive disorder. *Hum Mol Genet* 2008;17:717-723
22. Diaz-Anzaldua A, Joober R, Riviere JB, et al. Tourette syndrome and dopaminergic genes: a family-based association study in the French Canadian founder population. *Mol Psychiatry* 2004;9:272-277
23. Bloch MH, Landeros-Weisenberger A, Sen S, et al. Association of the serotonin transporter polymorphism and obsessive-compulsive disorder: systematic review. *Am*

J Med Genet B Neuropsychiatr Genet 2008;147B:850-858

24. Huang CH, Santangelo SL. Autism and serotonin transporter gene polymorphisms: a systematic review and meta-analysis. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:903-913
25. Leckman JF, Dolnansky ES, Hardin MT, et al. Perinatal factors in the expression of Tourette's syndrome: an exploratory study. *J Am Acad Child Adolesc Psychiatry* 1990;29:220-226
26. Pasamanick B, Kawi A. A study of the association of prenatal and paranatal factors with the development of tics in children; a preliminary investigation. *J Pediatr* 1956; 48:596-601
27. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
28. Santangelo SL, Pauls DL, Goldstein JM, et al. Tourette's syndrome: what are the influences of gender and comorbid obsessive-compulsive disorder? *J Am Acad Child Adolesc Psychiatry* 1994;33:795-804
29. Pringsheim T, Sandor P, Lang A, et al. Prenatal and perinatal morbidity in children with Tourette syndrome and attention-deficit hyperactivity disorder. *J Dev Behav Pediatr* 2009;30:115-121
30. Swedo SE, Leonard HL, Garvey M, et al. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *Am J Psychiatry* 1998;155:264-271
31. Singer HS, Giuliano JD, Hansen BH, et al. Antibodies against human putamen in children with Tourette syndrome. *Neurology* 1998;50:1618-1624
32. Rizzo R, Gulisano M, Pavone P, et al. Increased antistreptococcal antibody titers and anti-basal ganglia antibodies in patients with Tourette syndrome: controlled cross-sectional study. *J Child Neurol* 2006;21:747-753
33. Yeh CB, Wu CH, Tsung HC, et al. Antineural antibody in patients with Tourette's syndrome and their family members. *J Biomed Sci* 2006;13:101-112
34. Kawikova I, Leckman JF, Kronig H, et al. Decreased numbers of regulatory T cells suggest impaired immune tolerance in children with tourette syndrome: a preliminary study. *Biol Psychiatry* 2007;61:273-278
35. Morshed SA, Parveen S, Leckman JF, et al. Antibodies against neural, nuclear, cytoskeletal, and streptococcal epitopes in children and adults with Tourette's syndrome, Sydenham's chorea, and autoimmune disorders. *Biol Psychiatry* 2001;50:566-577
36. Cardona F, Orefici G. Group A streptococcal infections and tic disorders in an Italian pediatric population. *J Pediatr* 2001;138:71-75
37. Dale RC, Candler PM, Church AJ, et al. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. *J Neuroimmunol* 2006;172:187-197
38. Kansy JW, Katsovich L, McIver KS, et al. Identification of pyruvate kinase as an antigen associated with Tourette syndrome. *J Neuroimmunol* 2006;181:165-176
39. Kirvan CA, Swedo SE, Snider LA, et al. Antibody-mediated neuronal cell signaling in behavior and movement disorders. *J Neuroimmunol* 2006;179:173-179
40. Singer HS, Gause C, Morris C, et al. Serial immune markers do not correlate with clinical exacerbations in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections. *Pediatrics* 2008;121:1198-1205
41. Singer HS, Hong JJ, Yoon DY, et al. Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. *Neurology* 2005;65:1701-1707
42. Martino D, Dale RC, Gilbert DL, et al. Immunopathogenic mechanisms in tourette syndrome: A critical review. *Mov Disord* 2009;24:1267-1279
43. Leckman JF, Katsovich L, Kawikova I, et al. Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol Psychiatry* 2005;57:667-673
44. Gabbay V, Coffey BJ, Guttman LE, et al. A cytokine study in children and adolescents with Tourette's disorder. *Prog Neuro-psychopharmacol Biol Psychiatry* 2009;33: 967-971
45. Martino D, Church AJ, Defazio G, et al. Soluble adhesion molecules in Gilles de la Tourette's syndrome. *J Neurol Sci* 2005; 234:79-85
46. Mell LK, Davis RL, Owens D. Association between streptococcal infection and

obsessive-compulsive disorder, Tourette's syndrome, and tic disorder. *Pediatrics* 2005;116:56-60

47. Leslie DL, Kozma L, Martin A, et al. Neuropsychiatric disorders associated with streptococcal infection: a case-control study among privately insured children. *J Am Acad Child Adolesc Psychiatry* 2008;47:1166-1172
48. Kawikova I, Grady BPX, Tobiasova Z, et al. Children with Tourette's syndrome may suffer immunoglobulin A dysgammaglobulinemia. *Biol Psychiatry* 2009;67:679-683

Chapter

2

Role of perinatal adversities on tic severity and symptoms of attention deficit/hyperactivity disorder in children and adolescents with a tic disorder

Published in the Journal of Developmental and Behavioral Pediatrics 2010; 31:100-106

Netty G.P. Bos-Veneman

Anne Kuin

Ruud B. Minderaa

Pieter J. Hoekstra

Department of Psychiatry
University Medical Center Groningen
University of Groningen
the Netherlands

Abstract

Objective To investigate the role of perinatal adversities with regard to tic severity and comorbid attention deficit/hyperactivity disorder (ADHD) symptoms in children with a tic disorder.

Methods In 75 children and adolescents with a tic disorder we retrospectively assessed presence of pregnancy, delivery, and postnatal complications, and of prenatal exposure to smoking and alcohol. Children with and without these perinatal adversities were compared regarding tic and ADHD symptom severity. Furthermore, through linear regressions we investigated whether perinatal adversities would interact with presence in first-degree relatives of tic or any mental disorders with the tic or ADHD measure as outcome.

Results Presence of pregnancy and delivery complications was related to tic severity, and prenatal smoking exposure to severity of comorbid ADHD symptoms. The relationship between smoking exposure in utero and ADHD symptom severity appeared to be more pronounced in children with a positive family history of mental disorders.

Conclusion This study provides evidence of a role for perinatal adversities in the etiology of tic disorders. Children with perinatal adversities may be vulnerable to develop more severe tics or comorbid ADHD symptoms in the presence of a positive family history of mental disorders, suggesting a role for gene-environment interactions.

Introduction

Notwithstanding the unequivocal involvement of genetics[1], environmental factors also play a role in the etiology of tic disorders.[2] Specifically, perinatal adverse events have been found in most studies to be associated with tic disorders, although results have been somewhat inconsistent.[3-12]

In general, perinatal adversities have been found to both form a risk factor for tic disorders as such and to be associated with increased tic severity. An early study[12] demonstrated that the mothers of children with tics (N = 51, all under 14 years of age) showed significantly more pregnancy and delivery complications than the mothers of healthy children (N = 51); later a study[3], which involved seven pairs of monozygotic twins, reported that the co-twins with Tourette's disorder (TD) had lower birth weights than the unaffected co-twins. Another study[5], comparing 92 TD patients with 460 healthy controls (age range 5 to 31 years), however, did not find a relationship between the presence of TD and prenatal complications, gestational age, birth weight, and Apgar scores. Also, Khalifa et al.[6] reported no statistically significant differences between 25 children with TD and 25 healthy subjects (age range 7 to 15 years) with regard to reduced optimality of pregnancy, delivery, and the postnatal period, although the mothers of children with TD were two times more likely to have had pregnancy complications.

Severity of tics has been found to be higher when mothers had suffered from stress and severe hyperemesis during pregnancy in a study involving 31 children with TD between 7 and 17 years.[7] A twin study[8] of 16 pairs of monozygotic twins in whom at least one twin had TD (age range 8 to 26 years), demonstrated an association between the intra-pair differences in birth weight and tic severity scores. Recently, a relation between tic severity and prenatal maternal smoking, but not birth weight and perinatal complications, has been demonstrated in 180 patients with TD (age range 3 to 59) recruited for three genetic studies: a genetic study in Costa Rica (N = 53), a genetic study among individuals of Ashkenazi Jewish descent in the United States (N = 99), and an affected sibling pair study in the United States (N = 28).[9]

Three studies have investigated the role of perinatal adversities with regard to comorbid conditions in children with tic disorders.[9-11] Presence of comorbid obsessive-compulsive disorder (OCD) in 49 children with TD, aged 7 to 18 years, appeared to be associated with forceps delivery and prenatal exposure to smoking, coffee, or alcohol.[10] Mathews et al.[9] found evidence for a role of smoking exposure in utero in comorbid OCD, and an association of both birth weight and the number of prenatal problems with presence of comorbid attention deficit/hyperactivity disorder (ADHD) in children with TD; surprisingly, prenatal drug exposure was associated with a reduced risk of comorbid ADHD.[9] A recent study[11] demonstrated greater odds of comorbid ADHD in children with TD (N = 353, age range 5 to 17 years) who were born with a low birth weight or with exposure to smoking in utero.

In the present study, we investigated relations of perinatal adversities with severity of both tic and comorbid ADHD symptoms in children and adolescents with a tic disorder. We hypothesized that perinatal adversities would be related to increased severity of both tics and ADHD symptoms. We also investigated the role of perinatal adversities in interaction with a positive family history of both tic disorders as well as mental disorders in general, as a proxy for genetic vulnerability. We hypothesized that a positive family history of tic and mental disorders would interact with perinatal adversities to increase tic and ADHD symptom severity. The involvement of gene-environment interactions in the pathogenesis of tic disorders had been hypothesized by Leckman and colleagues almost 20 years ago[7], but has yet received little research attention. A recent study[9], however, found no interactions between a positive family history of tic and OCD symptoms and perinatal adversities with regard to tic severity and presence of comorbid OCD and ADHD, but had not taken family history for mental disorders in general into account.

Methods

Study sample

The study group involved all patients, aged 6 to 18 years, who had participated in an ongoing research project studying neuroimmunology and genetics of tic disorders. Children had been recruited through our outpatient child and adolescent psychiatry clinic or the Dutch TD Association and had to fulfill criteria of the Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR)[13] for either TD, chronic motor tic disorder, or chronic vocal tic disorder. The study had been approved by our Institutional Review Board. Informed consent from parents and children of 12 years or older, and assent from children between 6 and 12 years, was obtained.

Measures

Parents were interviewed regarding their child's tic severity by using the Yale Global Tic Severity Scale (YGTSS), both with regard to the current week and the week in which the tics were worst ever.[14] The YGTSS is a semi-structured interview, which records the number, frequency, intensity, complexity, and interference of motor and vocal tics separately. The sum of both scores is used as a measure of total tic severity.

ADHD symptom severity was investigated by using the parent version of the ADHD rating scale.[15] The ADHD rating scale is a DSM-IV based questionnaire containing 18 items on inattentive and hyperactive-impulsive symptoms to be scored on a four-point scale (0 = never, 1 = sometimes, 2 = often, 3 = very often).

Parents also completed questionnaires with regard to prenatal smoking and alcohol exposure as well as pregnancy, delivery, and first-week postnatal complications. We investigated prenatal smoking and alcohol exposure by asking the mother if she had smoked or used alcohol during pregnancy. Pregnancy complications could include

Table 1
Comparisons between patients with a tic disorder with and without a number of perinatal adversities regarding tic and ADHD severity.

	Pregnancy complications[a]						Delivery complications[b]					
	present		absent				present		absent			
	n	mean ± SD	n	mean ± SD	*t*	*p*	n	mean ± SD	n	mean ± SD	*t*	*p*
Current YGTSS	27		43				28		42			
motor		13.2 ± 4.8		11.7 ± 5.1	-1.25	.22		11.8 ± 5.7		12.6 ± 4.5	0.63	.53
vocal		9.0 ± 6.1		5.6 ± 5.1	-2.52	**.01**		7.7 ± 6.0		6.4 ± 5.5	-0.92	.36
total		22.3 ± 9.4		17.3 ± 8.4	-2.28	**.03**		19.5 ± 10.5		19.0 ± 8.4	-0.23	.82
Worst ever YGTSS	27		43				28		42			
motor		16.8 ± 4.3		15.8 ± 5.0	-0.84	.40		17.3 ± 4.6		15.5 ± 4.7	-1.63	.11
vocal		9.2 ± 7.6		9.4 ± 6.5	0.07	.94		11.4 ± 7.5		7.9 ± 6.2	-2.12	**.04**
total		26.0 ± 8.3		25.2 ± 8.6	-0.41	.68		28.7 ± 9.1		23.4 ± 7.3	-2.71	**.01**
ADHD rating scale	26		39				26		39			
hyperactive-impulsive		9.1 ± 5.8		9.1 ± 6.9	0.01	.99		9.9 ± 6.0		8.6 ± 6.8	-0.81	.42
inattentive		10.0 ± 4.7		12.1 ± 6.9	1.36	.18		13.0 ± 5.5		10.1 ± 6.4	-1.88	.07
total		19.1 ± 9.2		21.2 ± 12.1	0.76	.45		22.9 ± 9.3		18.7 ± 11.9	-1.53	.13

	First-week postnatal complications[c]					
	present		absent			
	n	mean ± SD	n	mean ± SD	*t*	*p*
Current YGTSS	18		51			
motor		12.5 ± 5.3		11.7 ± 4.11	0.61	.55
vocal		7.3 ± 5.6		6.9 ± 5.8	-0.26	.80
total		18.9 ± 8.8		19.4 ± 9.4	0.17	.87
Worst ever YGTSS	18		51			
motor		16.5 ± 4.5		16.3 ± 4.7	-0.16	.87
vocal		8.6 ± 7.7		9.6 ± 6.7	0.51	.61
total		25.1 ± 9.9		25.9 ± 7.9	0.33	.74
ADHD rating scale	17		47			
hyperactive-impulsive		8.3 ± 6.3		9.5 ± 6.6	0.66	.51
inattentive		12.7 ± 6.4		10.8 ± 6.1	-1.08	.28
total		21.0 ± 9.8		20.3 ± 11.6	-0.22	.83

YGTSS, Yale Global Tic Severity Scale; ADHD, attention deficit/hyperactivity disorder. [a]Including hypertension, infections, (pre)ecclampsia, psychosocial stress, or diabetes mellitus. [b]Including presence of meconium-stained amniotic fluid, premature rupture of the membranes, nuchal cord, fetal bradycardia, placenta praevia, or artificial delivery. [c]Including respiratory distress, hyperbilirubinemia, or hypothermia.

Table 1
continued.

	Prenatal smoking exposure						Prenatal alcohol exposure					
	present		absent				present		absent			
	n	mean ± SD	n	mean ± SD	*t*	*p*	n	mean ± SD	n	mean ± SD	*t*	*p*
Current YGTSS	11		59				8		62			
motor		14.1 ± 4.3		12.0 ± 5.1	-1.31	.19		10.1 ± 5.6		12.6 ± 4.9	1.31	.20
vocal		7.4 ± 7.3		6.9 ± 5.5	-0.26	.79		4.5 ± 5.1		7.2 ± 5.8	1.29	.20
total		21.5 ± 10.5		18.8 ± 8.8	-0.88	.38		14.6 ± 9.6		19.8 ± 8.9	1.54	.13
Worst ever YGTSS	11		59				8		62			
motor		15.3 ± 3.5		16.4 ± 4.9	0.72	.47		15.0 ± 2.6		16.4 ± 4.9	0.77	.44
vocal		9.8 ± 6.2		9.2 ± 7.1	-0.27	.79		5.9 ± 8.2		9.7 ± 6.7	1.50	.14
total		25.1 ± 7.8		25.6 ± 8.6	0.18	.86		20.9 ± 10.1		26.1 ± 8.0	1.68	.10
ADHD rating scale	11		54				8		57			
hyperactive-impulsive		11.4 ± 6.2		8.5 ± 6.4	-1.86	.07		8.0 ± 7.2		9.3 ± 6.4	0.52	.60
inattentive		14.0 ± 5.5		10.7 ± 6.2	-1.65	.10		13.8 ± 5.8		10.9 ± 6.2	-1.24	.22
total		26.4 ± 10.1		19.1 ± 10.9	-2.03	.05		21.8 ± 10.3		20.2 ± 11.2	-0.38	.71

YGTSS, Yale Global Tic Severity Scale; ADHD, attention deficit/hyperactivity disorder.

hypertension, infections, (pre)ecclampsia, psychosocial stress, or diabetes mellitus. Presence of meconium-stained amniotic fluid, premature rupture of the membranes, nuchal cord, fetal bradycardia, placenta praevia, or artificial delivery was classified as a delivery complication. Postnatal complications could include respiratory distress, hyperbilirubinemia, or hypothermia.

Finally, we asked parents to indicate whether or not a tic disorder or any mental disorder was present in a first-degree relative of the child through the following questions: Does the child have a parent or a sibling with tics? Does the child have a parent or a sibling with any other mental illness? Because of small sample sizes, separate mental illnesses could not be used in the analyses.

Statistical analyses

We made comparisons between patients with and without at least one pregnancy complication, with and without a delivery complication, with and without a postnatal complication, with and without prenatal smoking exposure, and with and without prenatal alcohol exposure. We tested between-group differences by using Student's *t* test regarding current and worst ever motor, vocal, and total YGTSS scores, and hyperactive-impulsive, inattentive, and total ADHD rating scale scores.

Table 2
Linear regression analysis for the hyperactive-impulsive ADHD rating scale score with regard to the interaction term of presence of first-degree relative with mental disorder and prenatal smoking exposure.

Independent variable	*b*	SE	β	*p*
Prenatal smoking exposure	-3.00	3.6	-.17	.41
Presence of first-degree relative with a mental disorder	2.98	1.8	.23	.10
Interaction term of presence of first-degree relative with a mental disorder and prenatal smoking exposure	9.88	4.6	.45	.04*

ADHD, attention deficit/hyperactivity disorder. *Adjusted R^2 = .18 (F = 4.91, df = 3, 50, p < .01).

Furthermore, those perinatal adversities that appeared to yield at least trend-level statistically significant (i.e. $p \leq .10$) between-group differences with regard to a tic or ADHD measure were used as independent variable together with interaction terms of the perinatal adversity with positive first-degree family history for a tic disorder and any mental disorder, respectively, with the tic or ADHD measure as outcome.

In all statistical analyses $p \leq .05$ was considered as significant and tests were two-sided. Given that we planned a total number of 45 between-group comparisons, the significance level corrected for the number of analyses would be 0.05 / 45 = 0.001. With regard to interaction terms, *p*-levels between .05 and .10 were regarded as indicating trend-level significance.

Results

The study involved 75 patients with a tic disorder (mean age 12.2 years [SD 3.3], 88.0% male). Most of the patients (n = 60) had been referred to our outpatient child and adolescent psychiatry clinic, the remaining (n = 15) were recruited through the Dutch TD Association. Patients fulfilled criteria for either TD (n = 62; 82.7%), chronic motor tic disorder (n = 12; 16.0%), or chronic vocal tic disorder (n = 1; 1.3%). At the time of investigation, psychotropic medication was used by 53% of patients (n = 40), either antipsychotic agents (n = 12), clonidine (n = 3), psychostimulants (n = 8), atomoxetine (n = 4), or a combination of two or more agents (n = 13). Mean current total, motor, and vocal YGTSS scores were 19.1 (SD 9.0, range 0 - 39), 12.3 (SD 4.9, range 0 - 22), and 6.8 (SD 5.7, range 0 - 19), respectively; respective mean worst ever YGTSS scores were 25.6 (SD 8.6, range 10 - 47), 16.3 (SD 4.7, range 0 - 25), and 9.3 (SD 7.0, range 0 - 23). Mean total, hyperactive-impulsive, and inattentive scores were 20.7 (SD 11.1, range 1 - 43), 9.2 (SD 6.6, range 0 - 23), and 11.4 (SD 6.1, range 0 - 23), respectively. The questionnaire regarding perinatal adversities was available from 70 patients. Prenatal exposure to smoking occurred in 11 patients (15.7%); prenatal alcohol exposure in 8 cases (11.4%); pregnancy complications in 27 patients (38.6%); delivery complications in 28 patients (40.0%); and postnatal complications in 18 patients (25.7%). Data regarding the presence of a tic disorder or any mental disorder in a first-degree

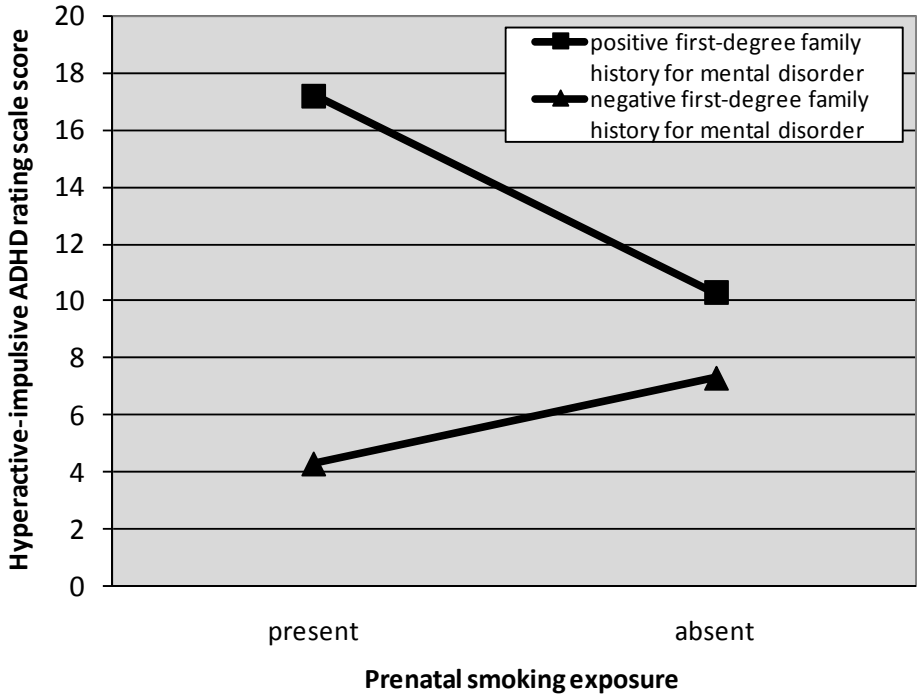

Figure. Role of prenatal smoking exposure regarding hyperactive-impulsive ADHD rating scale scores in patients with and without a first-degree relative with a mental disorder. ADHD, attention deficit/hyperactivity disorder.

relative of the child were available from 68 and 58 patients, respectively. A tic disorder in a first-degree relative was present in 21 patients (30.9%); any mental disorder in 24 patients (41.4%).

Table 1 shows between-group comparisons regarding presence or absence of a number of perinatal factors with regard to YGTSS and ADHD rating scale scores. Of note, patients with one or more delivery complication had significantly higher mean worst ever vocal and total YGTSS scores than patients without any delivery complication; current vocal and total YGTSS scores were significantly higher in patients with one or more pregnancy complication. Furthermore, patients who had been exposed to smoking in utero had significantly higher mean total ADHD rating scale scores. None of the between-group differences met the stringent significance level after correction for multiple testing.

Subsequently, those perinatal adversities that yielded at least trend-level statistically significant between-group differences with regard to a tic or ADHD measure were investigated in interaction with presence or absence of a tic disorder and any mental disorder in first-degree relatives. Table 2 shows the linear regression analysis for the hyperactive-impulsive subscale score of the ADHD rating scale including the interaction term of the presence of a first-degree relative with a mental disorder and prenatal smoking exposure. This interaction term was significantly related

to the hyperactive-impulsive ADHD rating scale score. The figure graphically displays the direction of effect: in patients with a first-degree relative with a mental disorder, smoking exposure during pregnancy was associated with a higher hyperactive-impulsive score: 17.2 (SD 1.8) versus 10.3 (SD 6.5), t = -4.07, p < .01. In contrast, in patients without a first-degree relative with a mental disorder prenatal smoking exposure was not associated with a higher hyperactive-impulsive score: 4.3 (SD 2.5) versus 7.3 (SD 6.0), t = 0.85, p = .45. Furthermore, the interaction term of presence of a first-degree relative with a mental disorder and prenatal smoking exposure was at trend-level significance related to the total ADHD rating scale score (b = 13.80, SE 8.1, β = .37, p = .09); in patients with a first-degree relative with a mental disorder, smoking exposure during pregnancy was associated with a higher total ADHD rating score: 32.4 (SD 5.9) versus 21.8 (SD 11.0), t = -2.05, p = .05. In contrast, in the group of patients without a first-degree relative with a mental disorder prenatal smoking exposure was not associated with a higher total ADHD rating score: 14.0 (SD 9.6) versus 17.2 (SD 10.2), t = 0.52, p = .61. No other statistically significant interaction terms of perinatal adversities with a positive first-degree family history for a tic or any mental disorder in association with a tic or ADHD measure were present.

Discussion

The present study suggests differential influences of pregnancy and delivery complications and smoking exposure in utero in children with a tic disorder: presence of pregnancy and delivery complications was related to tic severity, and prenatal smoking exposure to severity of ADHD symptoms. Moreover, we found preliminary evidence for possible gene-environment interactions: the relationship between smoking exposure in utero and ADHD symptom severity appeared to be more pronounced in children with a positive family history of mental disorders. We found no associations, however, of alcohol exposure in utero or postnatal complications with tic and ADHD symptom severity. The results of our study are in remarkable contrast with the findings of Mathews et al.[9] of an association of prenatal smoking exposure with increased tic severity, and no association of pregnancy and delivery complications with tic severity. While we have no direct explanation for this, it should be noted that, in contrast to our study that only involved children and adolescents, 28% of patients in the study of Mathews were adults with TD. Adults who continue to have tics may form a distinct subgroup in terms of clinical characteristics and etiology.[16]

Our finding that current tic severity is related to pregnancy complications is in line with an earlier study of Leckman and colleagues.[7] Leckman only regarded current tic severity rather than life-time worst ever tic severity. The use of worst ever tic severity may be a more representative measure of tic severity given the waxing and waning pattern of tics. The relation between delivery complications and worst ever tic severity has not been reported before.

Prenatal smoking exposure was related to comorbid ADHD symptom severity in the present study. This is in line with the findings of Pringsheim et al.[11] who recently reported a strong association between prenatal exposure to smoking and presence of ADHD. These findings are, again, contrary to the study of Mathews[9] who found that in utero drug exposure, including tobacco, was associated with a reduced risk of ADHD in patients with TD. In patients with ADHD as such, a wealth of studies have indicated an association between exposure to prenatal smoking and ADHD.[17,18] Results from animal studies indicate that prenatal nicotine exposure is associated with hyperactivity in the offspring.[19]

Our finding of no relations of postnatal complications with tic and ADHD symptoms severity is in line with several previous studies.[6,9,11] Also, in concordance with a previous study[11], prenatal alcohol exposure was not related to comorbid ADHD symptoms. To our knowledge no previous publications concerning in utero exposure to alcohol in relation to tic severity are available.

Children who had been exposed to smoking during pregnancy had more severe ADHD symptoms if they had a positive family history for mental disorders, which suggests the presence of gene-environment interactions. In a recent cohort of preadolescents from the general population, interactions between a family history of externalizing behavior and maternal prenatal smoking have been reported.[20] In the etiology of ADHD as such, evidence regarding a role of gene-environment (mainly prenatal smoking exposure) interactions has been increasing[17]: for example, children who had been exposed to prenatal smoking demonstrated more severe hyperactive-impulsive symptoms if they had the dopamine transporter-1 10-repeat allele.[21]

The effects of pregnancy and delivery complications and smoking exposure in utero may be understood through fetal hypoxia.[22] The basal ganglia, which have been implicated in the etiology of tic disorders[2], are particularly sensitive to hypoxia.[23] Pregnancy and delivery complications could result in damage of the basal ganglia in the course of acute metabolic insults, such as transient fetal hypoxia. Brake et al.[24] suggest dopamine dysregulation in the nucleus accumbens after perinatal complications involving hypoxia. Because of the involvement of dopaminergic pathways in tic disorders, this is an intriguing finding.[2] Whether pregnancy and delivery complications affect only a subgroup of, for example genetic vulnerable, patients with a tic disorder, should be investigated in future studies.

Nicotine crosses the placenta and the human fetus is exposed to even higher nicotine concentrations than the smoking mother.[25] Nicotine achieves its effects through nicotinic acetylcholine receptors (nAChRs). In the fetal brain, nAChRs are widely distributed and involved in the maturation of brain structures during development. Prenatal nicotine exposure can change the expression of nAChRs in the fetal brain possibly as a result of chronic hypoxia or the direct toxic effects of nicotine.[22,26] Animal studies have shown that nicotine exposure results in modulation of the dopaminergic system, probably mediated by subunits of nAChRs.[27,28]

Apart from the direct consequences of nicotine, associations between prenatal smoking exposure and psychiatric disorders may also be understood through factors

often found associated with smoking, such as low socioeconomic status and presence of psychiatric disorders in parents.[29] However, several studies[11,18] found that smoking during pregnancy increased the risk of ADHD, even after adjusting for these confounders. Therefore, even if we did not assess parents' socioeconomic status, it is likely that it is the true effect of smoking itself rather than confounding factors that best explains our results.

A number of limitations need to be acknowledged. First, the sample size of our study group is relatively small, and given the limited statistical power, none of our findings actually reached significance when taking multiple testing into account. Using a too stringent multiple testing correction method, however, increases the type II error rate leading to false negative findings. Thus, study findings need to be interpreted with caution and further research directed at replication of our findings and investigation of the underlying pathophysiological mechanisms is warranted. Another disadvantage of our study has been the retrospective collection of perinatal data as well as worst ever tic severity. It has been reported though that long-term maternal recall is accurate for many factors related to pregnancy and delivery.[30] A further limitation is the use of psychotropic medication in 53% of the patients, which may have affected current tic and ADHD symptom severity. Worst ever tic severity, however, was typically in a time when children did not use psychotropic medication. Furthermore, we investigated family history by parent report and not through direct examination of relatives. A meta analysis[31], however, has shown high reliability for data collected by using family history. Finally, we have not investigated the amount and duration of prenatal maternal smoking and alcohol use. Todd et al., however, have found that it is the presence of prenatal smoking rather than the number of cigarettes or trimester of exposure that is predictive of ADHD.[32]

In conclusion, this study confirms the earlier findings regarding the relation between pregnancy and delivery complications and tic severity[7], and underlines the importance of prenatal smoking exposure in comorbid ADHD symptoms in children with a tic disorder.[11] However, we could not replicate the findings of a relationship between prenatal smoking exposure and tic severity.[9] Future studies addressing gene-environment interactions may be a fruitful area.

References

1. Keen-Kim D, Freimer NB. Genetics and epidemiology of Tourette syndrome. J Child Neurol 2006;21:665-671
2. Hoekstra PJ, Anderson GM, Limburg PC, et al. Neurobiology and neuroimmunology of Tourette's syndrome: an update. *Cell Mol Life Sci* 2004;61:886-898
3. Leckman JF, Price RA, Walkup JT, et al. Nongenetic factors in Gilles de la Tourette's syndrome. *Arch Gen Psychiatry* 1987;44:100
4. Burd L, Severud R, Klug MG, et al. Prenatal and perinatal risk factors for Tourette disorder. *J Perinat Med* 1999;27:295-302
5. Klug MG, Burd L, Kerbeshian J, et al. A comparison of the effects of parental risk markers on pre- and perinatal variables in multiple patient cohorts with fetal alcohol syndrome, autism, Tourette syndrome, and sudden infant death syndrome: an enviromic analysis. *Neurotoxicol Teratol* 2003;25:707-717
6. Khalifa N, von Knorring AL. Tourette syndrome and other tic disorders in a total population of children: clinical assessment and background. *Acta Paediatr* 2005;94: 1608-1614
7. Leckman JF, Dolnansky ES, Hardin MT, et al. Perinatal factors in the expression of Tourette's syndrome: an exploratory study. *J Am Acad Child Adolesc Psychiatry* 1990; 29:220-226
8. Hyde TM, Aaronson BA, Randolph C, et al. Relationship of birth weight to the phenotypic expression of Gilles de la Tourette's syndrome in monozygotic twins. *Neurology* 1992;42:652-658
9. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
10. Santangelo SL, Pauls DL, Goldstein JM, et al. Tourette's syndrome: what are the influences of gender and comorbid obsessive-compulsive disorder? *J Am Acad Child Adolesc Psychiatry* 1994;33:795-804
11. Pringsheim T, Sandor P, Lang A, et al. Prenatal and perinatal morbidity in children with Tourette syndrome and attention-deficit hyperactivity disorder. *J Dev Behav Pediatr* 2009;30:115-121
12. Pasamanick B, Kawi A. A study of the association of prenatal and paranatal factors with the development of tics in children; a preliminary investigation. *J Pediatr* 1956; 48:596-601
13. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
14. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
15. DuPaul GJ. ADHD Rating Scale-IV: Checklists, Norms and Clinical Interpretation. New York: Power, T. J., Anastopoulos, A. D., and Reid, R.; 1998
16. Bloch MH, Leckman JF, Zhu H, et al. Caudate volumes in childhood predict symptom severity in adults with Tourette syndrome. *Neurology* 2005;65:1253-1258
17. Banerjee TD, Middleton F, Faraone SV. Environmental risk factors for attention-deficit hyperactivity disorder. *Acta Paediatr* 2007;96:1269-1274
18. Linnet KM, Wisborg K, Obel C, et al. Smoking during pregnancy and the risk for hyperkinetic disorder in offspring. *Pediatrics* 2005;116:462-467
19. Ajarem JS, Ahmad M. Prenatal nicotine exposure modifies behavior of mice through early development. *Pharmacol Biochem Behav* 1998;59:313-318
20. Buschgens CJ, Swinkels SH, van Aken MA, et al. Externalizing behaviors in preadolescents: familial risk to externalizing behaviors, prenatal and perinatal risks, and their interactions. *Eur Child Adolesc Psychiatry* 2009;18:65-74
21. Becker K, El Faddagh M, Schmidt MH, et al. Interaction of dopamine transporter genotype with prenatal smoke exposure on ADHD symptoms. *J Pediatr* 2008;152:263-269
22. Lv J, Mao C, Zhu L, et al. The effect of prenatal nicotine on expression of nicotine receptor subunits in the fetal brain. *Neurotoxicology* 2008;29:722-726
23. Pisani A, Bonsi P, Calabresi P.Calcium signaling and neuronal vulnerability to ischemia in the striatum. *Cell Calcium* 2004;36:277-284

24. Brake WG, Sullivan RM, Gratton A. Perinatal distress leads to lateralized medial prefrontal cortical dopamine hypofunction in adult rats. *J Neurosci* 2000;20:5538-5543
25. Luck W, Nau H, Hansen R, et al. Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther* 1985;8:384-395
26. Hellstrom-Lindahl E, Seiger A, Kjaeldgaard A, et al. Nicotine-induced alterations in the expression of nicotinic receptors in primary cultures from human prenatal brain. *Neuroscience* 2001;105:527-534
27. Marks MJ, Grady SR, Collins AC. Downregulation of nicotinic receptor function after chronic nicotine infusion. *J Pharmacol Exp Ther* 1993;266:1268-1276
28. Zoli M, Moretti M, Zanardi A, et al. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J Neurosci* 2002;22:8785-8789
29. Roza SJ, Verhulst FC, Jaddoe VW, et al. Maternal smoking during pregnancy and child behaviour problems: the Generation R Study. *Int J Epidemiol* 2009;38:680-689
30. Rice F, Lewis A, Harold G, et al. Agreement between maternal report and antenatal records for a range of pre and perinatal factors: the influence of maternal and child characteristics. *Early Hum Dev* 2007;83:497-504
31. Hardt J, Franke P. Validity, reliability and objectivity of the family history method in psychiatry: a meta analysis. *Eur Psychiatry* 2007;22:49-58
32. Todd RD, Neuman RJ. Gene-environment interactions in the development of combined type ADHD: evidence for a synapse-based model. *Am J Med Genet B Neuropsychiatr Genet* 2007;144B:971-975

Chapter

3

Associations between polymorphisms of SLC6A4 and MAOA genes and severity of tics and comorbid symptoms in children and adolescents with a tic disorder

This chapter has been submitted for publication

Netty G.P. Bos-Veneman

Ruud B. Minderaa

Pieter J. Hoekstra

Department of Psychiatry
University Medical Center Groningen
University of Groningen
the Netherlands

Abstract

The present study investigates relations of genetic polymorphisms and perinatal factors with phenotypic expression of tic disorders, including severity of tics and comorbid symptoms. We genotyped 109 children with a tic disorder with regard to the 5-hydroxytryptamine transporter-linked polymorphic region (5HTTLPR) including rs25531, and the monoamine oxidase upstream variable number of tandem repeats (MAOA uVNTR), and assessed presence of pregnancy and delivery complications, and of prenatal smoking exposure by parent questionnaires. Children with and without a 5HTTLPR long $(L)_A$ allele, and males with and without a MAOA uVNTR high-activity allele were compared regarding severity of tics and comorbid symptoms of attention deficit/hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), and autism spectrum disorder (ASD). Through linear regressions, we investigated whether perinatal adversities would interact with presence of an L_A or MAOA uVNTR high-activity allele with severity ratings of tics or comorbid symptoms as outcome. Presence of an L_A allele was associated with increased severity of tics and of comorbid ADHD, OCD, and ASD symptoms. Presence of a MAOA uVNTR high-activity allele was associated with decreased ASD symptom severity. Delivery complications were associated with ADHD symptom severity, and prenatal smoking exposure with ADHD and ASD symptom severity. The relationship between delivery complications and tic severity appeared to be most pronounced in males without MAOA uVNTR high-activity allele. In conclusion, this study provides evidence for a contribution of 5HTTLPR and the MAOA uVNTR to severity of tic and comorbid symptoms and for an interaction between MAOA and delivery complications in males with a tic disorder.

Introduction

Tic disorders, like Tourette's Disorder (TD), are childhood-onset neuropsychiatric conditions of largely unknown etiology. Genetic factors unequivocally contribute to the pathogenesis of tic disorders, although no independently replicated susceptibility genes have yet been identified.[1] The search for genes in tic disorders may be complicated by environmental factors associated with tics, such as perinatal adversities[2,3], that could modulate or interact with the effects of genes. Whereas many study have focussed on the identification of disease genes, in the current study we addressed possible relations of common genetic and perinatal factors with the phenotypic expression of tic disorders, i.e., severity of tics and comorbid symptoms.

Although the defining feature of tic disorders is the presence of sudden, rapid, recurrent, nonrhythmic motor movements, and/or vocalizations, a wide range of psychiatric conditions and symptoms often co-occur.[4] Attention deficit/hyperactivity disorder (ADHD) and obsessive-compulsive disorder (OCD) are highly comorbid with TD.[5] Moreover, patients with tic disorders may be prone to have features of autism spectrum disorders (ASD).[5,6] The possible etiological relationships between tic and comorbid OCD and ADHD have been studied extensively. ADHD and tic disorders appear to have independent etiologies, although a TD/OCD/ADHD subtype may exist representing a separate, more severe, nosological entity.[1,7] Findings of increased risk of early onset OCD in relatives of probands with TD indicate that tic disorders and OCD may have a shared genetic vulnerability.[1] Regarding ASD, a shared genetic etiology with tic disorders has been suggested.[6]

Serotonergic genes may be involved in tic disorders and comorbid symptoms, given that they have been implicated not only in tic disorders[8], but also in ADHD[9], OCD[10], and ASD.[11] Thereby, pharmacological studies have demonstrated a positive effect on tic severity of risperidone, a combined serotonin and dopamine blocking agent[12], and findings of (123I)β-CIT single photon emission computed tomography studies in patients with TD have pointed to a dysfunction of serotonergic neurotransmission.[13]

One gene of considerable interest is the solute carrier family 6, member 4, gene (SLC6A4, also named 5-hydroxytryptamine transporter [5HTT] or serotonine transporter gene), encoding the serotonine transporter that reuptakes serotonin from synaptic spaces. SLC6A4 contains a 44 base pairs (bp) functional polymorphism in the SLC6A4-linked polymorphic region, called 5HTTLPR, which modulates the transcriptional activity of SLC6A4.[14] The repetitive region that comprises 5HTTLPR exhibits an additional functional A→G single nucleotide polymorphism (SNP; rs25531) with the minor allele (G) almost always in phase with the long (L) 5HTTLPR allele.[15] Thus, 5HTTLPR is functionally triallelic: the L_A allele is the actual high-expressing variant, leading to more efficient reuptake of serotonin, whereas the L_G and short (S) alleles are low-expressing. Only one study has investigated genetic (but not triallelic) variation at 5HTTLPR in patients with tic disorders. Cavallini and colleagues[16] compared 52 patients with TD and 63 healthy subjects regarding 5HTTLPR, but found no

significant associations. Studies addressing relationships between 5HTTLPR and psychiatric conditions as such have shown inconsistent results. Two meta-analyses have reported the L allele as risk allele for ADHD[9,17], whereas another could not support this association.[18] Regarding OCD, one meta-analysis that included case-control studies has found a positive relation with S allele homozygosity and a negative association with heterozygosity at 5HTTLPR[19]; another found no relationship between 5HTTLPR and OCD when including both case-control and family-based studies but did identify an association with the L allele when including only family-based studies.[10] One study that took rs25531 into account has reported that the L_AL_A genotype was approximately twice as common in 169 adults with OCD than in 253 controls and, moreover, that the L_A allele was excessively transmitted in 175 trios consisting of probands with OCD and their parents.[15] However, an attempt to replicate this finding failed.[20] Regarding autism, one meta-analysis[11] has been published that reported preferential transmission of the S allele in family-based studies of United States mixed population samples, but no allelic distortion among European and Asian populations.

The gene encoding monoamine oxidase (MAO) A may also be of interest in tic disorders given that MAOA enzymatically degrades biogenic amines including dopamine and serotonin.[21] An upstream variable number of tandem repeats (uVNTR) functional polymorphism with 3, 3.5, 4, or 5 copies of the 30-bp repeat sequence affects the transcriptional activity of the MAOA gene: alleles with 3.5 or 4 repeats (R) are transcribed two to ten times more efficiently, resulting in an elevated MAOA activity compared to those with 3R or 5R.[22] A family-based association study, involving 110 patients with TD, has demonstrated excess transmission of high-activity alleles.[8] High-activity alleles have also been related to ADHD and OCD.[9,23] The 3R allele has been associated with ASD symptoms in boys with autism[24], but not to presence of autism in case-control and family-based studies.[25,26]

In the current study, we examined relations of genetic variation at the 5HTTLPR locus (including rs25531) and the MAOA uVNTR, with severity of tics and comorbid ADHD, OCD, and ASD symptoms in children and adolescents with a tic disorder. To date, no studies have directly addressed the involvement of gene-environment interactions in children with tic disorders, but our group did find indirect, preliminary evidence: smoking exposure in utero interacted with presence of a positive family history of mental disorders to increase ADHD symptom severity in children with a tic disorder.[27] This is the first molecular genetic study to investigate perinatal adversities in interaction with 5HTTLPR as well as the MAOA uVNTR regarding severity of tics and comorbid symptoms.

Methods

Study sample

The study involved 109 patients with a tic disorder between 6 and 18 years (mean age 12.3 years [SD 3.0]; 81.7% male), of which 75 patients had previously participated in a study investigating perinatal adversities.[27] Most of the patients (n = 85) had been referred to our outpatient child and adolescent psychiatry clinic, the remaining (n = 24) were recruited through the Dutch TD Association. Patients fulfilled criteria of the Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR)[28] for either TD (n = 96), chronic motor tic disorder (n = 11), or chronic vocal tic disorder (n = 2). At the time of investigation, parents indicated their child's use of psychotropic medication. Psychotropic medication was used by 60 patients, either an antipsychotic agent (n = 21), clonidine (n = 7), methylphenidate (n = 8), atomoxetine (n = 4), a selective serotonin reuptake inhibitor (n = 2), or a combination of two or more agents (n = 18).

Informed consent from parents and children of 12 years or older, and assent from children between 6 and 12 years was obtained. The study had been approved by our Institutional Review Board.

Clinical measures

Parents were interviewed regarding their child's tic severity, with regard to the week in which the tics were worst ever, by using the Yale Global Tic Severity Scale (YGTSS), a semi-structured interview which records the number, frequency, intensity, complexity, and interference of motor and vocal tics separately.[29] In a subgroup of patients (n = 38), worst ever tic severity had not been assessed; here, we used current tic severity ratings. Mean total, motor, and vocal YGTSS ratings were 23.1 (SD 9.5, range 4 - 47), 14.4 (SD 5.5, range 0 - 25), and 8.7 (SD 6.6, range 0 - 23), respectively.

ADHD symptom severity was measured by using the parent version of the ADHD rating scale (available from 66 patients), a DSM-IV based questionnaire containing 18 items on inattentive and hyperactive-impulsive symptoms to be scored on a four-point scale.[30] Respective mean total, hyperactive-impulsive, and inattentive scores were 20.2 (SD 11.2, range 1 - 43), 8.8 (SD 6.7, range 0 - 23), and 11.2 (SD 6.0, range 0 - 22).

The Children's Yale-Brown Obsessive-Compulsive Scale (CYBOCS), a semi-structured interview rating five items regarding current severity of obsessions and compulsions separately (i.e., time spent, interference, distress, resistance, and degree of control), was used to interview parents regarding their child's obsessive-compulsive symptom severity (available from 106 patients).[31] Mean total, obsessive, and compulsive CYBOCS ratings were 5.0 (SD 7.3, range 0 - 28), 1.8 (SD 3.9, range 0 - 16), and 3.2 (SD 4.6, range 0 - 16), respectively.

The parent version of the Children's Social Behavior Questionnaire (CSBQ) was administered to investigate severity of ASD features (available from 108 patients).[32] The CSBQ contains 49 items to be scored on a three-point scale that refer to six

subscales: not tuned (i.e., not optimally tuned to the social situation; mean 9.0 [SD 6.1, range 0 - 29]), reduced social interest (mean 4.5 [SD 4.6, range 0 - 17]), orientation problems (i.e., orientation problems in time, place, or activity; mean 5.4 [SD 4.7, range 0 - 23]), not understanding (i.e., difficulties in understanding of social information; mean 4.5 [SD 3.8, range 0 - 14]), resistance to changes (mean 2.3 [SD 4.5, range 0 - 27]), and stereotyped behavior (mean 3.6 [SD 3.3, range 0 - 13]). The sum of the subscales can be used as a measure of total ASD symptom severity (mean 29.3 [SD 19.0, range 1 - 88]).

Parents also completed questionnaires with regard to pregnancy and delivery complications, and maternal prenatal smoking (available from 86 patients). Pregnancy complications could include hypertension, infections, (pre)ecclampsia, psychosocial stress, or diabetes mellitus, and were present in 38 patients. Presence of meconium-stained amniotic fluid, premature rupture of the membranes, nuchal cord, fetal bradycardia, placenta praevia, or artificial delivery was classified as a delivery complication, present in 32 patients. Smoking during pregnancy was reported by 14 mothers.

Genotyping

DNA was extracted from blood samples or buccal swabs (Cytobrush®) using a manual salting out procedure.[33] The SLC6A4 and MAOA polymorphisms were genotyped in a quality-certified laboratory at the Department of Human Genetics of the Radboud University Medical Center in Nijmegen, the Netherlands.

Genotyping of 5HTTLPR was done by simple sequence length analysis. Polymerase chain reaction (PCR) was performed using 50 ng genomic DNA, 0.5 µM fluorescently labelled forward (FAM-5'-GGCGTTGCCGCTCTGAATGC-3') and reverse primer (5'-GAGGGACTGAGCTGGACAACCAC-3'), 0.25 mM deoxyribonucleotide triphosphates (dNTPs), 1x PCR optimization buffer A (30 mM Tris-HCl pH 8.5, 7.5mM (NH4)2SO4, 0.75 mM MgCl2), 10% dimethylsulfoxide (DMSO), and 0.4 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Cycling conditions were 12 minutes at 95°C, 35 cycles of 1 minute at 94°C, 1 minute at the optimized annealing temperature (57.5°C), and 2 minutes at 72°C, then followed by an extra 10 minutes at 72°C.

Genotyping of rs25531 was performed on a 7500 Fast Real-Time PCR System using 10 ng of genomic DNA, 0.25 µl of a custom-made Taqman assay (forty times; Applied Biosystems) that includes a forward (CCCTCGCGGCATCCC) and reverse primer (ATGCTGGAAGGGCTGCA), and two fluorescently labelled probes (VIC-CTGCACCCCCAGCAT, FAM-CTGCACCCCCGGCAT), 5 µl of Taqman Mastermix (twice; Applied Biosytems), and 3.75 µl of water. Genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems). The assay was validated by digesting the SLC6A4 PCR product with MspI (New England Biolabs, Ipswich, USA) and separating restriction fragments of 340 bp, 130 bp, and 60 bp for the L_A allele, of 175 bp, 165 bp, 130 bp, and 60 bp for the L_G allele, and of 300 bp, 130 bp, and 60 bp for the S allele, on a 2% agarose gel.

Table 1
Frequencies of 5HTTLPR genotypes and MAOA uVNTR alleles in our study group of patients with a tic disorder.

	Genotypes	n	%
5HTTLPR	L_AL_A	24	22.3
	L_AS	51	47.2
	L_AL_G	9	8.3
	SS	18	16.7
	SL_G	5	4.6
	L_GL_G	1	0.9
	total	108	100
	Alleles	**n**	**%**
MAOA uVNTR	3R	25	29.1
(males)	4R	60	69.8
	5R	1	1.1
	total	86	100

5HTTLPR, 5-hydroxytryptamine transporter-linked polymorphic region; MAO, monoamine oxidase; uVNTR, upstream variable number of tandem repeats; L, long allele; S, short allele; R, repeats.

The MAOA uVNTR was genotyped after amplification from 50 ng genomic DNA in a volume of 10 µl with 1x PCR buffer II (Applied Biosystems), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 10% DMSO, 0.4 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems), and 0.5 µM fluorescently labelled forward (FAM-5'-ACAGCCTGACCG TGGAGAAG-3') and reverse primer (5'-GAACGGACGCTCCATTCGGA).

Generally, 3% blanks as well as duplicates between plates were taken along as quality controls during genotyping. Subsequent determination of the length of the 5HTTLPR and MAOA uVNTR alleles was performed by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems) using standard conditions. Genotype and allele frequencies are represented in table 1.

Statistical analyses

Regarding 5HTTLPR, we made comparisons between patients with and without at least one L_A allele. Because MAOA is X-chromosomally located and the study sample involved only twenty females, we compared males with a high-activity allele (4R) of the MAOA uVNTR with males with a low-activity allele (3R and 5R). We made comparisons between patients with and without at least one pregnancy complication, with and without at least one delivery complication, and with and without prenatal smoking exposure. We tested between-group differences by using Student's *t* test regarding ratings of severity of tics and comorbid symptoms.

Furthermore, those perinatal adversities, that appeared to yield between-group differences at a significance level of at least $p \leq .20$ with regard to a tic or comorbid symptom severity measure were used in linear regression as independent variable together with interaction terms of the perinatal adversity and the presence of the risk allele (i.e., L_A for 5HTTLPR and the high-activity allele for the MAOA uVNTR) with the tic or comorbid symptom severity measure as outcome. Those regression analyses demonstrating a statistically significant interaction term in association with a tic or comorbid symptom severity measure were subsequently adjusted for gender and age in case of interactions with 5HTTLPR, or for age in case of interactions with the MAOA uVNTR.

Table 2
Comparisons between patients with and without minimally one 5HTTLPR L_A allele regarding severity of tics and comorbid symptoms.

	L_A			No L_A				
	n	mean	SD	n	mean	SD	*t*	*p*
YGTSS	85			23				
motor		15.2	5.5		12.0	4.4	-2.57	**.01**
vocal		9.0	6.7		7.7	6.1	-0.89	.38
total		24.2	9.3		19.6	9.3	-2.10	**.04**
ADHD rating scale	52			13				
inattentive		11.6	5.9		9.7	6.7	-1.04	.30
hyperactive-impulsive		9.7	6.6		5.8	6.3	-1.97	**.05**
total		21.5	10.9		15.9	12.2	-1.61	.13
CYBOCS	83			22				
obsessive		2.2	4.2		0.4	1.3	-3.34	**.001**
compulsive		3.5	4.8		1.5	3.5	-2.03	**.03**
total		5.8	7.8		1.9	4.3	-3.10	**.003**
CSBQ	80			22				
not tuned		9.4	6.0		7.6	6.2	-1.24	.22
reduced social interest		4.7	4.5		4.1	4.8	-0.46	.65
orientation problems		5.5	4.6		5.3	5.1	-0.22	.83
not understanding		4.8	4.1		3.4	2.1	-2.20	**.03**
resistance to changes		2.3	4.3		2.6	5.6	0.28	.78
stereotyped behavior		3.8	3.4		2.7	3.0	-1.39	.17
total		30.5	19.5		25.9	17.0	-1.01	.32

5HTTLPR, 5-hydroxytryptamine transporter-linked polymorphic region; L, long allele; YGTSS, Yale Global Tic Severity Scale; ADHD, Attention deficit/hyperactivity disorder; CYBOCS, Children's Yale-Brown Obsessive-Compulsive Scale; CSBQ, Children's Social Behavior Questionnaire.

In all statistical analyses $p \leq .05$ was considered as significant and tests were two-sided. Given that we planned a total number of 80 between-group comparisons, the significance level corrected for the number of analyses would be .05 / 80 = .0006.

Results

5HTTLPR

Table 2 shows comparisons between patients with and without at least one L_A allele with regard to tic and comorbid symptom severity measures. Of note, patients with an L_A allele had significantly higher mean motor and total YGTSS, hyperactive-impulsive ADHD rating scale, obsessive, compulsive, and total CYBOCS, and not understanding

Table 3
Comparisons between male patients with a high- and low-activity allele of the MAOA uVNTR regarding severity of tics and comorbid symptoms.

	High-activity allele			Low-activity allele				
	n	mean	SD	n	mean	SD	*t*	*p*
YGTSS	60			26				
motor		14.7	5.0		15.2	4.8	0.40	.69
vocal		8.3	6.9		9.5	6.3	0.79	.43
total		23.0	9.1		25.7	9.0	0.80	.43
ADHD rating scale	40			18				
inattentive		11.0	6.3		11.6	6.1	0.36	.72
hyperactive-impulsive		8.7	6.8		7.7	6.9	-0.53	.60
total		20.2	11.3		19.3	12.0	-0.25	.80
CYBOCS	58			26				
obsessive		1.6	3.8		1.9	3.9	0.31	.76
compulsive		3.0	4.5		3.0	4.6	0.07	.95
total		4.6	7.1		5.0	7.1	0.19	.85
CSBQ	55			25				
not tuned		8.0	4.7		9.7	7.4	1.01	.32
reduced social interest		3.9	4.0		6.8	5.4	2.20	**.04**
orientation problems		4.7	3.7		5.5	5.2	0.81	.42
not understanding		4.2	3.5		4.2	4.1	-0.02	.98
resistance to changes		1.5	1.4		2.6	5.3	1.05	.30
stereotyped behavior		3.3	3.1		3.6	3.1	0.41	.68
total		25.8	14.5		31.5	21.3	1.41	.16

MAO, monoamine oxidase; uVNTR, upstream variable number of tandem repeats; YGTSS, Yale Global Tic Severity Scale; ADHD, Attention deficit/hyperactivity disorder; CYBOCS, Children's Yale-Brown Obsessive-Compulsive Scale; CSBQ, Children's Social Behavior Questionnaire.

CSBQ ratings compared to patients without L_A allele. We found no other statistically significant between-group comparisons regarding 5HTTLPR and any tic or comorbid symptom severity measure. None of the between-group differences met the stringent significance level after correction for multiple testing.

MAOA uVNTR

Table 3 shows between-group comparisons of male patients with a high- and a low-activity allele regarding tic and comorbid symptom severity measures. Patients with a high-activity allele had significantly lower mean reduced social interest CSBQ ratings, but this difference did not meet the stringent significance level after correction for multiple testing. We found no other statistically significant between-group comparisons regarding the MAOA uVNTR and any tic or comorbid symptom severity measure.

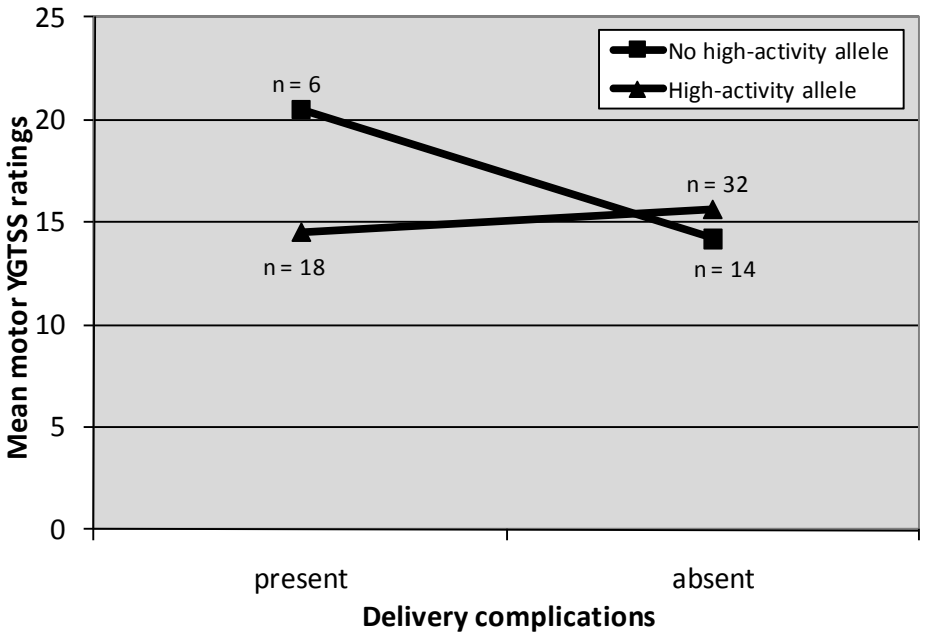

Figure. Role of delivery complications regarding motor YGTSS ratings in male patients with and without a high-activity allele of the monamine oxidase upstream variable number of tandem repeats.
YGTTS, Yale Global Tic Severity Scale.

Perinatal adversities

In line with our previous findings in mainly the same study sample[27], patients who had been exposed to smoking in utero had higher mean total ADHD rating scale scores (26.6 [SD 10.6] versus 18.7 [SD 10.9], t = -2.11, p = .04). Moreover, presence of a delivery complication and of prenatal smoking exposure were associated with higher inattentive ADHD rating scale scores (13.4 [SD 5.6] versus 9.8 [SD 6.0], t = -2.21, p = .03; and 14.6 [SD 5.4] versus 10.3 [SD 6.0], t = -2.13, p = .04, respectively). Smoking exposure in utero was also associated with higher orientation problems CSBQ ratings (6.6 [SD 3.8] versus 4.4 [SD 3.8], t = -1.99, p = .05). No other statistically significant between-group comparisons regarding any perinatal adversity and any symptom severity measure were present. None of the between-group differences met the stringent significance level after correction for multiple testing.

Gene-environment interactions

The interaction term of delivery complications and the MAOA uVNTR high-activity allele was significantly related to mean motor YGTSS ratings (b = -7.41, SE 2.6, β = -.69, p = .006), also after adjusting for age (b = -7.88, SE 2.6, β = -.73, p = .004). The figure graphically displays the direction of effect: in male patients without a high-activity allele, delivery complications were associated with higher mean motor YGTSS ratings (20.5 [SD 2.9] versus 14.2 [SD 4.1], t = -3.39, p = .003), while in the group of patients with a high-activity allele, delivery complications were not associated with mean motor

YGTSS ratings (14.5 [SD 5.4] versus 15.6 [SD 4.4], $t = 0.80$, $p = .43$). No other statistically significant interaction terms of perinatal adversities with the 5HTTLPR L_A allele or the MAOA uVNTR high-activity allele in association with a tic or comorbid symptom severity measure were present.

Discussion

The present study indicates influences of 5HTTLPR and the MAOA uVNTR on the severity of tics and comorbid symptoms in children with a tic disorder. Presence of at least one HTTLPR L_A allele was related to increased severity of tics and comorbid ADHD, OCD, and ASD symptoms. Presence of a MAOA uVNTR high-activity allele was associated with decreased severity of comorbid ASD features.

No studies regarding the relation between triallelic 5HTTLPR and tic and comorbid disorders had been performed so far. When L_G alleles remain unrecognized in SL and LL genotypes, the influence of 5HTTLPR on clinical phenotypes may be obscured. For example, a previous study[16] did not take the functional triallelic variation of 5HTTLPR into account and could not demonstrate an association of 5HTTLPR genotype and TD or comorbid OCD. By considering triallelic 5HTTLPR, however, we did find relations with severity of tics and comorbid symptoms. Regarding OCD as such, three studies have investigated possible associations with 5HTTLPR, including rs25531, and results are in line with our findings of a relation between the presence of an L_A allele and increased severity of OCD. Hu et al.[15] have found associations of L_A with OCD. Although a large case–control study could not replicate these findings[20], the same authors[34] in a subsequent study have provided evidence for an increased frequency of higher-expressing alleles in patients with OCD compared to healthy controls, when taking another functional 5HTTLPR variant, named rs25532, along with rs25531 into account.

The present finding of an inverse association between the MAOA high-activity alleles and severity of comorbid ASD features is consistent with a previous study demonstrating increased ASD symptom severity in boys with autism who exhibited the low-activity 3R allele.[24]

Previously, we had already reported the association between prenatal smoking exposure and ADHD symptom severity in mainly the same cohort.[27] The relations between delivery complications and ADHD symptoms, and between maternal smoking during pregnancy and ASD symptom severity are novel findings in this study sample. Evidence regarding a role of smoking exposure during pregnancy in the development of ASD features has been expanding.[35]

Most interestingly, we found evidence for a gene-environment interaction: the relationship between delivery complications and tic severity appeared to be most pronounced in boys without a MAOA uVNTR high-activity allele. These boys had more severe tics if they were born after a complicated delivery. No other studies have addressed the involvement of interactions between the MAOA uVNTR and perinatal adversities in children with tic disorders. However, an increased risk of antisocial

features has been associated with genotypes comprising low-activity alleles in boys who had been exposed to smoking in utero.[36]

The presently reported associations between 5HTTLPR as well as the MAOA uVNTR and severity of tics and comorbid ADHD, OCD, and ASD symptoms support the notion of serotonergic neurotransmission being involved in the phenotypic manifestation of tic disorders. Although serotonergic imbalance may contribute to the expression of tics and comorbid symptoms in a direct way, complex interactions exist between the serotonergic and the dopaminergic system.[12] Thereby, the direct effect of MAOA on dopamine may be of great importance.

A number of limitations need to be acknowledged. First, the sample size of our study group has been relatively small, and given the limited statistical power, none of our findings actually reached significance when taken multiple testing into account. Using a too stringent multiple testing correction method, however, increases the type II error rate, i.e., the risk of false negative findings. Clearly, study findings need to be interpreted with caution and further research directed at replication of our findings and investigation of the underlying pathophysiological mechanisms is warranted. Another disadvantage of our study has been the retrospective collection of worst ever tic severity. A further limitation is the use of psychotropic medication in 55% of the patients, which may have affected comorbid symptom severity. Worst ever tic severity, however, was typically in a time when children did not use psychotropic medication. Finally, other SNPs, for example rs25532, at the 5HTTLPR locus may also be involved, but have not been assessed in this study.

In conclusion, this study reveals relations between genetic variation at the 5HTTLPR locus, including rs25531, and increased severity of tics and comorbid ADHD, OCD, and ASD symptoms. Presence of a MAOA uVNTR high-activity allele was associated with decreased severity of comorbid ASD features, which points to a possible protective influence of MAOA regarding ASD symptoms in patients with a tic disorder. Moreover, delivery complications were associated with comorbid ADHD symptoms, and prenatal exposure to smoking with symptoms of ADHD and ASD. An interesting finding is that the relation between delivery complications and tic severity may be more pronounced in the absence of a MAOA uVNTR high-activity allele. Future research directed at replication of our findings and investigation of the underlying pathophysiological mechanisms is warranted.

References

1. O'Rourke JA, Scharf JM, Yu D, et al. The genetics of Tourette syndrome: a review. J Psychosom Res 2009;67:533-545
2. Leckman JF, Dolnansky ES, Hardin MT, et al. Perinatal factors in the expression of Tourette's syndrome: an exploratory study. *J Am Acad Child Adolesc Psychiatry* 1990; 29:220-226
3. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
4. Cavanna AE, Servo S, Monaco F, et al. The behavioral spectrum of Gilles de la Tourette syndrome. *J Neuropsychiatry Clin Neurosci* 2009;21:13-23
5. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
6. Comings DE, Comings BG. Clinical and genetic relationships between autism-pervasive developmental disorder and Tourette syndrome: a study of 19 cases. *Am J Med Genet* 1991;39:180-191
7. Rothenberger A, Roessner V, Banaschewski T, et al. Co-existence of tic disorders and attention-deficit/hyperactivity disorder-recent advances in understanding and treatment. *Eur Child Adolesc Psychiatry* 2007;16 Suppl 1:1-4
8. Diaz-Anzaldua A, Joober R, Riviere JB, et al. Tourette syndrome and dopaminergic genes: a family-based association study in the French Canadian founder population. *Mol Psychiatry* 2004;9:272-277
9. Gizer IR, Ficks C, Waldman ID. Candidate gene studies of ADHD: a meta-analytic review. *Hum Genet* 2009;126:51-90
10. Bloch MH, Landeros-Weisenberger A, Sen S, et al. Association of the serotonin transporter polymorphism and obsessive compulsive disorder: systematic review. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:850-858
11. Huang CH, Santangelo SL. Autism and serotonin transporter gene polymorphisms: a systematic review and meta-analysis. *Am J Med Genet B Neuropsychiatr Genet* 2008; 147B:903-913
12. Steeves TD, Fox SH. Neurobiological basis of serotonin-dopamine antagonists in the treatment of Gilles de la Tourette syndrome. *Prog Brain Res* 2008;172:495-513
13. Muller-Vahl KR, Meyer GJ, Knapp WH, et al. Serotonin transporter binding in Tourette Syndrome. *Neurosci Lett* 2005;385:120-125
14. Murphy DL, Fox MA, Timpano KR, et al. How the serotonin story is being rewritten by new gene-based discoveries principally related to SLC6A4, the serotonin transporter gene, which functions to influence all cellular serotonin systems. *Neuropharmacology* 2008;55:932-960
15. Hu XZ, Lipsky RH, Zhu G, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet* 2006;78:815-826
16. Cavallini MC, Di Bella D, Catalano M, et al. An association study between 5-HTTLPR polymorphism, COMT polymorphism, and Tourette's syndrome. *Psychiatry Res* 2000; 97:93-100
17. Faraone SV, Perlis RH, Doyle AE, et al. Molecular genetics of attention deficit/ hyperactivity disorder. *Biol Psychiatry* 2005; 57:1313-1323
18. Forero DA, Arboleda GH, Vasquez R, et al. Candidate genes involved in neural plasticity and the risk for attention-deficit hyperactivity disorder: a meta-analysis of 8 common variants. *J Psychiatry Neurosci* 2009;34:361-366
19. Lin PY. Meta-analysis of the association of serotonin transporter gene polymorphism with obsessive-compulsive disorder. P*rog Neuropsychopharmacol Biol Psychiatry* 2007;31:683-689
20. Wendland JR, Kruse MR, Cromer KR, et al. A large case-control study of common functional SLC6A4 and BDNF variants in obsessive-compulsive disorder. *Neuropsychopharmacology* 2007;32:2543-2551
21. Bortolato M, Chen K, Shih JC. Monoamine oxidase inactivation: from pathophysiology to therapeutics. *Adv Drug Deliv Rev* 2008;60:1527-1533
22. Sabol SZ, Hu S, Hamer D. A functional polymorphism in the monoamine oxidase A gene promoter. *Hum Genet* 1998;103:273-279
23. Karayiorgou M, Sobin C, Blundell ML, et al. Family-based association studies support a sexually dimorphic effect of COMT and

MAOA on genetic susceptibility to obsessive-compulsive disorder. *Biol Psychiatry* 1999;45:1178-1189

24. Cohen IL, Liu X, Schutz C, et al. Association of autism severity with a monoamine oxidase A functional polymorphism. *Clin Genet* 2003;64:190-197
25. Yirmiya N, Pilowsky T, Tidhar S, et al. Family-based and population study of a functional promoter-region monoamine oxidase A polymorphism in autism: possible association with IQ. *Am J Med Genet* 2002;114:284-287
26. Davis LK, Hazlett HC, Librant AL, et al. Cortical enlargement in autism is associated with a functional VNTR in the monoamine oxidase A gene. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:1145-1151
27. Bos-Veneman NG, Kuin A, Minderaa RB, et al. Role of perinatal adversities on tic severity and symptoms of attention deficit/hyperactivity disorder in children and adolescents with a tic disorder. *J Dev Behav Pediatr* 2010;31:100-106
28. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
29. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
30. DuPaul GJ. ADHD Rating Scale-IV: Checklists, Norms and Clinical Interpretation. New York: Power, T. J., Anastopoulos, A. D., and Reid, R.; 1998
31. Goodman WK, Price LH, Rasmussen SA, et al. The Yale-Brown Obsessive Compulsive Scale. I. Development, use, and reliability. *Arch Gen Psychiatry* 1989;46:1006-1011
32. Hartman CA, Luteijn E, Serra M, et al. Refinement of the Children's Social Behavior Questionnaire (CSBQ): an instrument that describes the diverse problems seen in milder forms of PDD. *Journal of Autism and Developmental Disorders* 2006;36:325-342
33. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 1988;16:1215
34. Wendland JR, Moya PR, Kruse MR, et al. A novel, putative gain-of-function haplotype at SLC6A4 associates with obsessive-compulsive disorder. *Hum Mol Genet* 2008;17:717-723
35. Indredavik MS, Brubakk AM, Romundstad P, et al. Prenatal smoking exposure and psychiatric symptoms in adolescence. *Acta Paediatr* 2007;96:377-382
36. Wakschlag LS, Kistner EO, Pine DS, et al. Interaction of prenatal exposure to cigarettes and MAOA genotype in pathways to youth antisocial behavior. *Mol Psychiatry* 2009;in press

Chapter

4

The DRD4 gene and severity of tics and comorbid symptoms: main effects and interactions with delivery complications

Accepted for publication in Movement Disorders

Netty G.P. Bos-Veneman

Ruud B. Minderaa

Pieter J. Hoekstra

Department of Psychiatry
University Medical Center Groningen
University of Groningen
the Netherlands

Abstract

In the current study we investigated the role of the dopamine receptor (DR) D4 48 base pairs (bp) variable number of tandem repeats (VNTR) and perinatal adversities regarding severity of tics and comorbid symptoms in children with tic disorders. We genotyped 110 children with tics with regard to the 48-bp VNTR and assessed presence of prenatal smoking exposure, and pregnancy and delivery complications by parent questionnaires. We examined associations between 2, 3, 4, and 7 repeat (R) alleles and severity of tics and comorbid obsessive-compulsive, depressive, anxious, and autistic symptoms. Through linear regressions, we investigated whether perinatal adversities and the 2R, 3R, 4R, and 7R alleles would interact with severity ratings of tics or comorbid symptoms as outcome. Presence of a 2R allele was related to more severe obsessive-compulsive symptoms, and presence of a 3R allele to increased severity of autistic features. Pregnancy complications were associated with decreased obsessive-compulsive symptom severity, and prenatal smoking exposure to more severe depressive and autistic symptoms. In children without a 3R allele delivery complications were associated with more severe tics, but in children with a 3R variant an inverse relation between delivery complications and tic severity was found. Moreover, the relation between delivery complications and internalizing symptom severity appeared to be most pronounced in children with a 2R allele. In conclusion, this study provides evidence for a role of the 48-bp VNTR in the etiology of tic and associated disorders, and for interactions with delivery complications regarding severity of tics and co-occurring internalizing symptoms.

Introduction

Tic disorders are often accompanied by a variety of psychiatric disorders and symptoms, such as attention deficit/hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), major depressive disorder (MDD), anxiety disorders, and autism spectrum disorder (ASD).[1] Whereas ADHD, OCD, and ASD may share an underlying genetic vulnerability with tic disorders[2,3], internalizing disorders in patients with tics may have an independent etiology.[4] However, different comorbidities may as well represent distinctive phenotypic subgroups. The search for genes could therefore be facilitated by subdividing patients with tics according to comorbid symptoms.[5]

Dopaminergic genes are suitable candidates for the study of tic disorders given that dopaminergic neurotransmission contributes to the etiology of tics.[6] A gene of particular interest is the dopamine receptor (DR) D4 gene which inhibits cyclic adenosine monophosphate (cAMP), and also influences arachidonic acid release and ion channels. A variable number of tandem repeats (VNTR), characterized by a sequence of 48 base pairs (bp) with two to eleven repeats (R), is located on the third exon. The functional significance of the DRD4 exon III 48-bp VNTR is still under debate[7], but it has been suggested that the 7R allele may lead to elevated cAMP levels.[8]

Studies regarding the 48-bp VNTR in tic and associated disorders have shown inconsistent results. A higher frequency of the 7R variant in patients with Tourette's disorder (TD) has been reported[9], but could not be confirmed in a later study.[10] Two family-based studies have found biased transmission in favor of the 7R allele, but two other studies found balanced transmission in probands with a chronic tic disorder.[10-13] In patients with OCD, lower frequencies of the 4R allele but no transmission disequilibrium[14], lower frequencies and absent transmission of the 2R variant[15] as well as reduced transmission of the 4R allele and slightly increased transmission of the 2R and 7R alleles[16] have been found. Associations of the 2R allele with (symptoms of) MDD and anxiety have also been reported.[17-19] However, in panic disorder and social phobia, balanced transmission of the 48-bp VNTR has been found.[20,21] Regarding ASD, only one case-control study has investigated the 48-bp VNTR, but showed no association with autism.[22]

Notwithstanding the unequivocal role of genetic factors, perinatal adversities may also contribute to, or interact with genes to modulate the severity of tic and co-occurring disorders.[23-25] Our group has provided indirect, preliminary evidence of the involvement of an interaction between smoking exposure in utero and the presence of a positive family history of mental disorders in symptom severity of ADHD in children with a tic disorder.[26]

In the current study, we examined relations of the DRD4 48-bp VNTR and perinatal adversities with severity of tics and comorbid OCD, MDD, anxiety, and ASD symptoms in children with a tic disorder. We also investigated whether perinatal adversities

would interact with the 48-bp VNTR to modulate severity of tics and comorbid symptoms.

Methods

Study sample

The study involved 110 patients with a tic disorder between 6 and 18 years (mean age 12.3 years [SD 3.0]; 81.8% male) of which 75 patients had previously participated in a study on perinatal adversities.[26] Most of the patients (n = 86) had been referred to our outpatient child and adolescent psychiatry clinic, the remaining (n = 24) were recruited through the Dutch TD Association. Patients fulfilled criteria of the Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR)[27] for either TD (n = 96; 87.3%), chronic motor tic disorder (n = 12; 10.9%), or chronic vocal tic disorder (n = 2; 1.8%). At the time of investigation, parents indicated their child's use of psychotropic medication. Psychotropic medication was used by 54.5% of patients (n = 60), either an antipsychotic agent (n = 21), clonidine (n = 7), methylphenidate (n = 8), atomoxetine (n = 4), a selective serotonin reuptake inhibitor (n = 2), or a combination of two or more agents (n = 18).

Informed consent from parents and children of 12 years or older, and assent from children between 6 and 12 years was obtained. The study had been approved by our Institutional Review Board.

Clinical measures

Parents were interviewed regarding their child's worst ever tic severity by using the Yale Global Tic Severity Scale (YGTSS).[28] However, assessment of worst ever tic severity had not yet been introduced at the first stage of the study and is therefore not available in a subgroup of patients (n = 39); here, we used current tic severity ratings. The YGTSS records the number, frequency, intensity, complexity, and interference of motor and vocal tics separately on a five-point scale. The sum of both scores is used as a measure of total tic severity, with maximum motor and vocal scores of 25, and total score of 50. Mean total, motor, and vocal YGTSS ratings were 23.0 (SD 9.5, range 4 - 47), 14.4 (SD 5.5, range 0 - 25), and 8.7 (SD 6.6, range 0 - 23), respectively.

The Children's Yale-Brown Obsessive-Compulsive Scale (CYBOCS) was used to interview parents regarding their child's obsessive-compulsive symptom severity (available from 107 patients).[29] Mean total, obsessive, and compulsive CYBOCS ratings were 5.0 (SD 7.3, range 0 - 28), 1.8 (SD 3.9, range 0 - 16), and 3.1 (SD 4.6, range 0 - 16), respectively.

The parent version of the Children's Social Behavior Questionnaire (CSBQ) was administered to investigate severity of ASD features (available from 104 patients).[30] The CSBQ contains 49 items to be scored on a three-point scale (0 = the behavior does not apply to the child, 1 = sometimes or somewhat applies, 2 = clearly or often applies) which refer to six empirically derived subscales: not tuned (i.e., not optimally tuned to

the social situation; mean 9.0 [SD 6.1, range 0 - 29]), reduced social interest (mean 4.5 [SD 4.6, range 0 - 17]), orientation problems (i.e., orientation problems in time, place, or activity; mean 5.4 [SD 4.7, range 0 - 23]), not understanding (i.e., difficulties in understanding of social information; mean 4.5 [SD 3.8, range 0 - 14]), resistance to changes (mean 2.3 [SD 4.5, range 0 - 27]), and stereotyped behavior (mean 3.6 [SD 3.3, range 0 - 13]). The sum of the subscales can be used as a measure of total ASD symptom severity (mean 29.4 [SD 19.0, range 1 - 88]).

To assess the severity of anxiety and depressive symptoms, we used the Revised Child Anxiety and Depression Scale (RCADS), a questionnaire with 47 items corresponding to DSM-IV MDD and anxiety disorders (i.e., separation anxiety disorder, social phobia, generalized anxiety disorder, OCD, and panic disorder) and scored on a three-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always).[31] As we added the RCADS to our clinical measures after the start of the study, only the parents of 63 patients filled out the questionnaire. We used the MDD subscale and the sum of the five anxiety subscales as severity measures of depressive and anxiety symptoms, respectively. Respective mean total, total anxiety, and MDD ratings were 27.1 (SD 19.0, range 2 - 82), 20.8 (SD 15.7, range 1 - 68), and 6.4 (SD 4.1, range 0 - 17).

Parents also completed questionnaires with regard to pregnancy and delivery complications, and maternal prenatal smoking (available from 87 patients). Long-term maternal recall has been demonstrated to be accurate for many perinatal adversities.[32] Pregnancy complications could include hypertension, infections, (pre)ecclampsia, psychosocial stress, or diabetes mellitus, and were present in 39 patients (44.8%). Presence of meconium-stained amniotic fluid, premature rupture of the membranes, nuchal cord, fetal bradycardia, placenta praevia, or artificial delivery was classified as a delivery complication, present in 33 patients (37.9%). Smoking during pregnancy was reported by 14 mothers (16.1%).

Genotyping

DNA was extracted from blood samples or buccal swabs (Cytobrush®) using a previously described manual salting out procedure.[33] The DRD4 exon III 48-bp VNTR was genotyped in a quality-certified laboratory at the Department of Human Genetics of the Radboud University Medical Center, Nijmegen, the Netherlands. Genotyping was performed after amplification from 10 ng genomic DNA in a 10 µl volume with 0.5 µM fluorescently labelled forward (Vic-5'-GCGACTACGTGGTCTACTCG-3') and reverse primer (5'-AGGACCCTCATGGCCTTG-3'), 0.4 mM

Table 1
Allele frequencies of the dopamine receptor D4 48 base pairs variable number of tandem repeats in our sample comprising children with a tic disorder.

Alleles	n	%
2R	18	8.2
3R	14	6.4
4R	141	64.1
5R	1	0.5
6R	1	0.5
7R	43	19.5
8R	2	0.9
Total	220	100

R, repeats.

deoxyribonucleotide triphosphates, and 0.5 U La Taq (Takara, Lonza Verviers Sprl, Verviers, Belgium), in GC I buffer (Takara) with 1 M betaine. The cycling conditions were 1 minute at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C, and an extra 5 minutes at 72°C. Three percent blanks as well as duplicates between plates were taken along as quality controls during genotyping. Subsequent determination of the length of the alleles was performed by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) using standard conditions. Allele frequencies are represented in table 1.

Statistical analyses

We made between-group comparisons between those individual 48-bp VNTR alleles with an allele frequency higher than 2%: 2R, 3R, 4R, and 7R. We tested between-group differences by using one-way independent analysis of variance (ANOVA) regarding the tic and comorbid symptom severity ratings. If a tic or comorbid symptom severity measure yielded a statistically significant difference between the individual alleles, Hochberg's GT2 Post hoc analyses were performed because of different sample sizes.

Pregnancy and delivery complications, and prenatal smoking exposure were dichotomized into presence and absence of pregnancy complications, presence and absence of delivery complications, and presence and absence of prenatal smoking exposure. We made between-group comparisons by using Student's *t* test, regarding the tic and comorbid symptom severity measures.

Furthermore, those perinatal adversities, that appeared to yield between-group differences meeting a significance level of at least $p < .20$ with regard to a tic or comorbid symptom severity measure, were used in linear regression as independent variable together with interaction terms of the perinatal adversity with the presence of the 2R, 3R, 4R, or 7R allele and the tic or comorbid symptom severity measure as outcome. Those regression analyses demonstrating a statistically significant interaction term in association with a tic or comorbid symptom severity measure were subsequently adjusted for gender and age.

In all statistical analyses $p \leq .05$ was considered as significant and tests were two-sided.

Results

DRD4 48-bp VNTR

Table 2 shows comparisons between patients with a 2R, 3R, 4R, and 7R allele, revealing statistically significant between-group differences regarding obsessive and total CYBOCS, and CSBQ subscale not understanding ratings. Post hoc analyses showed that the 2R allele was significantly associated with higher obsessive CYBOCS ratings compared to the 4R and 7R alleles, and with higher total CYBOCS ratings compared to

Table 2

Comparisons between 2R, 3R, 4R, and 7R alleles of the dopamine receptor D4 48 base pairs variable number of tandem repeats regarding severity of tics and comorbid symptoms in children with a tic disorder.

	2R			3R			4R			7R					
	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	df	*F*	*p*
YGTSS	14			6			92			27			3, 135		
motor		17.6	4.5		17.8	1.2		16.2	4.3		15.3	6.1		0.99	.40
vocal		11.7	7.4		8.2	9.0		8.8	6.7		10.4	6.6		1.06	.37
total		29.4	9.8		26.0	8.8		25.0	8.1		25.7	8.6		1.10	.35
CYBOCS	18			13			138			42			3, 208		
obsessive		4.8	5.4		2.9	5.6		1.8	3.7		0.4	1.2		6.41	**.0003**[1]
compulsive		4.3	4.9		5.1	5.9		3.1	4.5		2.3	4.1		1.69	.17
total		9.1	8.5		8.0	10.6		4.9	7.2		2.7	4.9		4.23	**.006**[2]
CSBQ	18			13			132			41			3, 200		
not tuned		7.4	6.2		10.6	5.8		9.3	6.0		8.6	6.1		0.90	.44
reduced social interest		5.6	6.1		4.8	5.0		4.5	4.5		3.8	3.9		0.68	.57
orientation problems		4.3	3.0		7.3	4.1		5.5	4.9		5.4	5.0		1.01	.39
not understanding		4.4	4.2		7.5	4.0		4.5	3.7		3.8	3.2		3.29	**.02**[3]
resistance to changes		1.4	1.5		1.8	1.4		2.5	4.6		2.6	5.8		0.36	.78
stereotyped behavior		3.3	3.6		4.8	4.4		3.7	3.3		3.3	2.9		0.72	.54
total		26.6	18.1		36.8	19.9		29.9	18.7		27.5	19.0		0.98	.40
RCADS	13			4			84			22			3, 119		
MDD		5.6	4.2		4.3	4.0		7.0	4.3		5.3	3.4		1.57	.20
total anxiety		19.8	19.6		7.3	6.8		22.4	15.5		18.8	14.4		1.44	.24
total		25.5	23.1		11.5	10.7		29.4	18.9		24.1	16.9		1.53	.21

R, repeats; YGTSS, Yale Global Tic Severity Scale; CYBOCS, Children's Yale-Brown Obsessive-Compulsive Scale; CSBQ, Children's Social Behavior Questionnaire; RCADS, Revised Child Anxiety and Depression Scale; MDD, major depressive disorder. Between-group differences were tested by using one-way independent analysis of variance. Between-group differences meeting the significance level ($p \leq .05$) are bold marked. [1]Hochberg's GT2 Post Hoc tests: mean obsessive CYBOCS difference between 2R and 4R: 3.0 (SE 0.9), $p = .008$; and between 2R and 7R: 4.4 (SE 1.0), $p = .0002$. [2]Mean total CYBOCS difference between 2R and 7R: 6.4 (SE 2.0), $p = .01$. [3]Mean not understanding CSBQ difference between 3R and 4R: 3.0 (SE 1.1), $p = .04$; and between 3R and 7R: 3.7 (SE 1.2), $p = .01$.

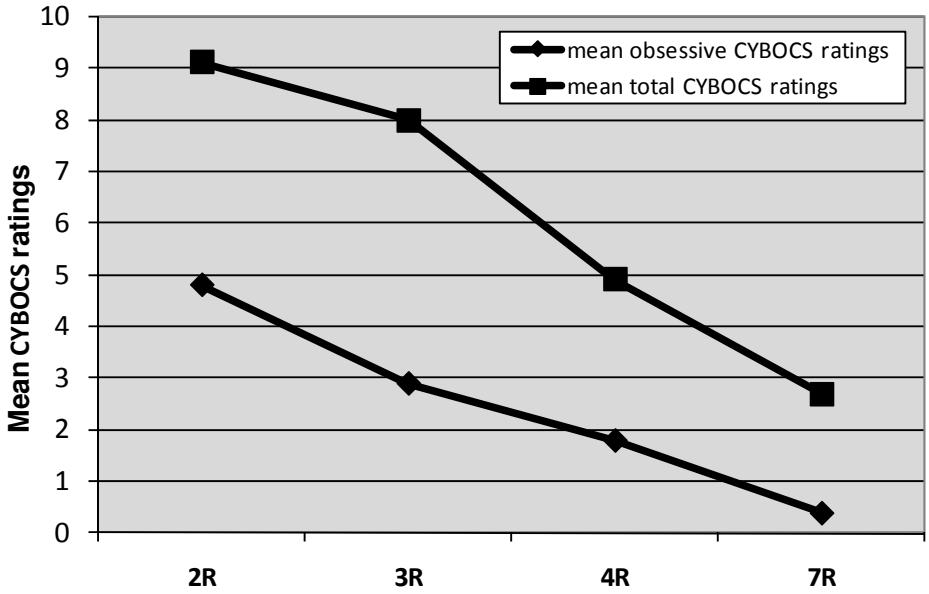

Figure. Associations between presence of 2R, 3R, 4R, and 7R alleles of the dopamine receptor D4 48 base pairs variable number of tandem repeats and severity of obsessive-compulsive symptoms in children with a tic disorder.
CYBOCS, Children's Yale-Brown Obsessive-Compulsive Scale; R, repeats.

the 7R allele (figure). CSBQ subscale not understanding ratings were higher in the presence of a 3R allele compared to the 4R and 7R alleles.

Perinatal adversities

Patients with pregnancy complications had lower mean compulsive CYBOCS ratings (2.0 [SD 3.7] versus 3.9 [SD 4.9], t = 2.02, p = .05). Patients who had been exposed to smoking in utero had higher mean MDD RCADS (9.1 [SD 4.1] versus 6.1 [SD 4.1], t = -2.05, p = .05) and CSBQ subscale orientation ratings (6.6 [SD 3.8] versus 4.4 [SD 3.7], t = -1.98, p = .05). No other statistically significant associations between a perinatal adversity and any symptom severity measure were present.

Gene-environment interactions

The interaction term of delivery complications and the 3R allele was significantly related to mean total YGTSS ratings (b = -15.87, SE 6.3, β = -.34, p = .01), also after adjusting for age and gender (b = -14.79, SE 6.3, β = -.32, p = .02). In the presence of a 3R allele, delivery complications were statistically significantly associated with lower mean total YGTSS ratings (25.6 [SD 10.8] versus 38.3 [SD 4.6], t = 2.63, p = .03), while in the absence of a 3R allele, delivery complications were associated with higher mean total YGTSS ratings (26.3 [SD 10.4] versus 23.2 [SD 8.0], t = -1.97, p = .05).

The interaction term of delivery complications and the 2R allele was statistically significantly associated with total anxiety (b = 28.41, SE 10.3, β = .33, p = .007) and total RCADS ratings (b = 33.40, SE 12.5, β = .32, p = .008), also after adjusting for age

and gender (*b* = 30.02, SE 10.2, β = .35, *p* = .004, and *b* = 35.06, SE 12.4, β = .33, *p* = .006, respectively). In the presence of a 2R allele, delivery complications were statistically significantly associated with higher mean total anxiety (44.8 [SD 16.4] versus 10.7 [SD 5.0], *t* = -4.90, *p* = .001), and total RCADS ratings (55.0 [SD 18.6] versus 14.3 [SD 6.8], *t* = -5.01, *p* = .001). In the absence of a 2R allele, delivery complications were not associated with mean total anxiety (25.6 [SD 19.8] versus 19.9 [SD 13.5], *t* = -1.40, *p* = .17), and total RCADS ratings (33.3 [SD 23.5] versus 26.0 [SD 16.6], *t* = -1.51, *p* = .14).

No other statistically significant interaction terms of perinatal adversities with DRD4 48-bp VNTR alleles in association with a tic or comorbid symptom severity measure were present.

Discussion

The present study demonstrates a contribution of the DRD4 48-bp VNTR to severity of tics and comorbid symptoms in children with a tic disorder. Presence of a 2R allele was related to more severe OCD symptoms, and presence of a 3R allele to increased severity of ASD symptoms. These findings support the involvement of dopaminergic neurotransmission in the etiology of comorbid OCD and ASD, as is also suggested by findings from pharmacological and neuroimaging studies[14,34,35]: effectiveness of antipsychotic medication (in combination with selective serotonin reuptake inhibitors) in patients with OCD and for aggression, irritability, and self-injurious behavior in patients with ASD[14,34,35], and higher dopamine transporter densities and down-regulation of D2 receptors in patients with OCD as demonstrated by positron emission tomography and single photon emission computer tomography studies.[34]

Tarnok et al.[10] have also investigated tic severity in relation to the 48-bp VNTR in 103 children with TD (mean age 13 ± 4.5 years), and found, in line with our study results, no relation between the 7R allele and tic severity. However, that study had not considered variants other than the 7R variant. Regarding comorbid OCD and ASD symptoms in patients with tic disorders, no studies had investigated possible relations with the 48-bp VNTR so far. All studies addressing OCD and ASD as such, have focussed on diagnostic entities, rather than severity of symptoms. Only one study has investigated possible relationships of the 48-bp VNTR with presence of autism (in 68 children compared to 826 healthy controls), but no association could be revealed.[22] Regarding OCD, slightly increased transmission of the 2R allele, and also of the 7R allele as well as reduced transmission of the 4R allele has been identified in 69 trios comprising Caucasian probands (mean age 13.2 ± 2.7 years) and their parents.[16] On the contrary, absent transmission of the 2R allele in 55 French probands with OCD (mean age 23.5 ± 10.3 years), and lower frequencies of the 2R allele and 2R-containing genotypes in the patients compared to 63 healthy controls have been found.[15] Mexican patients with OCD (N = 210; mean age 25.9 ± 10.4 years) had a lower frequency of the 4R allele compared to 202 controls, but no transmission

disequilibrium was detected.[14] These inconsistencies may at least partly be due to the inclusion of adults in the latter two studies. Moreover, the different allele frequencies in the samples complicate the comparison of the studies.

Our findings of smoking exposure in utero to be related to more severe depressive and autistic symptoms are in line with an expanding amount of studies suggesting that prenatal smoking exposure leads to vulnerability for these symptoms.[36-38] We also found, however, an *inverse* relation between pregnancy complications and OCD symptom severity. Two previous studies had indicated delivery complications and smoking exposure during pregnancy as risk factors for comorbid OCD in patients with TD.[24,39]

We also found evidence of gene-environment interactions in tic disorders: in the presence of delivery complications children without a 3R allele had more severe tics, whereas children with a 3R variant had decreased tic severity. Moreover, the relation between delivery complications and severity of internalizing symptoms appeared to be most pronounced in children with a 2R allele. No other studies have investigated interactions between the 48-bp VNTR and perinatal adversities in children with tic disorders. However, the presence of an interaction between prenatal exposure to smoking and the 7R allele has been suggested in children with ADHD, although studies have shown inconclusive results.[40,41]

A number of limitations need to be acknowledged. First, the sample size of our study group has been relatively small. Thereby, severity of internalizing features was only assessed in a subgroup of patients. Another disadvantage of our study has been the retrospective collection of worst ever tic severity. Finally, the use of psychotropic medication in 55% of the patients may have affected symptom severity.

In conclusion, the present study provides evidence for the 2R allele of the DRD4 48-bp VNTR as a genetic risk factor for OCD symptom severity, and the 3R allele for ASD symptom severity in children and adolescents with a tic disorder. The findings of interactions between delivery complications and the 3R allele regarding tic severity, and the 2R allele regarding depressive and anxious symptoms, would be strengthened by independent replication. Future studies should address further DRD4 polymorphisms and the pathophysiological mechanisms underlying the involvement of the DRD4 gene, also in interaction with environmental factors, in tic disorders.

References

1. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
2. McMahon WM, Carter AS, Fredine N, et al. Children at familial risk for Tourette's disorder: Child and parent diagnoses. *Am J Med Genet B Neuropsychiatr Genet* 2003;121B:105-111
3. Comings DE, Comings BG. Clinical and genetic relationships between autism-pervasive developmental disorder and Tourette syndrome: a study of 19 cases. *Am J Med Genet* 1991;39:180-191
4. Pauls DL, Leckman JF, Cohen DJ. Evidence against a genetic relationship between Tourette's syndrome and anxiety, depression, panic and phobic disorders. *Br J Psychiatry* 1994;164:215-221
5. Grados MA, Mathews CA. Latent class analysis of gilles de la tourette syndrome using comorbidities: clinical and genetic implications. *Biol Psychiatry* 2008;64:219-225
6. Swain JE, Scahill L, Lombroso PJ, et al. Tourette syndrome and tic disorders: a decade of progress. *J Am Acad Child Adolesc Psychiatry* 2007;46:947-968
7. Oak JN, Oldenhof J, Van Tol HH. The dopamine (4) receptor: one decade of research. *Eur J Pharmacol* 2000;405:303-327
8. Asghari V, Sanyal S, Buchwaldt S, et al. Modulation of intracellular cyclic AMP levels by different human dopamine D4 receptor variants. *J Neurochem* 1995;65:1157-1165
9. Comings DE, Gonzalez N, Wu S, et al. Studies of the 48 bp repeat polymorphism of the DRD4 gene in impulsive, compulsive, addictive behaviors: Tourette syndrome, ADHD, pathological gambling, and substance abuse. *Am J Med Genet* 1999;88:358-368
10. Tarnok Z, Ronai Z, Gervai J, et al. Dopaminergic candidate genes in Tourette syndrome: Association between tic severity and 3' UTR polymorphism of the dopamine transporter gene. *Am J Med Genet B Neuropsychiatr Genet* 2007;144B:900-905
11. Hebebrand J, Nothen MM, Ziegler A, et al. Nonreplication of linkage disequilibrium between the dopamine D4 receptor locus and Tourette syndrome. *Am J Hum Genet* 1997;61:238-239
12. Grice DE, Leckman JF, Pauls DL, et al. Linkage disequilibrium between an allele at the dopamine D4 receptor locus and Tourette syndrome, by the transmission-disequilibrium test. *Am J Hum Genet* 1996;59:644-652
13. Diaz-Anzaldua A, Joober R, Riviere JB, et al. Tourette syndrome and dopaminergic genes: a family-based association study in the French Canadian founder population. *Mol Psychiatry* 2004;9:272-277
14. Camarena B, Loyzaga C, Aguilar A, et al. Association study between the dopamine receptor D(4) gene and obsessive-compulsive disorder. *Eur Neuropsychopharmacol* 2007;17:406-409
15. Millet B, Chabane N, Delorme R, et al. Association between the dopamine receptor D4 (DRD4) gene and obsessive-compulsive disorder. *Am J Med Genet B Neuropsychiatr Genet* 2003;116B:55-59
16. Walitza S, Scherag A, Renner TJ, et al. Transmission disequilibrium studies in early onset of obsessive-compulsive disorder for polymorphisms in genes of the dopaminergic system. *J Neural Transm* 2008;115:1071-1078
17. Lopez LS, Croes EA, Sayed-Tabatabaei FA, et al. The dopamine D4 receptor gene 48-base-pair-repeat polymorphism and mood disorders: a meta-analysis. *Biol Psychiatry* 2005;57:999-1003
18. Guo G, Tillman KH. Trajectories of depressive symptoms, dopamine D2 and D4 receptors, family socioeconomic status and social support in adolescence and young adulthood. *Psychiatr Genet* 2009;19:14-26
19. Tochigi M, Hibino H, Otowa T, et al. Association between dopamine D4 receptor (DRD4) exon III polymorphism and Neuroticism in the Japanese population. *Neurosci Lett* 2006;398:333-336
20. Hamilton SP, Haghighi F, Heiman GA, et al. Investigation of dopamine receptor (DRD4) and dopamine transporter (DAT) polymorphisms for genetic linkage or association to panic disorder. *Am J Med Genet* 2000;96:324-330
21. Kennedy JL, Neves-Pereira M, King N, et al. Dopamine system genes not linked to social phobia. *Psychiatr Genet* 2001;11:213-217
22. Grady DL, Harxhi A, Smith M, et al. Sequence variants of the DRD4 gene in

autism: further evidence that rare DRD4 7R haplotypes are ADHD specific. *Am J Med Genet B Neuropsychiatr Genet* 2005;136B: 33-35

23. Pringsheim T, Sandor P, Lang A, et al. Prenatal and perinatal morbidity in children with Tourette syndrome and attention-deficit hyperactivity disorder. *J Dev Behav Pediatr* 2009;30:115-121
24. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
25. Leckman JF, Dolnansky ES, Hardin MT, et al. Perinatal factors in the expression of Tourette's syndrome: an exploratory study. *J Am Acad Child Adolesc Psychiatry* 1990;29:220-226
26. Bos-Veneman NG, Kuin A, Minderaa RB, et al. Role of perinatal adversities on tic severity and symptoms of attention deficit/hyperactivity disorder in children and adolescents with a tic disorder. *J Dev Behav Pediatr* 2010;31:100-106
27. American Psychiatric Association.Diagnostic and Statistical Manual of Mental Disorders, 4th edition,text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
28. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
29. Goodman WK, Price LH, Rasmussen SA, et al. The Yale-Brown Obsessive Compulsive Scale. I. Development, use, and reliability. *Arch Gen Psychiatry* 1989;46:1006-1011
30. Hartman CA, Luteijn E, Serra M, et al. Refinement of the Children's Social Behavior Questionnaire (CSBQ): an instrument that describes the diverse problems seen in milder forms of PDD. *Journal of Autism and Developmental Disorders* 2006;36:325-342
31. Chorpita BF, Moffitt CE, Gray J. Psychometric properties of the Revised Child Anxiety and Depression Scale in a clinical sample. *Behav Res Ther* 2005;43:309-322
32. Rice F, Lewis A, Harold G, et al. Agreement between maternal report and antenatal records for a range of pre and peri-natal factors: the influence of maternal and child characteristics. *Early Hum Dev* 2007;83:497-504
33. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215
34. Denys D, Zohar J, Westenberg HG. The role of dopamine in obsessive-compulsive disorder: preclinical and clinical evidence. *J Clin Psychiatry* 2004;65 Suppl 14:11-17
35. Chavez B, Chavez-Brown M, Sopko MA, Jr., et al. Atypical antipsychotics in children with pervasive developmental disorders. *Paediatr Drugs* 2007;9:249-266
36. Fergusson DM, Woodward LJ, Horwood LJ. Maternal smoking during pregnancy and psychiatric adjustment in late adolescence. *Arch Gen Psychiatry* 1998;55:721-727
37. Paz R, Barsness B, Martenson T, et al. Behavioral teratogenicity induced by nonforced maternal nicotine consumption. *Neuropsychopharmacology* 2007;32:693-699
38. Indredavik MS, Brubakk AM, Romundstad P, et al. Prenatal smoking exposure and psychiatric symptoms in adolescence. *Acta Paediatr* 2007;96:377-382
39. Santangelo SL, Pauls DL, Goldstein JM, et al. Tourette's syndrome: what are the influences of gender and comorbid obsessive-compulsive disorder? *J Am Acad Child Adolesc Psychiatry* 1994;33:795-804
40. Neuman RJ, Lobos E, Reich W, et al. Prenatal smoking exposure and dopaminergic genotypes interact to cause a severe ADHD subtype. *Biol Psychiatry* 2007;61:1320-1328
41. Altink ME, Arias-Vasquez A, Franke B, et al. The dopamine receptor D4 7-repeat allele and prenatal smoking in ADHD-affected children and their unaffected siblings: no gene-environment interaction. *J Child Psychol Psychiatry* 2008;49:1053-1060

Chapter

5

Role of single nucleotide polymorphisms of the SLC6A3 gene in severity of comorbid symptoms in patients with a tic disorder

This chapter has been submitted for publication

Netty G.P. Bos-Veneman

Ruud B. Minderaa

Pieter J. Hoekstra

Department of Psychiatry
University Medical Center Groningen
University of Groningen
the Netherlands

Abstract

Objective To investigate the role of single nucleotide polymorphisms (SNPs) of the solute carrier family 6, member 3, gene (SLC6A3) regarding severity of tics and comorbid symptoms in patients with a tic disorder.

Methods We genotyped 132 patients with tics with regard to three SNPs located at the 3' and 5' ends of SLC6A3 (rs40184, rs11564750, and rs2550946) and assessed severity of tics and symptoms of comorbid obsessive-compulsive disorder and attention deficit/hyperactivity disorder as well as presence of prenatal smoking exposure and pregnancy and delivery complications. Possible associations between SNP alleles and severity of tics and comorbid symptoms were addressed. Also, through linear regressions, we investigated whether SNP alleles would interact with perinatal adversities regarding tic and comorbid symptom severity.

Results Presence of an rs40184 G allele was associated with increased severity of inattentive symptoms. Thereby, pregnancy complications appeared to interact with rs11564750 and rs2550946 to decrease severity of comorbid obsessions and compulsions.

Conclusion This study provides preliminary evidence for a role of SLC6A3 in the expression of comorbid inattentive symptoms in patients with a tic disorder. Interactions between SLC6A3 and pregnancy complications may modulate severity of co-occurring obsessive-compulsive symptoms.

Introduction

Tic disorders are neuropsychiatric conditions characterized by the presence of sudden, rapid, recurrent, and nonrhythmic movements, and/or utterances. Many patients with tics are affected by a variety of comorbid psychiatric conditions and symptoms including obsessive-compulsive disorder (OCD) and attention deficit/hyperactivity disorder (ADHD).[1] Underlying etiological mechanisms are still under debate. Although genes indisputably contribute to the pathogenesis of tics, no genetic susceptibility regions have conclusively been identified.[2] Findings of family studies have suggested that tic disorders and at least some forms of OCD and ADHD may be different expressions of a shared genetic vulnerability[3-5], whereas other studies have indicated that tics and ADHD may have independent etiologies, and that patients with tics as well as ADHD and OCD may represent a separate nosological entity.[6] However, most studies have searched for genetic variants involved in tic disorders without taking comorbidities into account.

Postmortem and single photon emission computer tomography (SPECT) studies have indicated that the dopamine transporter may be involved in Tourette´s disorder.[7-9] Moreover, dopamine blocking agents are well established treatments for tic disorders. This has made the dopamine active transporter-1 gene (DAT1, alias solute carrier family 6, member 3 gene [SLC6A3]) that regulates synaptic concentrations of dopamine by reuptake into presynaptic ends an obvious candidate gene for tic disorders.[2] The majority of studies has confirmed a role of SLC6A3 in tic and co-occurring disorders, although results have been elusive.[10-24] Most work has addressed an SLC6A3 40 base pairs (bp) variable number of tandem repeats (VNTR) with 3 till 13 repeats (R) located at the 3' untranslated region. Associations between the 9R allele and increased tic severity in children with Tourette's disorder[10], and between the 10R allele and 10R/10R genotype and presence of a tic disorder have been demonstrated in both family-based and case-control studies[11,12], but could not be replicated in two other studies.[13,14] Heterozygosity for alleles of another SLC6A3 polymorphism, named DAT1 *Dde*I, has also been related to presence of Tourette's disorder.[13] Evidence for a role of SLC6A3 in OCD is much weaker. Here, two studies have found trends toward increased frequencies of the 9R allele of the 3' VNTR in patients with OCD compared to healthy controls[15,16], while two other investigations found no differences.[17,18] Also in 69 trios comprising probands with early onset OCD and their parents no evidence of transmission disequilibrium has been detected for alleles of the 3' VNTR.[19]

Several studies have also indicated a role for SLC6A3 polymorphisms in ADHD. The large scale international multi-site ADHD genetics study[20] investigated 32 single nucleotide polymorphisms (SNP) and four VNTR markers spanning SLC6A3 in 776 trios of patients with combined type ADHD and their parents and found nominal evidence of associations with six SNP markers and three VNTR markers. Two groups of markers located at the 3' and 5' flankings of the gene could be distinguished. Polymorphisms

within in each region, but not between the two regions, exhibited high linkage disequilibrium. In a subsequent investigation of the same research group[21], four SNP markers were addressed: two located at the 5' end (rs2550946 and rs11564750), one in intron 10 (rs3776153), and one at the 3' end of intron 14 (rs40184). ADHD appeared to be related to rs11564750 and rs3776153. Recently, an independent group has replicated the association between rs11564750 and ADHD in 450 trios comprising probands with ADHD and their parents.[22]

The main aim of the current study was to investigate possible associations between three SLC6A3 SNP markers (rs40184, rs11564750, and rs2550946) and severity of tics and comorbid OCD and ADHD symptoms in patients with a tic disorder. Genetic research in tic disorders may be complicated by substantial environmental influences that modulate or interact with gene effects. In a previous study, we have demonstrated that gene-environment interactions between the dopamine receptor D4 gene and delivery complications may be associated with tic and internalizing symptom severity in children with tic disorders.[25] Evidence pointing to a role of interactions between SLC6A3 and prenatal adversities in ADHD has been provided.[26-28] Therefore, we also explored possible interactions between the SLC6A3 SNP markers and perinatal adversities in patients with tics and comorbid symptoms.

Methods

Study sample

The study involved 132 patients with a tic disorder between 6 and 60 years (mean age 15.6 years [SD 10.2]; 14% of 18 years or older; 79.5% male), of which 65 patients had previously participated in a study investigating perinatal adversities.[29] Most of the patients (n = 96) had been referred to our outpatient child and adolescent psychiatry clinic, the remaining (n = 36) were recruited through the Dutch Tourette's disorder Association. Patients fulfilled criteria of the Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR)[30] for either Tourette's disorder (n = 116), chronic motor tic disorder (n = 15), or chronic vocal tic disorder (n = 1). At the time of investigation, the use of psychotropic medication was recorded (available from 125 patients). This was used by 64 patients, either an antipsychotic agent (n = 22), clonidine (n = 7), methylphenidate (n = 8), atomoxetine (n = 4), a selective serotonin reuptake inhibitor (SSRI; n = 3), or a combination of two or more agents (n = 20).

Informed consent from patients of 18 years or older, from parents and children of 12 years or older, and assent from children between 6 and 12 years was obtained. The study had been approved by our Institutional Review Board.

Clinical measures

Patients were interviewed regarding their tic severity, with regard to the week in which tics were worst ever, by using the Yale Global Tic Severity Scale (YGTSS), a semi-structured interview which records the number, frequency, intensity, complexity, and

interference of motor and vocal tics separately.[31] In a subgroup of patients (n = 57), worst ever tic severity had not been assessed; here, we used current tic severity ratings. Mean total, motor, and vocal YGTSS ratings were 22.6 (SD 9.6, range 4 - 50), 13.9 (SD 5.4, range 0 - 25), and 8.7 (SD 6.5, range 0 - 25), respectively.

Pediatric or adult versions of the Yale-Brown Obsessive-Compulsive Scale (YBOCS) as appropriate were used to interview patients regarding their current obsessive-compulsive symptom severity (available from 121 patients).[32] Mean total, obsessive, and compulsive YBOCS ratings were 5.0 (SD 7.5, range 0 - 28), 1.9 (SD 3.9, range 0 - 16), and 3.1 (SD 4.6, range 0 - 16), respectively.

ADHD symptom severity was measured by using the parent version of the ADHD rating scale (available from 65 children), a DSM-IV based questionnaire containing 18 items on inattentive and hyperactive-impulsive symptoms to be scored on a four-point scale.[33] Respective mean total, hyperactive-impulsive, and inattentive scores were 19.9 (SD 11.1, range 1 - 43), 8.7 (SD 6.6, range 0 - 23), and 11.0 (SD 5.9, range 0 - 22). ADHD symptom ratings were not available for the adult patients.

Patients also completed questionnaires with regard to pregnancy and delivery complications, and maternal prenatal smoking (available from 90 patients), as has been described before.[29] Pregnancy complications were present in 40 patients, delivery complications in 33 patients, and smoking exposure during pregnancy in 16 patients.

Genotyping

DNA was extracted from blood samples or buccal swabs (Cytobrush®) using a manual salting out procedure.[34] We genotyped one SNP from the 3' end (rs40184) and two SNPs markers located at the 5' end (rs11564750 and rs2550946) of the SLC6A3 gene by using the Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA, USA). Scan data were analyzed and genotyped in BeadStudio 3.0 (Illumina Inc., San Diego, CA, USA). Call rates were 98% for rs40184, 100% for rs11564750, and 99% for rs2550946. Genotyping accuracy was 100% as determined by concordance between DNA replicates. Minor allele frequencies and positions in the SLC6A3 gene are represented in table 1.

Table 1
Position in the solute carrier family 6, member 3, gene, alleles, and minor allele frequency of the investigated SNP markers.

SNP marker	Position in gene (bp)	Location	Alleles	MAF
rs40184	1,448,077	3´ flanking of intron 14	A/G	0.43
rs11564750	1,500,762	5´ flanking	C/G	0.11
rs2550946	1,503,513	5´ flanking	A/G	0.39

SNP, single nucleotide polymorphism; bp, base pairs; MAF, minor allele frequency.

Table 2
Comparisons between alleles of SNP markers of the solute carrier family 6, member 3, gene regarding severity of tics and comorbid OCD and ADHD symptoms in patients with a tic disorder.

	rs40184						rs11564750					
	A		G				C		G			
	n	mean ± SD	n	mean ± SD	*t*	*p*	n	mean ± SD	n	mean ± SD	*t*	*p*
YGTSS	117		145				31		233			
motor		13.7 ± 5.2		14.0 ± 5.6	-0.43	.67		13.7 ± 5.4		14.0 ± 5.5	-0.27	.79
vocal		8.1 ± 6.4		9.0 ± 6.5	-1.11	.27		8.1 ± 6.5		8.8 ± 6.5	-0.52	.61
total		21.8 ± 9.2		23.0 ± 9.8	-0.99	.32		21.8 ± 9.0		22.7 ± 9.7	-0.50	.62
YBOCS	102		138				27		215			
obsessive		1.9 ± 3.9		1.7 ± 3.7	0.52	.61		2.0 ± 4.2		1.8 ± 3.9	0.16	.88
compulsive		3.3 ± 4.8		2.8 ± 4.3	0.88	.38		4.3 ± 5.1		2.9 ± 4.5	1.51	.13
total		5.3 ± 7.6		4.4 ± 7.1	0.86	.39		6.3 ± 8.1		4.8 ± 7.4	0.99	.33
ADHD rating scale	54		74				15		115			
inattentive		9.5 ± 6.3		11.9 ± 5.5	-2.34	**.02**		11.1 ± 7.0		11.0 ± 5.8	0.10	.93
hyperactive-impulsive		8.1 ± 6.1		9.2 ± 7.0	-0.91	.37		10.0 ± 6.6		8.5 ± 6.6	0.82	.41
total		18.0 ± 10.9		21.2 ± 11.1	-1.61	.11		21.1 ± 11.4		19.8 ± 11.0	0.45	.65

	rs2550946					
	A		G			
	n	mean ± SD	n	mean ± SD	*t*	*p*
YGTSS	102		158			
motor		14.0 ± 5.4		13.8 ± 5.4	0.31	.76
vocal		8.7 ± 6.3		8.4 ± 6.5	0.34	.74
total		22.7 ± 9.3		22.2 ± 9.7	0.41	.69
YBOCS	90		148			
obsessive		2.1 ± 3.9		1.6 ± 3.8	1.01	.31
compulsive		3.6 ± 4.6		2.7 ± 4.5	1.44	.15
total		5.7 ± 7.4		4.3 ± 7.3	1.46	.15
ADHD rating scale	48		78			
inattentive		9.7 ± 6.4		11.6 ± 5.5	-1.79	.08
hyperactive-impulsive		8.0 ± 6.8		9.1 ± 6.6	-0.96	.34
total		18.1 ± 11.8		20.8 ± 10.7	-1.36	.18

SNP, single nucleotide polymorphism; OCD, obsessive-compulsive disorder; ADHD, attention deficit/hyperactivity disorder; YGTSS, Yale Global Tic Severity Scale; YBOCS, Yale-Brown Obsessive-Compulsive Scale.

Statistical analyses

We made comparisons between alleles of the three SNPs, and between patients with and without at least one pregnancy complication, with and without at least one delivery complication, and with and without prenatal smoking exposure by using Student's *t* test regarding ratings of tic and comorbid OCD and ADHD symptom severity.

A gene x environment interaction term was provided by the product of genetic and perinatal factors. Regarding rs40184 and rs2550946, presence of an A allele was given the value 0, and of a G allele the value 1; with regard to rs11564750, presence of a C allele was given the value 0, and of a G allele the value 1. Those perinatal adversities that appeared to yield between-group differences at a significance level of at least $p \leq .25$ with regard to a symptom severity measure were used in linear regression as independent variable together with these interaction terms with the symptom severity measures as outcome. Those regression analyses demonstrating a statistically significant interaction term in association with a symptom severity measure were subsequently adjusted for gender and age.

In all statistical analyses $p \leq .05$ was considered as significant and tests were two-sided.

Results

Main effects of SNP markers

Table 2 shows comparisons between the alleles of SNP markers rs40184, rs11564750, and rs2550946 with regard to the symptom severity ratings. Of note, presence of a G allele of SNP marker rs40184 was related to significantly higher mean inattentive ADHD rating scale scores. We found no other statistically significant between-group comparisons regarding any symptom severity measure.

Perinatal adversities

Patients who had been exposed to smoking in utero had lower mean motor YGTSS ratings (12.8 [SD 4.3] versus 15.8 [SD 5.1], $t = 2.22$, $p = .03$). No other statistically significant between-group comparisons regarding any perinatal adversity and any symptom severity measure were present.

Gene-environment interactions

The interaction term of pregnancy complications and rs11564750 was significantly related to mean compulsive and total YBOCS ratings ($b = 6.20$, SE 2.5, $\beta = .69$, $p = .01$, and $b = 9.61$, SE 4.1, $\beta = .64$, $p = .02$, respectively), also after adjusting for age and gender ($b = 6.19$, SE 2.5, $\beta = .69$, $p = .01$, and $b = 9.68$, SE 4.2, $\beta = .64$, $p = .02$, respectively). Figure 1 shows the direction of effect: in the presence of a C allele, pregnancy complications were associated with lower mean compulsive and total YBOCS ratings (0.2 [SD 0.4] versus 7.3 [SD 5.3], $t = 2.92$, $p = .002$, and 0.4 [SD 0.9]

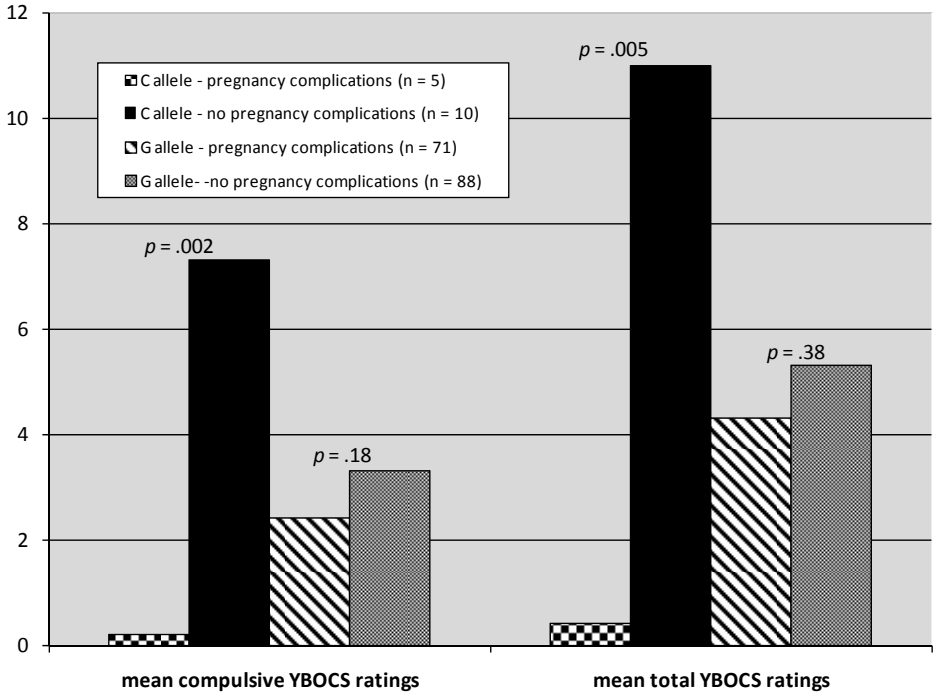

Figure 1. Associations between interaction terms of pregnancy complications and rs11564750 regarding comorbid OCD symptom severity in patients with a tic disorder.
OCD, obsessive-compulsive disorder; YBOCS, Yale-Brown Obsessive-Compulsive Scale.

versus 11.0 [SD 9.1], *t* = -3.67, *p* = .005); whereas in the presence of a G allele, pregnancy complications were not associated with compulsive and total YBOCS ratings (2.4 [SD 3.8] versus 3.3 [SD 4.6], *t* = 1.34, *p* = .18, and 4.3 [SD 6.5] versus 5.3 [SD 7.8], *t* 0.86, *p* = .38). Moreover, the interaction term of pregnancy complications and rs2550946 was significantly associated with mean total YBOCS ratings (*b* = 5.68, SE 2.3, β = .37, *p* = .01; after adjustment for age and gender: *b* = 5.83, SE 2.3, β = .38, *p* = .01). The direction of effect is represented in figure 2: in the presence of an A allele, pregnancy complications were related to lower mean total YBOCS ratings (3.6 [SD 5.2] versus 8.5 [SD 8.7], *t* = 2.70, *p* = .009); in the presence of a G allele, pregnancy complications were not associated with mean total YBOCS ratings (4.4 [SD 7.0] versus 3.5 [SD 6.2], *t* = -0.64, *p* = .52).

Regarding ADHD symptom severity, we found a statistically significant association of the interaction term of delivery complications and rs11564750 with mean inattentive ADHD rating scale scores (*b* = -8.69, SE 3.5, β = -.66, *p* = .02), also after adjusting for age and gender (*b* = -8.93, SE 3.5, β = -.67, *p* = .01). In the presence of a C allele, delivery complications were associated with higher mean inattentive ADHD rating scale scores (16.4 [SD 5.3] versus 5.6 [SD 3.6], *t* = -4.26, *p* = .002). However, in the presence of a G allele, delivery complications were at trend-level significance also associated with higher mean inattentive ADHD rating scale scores (11.4 [SD 5.2] versus 10.2 [SD 6.0], *t* = -1.79, *p* = .08).

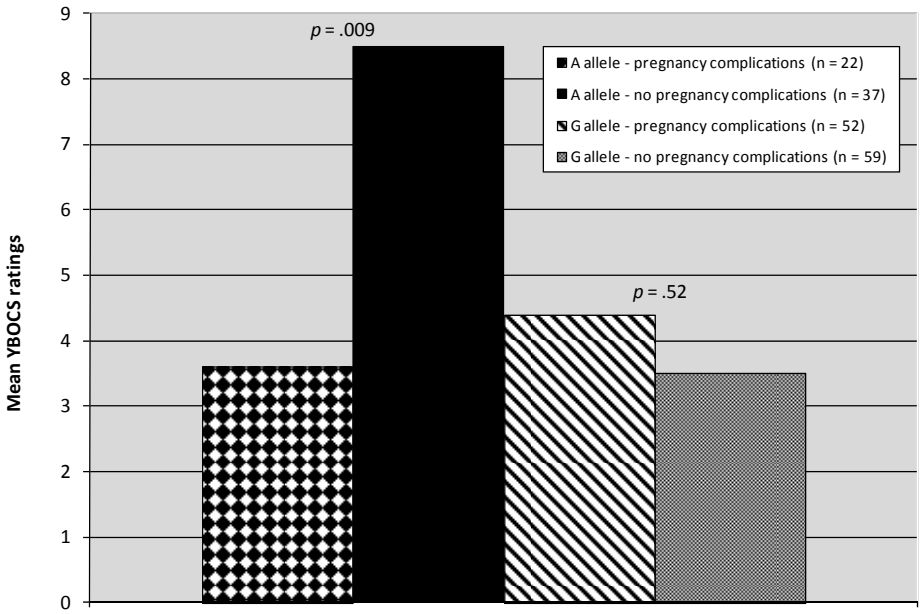

Figure 2. Associations between interaction terms of pregnancy complications and rs2550946 regarding comorbid OCD symptom severity in patients with a tic disorder.
OCD, obsessive-compulsive disorder; YBOCS, Yale-Brown Obsessive-Compulsive Scale.

No other statistically significant interaction terms of perinatal adversities with any SNP marker in association with any symptom severity measure were present.

5 Discussion

The current study suggests an association between the G allele of the SLC6A3 SNP rs40184 and increased severity of inattentive symptoms in patients with a tic disorder. No previous studies have addressed SLC6A3 SNP markers in patients with tics, but in a large cohort of children with combined type ADHD a relation between rs40184 and ADHD status had also been identified.[20] At least three studies in ADHD cohorts, however, have also detected associations between ADHD and SLC6A3 5´ end SNP markers, in contrast to the present study.[20-22] One explanation may be that our sample size has been too small to detect the previously reported associations. The presently found trend-significant association between rs2550946 and severity of inattentive symptoms (p = .08) supports this.

We did not find evidence of interactions between SNP markers of SLC6A3 and smoking or alcohol exposure in utero, in contrast to previous studies in ADHD cohorts.[26-28] However, we found preliminary evidence for a role of gene-environment interactions in that pregnancy complications appeared to interact with 5´ flanking SNP markers to decrease severity of obsessions and compulsions: in the presence of an rs11564750 C allele or rs2550946 A allele, patients with pregnancy complications surprisingly had less severe OCD symptoms, whereas in the presence of a G allele of

these SNP markers, pregnancy complications did not influence OCD symptom severity. In a previous study we had already found an inverse relation between pregnancy complications and OCD symptom severity in children with tics.[25] Also, a trend-level significant relation between the number of medications exposed to during pregnancy and decreased OCD symptoms had previously been reported in a large cohort of patients with Tourette's disorder.[35] These findings suggest that pregnancy complications in some cases may protect against expression of OCD symptoms in patients with tics. This may perhaps be understood in that (subclinical) obsessive-compulsive symptoms in some cases may actually be regarded as a healthy phenomenon with clear functionality for the individual.

The presently reported associations between SLC6A3 and co-occurring OCD and ADHD symptoms in patients with tics support the involvement of dopaminergic pathways, in line with findings from pharmacological and neuroimaging studies. In patients with OCD antipsychotic medication has shown to be efficacious in combination with SSRIs.[36] The dopamine transporter is a key target of methylphenidate, drug of first choice in ADHD.[37] Moreover, SPECT studies have demonstrated an increased dopamine transporter binding ratio in the basal ganglia in patients with OCD and ADHD.[36,38,39] Whereas a direct dopaminergic contribution to the pathogenesis of ADHD is obvious, dopamine may influence the expression of OCD by its interactions with the serotonin system.

A number of limitations need to be acknowledged. First, the sample size of our study group has been relatively small. Another disadvantage has been the retrospective collection of worst ever tic severity. In addition, the use of psychotropic medication in 51% of the patients may have affected comorbid symptom severity. Worst ever tic severity, however, was typically in a time when children did not use psychotropic medication. Thereby, severity of ADHD features was only assessed in a subgroup of patients. Finally, the investigated markers may not be risk variants, but may interact with other polymorphisms of SLC6A3.

In conclusion, this study addressed the possible role of SNP markers located at the 3' and 5' regions of SLC6A3, both separately as well in interaction with perinatal adversities, regarding severity of tics and co-occurring OCD and ADHD symptoms in patients with a tic disorder. The SLC6A3 polymorphism rs40184 appeared to be associated with increased severity of comorbid ADHD features. The findings of interactions between pregnancy complications and SNP markers located in the 5' region of SLC6A3 regarding severity of comorbid obsessions and compulsions would be strengthened by independent replication. To facilitate the search for genes future studies regarding complex disorders such as tic disorders should take the expression of comorbid symptoms and possible gene-environment interactions into account.

References

1. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
2. O'Rourke JA, Scharf JM, Yu D, et al. The genetics of Tourette syndrome: a review. J Psychosom Res 2009;67:533-545
3. McMahon WM, Carter AS, Fredine N, et al. Children at familial risk for Tourette's disorder: Child and parent diagnoses. *Am J Med Genet B Neuropsychiatr Genet* 2003; 121B:105-111
4. Grados MA, Riddle MA, Samuels JF, et al. The familial phenotype of obsessive-compulsive disorder in relation to tic disorders: the Hopkins OCD family study. *Biol Psychiatry* 2001;50:559-565
5. Stewart SE, Illmann C, Geller DA, et al. A controlled family study of attention-deficit/hyperactivity disorder and Tourette's disorder. *J Am Acad Child Adolesc Psychiatry* 2006;45:1354-1362
6. Rothenberger A, Roessner V, Banaschewski T, et al. Co-existence of tic disorders and attention-deficit/hyperactivity disorder-recent advances in understanding and treatment. *Eur Child Adolesc Psychiatry* 2007;16 Suppl 1:1-4
7. Cheon KA, Ryu YH, Namkoong K, et al. Dopamine transporter density of the basal ganglia assessed with [123I]IPT SPECT in drug-naive children with Tourette's disorder. *Psychiatry Res* 2004;130:85-95
8. Yoon DY, Gause CD, Leckman JF, et al. Frontal dopaminergic abnormality in Tourette syndrome: a postmortem analysis. *J Neurol Sci* 2007;255:50-56
9. Serra-Mestres J, Ring HA, Costa DC, et al. Dopamine transporter binding in Gilles de la Tourette syndrome: a [123I]FP-CIT/SPECT study. *Acta Psychiatr Scand* 2004;109:140-146
10. Tarnok Z, Ronai Z, Gervai J, et al. Dopaminergic candidate genes in Tourette syndrome: Association between tic severity and 3' UTR polymorphism of the dopamine transporter gene. *Am J Med Genet B Neuropsychiatr Genet* 2007;144B:900-905
11. Rowe DC, Stever C, Gard JM, et al. The relation of the dopamine transporter gene (DAT1) to symptoms of internalizing disorders in children. *Behav Genet* 1998; 28:215-225
12. Comings DE, Wu S, Chiu C, et al. Polygenic inheritance of Tourette syndrome, stuttering, attention deficit hyperactivity, conduct, and oppositional defiant disorder: the additive and subtractive effect of the three dopaminergic genes - DRD2, D beta H, and DAT1. *Am J Med Genet* 1996;67:264-288
13. Yoon DY, Rippel CA, Kobets AJ, et al. Dopaminergic polymorphisms in Tourette syndrome: association with the DAT gene (SLC6A3). *Am J Med Genet B Neuropsychiatr Genet* 2007;144:605-610
14. Diaz-Anzaldua A, Joober R, Riviere JB, et al. Tourette syndrome and dopaminergic genes: a family-based association study in the French Canadian founder population. *Mol Psychiatry* 2004;9:272-277
15. Billett EA, Richter MA, Sam F, et al. Investigation of dopamine system genes in obsessive-compulsive disorder. *Psychiatr Genet* 1998;8:163-169
16. Hemmings SM, Kinnear CJ, Niehaus DJ, et al. Investigating the role of dopaminergic and serotonergic candidate genes in obsessive-compulsive disorder. *Eur Neuro-psychopharmacol* 2003;13:93-98
17. Frisch A, Michaelovsky E, Rockah R, et al. Association between obsessive-compulsive disorder and polymorphisms of genes encoding components of the serotonergic and dopaminergic pathways. *Eur Neuro-psychopharmacol* 2000;10:205-209
18. Hemmings SM, Kinnear CJ, Lochner C, et al. Early- versus late-onset obsessive-compulsive disorder: investigating genetic and clinical correlates. *Psychiatry Res* 2004;128:175-182
19. Walitza S, Scherag A, Renner TJ, et al. Transmission disequilibrium studies in early onset of obsessive-compulsive disorder for polymorphisms in genes of the dopaminergic system. *J Neural Transm* 2008;115:1071-1078
20. Brookes K, Xu X, Chen W, et al. The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. *Mol Psychiatry* 2006;11:934-953
21. Brookes KJ, Xu X, Anney R, et al. Association of ADHD with genetic variants in the 5'-region of the dopamine transporter gene: evidence for allelic heterogeneity. *Am J Med*

Genet B Neuropsychiatr Genet 2008;147B: 1519-1523

22. Doyle C, Brookes K, Simpson J, et al. Replication of an association of a promoter polymorphism of the dopamine transporter gene and Attention Deficit Hyperactivity Disorder. *Neurosci Lett* 2009;462:179-181
23. Li D, Sham PC, Owen MJ, et al. Meta-analysis shows significant association between dopamine system genes and attention deficit hyperactivity disorder (ADHD). *Hum Mol Genet* 2006;15:2276-2284
24. Faraone SV, Perlis RH, Doyle AE, et al. Molecular genetics of attention-deficit/ hyperactivity disorder. *Biol Psychiatry* 2005; 57:1313-1323
25. Bos-Veneman NGP, Minderaa R, Hoekstra PJ. The DRD4 gene and severity of tics and comorbid symptoms: main effects and interactions with delivery complications. *Mov Disord* 2010;in press
26. Kahn RS, Khoury J, Nichols WC, et al. Role of dopamine transporter genotype and maternal prenatal smoking in childhood hyperactive-impulsive, inattentive, and oppositional behaviors. *J Pediatr* 2003; 143:104-110
27. Neuman RJ, Lobos E, Reich W, et al. Prenatal smoking exposure and dopaminergic genotypes interact to cause a severe ADHD subtype. *Biol Psychiatry* 2007;61:1320-1328
28. Todd RD, Neuman RJ. Gene-environment interactions in the development of combined type ADHD: evidence for a synapse-based model. *Am J Med Genet B Neuropsychiatr Genet* 2007;144B:971-975
29. Bos-Veneman NG, Kuin A, Minderaa RB, et al. Role of perinatal adversities on tic severity and symptoms of attention deficit/hyperactivity disorder in children and adolescents with a tic disorder. *J Dev Behav Pediatr* 2010;31:100-106
30. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
31. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
32. Goodman WK, Price LH, Rasmussen SA, et al. The Yale-Brown Obsessive Compulsive Scale. I. Development, use, and reliability. *Arch Gen Psychiatry* 1989;46:1006-1011
33. DuPaul GJ. ADHD Rating Scale-IV: Checklists, Norms and Clinical Interpretation. New York: Power, T. J., Anastopoulos, A. D., and Reid, R.; 1998
34. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 1988;16:1215
35. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
36. Denys D, Zohar J, Westenberg HG. The role of dopamine in obsessive-compulsive disorder: preclinical and clinical evidence. *J Clin Psychiatry* 2004;65 Suppl 14:11-17
37. Iversen L. Neurotransmitter transporters and their impact on the development of psychopharmacology. *Br J Pharmacol* 2006; 147 Suppl 1:S82-S88
38. van der Wee NJ, Stevens H, Hardeman JA, et al. Enhanced dopamine transporter density in psychotropic-naive patients with obsessive-compulsive disorder shown by [123I]{beta}-CIT SPECT. *Am J Psychiatry* 2004;161:2201-2206
39. Krause KH, Dresel SH, Krause J, et al. The dopamine transporter and neuroimaging in attention deficit hyperactivity disorder. *Neurosci Biobehav Rev* 2003;27:605-613

Chapter

6

Cytokines and soluble adhesion molecules in children and adolescents with a tic disorder

Accepted for publication in Progress in Neuropsychopharmacology and Biological Psychiatry

Netty G.P. Bos-Veneman[a]

Johan Bijzet[b]

Pieter C. Limburg[b]

Ruud B. Minderaa[a]

Cees G. Kallenberg[b]

Pieter J. Hoekstra[a]

[a] Department of Psychiatry
[b] Department of Rheumatology
and Clinical Immunology
University Medical Center Groningen
University of Groningen
the Netherlands

Abstract

Aim Dysregulation of the immune system may play a role in tic disorders. We screened for immune disturbances by investigating serum levels of cytokines and soluble adhesion molecules in patients with a tic disorder.

Methods Serum levels of interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, soluble IL-2 receptor (sIL2R), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, soluble vascular cell adhesion molecule-1 (sVCAM-1), and intercellular adhesion molecule-1 (sICAM-1) of 66 children and adolescents with a tic disorder and 71 healthy volunteers were compared. We also addressed possible relations between concentrations of the immune markers and severity of tics and comorbid obsessive-compulsive symptoms.

Results Median serum concentrations did not differ significantly between patients and healthy subjects. Serum IL-2 concentrations were positively associated with tic severity ratings; serum IL-12 concentrations negatively with severity ratings of obsessive-compulsive symptoms.

Conclusions These preliminary findings do not reveal major immune activation in children with a tic disorder but may suggest more subtle disturbances related to disease expression.

Introduction

Tic disorders are characterized by the presence of motor and/or vocal tics. However, the majority of patients with a tic disorder also suffer from other psychiatric conditions, mainly obsessive-compulsive disorder (OCD) and attention deficit/hyperactivity disorder (ADHD).[1] Notwithstanding the genetic background of tic disorders[2], environmental factors also account for at least a subgroup of patients with tics. A wealth of studies has addressed a broad range of immune related parameters in patients with tic disorders, but findings have been inconclusive so far.[3]

Most research has focussed on the hypothesis of autoimmune processes being involved in tic disorders, but at best provided indirect support for this by demonstrating antineuronal antibodies[4-6], decreased numbers of regulatory T cells (Treg) that maintain tolerance to self antigens[7], and increased single time point serum levels of antistreptococcal antibodies[5,8,9], and by identifying candidate auto-antigens[10-12] in patients with tics. However, not all of these findings have as yet been independently replicated.[3,13,14]

Other studies have addressed dysregulation of the immune system in patients with tic disorders, in particular altered cellular immunity. Studies using microarray gene expression profiling of peripheral blood cells have demonstrated associations of Tourette's disorder (TD) and comorbid ADHD with overexpression of natural killer (NK) and cytotoxic T cell genes.[15,16] Also, serum levels of the proinflammatory cytokines interleukin (IL)-12 and tumor necrosis factor (TNF)-α have been found to be higher in children with TD and/or OCD compared to age-matched control subjects.[17] Moreover, IL-12 and TNF-α levels were further increased during symptom exacerbations.[17] A recent study enrolled 32 children with TD, including 17 patients with comorbid OCD, and 16 healthy subjects.[18] Only the patients with TD and comorbid OCD had significantly elevated plasma IL-12 levels compared to controls, whereas IL-2 was significantly higher in patients with TD and OCD compared to patients without OCD. However, in a small group of children with OCD and/or a tic disorder in whom the onset and/or exacerbation of symptoms was related to group A β-hemolytic streptococcal infections (designated as PANDAS, i.e., pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections)[19], no relation could be demonstrated between symptom exacerbations and cytokine levels.[13]

Cellular adhesion molecules are expressed by peripheral blood mononuclear cells and endothelial cells. These glycoproteins interact with other cells and the extracellular matrix and facilitate endothelial adhesion of circulating leukocytes during inflammation. Higher levels of soluble adhesion molecules, such as soluble vascular cell adhesion molecule-1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1) may be due to an increased expression of cellular adhesion molecules, mediated by inflammatory cytokines, or due to endothelial damage.[20] In a study that included 51 adult patients with TD, 30 healthy adults, 33 children with TD, 19 children with post-streptococcal neuropsychiatric disorders (PANDAS or Sydenham's chorea), and 34

healthy children, serum levels of sVCAM-1 appeared to be increased in adults and children with TD compared to healthy controls, whereas children with post-streptococcal neuropsychiatric disorders had increased serum levels of sICAM-1 compared to healthy children.[21]

In the present study, we compared serum levels of the T helper (Th) 1 cytokines IL-2, IL-12, TNF-α, and interferon (IFN)-γ, and the soluble IL-2 receptor (sIL2R) as markers of cellular immunity, of the Th2 cytokines IL-4, IL-5, and IL-10 that are mainly involved in humoral immune responses, and of soluble adhesion molecules sVCAM-1 and sICAM-1 between children and adolescents with a tic disorder and healthy volunteers. We also investigated possible relationships between serum levels of these immunological markers and severity of tics and comorbid obsessive-compulsive symptoms.

Methods

Patient and control subject populations

The study involved 66 patients with a tic disorder (mean age 12.3 years [SD 3.2], range 6 - 18; 86.4% male) and 71 healthy volunteers (mean age 12.5 years [SD 2.7], range 6 - 18; 31.0% male). Gender ratios differed significantly between patients and controls (χ^2 = 43.0, df =1, $p < 0.001$). Patients fulfilled criteria of the Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised[22] for either TD (n = 45; 68.2%), chronic motor tic disorder (n = 19; 28.8%), or chronic vocal tic disorder (n = 2; 3.0%). At the time of investigation parents indicated their child's use of psychotropic medication. Psychotropic medication was used by 56% of patients (n = 37), either antipsychotic agents (n = 11), clonidine (n = 3), psychostimulants (n = 7), atomoxetine (n = 4), or a combination of two or more agents (n = 12). Most of the patients (n = 52) had been referred to our outpatient child and adolescent psychiatry clinic, the remaining (n = 14) were recruited through the Dutch TD Association. Healthy volunteers had been recruited through advertisements in local news papers. Exclusion criteria were presence of an active infection, inflammation, autoimmune disorder, or autism. Healthy children who had a first-degree family member with tics were excluded as well.

Informed consent from parents and children of 12 years or older, or assent from children between 6 and 12 years was obtained. The study had been approved by our Institutional Review Board.

Assessment instruments

Parents of the patients were interviewed regarding their child's tic severity by using the Yale Global Tic Severity Scale (YGTSS).[23] Mean total, motor, and vocal YGTSS ratings were 19.3 (SD 8.9, range 0 - 39), 12.4 (SD 4.8, range 0 - 21), and 6.9 (SD 5.8, range 0 - 19), respectively. The Children's Yale-Brown Obsessive-Compulsive Scale (CYBOCS) was used to interview parents regarding their child's obsessive-compulsive symptom severity.[24] Mean total, compulsive, and obsessive CYBOCS ratings were 5.1 (SD 6.8, range 0 - 28), 3.3 (SD 4.3, range 0 - 14), and 1.8 (SD 3.7, range 0 - 16), respectively.

Laboratory analyses

Serum samples were collected between April 2006 and October 2007, and stored at -20ºC until analysis in January 2008. Serum concentrations of IL-2, IL-4, IL-5, IL-10, IL-12, sIL2R, TNF-α, and IFN-γ were measured by using a multiplex cytokine array according to the manufacturer's protocol (Invitrogen corporation, Carlsbad, CA) and read on a Luminex100™ platform. Detection limits of the assay were: IL-2 0.5 pg/ml, IL-4 0.5 pg/ml, IL-5 0.1 pg/ml, IL-10 0.1 pg/ml, IL-12 2 pg/ml, sIL2R 5 pg/ml, IFN-γ 0.3 pg/ml, and TNF-α 0.1 pg/ml. When cytokine levels were below the limits of detection, cytokines were given the lowest detectable value. Serum concentrations of sICAM-1 and sVCAM-1 were measured using a quantitative enzyme-linked immunosorbent assay following the protocol of the manufacturer (R&D systems; http://www.rndsystems.com).

Data analyses

We made comparisons between patients and healthy subjects by using the Mann-Whitney *U* test with regard to serum concentrations of measured cytokines and soluble adhesion molecules. When cytokines were not detectable for more than 20% of either patients or healthy subjects, we subsequently investigated relations regarding the frequency of subjects with detectable levels by using Pearson Chi-Square test.

Because gender ratios differed between patients and healthy subjects, we also made comparisons between male and female healthy subjects. As psychotropic medication may have induced immune alterations, we also compared patients with and without psychotropic medication use. Here, we also used the Mann-Whitney *U* test.

We investigated possible relations of serum concentrations of cytokines and soluble adhesion molecules with severity ratings of tics and obsessive-compulsive symptoms by using Spearman's correlation coefficient.

In all statistical analyses $p \leq .05$ was considered as significant. Tests were two-sided. Given that we planned a total number of 10 patient-control comparisons and 60 correlations, the significance levels corrected for the number of analyses would be .05 / 10 = .005, and .05 / 60 = .0008, respectively.

Table 1
Serum concentrations of cytokines and soluble adhesion molecules in children with a tic disorder and healthy subjects.

	Patients n = 66 Median	**Healthy subjects** n = 71 Median	*U*	*z*	*p*
Soluble adhesion molecules[a]					
sVCAM-1	429	449	2197	-0.63	.53
sICAM-1	148	160	2251	-0.40	.69
Cytokines[b]					
IL-2	0.50	0.50	2099	-1.44	.15
IL-4	1.14	2.95	1926	-1.84	.07
IL-5	0.10	0.10	2160	-0.91	.36
IL-10	1.69	1.64	2291	-0.22	.82
IL-12	159	163	2255	-0.38	.71
sIL2R	203	219	2201	-0.61	.54
TNF-α	0.43	0.46	1966	-1.63	.10
IFN-γ	0.30	0.30	1957	-1.83	.07

U, Mann Whitney *U* test statistic; *z* , *z*-score; *p* , *p*-value; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule; IL, interleukin; sIL2R, soluble IL-2 receptor; TNF, tumor necrosis factor; IFN, interferon. [a]Measured in nanograms per milliliter. [b]Measured in picograms per milliliter.

Results

Table 1 shows comparisons regarding median serum concentrations of cytokines and soluble adhesion molecules between patients and healthy subjects. Median serum concentrations did not differ significantly between patients and healthy subjects. Since sizeable portions of patients and controls had undetectable levels of IL-2 (patients 82%, controls 73%), IL-4 (patients 44%, controls 30%), IL-5 (patients 92%, controls 82%), IFN-γ (patients 83%, controls 66%), and TNF-α (patients 21%, controls 14%), we subsequently tested possible relations of disease status with frequency of detectable levels. Patients had only a significantly lower frequency of detectable IFN-γ levels compared to controls (χ^2 = 5.3, df =1, *p* = 0.02).

No gender differences regarding median serum concentrations of cytokines and soluble adhesion molecules were present, with the exception of median serum sICAM-1 concentrations that were significantly higher in male healthy children compared to females (184.5 ng/ml versus 158.0 ng/ml, *U* = 356, *z* = -2.27, *p* = .02). Cytokine and soluble adhesion molecule levels did not differ between patients with and without psychotropic medication use, except median serum sIL2R concentrations that were

significantly higher in patients who used psychotropic medication (253.4 pg/ml versus 186.3 pg/ml, $U = 362$, $z = -2.07$, $p = .04$).

Serum IL-2 concentrations were positively associated with vocal YGTSS ratings ($r = .25$, $p = .05$); serum IL-12 concentrations negatively with compulsive and total CYBOCS ratings ($r = -.28$, $p = .03$ and $r = -.26$, $p = .04$, respectively). None of the correlations met the stringent significance level after correction for multiple testing. No other statistically significant associations between serum concentrations of cytokines and soluble adhesion molecules and symptom severity ratings were present.

Discussion

The present study suggests that children and adolescents with a tic disorder do not differ from healthy volunteers with regard to serum levels of several cytokines and soluble adhesion molecules. However, we found a positive association between serum IL-2 concentrations and tic severity ratings, and an inverse relation between serum IL-12 levels and severity of comorbid obsessive-compulsive symptoms, although these relations were not robust after corrections for multiple testing.

Our results are in line with the study of Gabbay et al.[18] who also did not find differences in plasma levels of TNF-α, IL-1β, IL-12, IL-6, and IL-2 between patients with TD and controls, but are in contrast with an earlier study[17] that reported higher serum levels of IL-12 and TNF-α in children with TD and/or OCD compared to control subjects. The associations between IL-2 and tic severity, and IL-12 and OCD symptom severity, had not been identified in previous studies.[17,18] Discrepancies between the studies may be due to different laboratory techniques and factors that may have influenced cytokine levels, for example stress and food intake. Furthermore, Leckman et al.[17] had also included patients with OCD only, but not excluded subjects with immunological or infectious disorder.

IL-2 and IL-12 are Th1 cytokines and as such mainly involved in cellular immune responses. Thereby, both cytokines contribute to the development of autoimmune responses. IL-2 plays an essential role in T cell activation and the differentiation of a subset of Tregs.[25,26] Interestingly, given the involvement of the dopamine system in tic disorders, IL-2 may influence dopaminergic pathways.[27] Although the relation between IL-2 and tic severity did not reach significance after multiple testing correction, the present finding weakly points to a role of cellular immune effector pathways in tic disorders. IL-12 induces IFN-γ production by Th1 and NK cells[28], but also contributes to autoimmunity by restoring T cell activation and effector functions in the presence of Tregs[29], and enhancing the affinity of cytotoxic T cells for peptides.[30] Tregs that usually inhibit such responses may be deficient in patients with TD.[7] Considering the enhancing effect of IL-12 on cellular immune and autoimmune responses, our finding of a negative relation between IL-12 and obsessive-compulsive symptoms in patients with tics, speaks against a role of these responses in comorbid OCD symptom severity.

The present study could also not confirm the earlier documented increased serum sVCAM-1 levels in children with TD.[21] However, previous findings may have been due to the older age of the patients compared to the healthy controls, as sVCAM-1 levels increase with age.[31]

A number of limitations of this study need to be addressed. Although the use of a multi-cytokine array is appealing, the sensitivity to detect all cytokines simultaneously is often low. Indeed, an obvious limitation of our study is that a number of cytokines could not be detected in sizeable portions of patients and controls. However, frequencies of subjects with cytokines in the detectable range generally did not differ between patients and controls which speaks against major changes in these cytokine levels in patients. Another potential limitation is that 56% of patients used psychotropic medication which may have induced anti-inflammatory side-effects and also affected symptom severity. Finally, the control group was not gender-matched.

Conclusions

The present study does not reveal major immune activation in patients with a tic disorder. The possible association between serum IL-2 levels and tic severity ratings could indicate involvement of cellular immune effector pathways in the pathogenesis of tic disorders. However, the inverse relation between serum IL-12 concentrations and severity of comorbid OCD symptoms is difficult to explain. These findings are very preliminary and replication is warranted. To facilitate the search for immunopathogenetic mechanisms underlying tics, future studies should focus on more homogenous patient subgroups defined by clinical and genetic features.

References

1. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
2. O'Rourke JA, Scharf JM, Yu D, et al. The genetics of Tourette syndrome: a review. *J Psychosom Res* 2009;67:533-545
3. Martino D, Dale RC, Gilbert DL, et al. Immunopathogenic mechanisms in tourette syndrome: A critical review. *Mov Disord* 2009;24:1267-1279
4. Singer HS, Giuliano JD, Hansen BH, et al. Antibodies against human putamen in children with Tourette syndrome. *Neurology* 1998;50:1618-1624
5. Rizzo R, Gulisano M, Pavone P, et al. Increased antistreptococcal antibody titers and anti-basal ganglia antibodies in patients with Tourette syndrome: controlled cross-sectional study. *J Child Neurol* 2006;21:747-753
6. Yeh CB, Wu CH, Tsung HC, et al. Antineural antibody in patients with Tourette's syndrome and their family members. *J Biomed Sci* 2006;13:101-112
7. Kawikova I, Leckman JF, Kronig H, et al. Decreased numbers of regulatory T cells suggest impaired immune tolerance in children with tourette syndrome: a preliminary study. *Biol Psychiatry* 2007;61:273-278
8. Morshed SA, Parveen S, Leckman JF, et al. Antibodies against neural, nuclear, cytoskeletal, and streptococcal epitopes in children and adults with Tourette's syndrome, Sydenham's chorea, and autoimmune disorders. *Biol Psychiatry* 2001; 50:566-577
9. Cardona F, Orefici G. Group A streptococcal infections and tic disorders in an Italian pediatric population. *J Pediatr* 2001;138:71-75
10. Dale RC, Candler PM, Church AJ, et al. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. *J Neuroimmunol* 2006;172:187-197
11. Kansy JW, Katsovich L, McIver KS, et al. Identification of pyruvate kinase as an antigen associated with Tourette syndrome. *J Neuroimmunol* 2006;181:165-176
12. Kirvan CA, Swedo SE, Snider LA, et al. Antibody-mediated neuronal cell signaling in behavior and movement disorders. *J Neuroimmunol* 2006;179:173-179
13. Singer HS, Gause C, Morris C, et al. Serial immune markers do not correlate with clinical exacerbations in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections. *Pediatrics* 2008;121:1198-1205
14. Singer HS, Hong JJ, Yoon DY, et al. Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. *Neurology* 2005;65:1701-1707
15. Tang Y, Gilbert DL, Glauser TA, et al. Blood gene expression profiling of neurologic diseases: a pilot microarray study. *Arch Neurol* 2005;62:210-215
16. Lit L, Gilbert DL, Walker W, et al. A subgroup of Tourette's patients overexpress specific natural killer cell genes in blood: a preliminary report. *Am J Med Genet B Neuropsychiatr Genet* 2007;144:958-963
17. Leckman JF, Katsovich L, Kawikova I, et al. Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol Psychiatry* 2005;57:667-673
18. Gabbay V, Coffey BJ, Guttman LE, et al. A cytokine study in children and adolescents with Tourette's disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2009;33:967-971
19. Swedo SE, Leonard HL, Garvey M, et al. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *Am J Psychiatry* 1998;155:264-271
20. Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta* 2006;368:33-47
21. Martino D, Church AJ, Defazio G, et al. Soluble adhesion molecules in Gilles de la Tourette's syndrome. *J Neurol Sci* 2005; 234:79-85
22. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
23. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
24. Goodman WK, Price LH, Rasmussen SA, et al. The Yale-Brown Obsessive Compulsive

Scale. I. Development, use, and reliability. *Arch Gen Psychiatry* 1989;46:1006-1011

25. Ma A, Koka R, Burkett P. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu Rev Immunol* 2006;24: 657-679
26. Malek TR. The biology of interleukin-2. *Annu Rev Immunol* 2008;26:453-479
27. Dunn AJ. Effects of cytokines and infections on brain neuro-chemistry. *Clin Neurosci Res* 2006;6:52-68
28. Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 2003;19:641-644
29. King IL, Segal BM. Cutting edge: IL-12 induces CD4+. *J Immunol* 2005;175:641-645
30. Markiewicz MA, Wise EL, Buchwald ZS, et al. IL-12 enhances CTL synapse formation and induces self-reactivity. *J Immunol* 2009; 182:1351-1361
31. Blann AD, Daly RJ, Amiral J. The influence of age, gender and ABO blood group on soluble endothelial cell markers and adhesion molecules. *Br J Haematol* 1996; 92:498-500

Chapter

7

Altered immunoglobulin profiles in children and adolescents with a tic disorder

This chapter is adapted from the article submitted with the title 'Altered immunoglobulin profiles in children with Tourette syndrome'

Netty G.P. Bos-Veneman[a*]

Renske Olieman[b*]

Zuzana Tobiasova[b*]

Pieter J. Hoekstra[a]

Lilly Katsovitch[b]

Alfred L. M. Bothwell[b]

James F. Leckman[c]

Ivana Kawikova[b]

[a]Department of Psychiatry
University Medical Center Groningen
University of Groningen
the Netherlands
[b]Department of Immunobiology, and
[c]Yale Child Study Center
Yale University School of Medicine
New Haven, CT

*Authors who contributed equally to the article

Abstract

Background Two epidemiological studies have suggested an increased frequency of infections in patients with a tic disorder, implying some form of immune deficiency. We investigated whether patients of two independent study samples have underlying altered immunoglobulin (Ig) profiles and whether Ig profiles change during tic exacerbations.

Methods In the primary sample, consisting of 21 children and adolescents with a tic disorder and 21 healthy age-matched controls, we compared serum levels of IgG1, IgG2, IgG3, IgG4, IgM, and IgA. Those Ig levels that yielded between-group differences were subsequently compared between 53 patients and 53 healthy age-matched subjects of the replication sample. We also investigated possible relations between Ig concentrations and tic severity ratings. Of 13 additional patients, we compared Ig concentrations between time points before, during, and after exacerbations of tics.

Results IgG3 levels were significantly lower in patients compared to healthy children of the primary sample (medians 0.28 versus 0.49 mg/ml, $p = .04$), while levels of IgG2, IgG4, and IgM were decreased at trend-level ($p \leq .10$). Decreased IgG3 (medians 0.45 versus 0.52 mg/ml; $p = .05$) and IgM (medians 0.30 versus 0.38 mg/ml; $p = .04$) levels were confirmed in the replication sample. Ig levels did not correlate with tic severity. There was a trend to elevated IgG1 during symptom exacerbations ($p = .09$).

Conclusion Children with a tic disorder had decreased serum IgG3 and IgM levels, which may be related to increased susceptibility to recurrent infections. Serum IgG1 levels were elevated during tic exacerbations.

Introduction

Tic disorders, such as Tourette's disorder (TD), are neuropsychiatric conditions characterized by the presence of involuntary, rapid, recurrent, non-rhythmic movements, and/or vocalizations. Although the exact etiological mechanisms remain in doubt, both genetic and environmental factors have been demonstrated to contribute to the development of tics.[1]

In 1998 Swedo and colleagues proposed a putative subgroup of neuropsychiatric disorders, designated as Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS), characterized by the sudden onset and/or symptom exacerbations of obsessive-compulsive disorder (OCD) and/or tic disorder which are temporally related to group A ß-hemolytic streptococcal (GABHS) infections.[2] Introduction of the concept of PANDAS has greatly fuelled research into the possible role of infections and (auto)immunity in tic disorders. Although the involvement of the immune system in tic disorders is still controversial[3,4], indirect support has been provided by studies reporting on presence of antineuronal antibodies[5-7], decreased numbers of regulatory T cells[8], and increased single time point serum levels of antistreptococcal antibodies[9], soluble adhesion molecules[10], and proinflammatory cytokines.[11]

Perhaps the most solid evidence in favor of a role for the immune system in tic disorders is formed by evidence suggesting that children with tics may be more vulnerable to infections. Two epidemiological studies have demonstrated that patients with newly diagnosed tic disorder or OCD were more likely to have had a GABHS infection in the previous year.[12,13] Immunoglobulin (Ig) profiles may be an important factor related to susceptibility to infections. This is most evident in patients with Ig deficiencies who have consistently been shown to be vulnerable to recurrent infections.[14-16] A recent study[17] has compared plasma IgG and IgM levels of 59 children with autism, 25 healthy controls, and 24 patients with TD, but demonstrated no differences. In a preliminary report[18], we also found no differences regarding plasma levels of IgG and IgM in patients with TD and/or OCD compared to healthy subjects, but IgA concentrations appeared to be decreased in patients. Moreover, patients fulfilling PANDAS criteria showed decreased IgA levels compared to non-PANDAS cases.

In the present study, we compared serum levels of IgG subclasses IgG1, IgG2, IgG3, and IgG4, of IgM and IgA between children and adolescents with a tic disorder and healthy children. We also investigated possible relations of Ig levels with severity of tics. Finally, we made comparisons of serum Ig concentrations between time points before, during, and after an exacerbation of tics.

Methods

Study samples

The study involved two independent cross-sectional samples and one longitudinal sample. Table 1 shows demographic and clinical characteristics of the study samples.

Cross-sectional samples. Our primary sample involved 21 pediatric patients who fulfilled Diagnostic and Statistical Manual of Mental Disorders fourth edition, text revised (DSM-IV-TR) criteria[19] for a tic disorder and 21 individually age-matched healthy volunteers. Patients had been recruited through the Yale Child Study Center, New Haven, CT; healthy volunteers by the use of telemarketing lists of telephone numbers. Exclusion criteria included an intelligence quotient of <75, presence of a serious medical illness, major sensory handicaps (e.g., blindness, deafness), major neurological diseases (including a seizure disorder), head trauma resulting in loss of consciousness, or a current (past 6 months) psychiatric disorder that could interfere with participation, such as major depression, psychosis, or an autism spectrum disorder. Information concerning the patients, including assessment of PANDAS status, was collected in a two-stage process as previously described.[11]

The replication sample consisted of 53 pediatric patients who fulfilled DSM-IV criteria for a tic disorder and 53 individually age-matched healthy volunteers. Patients had either been recruited through the Groningen University Child and Adolescent Psychiatry outpatient clinic in the Netherlands (n = 40) or through the Dutch TD Association (n = 13); healthy volunteers had been recruited through advertisements in local news papers. Patients were asked whether the onset or exacerbation of tics were temporarily associated with GABHS infections. Exclusion criteria were presence of an active infection, inflammation, atopic conditions, an autoimmune disorder, or autism.

In both samples, tic severity was assessed by using the Yale Global Tic Severity Scale (YGTSS). This is a semi-structured interview which records the number, frequency, intensity, complexity, and interference of motor and vocal tics separately.[20]

Longitudinal sample. Sera of 13 additional patients with a tic disorder were available before, during, and after an exacerbation of tics. These patients had been followed prospectively for periods ranging from 4 to 24 months at the Yale Child Study Center as part of a larger longitudinal study[11], involving monthly clinical assessments and collection of serum specimens both at regular four-month intervals and in case of an exacerbation. An exacerbation of tics was defined as a total YGTSS score exceeding the previous monthly score by nine points and the total YGTSS score exceeding 19.

Informed consent from parents and children of 12 years or older, and assent from children between 6 and 12 years had been obtained. The study had been approved by the relevant Institutional Review Boards.

Table 1

Demographic and clinical characteristics of patients with a tic disorder and healthy subjects.

	Primary sample		**Replication sample**		**Longitudinal sample**
	Patients n = 21	**Healthy subjects** n = 21	**Patients** n = 53	**Healthy subjects** n = 53	n = 13
Male, n (%)	17 (81.0)	13 (61.9)	45 (84.9)	22 (41.5)	9 (69.0)
Age, Mean (SD), range (years)	11.9 (2.6), 8-16	12.2 (2.7), 7-17	12.3 (3.2), 6-18	12.2 (3.0), 6-18	10.6 (1.7) 9-14
	n (%)		**n (%)**		**n (%)**
Type of tic disorder					
Tourette's disorder	18 (85.7)		52 (98.1)		12 (92.3)
Chronic motor tic disorder	3 (14.3)		1 (1.9)		1 (7.7)
Patients fulfilling PANDAS criteria	3 (14.3)		0		5 (38.5)
Psychotropic medication					
Antipsychotic agents	1 (4.8)		9 (17.0)		0
Antidepressive agents	2 (9.5)		1 (1.9)		1 (7.7)
Clonidine	5 (23.8)		3 (5.7)		5 (3.8)
Psychostimulants	0		4 (7.5)		0
Atomoxetine	0		4 (7.5)		0
Combination of two or more agents	8 (38.1)		12 (22.6)		6 (4.6)
No psychotropic medication	5 (23.8)		20 (37.8)		1 (7.7)
	Mean (SD), range		**Mean (SD), range**		**Mean (SD), range**
YGTSS ratings					
Total	18.5 (7.6), 5-32		22.1 (8.1), 3-39		
Motor	10.3 (4.5), 0-18		13.7 (4.2), 0-22		
Vocal	8.2 (4.8), 0-15		8.5 (5.5), 0-19		
Δ YGTSS before – during exacerbation					15.6 (7.5), 9-31
Δ YGTSS after – during exacerbation					10.4 (5.5), 2-20

PANDAS, Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections; YGTSS, Yale Global Tic Severity Scale.

Analysis of serum samples

Ig concentrations were measured in archived sera using Luminex based Ig Isotyping reagent kit for measurement of IgG1, IgG2, IgG3, IgG4, IgM, and IgA (Bio-Rad Laboratories, Hercules, CA, catalog number 171-A3001M) in 10,000 fold diluted serum samples according to manufacturer's instructions. Briefly, anti-isotype-conjugated beads were pipetted in a 96-well pre-wet filtration plate (Catalog number MSBVN1210) and incubated with 50 µl (10,000 times diluted) sample on a microplate shaker (IKA NTS 2/4 digital microtiter, catalog number 3208000) followed by three washes using multiscreen resist vacuum manifold (Millipore, Billerica, MA, catalog number MSVMHTS00). Then biotinylated detection antibody solution was added, incubated for 30 minutes on a microplate shaker, and washed three times. Subsequently, streptavidin-phycoerythrin was added and incubated for 10 minutes while shaking and then washed three times. The fluorescence signal was analyzed using a Bioplex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA, catalog number 171-000201) and Bioplex manager software (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

In the primary cross-sectional sample, we made comparisons between patients and healthy subjects by using the Mann-Whitney test with regard to the measured serum Ig levels. We investigated possible relations between serum Ig concentrations, and mean total, vocal, and motor YGTSS ratings by using Spearman's correlation coefficient. Those Ig levels that yielded at least trend-level statistically significant (i.e., $p \leq .10$) between-group differences or correlations with tic severity were subsequently analyzed in the replication sample.

Because gender ratios differed between patients and healthy children of the replication sample, we also made comparisons between male and female healthy children regarding serum Ig concentrations. As psychotropic medication may have induced immune alterations, we also compared patients with and without psychotropic medication use.

With regard to the longitudinal sample, we made comparisons of serum Ig concentrations between time points before, during, and after an exacerbation of tics by conducting repeated-measures analysis of variance (ANOVA). If serum concentrations yielded at least trend-level statistically significant differences between time points, Bonferroni Post Hoc Analyses were performed.

In all statistical analyses $p \leq .05$ was considered as significant, and $p \leq .10$ as trend-level significant, to avoid type II (false negative) errors. Tests were two-sided.

Table 2a

Serum Ig levels of patients with a tic disorder and healthy subjects of the primary sample.

Serum Ig level (mg/ml)	Patients n = 21 Median (p25/p75)	Healthy subjects n = 21 Median (p25/p75)	*U*	*z*	*p*
IgG1	8.46 (6.14/13.1)	9.61 (8.18/13.8)	175	-1.15	.25
IgG2	2.30 (1.07/3.69)	3.22 (2.05/6.24)	150	-1.79	*.07*
IgG3	0.28 (0.18/0.64)	0.49 (0.37/0.61)	137	-2.10	**.04**
IgG4	0.23 (0.09/0.48)	0.45 (0.17/0.63)	152	-1.72	*.09*
IgM	0.34 (0.23/0.48)	0.42 (0.37/0.60)	149	-1.80	*.07*
IgA	1.11 (0.70/1.98)	1.32 (0.96/1.79)	196	-0.62	.54

Ig, immunoglobulin.

Table 2b

Serum Ig levels of patients with a tic disorder and healthy subjects of the replication sample.

Serum Ig level (mg/ml)	Patients n = 53 Median (p25/p75)	Healthy subjects n = 53 Median (p25/p75)	*U*	*z*	*p*
IgG2	1.76 (1.12/2.75)	1.88 (1.13/3.34)	1313	-0.58	.56
IgG3	0.45 (0.31/0.59)	0.52 (0.34/0.75)	1095	-1.96	**.05**
IgG4	0.25 (0.14/0.54)	0.18 (0.06/0.32)	1038	-2.31	**.02**
IgM	0.30 (0.24/0.44)	0.38 (0.29/0.46)	1083	-2.03	**.04**

Ig, immunoglobulin.

Table 3

Serum Ig levels before, during, and after a tic exacerbation in 13 patients with a tic disorder.

Serum Ig level (mg/ml)	Before exacerbation Mean (SD)	During exacerbation Mean (SD)	After exacerbation Mean (SD)	*F*	df	*p*
IgG1	11.8 (3.29)	14.3 (3.79)	11.4 (5.88)	2.63	2, 24	*.09*[a]
IgG2	2.82 (0.99)	3.48 (1.69)	3.07 (2.11)	1.09	2, 23	.35
IgG3	0.65 (0.36)	0.79 (0.47)	0.65 (0.44)	2.94	2, 24	.17
IgG4	0.39 (0.35)	0.42 (0.27)	0.35 (0.27)	0.93	2, 24	.41
IgM	0.42 (0.20)	0.50 (0.24)	0.46 (0.32)	1.33	2, 24	.28
IgA	1.34 (0.66)	1.74 (0.93)	1.38 (0.69)	2.86	2, 24	*.08*[b]

Ig, immunoglobulin. [a]Bonferroni Post Hoc test: mean difference between before and during exacerbation: -2.56 (SE 1.0), p = .08; mean difference between during and after exacerbation: 2.88 (SE 1.5), p = .24. [b]Bonferroni Post Hoc test: mean difference between before and during exacerbation: -0.40 (SE 0.2), p = .19; mean difference between during and after exacerbation: 0.36 (SE 0.2), p = .34.

Results

Cross-sectional findings

Table 2a shows comparisons regarding serum Ig concentrations between patients and healthy subjects of the primary sample. Of note, median serum IgG3 concentrations were significantly lower, and median serum IgG2, IgG4, and IgM concentrations at trend-level significantly lower in patients compared to healthy subjects (figures 1 and 2). No associations between serum Ig concentrations and YGTSS ratings were present. In the replication sample, median serum IgG3 and IgM concentrations were significantly lower in patients compared to healthy children (table 2b, figures 1 and 2). However, in contrast with the findings of the primary sample, median serum IgG4 concentrations were significantly higher in patients.

No gender differences regarding Ig levels in both samples were present, with the exception of serum IgM concentrations that were significantly lower in male healthy children compared to females (0.32 versus 0.40 mg/ml, U = 232, z = -1.97, p = .05) of the replication sample.

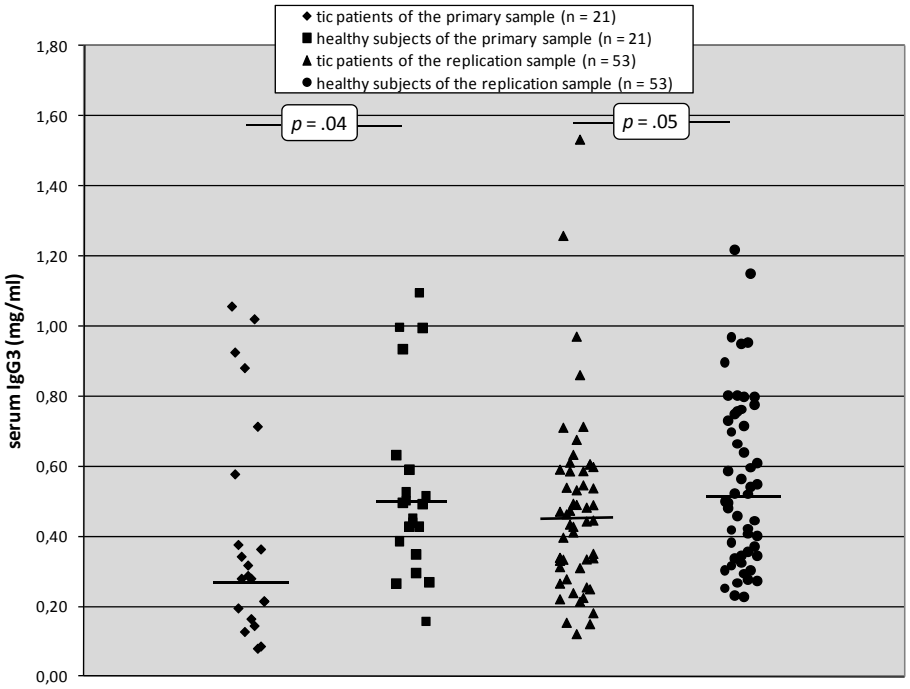

Figure 1. Serum IgG3 levels in children with a tic disorder and healthy subjects of the primary and replication samples. The bars represent the medians in each group.
Ig, immunoglobulin.

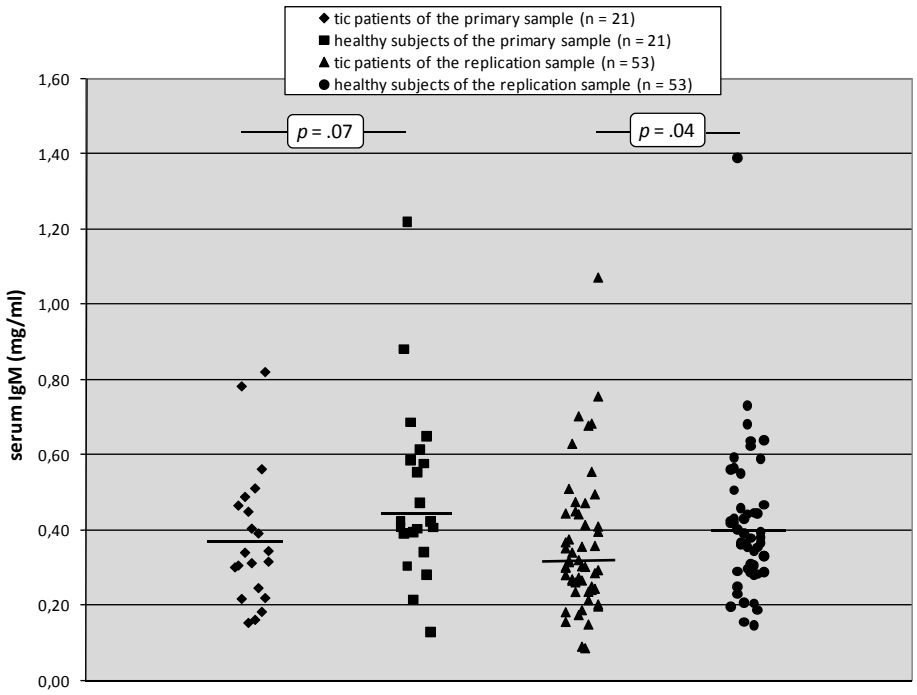

Figure 2. Serum IgM levels in children with a tic disorder and healthy subjects of the primary and replication samples. The bars represent the medians in each group.
Ig, immunoglobulin.

In the primary sample, no differences between patients with and without psychotropic medication use were present. Patients of the replication sample that used psychotropic medication, had only significantly lower serum levels of IgG4 compared to patients without psychotropic medication use (respective medians 0.22 mg/ml versus 0.32 mg/ml, $U = 224$, $z = -1.95$, $p = .05$).

Longitudinal findings

Table 3 shows serum Ig concentrations before, during, and after a tic exacerbation. Of note, mean serum IgG1 and IgA concentrations were at trend-level significantly elevated during exacerbation of tics. In Post Hoc analyses, the trend to an increase of IgA level during exacerbation did not remain present. However, the difference between IgG1 level during exacerbation and IgG1 level before, but not after, exacerbation remained at trend-level statistically significant.

Discussion

The present study demonstrates lower IgG3 and IgM levels in children and adolescents with a tic disorder in comparison to age-matched healthy subjects in two independent patient-control comparisons. Tic severity ratings were not related to serum Ig concentrations. However, there was an overall tendency of increased Ig levels during exacerbations with IgG1 elevations actually approaching significance.

The observed differences regarding IgG3 and IgM do not correspond to fully expressed deficiencies, defined as a decrease of two standard deviations below the mean of appropriate age. Clinical consequences of the observed changes may be extrapolated from the presentation of isolated IgG3 and IgM deficiencies with recurrent infections affecting mainly the upper respiratory tract.[14-16,21-24] Since patients with a tic disorder may suffer more frequently from upper respiratory tract infections[13,14], the decrease in IgG3 and IgM may be closely related to this susceptibility. Among all Ig subclasses, IgG3 and pentameric IgM are the most potent activators of the classical complement cascade that leads to the formation of membrane attack complexes and the destruction of microbial pathogens. Both IgG3 and monomeric IgM play a role in phagocytosis.[25,26] Because both IgG3 and IgM are involved in immune responses against a wide range of microorganisms[23,24,26-28], the findings of lower IgG3 and IgM levels may indicate that the clearance of multiple microorganisms is compromised in patients with tic disorders. In agreement with this, common cold and Mycoplasma pneumonia have previously been related to tics.[29,30]

In contrast with our finding of lower IgM levels in patients with tics, Libbey et al. have not reported differences between patients with TD and healthy controls.[17] In our study, the decrease of serum IgM in patients with a tic disorder may have been confounded by gender, given the preponderance of male patients compared to the healthy subjects in the replication sample, and the fact that IgM levels of the healthy male subjects were significantly lower than those of the healthy female subjects. However, in the initial sample no gender differences regarding serum IgM concentrations were present while differences between patients and controls were similar as in the replication sample, altogether suggesting a true effect of clinical status.

We could not replicate our previous preliminary findings of decreased IgA levels in patients with TD and/or OCD.[18] We do not have a full explanation for this. However, the previous study has also included patients with OCD but no tics, possibly leading to a more heterogeneous study sample. Moreover, 39% of patients fulfilled PANDAS criteria and in these cases IgA levels were even further decreased. In the present study only three patients of the initial sample and none of the replication sample fulfilled PANDAS criteria. Clearly, more work is warranted with regard to comparisons between PANDAS and non-PANDAS cases.

The tendency of increased Ig levels during exacerbations, mainly of IgG1, suggests that, at least in some patients, humoral immunity might be related to symptom

elicitation. No previous longitudinal studies regarding Igs in neuropsychiatric disorders have been performed. In a mouse model of PANDAS, mice immunized with GABHS-immunization exhibited repetitive behavior that correlated with elevated anti-neuronal IgG1 in their serum and the presence of immune deposits in brain tissue. Naive mice that received IgG from PANDAS mice also developed behavioral changes, and the depletion of IgG from donor sera abrogated the behavior changes.[31]

A number of limitations need to be acknowledged. First, the sample sizes of the study groups have been relatively small especially with regard to the longitudinal sample. Because of the small sample sizes, we did not apply a multiple comparison correction, in order to preserve statistical power, but this may have increased the type I error rate. Second, the control group of the replication sample was not fully gender-matched. Another disadvantage of our study has been the difference in exclusion criteria between the initial and replication sample. An important limitation is the use of psychotropic medication in 76% and 62% of the patients of the initial and replication sample, respectively, which may have induced anti-inflammatory side-effects and affected tic severity. Finally, we did not have any data on infection history of the subjects. It would be interesting in future longitudinal studies to directly investigate the possible impact of lowered Ig levels in children with a tic disorder on susceptibility to infections and possibly associated fluctuations in tic severity.

Although the exact pathophysiological mechanisms remain unclear, the present study indicates that at least a subgroup of children with tic disorders may have a dysregulated immune system. The strongest evidence to support this are the lower IgM and IgG3 levels which were actually present in two independent samples. Future studies should address B cell function to identify whether this is due to decreased production, increased degradation, or increased binding of the Igs to a yet unknown site. The increased IgG1 levels during exacerbations of tics may provide additional support but should be confirmed in more large scale longitudinal studies.

References

1. Hoekstra PJ, Anderson GM, Limburg PC, et al. Neurobiology and neuroimmunology of Tourette's syndrome: an update. *Cell Mol Life Sci* 2004;61:886-898
2. Swedo SE, Leonard HL, Garvey M, et al. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *Am J Psychiatry* 1998;155:264-271
3. Singer HS, Gause C, Morris C, et al. Serial immune markers do not correlate with clinical exacerbations in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections. *Pediatrics* 2008;121:1198-1205
4. Martino D, Dale RC, Gilbert DL, et al. Immunopathogenic mechanisms in tourette syndrome: A critical review. *Mov Disord* 2009;24:1267-1279
5. Singer HS, Hong JJ, Yoon DY, et al. Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. *Neurology* 2005;65:1701-1707
6. Morer A, Lazaro L, Sabater L, et al. Antineuronal antibodies in a group of children with obsessive-compulsive disorder and Tourette syndrome. *J Psychiatr Res* 2008;42:64-68
7. Singer HS, Giuliano JD, Hansen BH, et al. Antibodies against human putamen in children with Tourette syndrome. *Neurology* 1998;50:1618-1624
8. Kawikova I, Leckman JF, Kronig H, et al. Decreased numbers of regulatory T cells suggest impaired immune tolerance in children with tourette syndrome: a preliminary study. *Biol Psychiatry* 2007; 61:273-278
9. Morshed SA, Parveen S, Leckman JF, et al. Antibodies against neural, nuclear, cytoskeletal, and streptococcal epitopes in children and adults with Tourette's syndrome, Sydenham's chorea, and autoimmune disorders. *Biol Psychiatry* 2001; 50:566-577
10. Martino D, Church AJ, Defazio G, et al. Soluble adhesion molecules in Gilles de la Tourette's syndrome. *J Neurol Sci* 2005; 234:79-85
11. Leckman JF, Katsovich L, Kawikova I, et al. Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol Psychiatry* 2005;57:667-673
12. Leslie DL, Kozma L, Martin A, et al. Neuropsychiatric disorders associated with streptococcal infection: a case-control study among privately insured children. *J Am Acad Child Adolesc Psychiatry* 2008;47:1166-1172
13. Mell LK, Davis RL, Owens D. Association between streptococcal infection and obsessive-compulsive disorder, Tourette's syndrome, and tic disorder. *Pediatrics* 2005; 116:56-60
14. Yel L, Ramanuja S, Gupta S. Clinical and Immunological Features in IgM Deficiency. *Int Arch Allergy Immunol* 2009;150:291-298
15. Goldstein MF, Goldstein AL, Dunsky EH, et al. Selective IgM immunodeficiency: retrospective analysis of 36 adult patients with review of the literature. *Ann Allergy Asthma Immunol* 2006;97:717-730
16. Ozkan H, Atlihan F, Genel F, et al. IgA and/or IgG subclass deficiency in children with recurrent respiratory infections and its relationship with chronic pulmonary damage. *J Investig Allergol Clin Immunol* 2005;15:69-74
17. Libbey JE, Coon HH, Kirkman NJ, et al. Are there altered antibody responses to measles, mumps, or rubella viruses in autism? *J Neurovirol* 2007;13:252-259
18. Kawikova I, Grady BPX, Tobiasova Z, et al. Children with Tourette's syndrome may suffer immunoglobulin A dysgamma-globulinemia. *Biol Psychiatry* 2009;67:679-683
19. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR). Washington, DC: American Psychiatry Association; 2000
20. Leckman JF, Riddle MA, Hardin MT, et al. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28:566-573
21. Goldstein MF, Goldstein AL, Dunsky EH, et al. Pediatric selective IgM immuno-deficiency. *Clin Dev Immunol* 2008; 2008:624850
22. Bernstein JM. Immunologic aspects of otitis media. *Curr Allergy Asthma Rep* 2002;2:309-315
23. Oxelius VA, Hanson LA, Bjorkander J, et al. IgG3 deficiency: common in obstructive lung disease. Hereditary in families with

immunodeficiency and auto-immune disease. *Monogr Allergy* 1986;20:106-115

24. Noyes J, Woodmansee D, Chervinsky P. IgG subtype abnormalities with normal total IgG in a clinical allergy practice. *Ann Allergy* 1986;57:273-275
25. Shibuya A, Sakamoto N, Shimizu Y, et al. Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nat Immunol* 2000;1:441-446
26. Meyts I, Bossuyt X, Proesmans M, et al. Isolated IgG3 deficiency in children: to treat or not to treat? Case presentation and review of the literature. *Pediatr Allergy Immunol* 2006;17:544-550
27. Ferrante A, Beard LJ, Feldman RG. IgG subclass distribution of antibodies to bacterial and viral antigens. *Pediatr Infect Dis J* 1990;9:S16-S24
28. Goldblatt D, Scadding GK, Lund VJ, et al. Association of Gm allotypes with the antibody response to the outer membrane proteins of a common upper respiratory tract organism, Moraxella catarrhalis. *J Immunol* 1994;153:5316-5320
29. Hoekstra PJ, Manson WL, Steenhuis MP, et al. Association of common cold with exacerbations in pediatric but not adult patients with tic disorder: a prospective longitudinal study. *J Child Adolesc Psychopharmacol* 2005;15:285-292
30. Muller N, Riedel M, Blendinger C, et al. Mycoplasma pneumoniae infection and Tourette's syndrome. *Psychiatry Res* 2004;129:119-125
31. Yaddanapudi K, Hornig M, Serge R, et al. Passive transfer of streptococcus-induced antibodies reproduces behavioral disturbances in a mouse model of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection. *Mol Psychiatry* 2009;in press

Chapter 8

Appendices

Summary and general discussion

Tic disorders are neuropsychiatric conditions characterized by the presence of rapid, recurrent, non-rhythmic movements, and/or vocalizations. Tics itself often cause no hinder. However, many patients suffer from comorbid (symptoms of) attention deficit/hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), internalizing disorders, or an autism spectrum disorder (ASD).[1] The exact etiological mechanisms underlying tics and comorbid symptoms remain in doubt. Since in the 1960s neuroleptic medication has proved to be efficacious in patients with a tic disorder, many researchers have started to investigate possible neurobiological pathways involved in tics.[2]

It has been noticed for many years that tics run in families and a wealth of studies have established their genetic background.[3,4] In the view of findings of pharmacological and biochemical studies in patients with tic disorders, genes encoding serotonergic and dopaminergic neurotransmission are of considerable interest.[5-7] However, no independently replicated genetic vulnerability regions have been identified yet. The unravelling of neurobiological pathways involved in tics may be hindered by the clinical heterogeneity of tic disorders and possible modulation of gene effects by environmental factors, such as perinatal adversities and infections. Perinatal adverse events have been found to be associated with tic disorders as well as comorbid OCD and ADHD, although results have been somewhat inconsistent.[8-12] Regarding the suggested involvement of the immune system in tic disorders[13] some evidence has been provided that points to an altered (cellular) immunity[14] and compromised immune system[15] leading to increased vulnerability to infections in patients with tics.

The general aim of the studies described in this thesis was to examine the possible contribution of neurobiological factors to the development of tic disorders. Specifically, we have investigated possible relations between perinatal adversities and severity of tics and comorbid symptoms. Moreover, possible associations of polymorphisms located at dopaminergic and serotonergic genes with severity of tics and comorbid symptoms have been addressed, both separately as well as in interaction with perinatal adversities. Regarding the immune system we have studied possible immune disturbances and immunoglobulin (Ig) profiles in children and adolescents with a tic disorder.

Perinatal adversities

Chapters 2, 3, 4, and 5 describe our studies regarding perinatal adversities in patients with tics and comorbid symptoms of OCD, ADHD, internalizing disorders, and/or ASD. We assessed presence of pregnancy and delivery complications, and prenatal exposure to smoking. In the study we report on in *chapter 2,* we also assessed presence of

postnatal complications and maternal alcohol use during pregnancy. Patients with and without these perinatal adversities were compared regarding the various symptom severity ratings. We have detected that children with tics who had been exposed to smoking during pregnancy had more severe ADHD, depressive, and autistic symptoms, but decreased tic severity; children with pregnancy complications had more severe tics, but decreased OCD symptom severity; children born after a complicated delivery had increased severity of tics and ADHD symptoms. In line with findings of previous studies[8-11], the present results suggest that smoking exposure in utero may contribute to the severity of comorbid symptoms in children with tics, whereas pregnancy and delivery complications may mainly influence severity of tics and co-occurring ADHD and OCD features. The inverse relation between tic severity and prenatal smoking exposure is difficult to relate to earlier studies, and should be interpreted with caution.

In 75 children and adolescents with a tic disorder, we have investigated by using linear regressions whether perinatal adversities would interact with presence of tic or any mental disorders in first-degree relatives with the tic or comorbid ADHD severity measure as outcome (*chapter 2*). We found evidence of an interaction between smoking exposure in utero and a positive family history of mental disorders regarding ADHD symptom severity: in patients with a first-degree relative with a mental disorder, smoking exposure during pregnancy was associated with a higher hyperactive-impulsive score, whereas in patients without a first-degree relative with a mental disorder, prenatal smoking exposure was not associated with ADHD symptom severity. These findings suggest that gene-environment interactions may be involved in tics.

Genetic factors and gene-environment interactions

In *chapters 3, 4, and 5* we present the findings of three molecular genetic studies that have addressed possible main effects of the 5-hydroxytryptamine transporter-linked polymorphic region (5HTTLPR) including rs25531, monoamine oxidase (MAO) A upstream variable number of tandem repeats (uVNTR), dopamine receptor (DR) D4 48 base pairs (bp) VNTR, and three single nucleotide polymorphisms (SNP) located at the solute carrier family 6, member 3 gene (SLC6A3) regarding severity of tics and co-occurring symptoms. Subsequently, through linear regressions we have examined whether these polymorphisms may interact with perinatal adversities to influence tic or comorbid symptom severity.

The first study (*chapter 3*) has investigated possible relations of 5HTTLPR and the MAOA uVNTR with severity of tics and comorbid ADHD, OCD, and ASD symptoms. We genotyped 109 children and adolescents with a tic disorder and compared children with and without a 5HTTLPR long $(L)_A$ allele, and males with and without a MAOA uVNTR high-activity allele regarding severity of tics and comorbid symptoms. Presence of an L_A allele was associated with increased severity of tics and comorbid ADHD, OCD, and ASD symptoms suggesting a shared genetic vulnerability located at 5HTTLPR underlying tics and a wide range of comorbid disorders. Presence of a MAOA uVNTR

high-activity allele was related to decreased ASD symptom severity which may point to a possible protective influence of MAOA regarding ASD symptoms in boys with a tic disorder. Intriguing, the relationship between delivery complications and tic severity appeared to be most pronounced in males without MAOA uVNTR high-activity allele: in the absence of a high-activity allele, delivery complications were associated with higher tic severity ratings, while in presence of a high-activity allele, delivery complications were not associated with tic severity. In conclusion, this study provides evidence for a contribution of 5HTTLPR and the MAOA uVNTR to severity of tics and comorbid features. Moreover, the MAOA polymorphism may interact with delivery complications to modulate tic severity.

Chapter 4 documents the findings regarding relations between the DRD4 48-bp VNTR and severity of tics and comorbid obsessive-compulsive, depressive, anxious, and autistic symptoms. In 110 children and adolescents with tics we examined associations between the 2, 3, 4, and 7 repeat (R) alleles of the DRD4 48-bp VNTR and severity of tics and comorbid symptoms. The 2R allele appeared to be a genetic risk factor for OCD symptom severity, and the 3R allele for ASD symptom severity in children with a tic disorder. In children without a 3R allele delivery complications were associated with more severe tics, but in children with a 3R variant an inverse relation between delivery complications and tic severity was found. Moreover, the relation between delivery complications and internalizing symptom severity appeared to be most pronounced in children with a 2R allele. These data suggest that the 48-bp VNTR may influence severity of co-occurring OCD and ASD symptoms, and hints at interactions of the 48-bp VNTR with delivery complications regarding severity of tics and comorbid internalizing symptoms.

Results regarding the SLC6A3 gene and severity of tics and comorbid OCD and ADHD features are described in *chapter 5.* In 132 patients with tics three SNP markers located at the 3' and 5' ends of SLC6A3 (rs40184, rs11564750, and rs2550946) were genotyped. Possible associations between alleles of the SNP markers and severity of tics and comorbid symptoms were addressed. Presence of an rs40184 G allele was associated with increased severity of inattentive symptoms. Moreover, pregnancy complications appeared to interact with rs11564750 and rs2550946 to decrease severity of comorbid obsessions and compulsions. This study provides preliminary evidence for a role of the SLC6A3 gene in the expression of comorbid inattentive symptoms and of gene-environment interactions between SLC6A3 and pregnancy complications regarding severity of obsessive-compulsive symptoms in patients with a tic disorder.

In conclusion, the findings of our genetic molecular studies support the notion of serotonergic and dopaminergic genes being involved in the phenotypic expression of tic and comorbid disorders, and suggest that these genes may interact with perinatal adversities to modulate severity of tics and co-occurring symptoms.

Immune system

The other main topic of this thesis was the relation between tics and the immune system, in particular the presence of immune disturbances and alterations of Ig profiles in children and adolescents with a tic disorder.

Chapter 6 reports on our findings regarding cytokines and soluble adhesion molecules in tic disorders. We compared serum levels of interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, soluble IL-2 receptor (sIL2R), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, soluble vascular cell adhesion molecule-1 (sVCAM-1), and intercellular adhesion molecule-1 (sICAM-1) of 66 children and adolescents with a tic disorder and 71 healthy volunteers. We also investigated possible relations between concentrations of the immune markers and severity ratings of tics and comorbid obsessive-compulsive symptoms. Serum levels of the immune markers did not differ between patients and healthy children. Serum IL-2 concentrations were positively associated with tic severity which could indicate involvement of cellular immune effector pathways in tic disorders. However, serum IL-12 concentrations were negatively related to severity of comorbid obsessive-compulsive symptoms which is difficult to explain. In conclusion, these preliminary findings do not reveal major immune activation in children with a tic disorder, but replication is warranted.

Chapter 7 contains the results of our study regarding Ig profiles (i.e., serum levels of IgG1, IgG2, IgG3, IgG4, IgM, and IgA) in children and adolescent with tics of two independent study samples as well as during tic exacerbations. In the primary sample, consisting of 21 children and adolescents with a tic disorder and 21 healthy age-matched controls, serum IgG3 levels were significantly lower in patients while serum levels of IgG2, IgG4, and IgM were decreased at trend-level significance. Decreased serum IgG3 and IgM levels in patients were confirmed in the replication sample comprising 53 patients and 53 healthy age-matched subjects. In 13 additional patients, we compared Ig concentrations between time points before, during, and after exacerbations of tics and found a trend toward elevated IgG1 during tic exacerbations. These findings suggest that children with a tic disorder may have changed Ig profiles, comprising decreased serum IgG3 and IgM levels that may be related to increased susceptibility to recurrent infections. The finding of elevated serum IgG1 levels during tic exacerbations would be strengthened by independent replication.

Future perspectives

Considering the results described in this thesis, future studies should further explore the role of perinatal, genetic, and immunological factors in tic disorders. First, replication of the results described in this thesis is warranted in larger study samples that take into account clinical phenotype, including comorbidities, and psychotropic medication use. Large scale, multi-center, prospective studies are preferred. Following

children at risk for a tic disorder (i.e., having a parent or sibling with tics) from pregnancy till adulthood may provide insight into the contribution of perinatal and immunological factors in genetically susceptible children. Moreover, cellular-molecular studies are needed. For example, considering the relations between perinatal adversities and tic and comorbid disorders, it is worthwhile to perform studies aimed at unravelling the exact pathophysiological pathways. Thereby, since the functional significances of the genetic polymorphisms studied in this thesis are still under debate, studies on this topic are warranted. With regard to our findings of lower Ig levels, it would be interesting to study the pathophysiological mechanisms leading to these lower levels in children with tics. While genes and environmental influences may interact and therefore complicate the search for neurobiological factors involved in tic disorders, future studies should take these interactions into account.

Concluding remarks

Although neurobiological factors have been suggested to contribute to the pathogenesis of tic disorders, the exact mechanisms so far have remained elusive. This thesis describes associations of tic disorders with perinatal adversities, serotonergic and dopaminergic genes, and immunological factors. Future research directed at replication of our findings and investigation of the underlying pathophysiological mechanisms is warranted. Interactions between genes and environmental factors may be a fruitful research area. To facilitate the unravelling of neurobiological pathways involved in tics, the phenotypic expression of tic disorders and comorbid symptoms should receive more attention in neurobiological research.

References

1. Freeman RD. Tic Disorders and ADHD - answers from a world-wide clinical data set on Tourette Syndrome. *Eur Child Adolesc Psychiatry* 2007;16:I/15-I/23
2. Hoekstra PJ, Anderson GM, Limburg PC, et al. Neurobiology and neuroimmunology of Tourette's syndrome: an update. *Cell Mol Life Sci* 2004;61:886-898
3. O'Rourke JA, Scharf JM, Yu D, et al. The genetics of Tourette syndrome: a review. *J Psychosom Res* 2009;67:533-545
4. Keen-Kim D, Freimer NB. Genetics and epidemiology of Tourette syndrome. *J Child Neurol* 2006;21:665-671
5. Muller-Vahl KR, Meyer GJ, Knapp WH, et al. Serotonin transporter binding in Tourette Syndrome. *Neurosci Lett* 2005;385:120-125
6. Swain JE, Scahill L, Lombroso PJ, et al. Tourette syndrome and tic disorders: a decade of progress. *J Am Acad Child Adolesc Psychiatry* 2007;46:947-968
7. Steeves TD, Fox SH. Neurobiological basis of serotonin-dopamine antagonists in the treatment of Gilles de la Tourette syndrome. *Prog Brain Res* 2008;172:495-513
8. Pringsheim T, Sandor P, Lang A, et al. Prenatal and perinatal morbidity in children with Tourette syndrome and attention-deficit hyperactivity disorder. *J Dev Behav Pediatr* 2009;30:115-121
9. Leckman JF, Dolnansky ES, Hardin MT, et al. Perinatal factors in the expression of Tourette's syndrome: an exploratory study. *J Am Acad Child Adolesc Psychiatry* 1990; 29:220-226
10. Santangelo SL, Pauls DL, Goldstein JM, et al. Tourette's syndrome: what are the influences of gender and comorbid obsessive-compulsive disorder? *J Am Acad Child Adolesc Psychiatry* 1994;33:795-804
11. Mathews CA, Bimson B, Lowe TL, et al. Association between maternal smoking and increased symptom severity in Tourette's syndrome. *Am J Psychiatry* 2006;163:1066-1073
12. Pasamanick B, Kawi A. A study of the association of prenatal and paranatal factors with the development of tics in children; a preliminary investigation. *J Pediatr* 1956;48:596-601
13. Martino D, Dale RC, Gilbert DL, et al. Immunopathogenic mechanisms in tourette syndrome: A critical review. *Mov Disord* 2009;24:1267-1279
14. Leckman JF, Katsovich L, Kawikova I, et al. Increased serum levels of interleukin-12 and tumor necrosis factor-alpha in Tourette's syndrome. *Biol Psychiatry* 2005;57:667-673
15. Kawikova I, Grady BPX, Tobiasova, Z, et al. Children with Tourette's syndrome may suffer immunoglobulin A dysgamma-globulinemia. *Biol Psychiatry* 2009;67:679-683

Samenvatting in het Nederlands

Tic-stoornissen zijn neuropsychiatrische aandoeningen die gekenmerkt worden door herhaald optredende, niet-ritmische bewegingen en geluiden, zogenaamde motorische en vocale tics. In de meeste gevallen leiden tics niet tot beperkingen. Echter, een groot deel van patiënten met tics heeft ook last van (symptomen van) aandachtstekortstoornis met hyperactiviteit (ADHD), obsessieve-compulsieve stoornis (OCD), internaliserende stoornissen en/of een autisme spectrumstoornis (ASD). Het is nog onduidelijk waardoor tics en deze bijkomende symptomen ontstaan. Sinds de ontdekking in de jaren zestig dat patiënten met een tic-stoornis baat kunnen hebben bij het gebruik van neuroleptica, is er veel onderzoek verricht naar mogelijke neurobiologische factoren die zouden kunnen bijdragen aan de ontwikkeling van tics.

Ondanks dat genetische factoren ontegenzeggelijk een rol spelen, is nog niet duidelijk welke genen leiden tot een verhoogde kwetsbaarheid voor tic-stoornissen. Omdat farmacologische en biochemische studies erop wijzen dat de neurotransmitters serotonine en dopamine betrokken zijn, is het zinvol om serotonerge en dopaminerge genen te bestuderen bij patiënten met tics. De zoektocht naar genen wordt mogelijk bemoeilijkt door het bestaan van gen-omgevingsinteracties waarbij omgevingsfactoren die van belang kunnen zijn bij tics, zoals perinatale en immunologische factoren, geneffecten moduleren. Er zijn aanwijzingen dat complicaties tijdens de zwangerschap en bevalling een rol spelen bij tic-stoornissen en comorbide OCD en ADHD. Wat betreft het afweersysteem wordt gesuggereerd dat patiënten met tics een veranderde (cellulaire) afweer en/of een verminderde humorale immuniteit met als gevolg een verhoogde vatbaarheid voor infecties zouden kunnen hebben.

Het doel van dit proefschrift is om de rol van genetische en omgevingsfactoren in tic- en comorbide stoornissen te verhelderen. In het bijzonder hebben polymorfismen van een aantal dopaminerge en serotonerge genen, perinatale omstandigheden en immunologische factoren onze interesse.

Perinatale factoren

In de studies beschreven in de *hoofdstukken 2, 3, 4 en 5* hebben we de perinatale omstandigheden, te weten de aanwezigheid van zwangerschaps- en bevallingscomplicaties en prenatale blootstelling aan roken, van patiënten met een tic-stoornis in kaart gebracht. In *hoofdstuk 2* wordt ook gerapporteerd over postnatale complicaties en alcoholgebruik tijdens de zwangerschap. Patiënten met en zonder deze perinatale factoren werden vergeleken wat betreft de ernst van tics en comorbide symptomen van OCD, ADHD, internaliserende stoornissen en ASD. Zwangerschapcomplicaties bleken verband te houden met ernstigere tics, maar minder ernstige OCD-symptomen. Kinderen met tics die geboren zijn na een

gecompliceerde bevalling hadden hogere tic- en ADHD-symptoomscores. Patiënten van wie de moeder rookte tijdens de zwangerschap hadden ernstigere ADHD-, depressieve en autistische symptomen, maar minder ernstige tics. Blijkbaar speelt het in de baarmoeder blootgesteld worden aan roken een rol in de expressie van comorbide symptomen, en zwangerschaps- en bevallingscomplicaties in de ernst van tics en van symptomen van OCD en ADHD.

Vervolgens vonden we (*hoofdstuk 2*) in een groep van 75 kinderen en adolescenten met een tic-stoornis aanwijzingen dat een interactie tussen blootstelling aan roken tijdens de zwangerschap en een positieve familie-anamnese van psychische aandoeningen mogelijk van belang is voor de ernst van ADHD-symptomen: alleen in de groep patiënten met een eerstegraads familielid met psychische stoornissen leidde prenatale blootstelling aan roken tot hogere hyperactieve-impulsieve symptoomscores. Deze bevinding wijst indirect op een rol van gen-omgevingsinteracties in tic- en comorbide stoornissen.

Genetische factoren en gen-omgevingsinteracties

In de *hoofdstukken 3, 4 en 5* beschrijven wij de resultaten van drie studies naar een mogelijke betrokkenheid van serotonerge en dopaminerge genen bij tic-stoornissen. We hebben gezocht naar invloeden van 5HTTLPR (5-hydroxytryptamine transporter-linked polymorphic region), MAOA uVNTR (monoamine oxidase A upstream variable number of tandem repeats), DRD4 48-bp (dopamine receptor D4 gen 48 base paren) VNTR, en drie SNP-markers (single nucleotide polymorphisms) van het SLC6A3-gen (solute carrier family 6, member 3) op de ernst van tics en comorbide symptomen van ADHD, OCD, internaliserende stoornissen en ASD. Daarna hebben we middels lineaire regressies bestudeerd of deze polymorfismen in interactie met perinatale factoren (te weten roken tijdens de zwangerschap, zwangerschaps- en bevallingscomplicaties) gerelateerd zijn aan de ernst van tics of comorbide symptomen.

De eerste studie (*hoofdstuk 3*) rapporteert relaties tussen het 5HTTLPR lange $(L)_A$ allel en ernstigere tics en comorbide ADHD-, OCD- en ASD-symptomen, en tussen MAOA uVNTR hoog-activiteitsallelen en minder ernstige ASD-kenmerken in een groep van 109 kinderen en adolescenten met een tic-stoornis. Daarnaast bleek dat in afwezigheid, maar niet in aanwezigheid, van een MAOA uVNTR hoog-activiteitsallel bevallingscomplicaties gerelateerd waren aan ernstigere tics. Deze studie wijst erop dat 5HTTLPR en de MAOA uVNTR en een interactie tussen MAOA en bevallingscomplicaties mogelijk bijdragen aan de expressie van tics en comorbide kenmerken bij kinderen met een tic-stoornis.

Hoofdstuk 4 beschrijft ons onderzoek in een groep van 110 kinderen en adolescenten met tics naar de 2-repeats (R), 3R-, 4R- en 7R-allelen van de DRD4 48-bp VNTR in relatie tot de ernst van tics en comorbide obsessieve-compulsieve, internaliserende en autistische symptomen. Aanwezigheid van een 2R-allel bleek gerelateerd aan ernstigere dwanggedachten en -handelingen, en van een 3R-allel aan

ernstigere autistische kenmerken. Bij kinderen zonder 3R-allel vonden we een relatie tussen bevallingscomplicaties en ernstigere tics, terwijl in aanwezigheid van een 3R-allel bevallingscomplicaties gerelateerd waren aan lagere tic-scores. Daarbij waren bevallingscomplicaties en internaliserende symptoomscores slechts met elkaar gerelateerd als er een 2R-allel aanwezig was. Kortom, deze studie suggereert dat de 48-bp VNTR de ernst van comorbide symptomen bij kinderen met tics beïnvloedt, en dat de interactie tussen de 48-bp VNTR en bevallingscomplicaties mogelijk een rol speelt in de ernst van tics en internaliserende symptomen.

Voor de laatste genetische studie (*hoofdstuk 5*) werden bij 132 patiënten met een tic-stoornis drie SNP-markers, die gelokaliseerd zijn in de 3' en 5' regio's van SLC6A3 (rs40184, rs11564750 en rs2550946), gegenotypeerd. We onderzochten mogelijke associaties tussen de allelen van deze SNP's en de ernst van tics en comorbide OCD- en ADHD-symptomen. In aanwezigheid van een rs40184 G-allel waren comorbide concentratieproblemen ernstiger. Tevens vonden we aanwijzingen dat interacties tussen zwangerschapscomplicaties en zowel rs11564750 als rs2550946 een rol spelen in het verminderen van de ernst van dwanggedachten en –handelingen. SLC6A3 heeft dus mogelijk een aandeel in de expressie van comorbide ADHD-kenmerken en moduleert de relatie tussen zwangerschapscomplicaties en ernst van OCD-symptomen bij patiënten met een tic-stoornis.

De bevindingen van de drie moleculair–genetische studies wijzen erop dat serotonerge en dopaminerge genen waarschijnlijk betrokken zijn bij de fenotypische expressie van tics en comorbide symptomen. Tevens hebben interacties tussen deze genen en perinatale factoren mogelijk een aandeel. Omdat onze onderzoeksgroepen relatief klein waren, moeten onze resultaten met de nodige voorzichtigheid worden geïnterpreteerd. Daarnaast is van groot belang dat onderliggende pathofysiologische mechanismen verder opgehelderd worden.

Immunologische factoren

In de *hoofdstukken 6 en 7* staan de bevindingen van twee immunologische studies beschreven. In de eerste studie hebben we gekeken naar algemene immuunmarkers, namelijk cytokines en oplosbare adhesiemoleculen. Bij 66 kinderen en adolescenten met een tic-stoornis en 71 gezonde vrijwilligers hebben we serumconcentraties van interleukine (IL)-2, IL-4, IL-5, IL-10, IL-12, oplosbare IL-2 receptor (sIL2R), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, oplosbare vasculaire cel-adhesiemolecuul-1 (sVCAM-1) en intercellulaire adhesiemolecuul-1 (sICAM-1) vergeleken en onderzocht of er relaties zijn tussen serumconcentraties van deze immuunmarkers en ernst van tics en comorbide OCD-symptomen. Serumconcentraties van de immuunmarkers verschilden niet tussen patiënten en controles. Serumconcentraties van IL-2 bleken positief geassocieerd met tic-ernst, serumconcentraties van IL-12 negatief met ernst van obsessies en compulsies. De resultaten van deze studie wijzen niet op een belangrijke rol van het immuunsysteem in tic-stoornissen, maar gezien de relatie

tussen IL-2 en de ernst van de tics zou de cellulaire afweer een bijdrage kunnen leveren aan de fenotypische expressie van tics.

Naast de hypothese dat infecties leiden tot tics laat een recente studie zien dat patiënten met tics mogelijk gevoeliger zijn voor infecties, hetgeen veroorzaakt kan worden door afwijkende antistofprofielen. Antistoffen worden ook wel immunoglobulines (Ig) genoemd. Er zijn verschillende subklassen die aangeduid worden met letters, en bij een nog verdere onderverdeling met cijfers, zoals IgA, IgM, IgG, IgG1, IgG2, IgG3 en IgG4. In *hoofdstuk 7* beschrijven wij ons onderzoek naar Ig-profielen (serumconcentraties van IgG1, IgG2, IgG3, IgG4, IgM en IgA) en tic-stoornissen. Allereerst vergeleken wij in twee onafhankelijke studiegroepen de serum Ig-concentraties van kinderen en adolescenten met tics en gezonde vrijwilligers. In de primaire studiegroep waren de serum IgG3-concentraties van de 21 patiënten significant lager dan van de 21 gezonde kinderen, terwijl IgG2-, IgG4- en IgM-concentraties een trend tot lagere waarden in de patiëntengroep lieten zien. Ook in de replicatiegroep waren de IgG3- en IgM-serumwaarden van de 53 patiënten verlaagd in vergelijking met de 53 gezonde controles. Daarna onderzochten we in een afzonderlijke groep van 13 patiënten Ig-concentraties voor, tijdens en na een exacerbatie van tics. Tijdens exacerbaties was er een trend tot verhoging van IgG1-waarden. Kennelijk hebben kinderen met een tic-stoornis lagere serumconcentraties van IgG3 en IgM, hetgeen zou kunnen leiden tot een verhoogde kwetsbaarheid voor infecties. Het is zinvol om, gezien de verhoogde serumconcentraties van IgG1 tijdens exacerbaties van tics, een vergelijkbare studie in een grotere patiëntengroep te verrichten.

Conclusie

Hoewel er veel aanwijzingen zijn dat tic-stoornissen een neurobiologische basis hebben, blijven de exacte mechanismen onduidelijk. Dit proefschrift heeft associaties van tics met perinatale factoren, serotonerge en dopaminerge genen en immunologische factoren beschreven. Toekomstige studies, die deze bevindingen repliceren en de onderliggende pathofysiologische mechanismen ophelderen, zijn nodig. Daarnaast is verder onderzoek naar gen-omgevingsinteracties zinvol. Om het ontrafelen van bij tic-stoornissen betrokken neurobiologische factoren te vergemakkelijken, zou er meer aandacht moeten komen voor de fenotypische expressie van tics en comorbide symptomen.

Dankwoord

"Vroeger dacht ik dat mijn beperkingen mijn grenzen waren." (Loesje)

Veel lieve, inspirerende en wijze mensen hebben mij gemotiveerd om over mijn "beperkingen" heen te kijken, grenzen te verleggen en een prestatie te leveren die ik niet voor mezelf weggelegd zag: het schrijven van een proefschrift. Ik wil graag degenen die mij hierbij hebben gesteund, bedanken.

De omslag van dit proefschrift is een ode aan de *kinderen en jongeren* die mij geïnspireerd en gemotiveerd hebben om dit promotie-onderzoek te verrichten: kinderen in mijn directe omgeving, maar vooral de ruim 160 dappere jongens en meisjes bij wie ik bloed en een niet te onderschatten vragenlijstpakket mocht afnemen. Jongelui met tics, die zo graag willen weten waarom ze tics hebben; jongelui zonder tics, die solidair zijn met leeftijdgenoten die last hebben van tics. Ik heb mij verbaasd over de inzet van al deze kinderen en hun ouders. Duizendmaal dank voor jullie bijdrage en wijze lessen!

Zonder promotores geen promotie. Twee bijzonder vriendelijke, enthousiaste hoogleraren hebben mij de afgelopen jaren bijgestaan. *Ruud Minderaa* bood mij de mogelijkheid om poliklinisch werk te combineren met promotie-onderzoek. Zijn deur stond altijd open, en zelfs toen ik besloot dat mijn toekomst niet in de psychiatrie lag, had hij bemoedigende, ondersteunende woorden voor me. Ik had het voorrecht *Cees Kallenberg* als tweede promotor te hebben omdat het de bedoeling was dat dit proefschrift vooral immunologische studies zou gaan bevatten. Vrijwel alles liep de afgelopen jaren anders dan gedacht, en mijn aandachtsgebied verschoof naar de genetica. Desondanks bleef Cees steeds betrokken en enthousiast. Ik waardeer de inspirerende, coachende en kritische gesprekken die we gevoerd hebben.

Van onschatbare waarde is de samenwerking met *Pieter Hoekstra*, mijn copromotor, geweest. Mijn onderzoek was een voortzetting op zijn proefschrift. Toen de immunologie niet genoeg leek op te leveren voor een proefschrift, kwam hij al gauw met nieuwe onderzoeksideeën. Zijn inspanningen, creativiteit en vermogen om nieuwe onderzoeksprojecten te ontwikkelen en samenwerkingsverbanden tot stand te brengen, hebben me regelmatig verwonderd. Naast zijn professionele vaardigheden is Pieter ook erg aardig, laagdrempelig, belangstellend, scherpzinnig, humoristisch en mogelijk een tikje chaotisch. Ik heb geboft met en veel geleerd van onze samenwerking.

Prof. dr. Jan Buitelaar, prof. dr. Piet Limburg en prof. dr. Tineke Oldehinkel wil ik bedanken voor hun bereidheid om deel uit te maken van de beoordelingscommissie. *Piet* was tevens nauw (en kritisch!) betrokken bij de opzet en uitvoering van de immunologische analyses. Na het eerste brainstormen kwam ik al gauw in contact met *Johan Bijzet*, die de eerste analyses uitvoerde en mij zelfs begeleidde op een tripje naar Londen om enzymen te bemachtigen. Later werd ook *Hannie Westra* betrokken.

Samenwerken met hen is erg plezierig en ontspannen. Helaas is er in dit boekje weinig terug te vinden van al het werk dat door hen, met hulp van hun collega's, verricht is. Juist daarom, een extra applaus voor het lab!

Mijn eerste onderzoeksjaar had ik geen idee van het bestaan van andere onderzoekers. Dat veranderde toen ik werd verhuisd naar de "onderzoeksgang" waar alle deuren altijd open staan en die een flink aantal inspirerende, talentvolle, vriendelijke mensen bleek te herbergen. Om een paar te noemen: *Annelies de Bildt, Agnes Brunnekreef, Barbara van den Hoofdakker, Catharina Hartman, Harma Moorlag, Liza Muskee, Marjan Houwing, Myriam Harfterkamp, Neeltje Valkhof*: stuk voor stuk mensen van wie ik geleerd en genoten heb. In het bijzonder heb ik geboft met ons "AIO-clubje": *Christina Mathyssek*: kort maar krachtig heb ik haar leren kennen, zelden zie je mensen met zoveel power, inzet en ambitie; *Esther Sportel*: alomtegenwoordig, vol optimisme, gezelligheid en betrokkenheid; *Judith Nijmeijer*, mijn lieve (LAT-) kamergenoot, heeft mijn lief en leed de afgelopen jaren gedeeld, en mij, als ik weer eens bedacht had om toch maar te stoppen, gemotiveerd om door te gaan. Ik gun haar haar boekje enorm; *Julie Karsten*: erg attent, betrokken en vrijgevig, ze trakteert 's maandags omdat het maandag is!; *Sanne Kuijper*: een gezellige, optimistische doorzetter en heerlijk mens, met altijd een hartelijk woord paraat; *Yvonne Groen*: gedreven, enthousiaste medemoeder en binnenkort prof (?!); *Vera Dekker*: vriendelijk, ontspannen en gezellig, een aanwinst voor het clubje! Zonder deze dames was ik vast een holbewoner geworden: dank voor de inspiratie, ontspanning, afleiding en hulp.

Een aantal collega's heeft mij bijgestaan in de verschillende onderzoeksfasen. Allereerst mijn supervisoren van het eerste uur: *Wies Buhre* en *Catrien Reichart*. Toen ik me afvroeg of ik genoeg hersens zou hebben voor een promotietraject, waren zij alleen maar enthousiast en vol vertrouwen waardoor ik de uitdaging durfde aan te gaan. De eerste jaren vormden *Pieter, Jetty Noltes* en *Wilma Kamp* met mij het expertiseteam tic-stoornissen. Samen gingen we (drie dagen voor het begin van mijn zwangerschapsverlof!) naar Utrecht om een groep kinderen te zien voor het onderzoek. Veel van wat ik nu weet over tics heb ik van deze drie mensen geleerd, in het bijzonder van *Jetty* die mijn poliklinische werk superviseerde. Naast dat zij een wijze psychiater en topsupervisor is, is zij ook een boeiend en prachtig mens. *Mascha van den Akker* nam de (mijns inziens gruwelijke) klus op zich om alle vragenlijsten in te scannen en verzorgde alle logistiek omtrent de iDISC-vragenlijst. Ik weet niet meer waarvoor ik *Mark-Peter* wel en niet mag bedanken, maar ben erg dankbaar voor al zijn hulp en advies. Onverwachts kreeg ik hulp van een enthousiaste, nauwkeurige en ontzettend aardige studente, *Anne Kuin*, die haar wetenschappelijke stage wilde besteden aan het in kaart brengen van de perinatale gegevens van onze patiëntengroep. Anne heeft heel wat uurtjes tussen oude dossiers op een stoffige zolder gezeten. Door deze inspanning kwamen er zoveel nieuwe mogelijkheden dat ik weer begon te geloven in de haalbaarheid van een proefschrift. Ik ben de *Stichting Gilles de la Tourette* zeer erkentelijk voor het werven van een deel van de kinderen. Vermeldenswaardig is de samenwerking met middelbare scholiere *Diede*, die op eigen initiatief 30 jongelui rekruteerde om bloed te geven voor het onderzoek! Door de

samenwerking met *Ivana Kawikova* van Yale University kwam de immunoglobuline-studie tot stand. I have never met *Ivana* face to face, but our working together was an intense and valuable experience. Ivana introduced to me a new point of view regarding tics and infections, and initiated, planned and performed the immunoglobulin analyses. It was a privilege to have worked with *her, Renske Olieman* and *Zuzanna Tobiasova.*

In een drukke, hectische tijd als de afgelopen jaren is een liefdevolle thuishaven onontbeerlijk. Vooral het laatste jaar heb ik mijn *familie en vrienden* schandelijk verwaarloosd, maar steeds heb ik me gesteund gevoeld. Ik zie ernaar uit meer tijd met hen door te brengen. Zonder de anderen te tekort te doen, wil ik een paar naasten in het bijzonder noemen. *Mijn moeder* is uniek in haar soort. Mijn hele school-, studie- en werkcarrière stond zij aan de zijlijn te juichen. Bij alle pieken en dalen lag er een kaartje in de brievenbus. Zonder haar was ik niet geworden die ik ben. *Mijn vader* is eveneens niet te evenaren. Wat wij gepresteerd hebben de afgelopen twee jaar overstijgt elke andere prestatie. Wat ben ik dankbaar dat ik hem nu kan zien stralen van trots. *Mijn schoonouders* zijn van het "nait soezen, deurbroezen", en ik leer dagelijks van ze. Lievere mensen zijn er niet, ze staan werkelijk altijd voor ons klaar. Mijn broer *Adry* wil ik danken voor zijn bereidheid mijn altijd bereikbare (helemaal anno 2010), persoonlijke helpdesk te zijn. Tot slot: logistieke ondersteuning van het gezin is een must voor ouders om hun werk goed te kunnen doen. Mijn grote dank gaat vooral uit naar *Alex en Kirsten, Jolanda, Klazina, mama en Henk, pa en ma*.

De mooiste promotie van de afgelopen jaren was mijn promotie tot moeder van twee wonderbaarlijke mensjes. *Gijs en Katy* hebben mij geleerd wat een moederinstinct is, en daardoor hebben ze me meer dan eens doen twijfelen aan de juistheid van mijn keuze een werkende, promoverende moeder te zijn. Toch gaven juist zij mij ook de motivatie om af te maken waar ik aan begonnen was. Ze zijn mijn alles.

Het is een mirakel dat tijdens alle stress, klaagzangen, gemopper en getier er steeds één was die volkomen rustig, domweg in mij bleef geloven en in elke onderzoeksfase met me meedacht. *Gaaijo*, de liefde van mijn leven, heeft de afgelopen vijf jaar een heleboel met me beleefd. De komende vijf jaren worden vast niet rustiger. In this crazy life we lead, it's you I need……

De dag van mijn promotie wordt nog mooier door de aanwezigheid van mijn twee paranimfen. *Carly*, mijn lieve, mooie en niet eens zo hele kleine zusje, kwam halverwege dit promotietraject mijn leven binnenwandelen. Het is een feest om haar om je heen te hebben, en wat ben ik blij dat zij mijn paranimf wilde zijn. *Inge* leerde ik kennen als arts-assistent kindergeneeskunde. We kozen andere werkrichtingen maar de vriendschap werd hechter. Nu is de tijd gekomen dat ik haar gelijk moet geven: ik moet huisarts worden.

List of publications

Veneman NGP, Tinga DJ, Kragt H, Brandsma G, van der Leij G. The efficacy of intratubal silicone in the Ovabloc hysteroscopic method of sterilization. *Acta Obstet Gynecol Scand* 1999;78:824-825

De Waard PE, Veneman NGP, Schirm J, Benne CA, Woltil HA. Metapneumovirus infectie: Humaan voor jonge kinderen met acute luchtweginfectie? *Tijdschr Kindergeneeskd* 2004;Suppl 1:18

Veneman NGP, Waalkes HJ, van Raaij JJ, Brouwer RW. Septische polyarthritis ten gevolge van een geïnfecteerde, ingegroeide nagel bij een voorheen gezonden jongen. *Ned Tijdschr Geneeskd* 2006;150:973-6

Veneman NGP, Waalkens HJ, Tamminga RY. Anemie bij adoptiekinderen, niet altijd ijzerdeficiëntie. *Ned Tijdschr Geneeskd* 2006;150:369-72

Bos NGP, Hoekstra PJ. Ticstoornissen bij kinderen: een update. *Tijdschrift voor Neuropsychiatrie en Gedragsneurologie* 2007;6:93-197

Bos NGP, Hoekstra PJ. Ticstoornissen bij kinderen: welke behandelingen zijn bewezen effectief? *Kind en Adolescent Praktijk* 2008;2:78-84

Bos-Veneman NGP, Kuin A, Minderaa RB, Hoekstra, PJ. Role of perinatal adversities on tic severity and symptoms of attention deficit/hyperactivity disorder in children and adolescents with a tic disorder. *J Dev Behav Pediatr* 2010;31:100-106

Bos-Veneman NGP, Minderaa RB, Hoekstra, PJ. The DRD4 gene and severity of tics and comorbid symptoms: main effects and interactions with delivery complications. *Movements Disorders* 2010;in press

Bos-Veneman NGP, Bijzet J, Limburg PC, Minderaa RB, Kallenberg CG, Hoekstra PJ. Cytokines and soluble adhesion molecules in children and adolescents with a tic disorder. *Progress in Neuropsychopharmacology and Biological Psychiatry*; accepted for publication

Bos-Veneman NGP, Minderaa RB, Hoekstra, PJ. Associations between polymorphisms of SLC6A4 and MAOA genes and severity of tics and comorbid symptoms in children and adolescents with a tic disorder. Submitted for publication

Bos-Veneman NGP, Minderaa RB, Hoekstra, PJ. Role of single nucleotide polymorphisms of the SLC6A3 gene in severity of comorbid symptoms in patients with a tic disorder. Submitted for publication

Bos-Veneman NGP, Olieman R, Tobiasova Z, Hoekstra PJ, Katsovitch L, Bothwell ALM, Leckman JF, Kawikova I. Altered immunoglobulin profiles in children with Tourette syndrome. Submitted for publication

Exon Expression and Alternatively Spliced Genes in Tourette Syndrome Subjects. Tian Y, Liao I, Gunther J, Ander BP, Liu D, Lit L, Jicking G, Corbett BA, Bunge SA, Bos-Veneman NGP, Hoekstra PJ, Sharp FR. Submitted for publication

The role of the BDNF Val66Met and COMT Val158Met polymorphism in symptom dimensions of Tic Disorders: Preliminary results. Katerberg H, Cath DC, Hoekstra PJ, de Jonge P, Tijssen MAJ, Bos-Veneman NGP, Sondervan D, Minderaa RB, den Boer JA, Heutink P. In preparation