ORIGINAL ARTICLE

The VNTR Polymorphism of the Serotonin Transporter Gene Predisposes Czech Boys to Hyperkinetic Disorders

Ivana Drtilkova [1], Marketa Neumannova [1], Pavel Theiner [1], Alena Filova [1], Jan Lochman [2], Lukas Castulik [2], Omar Sery [2]

[1] Department of Psychiatry, Masaryk University, Faculty of Medicine, Brno, Czech Republic; [2] Laboratory of Neurobiology and Molecular Psychiatry, Department of Biochemistry, Masaryk University, Faculty of Science, Brno, Czech Republic

Correspondence to: Prof. Ivana Drtílková, MD., PhD., Psychiatricka klinika FN Brno, Jihlavska 20, 625 00 Brno, Czech Republic. TEL.: +420 53223 2056 (office); FAX: +420 53223 2057; E-MAIL: idrtilkova@fnbrno.cz

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Abstract

Impulsive and hyperactive behavior is related to a central serotonin dysfunction and the results of recent studies indicate the possible relationship between polymorphisms of the serotonin transporter gene and hyperkinetic disorder/ADHD.
The sample included 90 boys with ADHD and the control group consisted of 82 boys.
The diagnosis was based on the ICD-10 criteria and the Conners' Parent Rating Scales. Attention was evaluated using the Shape Discrimination Test; TE-NA-ZO (Familiar Figures Finding Test) was used for impulsivity measures and a computerized set of Neurobehavioral Evaluation System (NES-2) was used for evaluation of both. The detection of the VNTR length polymorphism of the 5-HTT gene was done using the fragment analysis. The GeneMapper software was used for results analysis.
Thirty-eight boys fulfilled the criteria of the Disturbance of activity and attention subtype (ICD-10; F90.0) and 52 of the Hyperkinetic conduct disorder (ICD-10; F90.1). The short S-allele and the SS-genotype was significantly more frequently ($P<0.01$) found in boys with hyperkinetic disorder compared to the control group. The results show a relationship between hyperkinetic disorder and the short alleles in contrast to studies published so far. It may be hypothesized that dysregulation in the 5-HT system could mainly influence the development of emotional and behavioral regulation in children with hyperkinetic disorder and reflects the symptomatology of hyperkinetic disorders as defined in ICD-10 rather than the narrower spectrum of symptoms of ADHD defined in DSM-IV.

Abbreviations:
serotonin (5-HT); serotonin-transporter-linked promoter region(5-HTTLPR); attention deficit/hyperactivity disorder (ADHD); ankyrin repeat and kinase domain containing 1 (ANKK1); brain-derived neurotrophic factor (BDNF); confidence interval (CI); dopamine receptor D2 (DRD2); Diagnostic and Statistic Manual (DSM); International Classification of Diseases (ICD); long (L); Neurobehavioral evaluation system (NES); oppositional defiant disorder (ODD); short (S); standard deviation (SD); serotonin transporter (SLC6A4); single nucleotide polymorphism (SNP); tumor necrosis factor (TNF); variable number tandem repeat (VNTR).

Introduction

Genetic factors play an important role in the etiopathogenesis of hyperkinetic disorder/ADHD, which has been documented in many recent molecular genetic studies that have found a relationship between the disorder and several gene polymorphisms that influence neurotransmitters (catecholamines in particular) in the central nervous system as well as brain development. In recent years our attention has been drawn to the study of the relations between hyperkinetic disorder and the TaqI A polymorphism of the ANKK1 gene (formerly called dopamine receptor gene DRD2) where we found a significant association (Šerý *et al* 2006; Drtílková *et al* 2008; Paclt *et al* 2010). We also found a relation between results of some neuropsychological tests and the polymorphisms of several genes (TNF alpha and BDNF) that affect neurodevelopmental processes in brain in children with ADHD (Drtílková *et al* 2008).

Recently, we have drawn attention to the study of serotonergic system in the previous sample of patiens, in the concrete the generally studied polymorphism of 44-bp deletion in the promoter area of the serotonin transporter gene (5-HTTLPR, SLC6A4 gene). Serotonin transporter influences serotonin reuptake from synaptic cleft. Serotonin transporter is localized on the presynaptic membrane and is the target for many tricyclic antidepressants and is also able to mediate the effect of amphetamines and cocaine. The gene for serotonin transporter is located on chromosome 17 and is composed of 14 exons. Its long (L/L) and short (S/S) allelic variants are related to a different ability for transcription of the gene. The short alleles are connected with a reduced expression of serotonin transporter and thus reduced serotonin reuptake from synaptic clefts.

The serotonin transporter gene has been often correlated with regulation of affectivity and particularly with depressive disorders (Kato 2007). A review on the relationship between the serotonin transporter gene and psychiatric disorders was published in the Czech Republic by Kuželová *et al* (2010). Original results in Czech population were published by Kopečková *et al* (2008).

The relation between the 5-HTTLPR gene and depressive disorder was studied by several authors. Statistic analysis revealed that the short allele of the serotonin transporter gene, which results from a 44 base pairs deletion in promoter area of the gene (Levinson 2006), causes susceptibility to depression after environmental stress or in people with childhood abuse in their history. After suffering trauma, bearers of the short allele (S/S) develop clinical symptoms of depression in 43%, whilst long-allele homozygotes only in 17%. Bellivier *et al* (2000) in an extensive study of 2,539 patients with suicidal tendencies found a correlation between the S/S genotype and suicidal behavior. Some authors suggest that the 5-HTTLPR gene cannot be considered as exclusively "depressive gene" because an association with cognitive impulsivity, which is typical for ADHD individuals, has been also found (Haberstick *et al* 2006).

Lesch (2005) described higher levels of impulsive aggression, drug abuse, chronic alcoholism, antisocial traits and increased suicidal tendencies in patients with the S/S genotype.

Hariri *et al* (2002) found increased fear and anxiety reactivity of the amygdala in patients with the S/S genotype. Jorm *et al* (2000) described an association of the S/S genotype with anxiety in children aged 13–16. Stein *et al* (2008) found an increased traumatic responsivity to abuse. Sysoeva *et al* (2009) mentions a correlation between the S/S genotype and latent aggression, whilst in the L/L genotype the aggression is straightforward.

Serotonin system and ADHD

Preclinical studies have shown that microinjections of agonists of various serotonin receptors into particular brain structures had positive modulatory effect on the activity of mesencephalic dopaminergic system. It is possible that some clinical dispositions for ADHD are associated with variants of genes of the serotonergic system and can thus hypothetically influence the development of emotional and behavioral regulation in ADHD (Hawi *et al* 2002).

A decrease in the central serotonergic activity was described in connection with deficiency in impulse control in animals, children and adults. In pre-clinical studies mice with knock-out 5-HT_{1B} gene showed hyperactivity, aggression, reduced anxiety, increased vulnerability and increased alcohol and cocaine self-administration.

Gao *et al* (2004) found decreased levels of serotonin in children with ADHD and co-morbid ODD compared to "pure" ADHD, which suggests that the serotonin activity is related to conduct disorders (impulsivity) rather than to hyperkinetic symptoms. Reduced 5-HT activity is usually correlated with impulsive-aggressive behavior whereas cognitive impulsivity is related to increased 5-HT levels. Both types of impulsivity may be parts of the ADHD symptoms that are conditioned by dysregulation in the 5-HT system.

Gizer *et al* (2009), in a metaanalysis of studies aimed at candidate genes of ADHD, states that the results of studies targeted at serotonin transporter gene polymorphisms are highly inconsistent; the long, 16 repetition variant (L) with increased activity is associated with ADHD in many studies whereas in several studies an association with the short variant was found.

Fowler *et al* (2009) state that data from three published studies show an increased frequency of the long allele in children with ADHD (χ^2=7.14, 1df, p=0.008) as well as prevailing prevalence of the long/long genotype (OR=1.33, 95% CI 0.11 – 1.66; p=0.01). Manor *et al* (2001) investigated the relation between 44-bp deletion in the promoter area of the serotonin transporter gene and ADHD because this deletion was associated with

reduced transcription and reduced levels of serotonin transporter. Ninety-eight families were investigated in total. The study found a relation between ADHD (combined subtype) and deletion homozygotes. Retz *et al* (2002) found a relation between the insertion allele of the above mentioned polymorphism in the promoter area of the serotonin transporter gene and a high score in the Wender Utah Rating Scale, a scale for evaluating ADHD symptoms in patient's history. Moreover, a relation between this polymorphism and social orientation was found. Zoroglu *et al* (2002) studied a relation between two polymorphisms of the serotonin transporter gene (5-HTTLPR and repetitive sequence – variable number tandem repeat, VNTR) and ADHD in a group of 71 ADHD children and 128 controls in Turkish population. The deletion genotype short/short (S/S) of the 5-HTTLPR was found significantly less frequently in children with ADHD. The VNTR STin2.12/12 genotype was significantly less frequent in children with ADHD than in controls. Zoroglu *et al* (2002) concluded that the lower frequency of the S/S genotype of the 5-HTTLPR and the VNTR STin2.12/12 are associated with higher risk of ADHD.

Kent *et al* (2002) studied the 5-HTTLPR polymorphism in 113 ADHD families and found a relation between the insertion allele of this polymorphism and ADHD. Kent *et al* (2002) studied also other two polymorphisms of serotonin transporter – a VNTR in the second intron and a single nucleotide polymorphism at the 3' non-transcribed end (3' UTR SNP) of the serotonin transporter gene. A relation between the T-allele of the above mentioned SNP and ADHD was found. Subsequent analysis proved a significantly preferred transmission of the T-allele of the SNP, the insertion (long) allele of the 5-HTTLPR polymorphism and the 10-allele of the VNTR polymorphism among children with ADHD. Langey *et al* (2003) replicated the study of VNTR polymorphisms in the second intron of the serotonin transporter with negative results. Retz *et al* (2004) studied aggressive behavior and the serotonin transporter gene polymorphism and found a relation between the deletion S-allele of this polymorphism as well as the S/S genotype and repeated physical aggression among adult probands with ADHD.

Cadoret *et al* (2003) studied the relation between the 5-HTTLPR polymorphism and ADHD and found a correlation between this polymorphism and aggression, conduct disorders and attention deficit. A relation between the deletion allele and the above mentioned symptoms was found in boys; on the contrary, in girls with the S/S and S/L genotypes these symptoms were suppressed. Another study (Curran *et al* 2005) found an association of the L-allele of the 5-HTTLPR polymorphism with the predominantly hyperactive-impulsive subtype of ADHD only (p=0.019), whereas the frequency of the S/S genotype was the same in all three subtypes. Grevet *et al* (2007) found a higher frequency of the S/S genotype in the predominantly inattentive subtype of ADHD. Grizenko *et al* (2009), in their controlled study with 371 children with ADHD (6 – 12 years old), found a significant linkage of the L/L genotype to combined subtype of ADHD only, but not to the predominantly inattentive subtype.

It may be concluded that the serotonin transporter gene significantly contributes to the pathogenesis of ADHD.

Material & Methods

The sample of children with hyperkinetic disorder consisted of 90 boys either hospitalized or in outpatient treatment at the child ward of the Department of Psychiatry of the Faculty Hospital in Brno, Czech Republic, and of boys hospitalized in the Child Psychiatric Hospital of Velka Bites, Czech Republic. Exclusion criteria for both groups included genetic abnormalities, epilepsy, mental retardation, schizophrenia, pervasive developmental disorders and severe physical diseases.

The average age of the sample was 9.97 years (SD=1.66). The research included only boys owing to the fact that estrogens may play a role in the pathogenesis of hyperkinetic disorders (mostly due to the impact of estrogens on neurogenesis and gene expression, e.g. of dopaminergic receptors). The selection according to age and gender enabled to obtain relatively homogenous groups for genetic analysis. Legal guardian of each proband had to sign the informed consent prior to study enrollment.

The control group consisted of 82 age-matched boys from basic schools of the Brno region.

In the group of hyperkinetic boys a detailed pedopsychiatric assessment and diagnostic process according to the criteria of the International Classification of Diseases, 10th Revision (ICD-10), was carried out. The exact subtype of hyperkinetic disorder (F90.0 – Disturbance of activity and attention, and F90.1 – Hyperkinetic conduct disorder) was defined. Parents were interviewed for a detailed psychiatric history of the child with special interest in the course of the disease and its clinical manifestations through the lifespan. Two questionnaires were used for the detection and quantification of hyperkinetic disorders – the Conners' Parent Rating Scale – CPRS and the Conners' Teacher Rating Scale – CTRS (Conners *et al* 1998a,b), which enabled an easy detection and quantification of behavioral psychopathology. The CPRS includes 90 items which examine conduct disorders, anxiety, impulsivity, hyperactivity, learning difficulties, perfectionism, antisocial symptoms and muscle tension. The CTRS has 39 items grouped in "class behavior", "group behavior" and "attitude towards authorities" and investigates conduct disorders, inattention, tension, anxiety and hyperactivity.

Attention was assessed using the Shape discrimination test (Švancara 1976) and impulsivity using the TE-NA-ZO, which is an acronym of the Czech words

Familiar figures finding test (Psychodiagnostické a didaktické testy, Müllner *et al* 1984), and Neurobehavioral evaluation system (NES 2) – a computerized test battery (Letz 1998). This set proved sensitivity to mild dysfunctions of the nervous system. From the test battery only 5 tests were used in this study – Finger Tapping, Hand-Eye Coordination, Digit Span, Pattern Comparison and Switching Attention.

Genetic assessment

The detection of the VNTR polymorphism of the serotonin transporter gene was performed using the fragment analysis with the Genetic Analyzer 3130 machine. Fprimer FAM ATA CTG GTA GGG TGC AAG GAG A and Rprimer CTC TGA ATG CCA GCA CCT AAC were used for PCR. The reaction mixture of the total volume of 10 µl contained a 100 nM concentration of each primer, 1× reaction buffer for DynaZyme polymerase (Finnzymes), 0.5 mM dNTP and 1 U of DynaZyme polymerase. The amplification reaction ran in the Veriti thermal cycler (Applied Biosystems). After an initial denaturating during 2.5 min at 94 °C DNA was amplified in three-step cycles: denaturating of 30s at 94 °C, annealing of 30s at 57 °C and elongation of 60s at 72 °C. After 35 cycles the final elongation during 30 minutes at 72°C was used. PCR products were analyzed in the 3130 genetic analyzer as follows: 0.5 µl of the PCR product was mixed with 9.3 µl of HiDi Formamide and 0.2 µl of the LIZ500 length standard. The analysis was performed according to the standard protocol for fragment analysis with the DS33 calibration. The results were evaluated using the GeneMapper software (Applied Biosystems).

Tabulated data were transformed into contingency tables with numbers of subjects corresponding to individual genotypes. These tables were the source for calculations of genotype and phenotype frequencies. The genotype frequency calculation used the χ^2- test, allelic frequency calculations used the Fisher's exact test. The probability of $p<0.05$ was considered a significant result for the studied genotype and allelic frequencies of the two polymorphisms (Table 1).

Statistics

The statistical analysis of the results was performed using the CSS Statistica program (StatSoft, USA). Average age of the hyperkinetic group as well as the control group was drawn from the tabulated data. Further, contingency tables with numbers of subject with individual genotypes were formed. They were a source for genotype and allelic frequencies calculations. χ^2-test was used for calculating the genotype frequencies of the studied polymorphisms and the Fisher's exact test for calculating the allelic frequencies of the polymorphisms. The probability $p<0.05$ was considered a significant result for the studied genotype and allelic frequencies of the two polymorphisms.

TABLE 1. VNTR polymorphism of the serotonin transporter gene.

group	Number of subjects with particular genotypes			total	F. exact
	LL	LS	SS		
control	38	36	8	82	p=0.008
hyperkinetic	33	31	26	90	

Results

From the total of 90 boys with hyperkinetic disorder 38 fulfilled the criteria for Disturbance of activity and attention (F90.0) and 52 fulfilled the criteria for Hyperkinetic conduct disorder (F90.1).

Within the association study a significant relation between the VNTR polymorphism of the serotonin transporter gene and hyperkinetic disorder was found. The short S-allele and the S/S genotype was found significantly more frequently ($p<0.01$) in boys with hyperkinetic disorder compared with the control group (Risk Ratio = 1.2673, 95% CI of RR = 1.0684 to 1.5032, Odds Ratio = 1.843, 95% CI of OR = 1.1863 to 2.8631). The results show connection between the hyperkinetic disorder and short alleles unlike to so far published articles, which used diagnostic criteria for ADHD.

Discussion

Although higher number of studies found a relation between ADHD and the long allele (5-HT L/L) and lower frequency of the short allele (5-HT S/S), studies aimed at the detection of serotonin transporter gene polymorphisms in the ADHD subtypes show large inconsistency (Curran *et al* 2005; Grevet *et al* 2007; Grizenko *et al* 2009). The authors conclude that the predominantly inattentive subtype and the subtypes with hyperactivity and impulsivity may have different etiological basis, which is suggested by the different distribution of the L/L and S/S genotypes of the serotonin transporter gene (Thapar *et al* 2006; McLoughlin *et al* 2007).

Our results may be influenced by different definition of the subtypes of hyperkinetic disorder and ADHD according to ICD-10 and DSM-IV criteria respectively. The DSM-IV predominantly inattentive and predominantly hyperactive-impulsive subtypes of ADHD do not exist in ICD-10. On the contrary, the ICD-10 category of Hyperkinetic conduct disorder is not represented among the DSM-IV subtypes of ADHD.

The L-allele of the serotonin transporter gene is usually connected with ADHD, whereas the S-allele rather with comorbid conditions in ADHD (mood disorders, conduct problems, temper tantrums, persistent aggression etc.).

Our results largely correspond with findings of the S/S genotype in studies that found increased physical aggression in adult probands with ADHD (Retz *et al* 2004) and in studies that found a relation to impulsive aggression, drug and alcohol abuse, antisocial behavior and suicidal tendencies (Lesch 2005). A correlation between the S/S genotype and latent aggression was also described by Sysoeva *et al* (2009). Only one study described a relation between the S/S genotype and the predominantly inattentive subtype of ADHD (Grevet *et al* 2007). Nikolas *et al* (2010) states that both increased and decreased activity of 5-HT functions in children and adolescents are connected with occurrence of impulsivity and aggression – contrary to adults where only decreased 5-HT activity has been described. The author hypothesizes that both genotypes of 5-HTTLPR (L and S) may represent a risk for ADHD and comorbid conditions.

Conclusion

Considering the published results it is possible to conclude that a significant superiority of the S/S genotype in our study reflects more the symptoms of hyperkinetic disorders defined in ICD-10 rather than the narrower spectrum of symptoms of ADHD defined in DSM-IV. Hyperkinetic disorders categories (F90.0 and F90.1) include, in addition to the classical triad of symptoms (attention deficit, hyperactivity and impulsivity), also hyperkinetic conduct disorder, not included in the diagnostic criteria of DSM-IV, with individuals presenting impulsive, oppositional and aggressive behavior.

It may be hypothesized that a dysregulation in the 5-HT system could mainly influence the development of emotional and behavioral regulations in children with hyperkinetic disorder.

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