COMBINED EFFECT OF PROMOTER POLYMORPHISMS IN THE DOPAMINE D4 RECEPTOR AND THE SEROTONIN TRANSPORTER GENES IN HEROIN DEPENDENCE

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A DPAMIN D4 RECEPTOR ÉS A SZEROTONIN TRANSZPORTER GÉNEK PROMOTER POLIMORFIZMUSAINAK KOMBINÁLT HATÁSA HEROINFÜGGÉSBEN

Munkánk során a dopamin D4 receptor (DRD4) és a szerotonin transzporter (SERT) gének polimorfizmusait vizsgáltuk, mint a kábítószerfüggés lehetséges genetikai rizikófaktorait. Esetkontroll vizsgálatunkba egy meglehetősen nagy számú (n=362) egészséges kontroll populációt, illetve kezdetként egy 73 személyből álló kábítószerfüggő csoportot (köztük 53 fő volt heroinfüggő) vontunk be. A DRD4 gén polimorfizmusainak (exon 3 48 bp VNTR; -521 C/T SNP és 120 bp duplikáció az 5' régióban) és a SERT gén polimorfizmusainak (5-hydroxytriptamin transporter linked polymorphic region [5-HTTLPR] az 5' régióban, illetve az intron 2 VNTR [STin2]) vizsgálatára számos, munkacsoportunk által kidolgozott genotipizálási módszert alkalmaztunk. A heroinfüggő alcsoportban szignifikáns asszociációt találtunk a DRD4 promoter régiójának -521 C/T SNP jével (p=0.044). A többi vizsgált polimorfizmus esetében nem találtunk szignifikáns asszociációt, azonban a DRD4 -521 C/T SNP és a 5-HTTLPR polimorfizmus interakcióját figyeltük meg. A -521 CC vs. CT vagy TT genotípusok és a heroindependencia közötti asszociációt felerősítette a rövid HTTLPR allél jelenléte (s [short] vagy 14-szeres ismétlődés, p<0.01). A -521 CC genotípus esetében megfigyelt 2.14-es odds ratio 4.82-re emelkedett a -521 CC és 5-HTTLPR ss dupla homozigóták esetében, amely aláhúzza az agyi dopaminerg és szerotonerg rendszerek polimorfizmusai kombinált analízisének fontosságát a heroinfüggésben. Ugyanakkor a viszonylag alacsony mintaszám miatt ezen eredményeket óvatossággal kell értékelni.

KULCSSZAVAK: heroin abúzus, genetikai rizikófaktorok, dopamin, szerotonin transzporter

SUMMARY

Dopamine D4 receptor (DRD4) and serotonin transporter (SERT) gene polymorphisms were studied, as possible genetic risk factors for substance dependence. The case-control study involved a large cohort (n=362) of healthy Caucasian population, and an initial sample of 73 substance dependent patients (including a subgroup of 53 heroin dependents). Improved methods

Abbreviations:
SNP – single nucleotide polymorphism, bp - base pair, PCR – polymerase chain reaction, VNTR – variable number of tandem repeats, DRD4 – dopamine D4 receptor, DRD4 VNTR – the 48 bp repeat polymorphism in the third exon of DRD4 gene, 5-HTTLPR – 5-hydroxytriptamine transporter linked polymorphic region, SERT - serotonin transporter, STin2 - serotonin transporter gene intron 2 polymorphism.

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were applied for genotype detection of the DRD4 polymorphisms (exon 3 48 bp VNTR; -521 C/T SNP and 120 bp duplication in the 5' flanking region) and the SERT gene polymorphisms (5-hydroxytriptamin transporter linked polymorphic region [5-HTTLPR] in the 5' flanking region and the intron 2 VNTR [STin2]). Association between the -521 C/T SNP of the DRD4 promoter region and substance dependence was significant in the subgroup of heroin dependents (p=0.044). The other analyzed polymorphisms did not show any significant association, but an interaction between -521 C/T SNP of DRD4 and the 5-HTTLPR polymorphisms was observed. Association between the -521 CC vs. CT or TT genotypes and heroin dependence was enhanced in the presence of short (s or 14-repeat) 5-HTTLPR allele (p0.01). The odds ratio of 2.14 observed for the -521 CC genotype increased to 4.82 in double homozygotes of -521 CC and 5-HTTLPR ss, emphasizing the importance of combined analysis of polymorphisms in the dopaminergic and serotonergic systems in heroin dependence. However, due to the limited size of our sample these results should be interpreted with caution.

KEYWORDS: substance dependence, association study, DRD4, SERT, 5-HTTLPR

INTRODUCTION

Substance dependence is a major social and health problem worldwide. It is generally accepted that genetic and environmental risk factors contribute to the development of drug addiction, however, at present, little is known about the exact nature and effects of these genetic components. Neurobiological models emphasize the key role of the reward system through the dopaminergic mesocorticolimbic pathway, which is modulated by a number of other stimulatory and inhibitory neurotransmitters. The most extensively studied modulator is the serotonin system with its complex, mutual interactions with the dopamine network. Different drugs of abuse act at various points of these systems, but eventually they all result in an elevated dopamine level in the nucleus accumbens . In a study of combined dopamine and serotonin transporter (DAT -/-, SERT -/-) knockout mice cocaine-conditioned place preference was eliminated indicating that the serotonin transporter plays a crucial role in the rewarding/reinforcing effects of the drug, emphasizing the interaction of the dopaminergic and serotonergic systems.

Genetic polymorphisms of several components of the reward system (receptors, transporters, enzymes in neurotransmitter metabolism) have been widely studied for associations with various personality traits, as well as psychiatric disorders. Among these, close attention has been paid to the highly polymorphic dopamine D4 receptor (DRD4) and the serotonin transporter (SERT) genes. The human personality trait of Novelty Seeking has been associated with a VNTR in the third exon of the dopamine D4 receptor gene , however, these results are still controversial . An association of substance dependence and the above VNTR was first proposed by Kotler et al., and replicated by others , but contradictory results were also obtained. Heroin dependence was found to be associated with the STin2 polymorphism of the SERT gene , but not with the 5-HTTLPR .

Here, we present an analysis of DRD4 and SERT gene polymorphisms as possible risk factors for substance dependence, in a case-control study of 73 substance dependent subjects (including 53 heroin dependents) and 362 healthy Caucasian (Hungarian) controls. The investigated polymorphic regions included the coding region (exon 3 VNTR) and the 5' upstream region (-521 CT and 120 bp duplication) of the DRD4 gene, the 5' upstream region (5-HTTLPR), and the intron 2 polymorphism (STin2) of the SERT gene.

METHODS

Subjects

Patients: Seventy three Hungarian substance dependent patients (50 males and 23 females: 68.5% vs. 31.5%) were comprised in the study from the National Institute of Psychiatry and Neurology, Budapest. The diagnosis was made based on the DSM-IV criteria (American Psychiatric Association, 1994). Patients were divided into two subgroups according to the abused substance (53 heroin dependent individuals and 20 addicts of other non-opiate drugs, such as amphetamine, cocaine, cannabis, LSD, etc.). The age of subjects at diagnosis was 15-70 years (mean age 28.23±10.63 years). Four subjects suffering from a major psychiatric disorder (schizophrenia, major depression and alcohol dependence) with possible involvement of the studied neurotransmitter systems were excluded from the initial cohort of 77 patients.

Control subjects: Genotype data of 362 sexually matched, healthy individuals (248 males and 114 females: 68.5% vs. 31.5%) was used to construct a large normative sample for determination of control genotype and allele frequencies. The research protocol has been approved by the Ethical Committee of Semmelweis University and the Institute of Psychology, Hungarian Academy of Sciences.

Genotyping

Genomic DNA was extracted from buccal swabs and approximately 1 ng DNA was used as template for each of the tested polymorphisms, performed as described earlier (*DRD4 gene:* -521 C/T SNP, 120 bp duplication, DRD4 VNTR; *SERT gene:* STin2 and 5-HTTLPR). The -521 CT polymorphism was determined by two independent methods using a newly described primer pair , and only genotypes with identical results were accepted. Using this improved method the genotype distribution of the control population corresponds to the Hardy-Weinberg equilibrium (-521 C/T: ^{2}=0.02, p=0.99; 5-HTTLPR: ^{2}=1.46, p=0.48).

Statistical analysis

SPSS 11.5 for Windows was used for all statistical analyses. P-values 0.05 were considered significant and were not adjusted for multiple testing.

RESULTS

Obtained genotype and allele frequencies of the polymorphisms in the DRD4 and SERT genes are listed in Table 1 and Table 2, respectively. A significant association was found between the -521 C/T polymorphism and heroin dependence (^{2}=6.24, df=2, p=0.044). The -521 CC genotype was more frequent in the heroin group than in controls (35.9% vs. 20.7%, odds ratio: 2.14). When non-opiate drug dependent individuals were also included in the sample (Table 1. "Substance dependent") no significant difference was observed between cases and controls (^{2}=4.23, df=2, p=0.12). A tendency for difference was also obtained in the allele distribution in case of the heroin dependent subgroup (^{2}=3.01, df=2, p=0.083) Analysis of other polymorphic regions in the two genes did not result in any significant association.

An interaction was found between the -521 C/T promoter polymorphism of the DRD4 gene and the 5-HTTLPR promoter polymorphism of the SERT gene. When -521 C/T genotypes were further analyzed in combination with the 5-HTTLPR polymorphism (Table 3.), the association of the -521 CC vs. CT or TT groups with heroin dependence was enhanced in the presence of the 5-HTTLPR short (14 repeat) allele (^{2}=9.89, df=1, p=0.0017). The odds ratio for heroin dependence was 2.86 in the presence of the -521 CC genotype and at least one 5-HTTLPR s allele, and it is increased to 4.82 in case of CC and ss double homozygotes, which estimate the combined effect of the two risk factors. This interaction was also seen in the whole group of substance dependents (^{2}=6.71, df=1, p=0.0096, OR=2.26).

No significant difference was found between heroin or substance dependents and controls regarding the DRD4 exon 3 VNTR genotypes when grouped according to the presence or absence of the seven repeat allele (7- vs. 7+, Table 1.). Allele frequencies with a repeat number from 2 to 9 are also presented in Table 1, showing a similar distribution between cases and controls.

DISCUSSION

The results presented here show the significance of genetic variations in both the dopamine and the serotonin system as risk factors for drug dependence. The subtelomeric region of chromosome 11p, where the DRD4 gene is located was identified by single-point analysis in a recent genome-wide search for quantitative trait loci influencing substance dependence vulnerability. Among the numerous polymorphisms of the DRD4 gene, the -521 C/T promoter polymorphism was previously shown to play a role in the personality dimension of Novelty Seeking and in disorganized early attachment in our laboratory. According to our knowledge, the only association study between -521 C/T polymorphisms and heroin abuse has so far been reported about 387 Chinese cases , where no significant difference was found for either allele or genotype frequencies of the DRD4 exon 3 VNTR and the -521 C/T polymorphisms. Their data, however, also showed a small, but non-significant increase of the -521 C allele and CC genotype frequencies in heroin abusers, as well as in the injector (but not the inhaler) subgroup.

The statistical association between the -521 C/T promoter polymorphism and heroin dependence found in the present study is in agreement with previous findings of association between the personality trait of Novelty Seeking and the -521

Table 1. Genotype and allele frequencies of polymorphisms in the dopamine D4 gene and its promoter region. Repeat polymorphisms are depicted by the number of repeats. DRD4 exon 3 VNTR genotypes are also grouped according to the absence (7 -) or presence (7 +) of the seven repeat allele.

Polymorphism	***Control***	***Substance dependent***		***Heroin dependent***	
- 521 C/T	n=362	n=73		n=53	
CC	20.7%	30.1%		35.9%	^{2} =6.24
CT	50.0%	38.4%	n.s.	37.7%	p=0.044
TT	29.3%	31.5%		26.4%	^{2} =3.01
C	45.7%	49.3%	n.s.	54.7%	p=0.083
T	54.3%	50.7%		45.3%	
120 bp dup.	n=362	n=73		n=53	
1/1	3.6%	2.7%	n.s.	3.8%	n.s.
1/2	27.9%	32.9%		26.4%	
2/2	68.5%	64.4%		69.8%	
1	17.5%	19.2%	n.s.	17.0%	n.s.
2	82.5%	80.8%		83.0%	
exon 3 VNTR	n=362	n=71		n=52	
7-	63.5%	64.8%	n.s.	65.4%	n.s.
7+	36.5%	35.2%		34.6%	
2	9.3%	5.0%	–	4.8%	–
3	3.7%	4.9%		6.7%	
4	64.1%	67.6%		68.3%	
5	0.8%	0.7%		0%	
6	0.4%	0%		0%	
7	20.6%	19.0%		19.2%	

Table 2. Genotype and allele frequencies in the serotonin transporter gene promoter region (5-HTTLPR) and second intron (STin2). Genotype and allele frequencies are depicted by the number of repeats (5-HTTLPR 14: short allele, 16: long allele)

Polymorphism	***Control***	***Substance dependent***		***Heroin dependent***	
5-HTTLPR	n=362	n=70		n=52	
14/14	17.7%	24.3%	n.s.	23.1%	n.s.
14/16	45.0%	44.3%		48.1%	
16/16	37.3%	31.4%		28.8%	
14	40.2%	46.4%	n.s.	47.1%	n.s.
16	59.8%	53.6%		52.9%	
STin2	n=362	n=73		n=53	
9/10	0.6%	1.4%	n.s.	0%	n.s.
9/12	2.2%	2.7%		1.9%	
10/10	11.6%	9.6%		9.4%	
10/12	47.2%	46.6%		54.7%	

Table 3. Interaction of the –521 C/T polymorphism in the dopamine D4 receptor promoter and the 5-HTTLPR polymorphism in the serotonin transporter promoter region

Genotypes		Control n=362	Substance dependent n=70		Heroin dependent n=52	
-521 C/T	5-HTTLPR					
CC	14/14+14/16	12.4%	24.3%	^{2}=6.71 P=0.0096	28,8%	^{2}=9.89 P=0.0017
all other genotypes		87.6%	75.7%		71.2%	

CC genotype provided that substance users have higher Novelty Seeking scores than controls . As the -521 C allele was shown to cause a considerable increase in the transcriptional activity of the DRD4 promoter in a heterologous *in vitro* assay, one could hypothesize that individuals carrying only C alleles, and therefore having a higher DRD4 expression in brain areas including the reward system, would require more novel stimuli, which could lead to an increased risk of using and abusing heroin.

When trying to get an insight into the complex functioning of the human brain, interactions between different monoamine neurotransmitter systems need to be taken into account, rather than considering these systems individually. According to this concept, dopamine and serotonin levels in various brain areas are more informative if their relative levels are also compared. Our findings of the statistical interaction between the dopaminergic and the serotonergic polymorphisms is in agreement with emerging evidence that serotonergic neurons can modulate dopamine function at various levels, including the mesocorticolimbic system involved in reward . Transfection assays and in vivo SPECT studies of the SERT promoter activity revealed that the short variant of 5-HTTLPR is associated with low transporter expression. In light of our findings on the interaction of the -521 CC and 5-HTTLPR ss polymorphisms in heroin dependence, one may propose that increased synaptic serotonin concentration due to lower expression of SERT could result in greater stimulation of dopamine release in areas responsible for reward including the nucleus accumbens. This would elevate synaptic dopamine concentration, which, in addition to the more abundantly available postsynaptic D4 receptors could make downstream signaling of the neurons more effective. Thus, polymorphisms of the dopamine and serotonin systems can influence individual sensitivity to the rewarding actions of the drug.

The presented initial sample of substance dependents is relatively low in number and vulnerable to Type I error of false positive results. The control group of healthy Hungarian individuals, however, is a large and homogeneous sample where the -521 C/T and the 5-HTTLPR genotype frequencies correspond to the Hardy-Weinberg equilibrium (see Methods). Attempts were also made to verify the accuracy of genotyping methods, especially in case of the -521 C/T SNP , which lies in a highly polymorphic region. In other association studies, family-based analyses are often preferred in order to avoid population stratification, but it is often complicated to carry out these studies in case of substance dependents' families. Moreover, searching for patients with complete and co-operating families might result in a drop out of the most serious cases, thus distorting the results. Therefore, replications by case-control studies on other homogenous populations, as well as extended studies using a larger number of subjects would be necessary to confirm the role of the -521 C/T polymorphism and its interaction with the 5-HTTLPR as possible genetic risk factors for substance dependence.

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