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The Association Study of DRD2, ACE and AGT Gene Polymorphisms and Metamphetamine Dependence

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Summary

We investigated the association between metamphetamine dependence and TaqI A polymorphism of the dopamine receptor D2 gene (DRD2), I/D polymorphism in angiotensin-converting enzyme (ACE) and M235T polymorphism of the angiotensinogen gene (AGT) in 93 unrelated metamphetamine-dependent subjects and 131 controls. Our results did not prove any association of TaqI A polymorphism of the DRD2 gene, I/D polymorphism of ACE gene, and M235T polymorphism of AGT gene with the metamphetamine dependence in Caucasians of Czech origin. However, a significant difference in allele I frequency between male and female control groups for the I/D ACE polymorphism ($p<0.03$) was found.

Key words

TaqI A polymorphism • DRD2 • ACE • M235T • Angiotensinogen • Metamphetamine dependence

Introduction

In recent years, a dramatic escalation in the use of illicit drugs in the Czech Republic has been observed. The majority of newly drug-dependent subjects are teenagers and the mostly used drugs are metamphetamines. Social, psychological and biological factors are the cause of the metamphetamine dependence. Hereditary factors are also involved in the dispositions to the illicit drug use and abuse (Yates *et al.* 1996, van den Bree *et al.* 1998).

Dopamine plays a key role in mediating effects of every drug of abuse. The role of dopamine neurotransmission in appetitive mechanisms has been supported by observations indicating that major drugs of abuse exert their addictive properties through dopamine mechanisms in the nucleus accumbens and prefrontal cortex (Schultz *et al.* 1997). Amphetamine non-selectively elevates synaptic levels of dopamine, noradrenaline and also of serotonine, but the rewarding effects of this agent are attenuated by selective dopamine antagonists and not by selective noradrenergic or serotonergic antagonists. Dopamine is found in a limited number of systems in the brain. It is the mesolimbic and mesocortical dopamine systems, which project primarily from the ventral tegmental area to the nucleus accumbens and frontal cortex, respectively, that appear to be involved in psychomotor stimulant reward function (Wise 1998). The primary disturbance in the dopamine system is assumed to be involved in the dispositions to the drug

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dependence. From this point of view, the association studies explored the genetic dispositions to the drug dependence using genes that participate in the regulation of the dopamine system.

Since 1990, the most frequently studied polymorphism in the area of studies on the drug dependence is the TaqI A DRD2 polymorphism. Blum *et al.* (1990) were the first to report this association with alcoholism. Many authors have supported the association between this polymorphism and drug dependence since then (Comings *et al.* 1991, Arinami *et al.* 1993, Noble *et al.* 1993, Kono *et al.* 1997), while others have not found such an association (Amadéo *et al.* 1992, Gelernter *et al.* 1999).

Angiotensin-converting enzyme (ACE) and angiotensinogen (AGT) genes are also involved in the regulation of dopamine transmission. The role of the angiotensin system in the brain has recently been discussed (for review see Wright and Harding 1997). The mRNAs for renin, AGT and ACE were detected in the brain. Angiotensin II (AII) produces a variety of behavioral effects, such as stimulation of thirst (el Ghissassi *et al.* 1995), modulation of salt appetite (Liénard *et al.* 1996), increase in locomotor and exploratory activity and enhancement of stereotyped behavior (Braszko *et al.* 1987). Intracerebrovascular (ICV) administration of angiotensin II improves learning and memory of active and passive avoidance (Baranowska *et al.* 1983, Braszko *et al.* 1991). The effect of AII on memory is mediated by the dopaminergic system (Winnicka *et al.* 1997). An interaction between the brain renin-angiotensin system (RAS) and the classical neurotransmitter systems (cholinergic, noradrenergic, dopaminergic, GABAergic, opioid) has been demonstrated (Barnes *et al.* 1990, Braszko and Wisniewski 1990, Dwoskin *et al.* 1992, Hadjiivanova and Georgiev 1998). Angiotensin AT_1 receptor antagonist DuP753 alters dopaminergic functions in rat striatum (Dwoskin *et al.* 1992).

From studies mentioned above it is obvious that the renin-angiotensin system participates in dopamine functions in the brain and even directly in the striatum, where the dopamine system is involved in psychostimulant reward mechanisms. From this point of view, we have tried for the first time to launch an association study between the polymorphisms of DRD2, ACE and AGT genes and metamphetamine dependence.

Methods

Subjects

The study group included 225 subjects, who were unrelated Caucasians from the Czech Republic. No minorities were included. The group consisted of 55 males and 38 females diagnosed as metamphetamine-dependent (mean age ± S.E.M., 21.6 ± 4.5 yrs). The diagnosis of metamphetamine dependence was based on clinical assessment according to the criteria of DSM-IV (APA 1994). The control subjects were 63 psychiatrically normal males and 69 females not addicted to tobacco smoking, alcohol and coffee drinking, and drug usage (mean age ± S.E.M., 21.3 ± 3.4 years).

Samples of venous blood were collected from all subjects after their written informed consent had been obtained.

Genotype determination

Individual genomic DNA samples were extracted from the blood using the Micromix200 kit (Talent, Italy). The kit Micromix200 is optimized for genomic DNA extraction with a high yield and purity from whole blood. The DNA can be used for the polymerase chain reaction (PCR).

PCR detection of the DRD2 polymorphism was performed using primers, described by Grandy *et al.* (1993). The primer sequences were 5′- CCG TCG ACC CTT CCT GAG TGT CAT CA -3′ and 5′- CCG TCG ACG GCT GGC CAA GTT GTC TA -3′. Amplification reactions were carried out in a volume of 50 μl, containing 100 ng genomic DNA, 200 μM each dNTP, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 % Triton X-100, 2 μM of each of primers and 2.5 units of RecTaq DNA polymerase (Top-Bio). After initial denaturation at 94 °C for 1 min, DNA was amplified in three-step cycles as follows: denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s, using the Techne Progene thermal cycler (Cambridge, England). After 35 cycles, a final extension time of 5 min was applied at 72 °C. Length of the amplified fragment was 318 bp. The PCR products were analyzed by electrophoresis on a 2 % agarose gel containing ethidium bromide and were visualized under UV light. The PCR products were digested with 8 units of TaqI restriction enzyme (New England BioLabs) at 65 °C for 5 h. Digestion products were analyzed by agarose gel electrophoresis as described above. The amplified fragment is cleaved by TaqI restrictase to

308 bp and two fragments with the length of 6 bp and 4 bp. The fragment with the length of 308 bp can be cleaved to two fragments with the length of 180 bp and 128 bp. The uncut product of 308 bp identified A1/A1 genotype, the A1/A2 genotype was characterized by three fragments 308 bp, 180 bp and 128 bp, and the A2/A2 genotype was indicated by two fragments 180 bp and 128 bp.

The analysis of I/D ACE gene polymorphism was performed according to Rigat *et al.* (1992). Oligonucleotides 5′- CTG GAG ACC ACT CCC ATC CTT TCT -3′ and 5′- GAT GTG GCC ATC ACA TTC GTC AGA T -3′ were used as primers. The PCR reaction was performed in a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl buffer pH 8.4, 5 mM $MgCl_2$, 0.2 µM of primers, 0.5 mM of each dNTP, 100 ng genomic DNA and 1.2 U of Taq polymerase. The DNA fragments were amplified for 30 cycles with denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 45 s, after initial denaturation at 95 °C for 2 min and with final elongation at 72 °C for 9 min. PCR products were analyzed using agarose gel electrophoresis, i.e. a 192 bp fragment in the absence and a 480 bp fragment in the presence of insertion. Single bands were detected using ethidium bromide in UV light.

The analysis of M235T polymorphism of the angiotensinogen gene was performed using the method described by Russ *et al.* (1993). The primer sequences (one with two mismatches located at position 4 and 5 from its 3′- end) were 5′-CAG GGT GCT GTC CAC ACT GGA CCC C -3′ and 5′- CCG TTT GTG CAG GGC CTG GCT CTC T -3′. The PCR reaction was performed in a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl buffer pH 8.4, 15 mM $MgCl_2$, 1 µM of primers, 0.5. mM of each dNTP, 100 ng genomic DNA and 1 U of Taq polymerase. Cycling conditions were: initial denaturation at 94°C for 3 min, 35 cycles for 94 °C 30 s, 69 °C for 1 min, final extension at 69 °C for 7 min. The amplification yields a product of 165 bp. 15 µl of PCR product was digested with 5U of AspI restrictase for 3 h. The uncut product of 165 bp identified M allele and the cut product of 141 bp identified the T allele.

Table 1. Genotype distribution and A1 allele frequencies of TaqI A polymorphism of the DRD2 gene.

	GENOTYPE			Total	Allele A1
	A1A1	**A1A2**	**A2A2**		**frequency**
Dependent males	2	22	31	55	0.24
Dependent females	0	12	26	38	0.16
Male controls	2	21	40	63	0.20
Female controls	2	32	35	69	0.26
Dependent males + females	2	34	57	93	0.20
Total controls	4	53	75	132	0.23

Table 2. Genotype distribution and I allele frequencies of I/D ACE gene polymorphism.

	GENOTYPE			Total	Allele I
	II	**ID**	**DD**		**frequency**
Dependent males	8	34	10	52	0.48
Dependent females	11	17	7	35	0.56
Male controls	17	34	9	60	0.57*
Female controls	15	27	24	66	0.43*
Dependent males + females	19	51	17	87	0.51
Total controls	32	61	33	126	0.50

** Significant difference ($p < 0.03$) between male and female controls.*

Results

A total of 225 unrelated subjects were studied for three polymorphic sites: TaqI A polymorphism of the DRD2 gene, I/D ACE polymorphism and M235T polymorphism of the AGT gene. Tables 1-3 present genotyping data for these polymorphisms in metamphetamine-dependent subjects and controls. Comparison of the allelic variant frequencies was performed by Fisher's exact test. No significant differences in allele frequencies between metamphetamine-dependent and control subjects could be seen for any of the three polymorphic sites. However, a significant difference in allele frequency between male and female control groups for the I/D ACE polymorphism ($p<0.03$) was found. An increase in frequency of the allele I of the I/D ACE polymorphism was observed in male controls (0.57) when compared with female controls (0.43). The opposite tendency in allele I frequency, although not statistically significant ($p=0.17$), was found in metamphetamine-dependent groups, where metamphetamine-dependent males had a smaller allele I frequency (0.48) when compared with metamphetamine-dependent females (0.56).

The comparison of double homozygotes to the other double homozygotes in all three studied polymorphisms was also performed by Fisher's exact test that did not reveal any significant difference.

The Hardy-Weinberg equilibrium was tested by the χ^2 test. The genotypic frequencies of the TaqI A polymorphism of the DRD2 gene were found in Hardy-Weinberg disequilibrium among metamphetamine-dependent females ($p<0.05$). The genotypic frequencies of the I/D ACE polymorphism deviated significantly from those expected according to the Hardy-Weinberg equilibrium among metamphetamine-dependent males ($p<0.004$) and in the whole metamphetamine-dependent group ($p<0.04$). The genotypic frequencies of the M235T polymorphism of the AGT gene deviated from Hardy-Weinberg equilibrium among control males ($p<0.04$). The genotypic frequencies of all three polymorphisms among the cases and controls of the other groups did not deviate from the Hardy-Weinberg equilibrium.

Table 3. Genotype distribution and M allele frequencies of the M235T polymorphism of the AGT gene.

	GENOTYPE			Total	Allele M
	MM	MT	TT		frequency
Dependent males	16	30	7	53	0.58
Dependent females	16	16	4	36	0.67
Male controls	23	23	14	60	0.58
Female controls	18	35	12	65	0.55
Dependent males + females	32	46	11	89	0.62
Total controls	41	58	26	125	0.56

Discussion

Studies of the causes leading to the drug dependence have been directed to the exploration of disturbances of the dopaminergic system because all drugs of abuse increase the level of dopamine in certain brain areas that are involved in reward mechanisms (Wise 1998, Di Chiara and Imperato 1988, Schultz 1997).

The possible role of TaqI A polymorphism of the DRD2 gene in influencing the dispositions to alcoholism, drug dependence and some psychic diseases has already been frequently discussed in recent years. Some authors support the suggestion that the allele A1 of TaqI A polymorphism of the DRD2 gene associates with alcohol or drug dependence, others do not support the hypothesis on the basis of their results (see the Introduction). It is known that this polymorphism is located in the 3' end region of the DRD2 gene and its participation in the regulation of the gene expression is only hypothetical at present because it has not been found in any regulatory regions of the DRD2 gene. The DRD2 gene itself has some secrets. Its length is estimated to be more than 270 kilobases and it has not been sequenced in its total length. Authors, who refer the relationship of TaqI A polymorphism to alcohol dependence, do postulate that this polymorphism might be due to linkage

disequilibrium with some hitherto unknown functional polymorphism in the promoter/regulatory gene element that affects dopamine D2 receptor expression (Pohjalainen 1999). The information about the relationship between allele A1 and phenotypic expression of DRD2 gene are controversial. Although some authors found the connection between the phenotypic expression of the DRD2 receptors and the allele A1 in the human brain, when allele A1 decreases B_{max} in the caudate nucleus (Noble *et al.* 1991) or the D2 receptor availability (B_{max}/K_d) in the striatum of the brain (Pohjalainen *et al.* 1998), other authors have not confirmed these results (Laruelle *et al.* 1998).

Noble (1998) carried out a meta-analysis of the results concerning the relationship of the allele A1 to alcohol dependence and concluded that the results of these studies depend on the criteria of the selection not only of the group of alcoholics but also of the control group.

In our study, we were the first to assess the relationship between metamphetamine dependence and allele A1 of TaqI A polymorphism. However, we did not find any association. The frequency of allele A1 among controls in our study is not different from frequencies in controls published by other authors (for review Noble, 1998). The frequency of allele A1 in metamphetamine-dependent subjects (0.20) did not statistically differ from that in control subjects (0.23). Almost the same results were published by Gelernter *et al.* (1999) among European-Americans in the groups of cocaine-dependent and control subjects (0.23 and 0.19, respectively). Allele A1 does not participate in the dispositions to the metamphetamine dependence of the population in the Czech Republic. Our negative results can be caused by the following factors: i) Genetic predisposition to the metamphetamine dependence is likely to differ from the dependence on alcohol or other drugs of abuse (Šerý *et al.* 1998), ii) The criteria of DSM-IV could be insufficient for selecting metamphetamine-dependent subjects for molecular genetics studies. Noble (1998) published a paper on the importance of selection criteria for this kind of research.

Plasma and tissue ACE concentrations have been found to track with the deletion (D) allele of the I/D ACE polymorphism. The plasma values decrease from genotype DD to II (Costerousse *et al.* 1993). I/D ACE polymorphism in the brain was studied only three times. Arinami *et al.* (1996) suggested that it is one of the genetic factors for an interindividual variability of brain substance P levels, and that the ACE polymorphism may contribute to the susceptibility to affective disorders. Amouyel *et al.* (1996) found that the incidence of D allele and DD genotype of ACE gene was higher in the group with cognitive impairment. Hollá *et al.* (1999) studied I/D ACE polymorphism and M235T polymorphism of the AGT gene in healthy volunteers and they found that DDMT and IIMT genotype combinations of ACE and AGT genes are associated with type A behavior pattern score assessed by means of an extended Bortner scale. The I allele frequencies published by Arinami (Japanese population), Amouyel and by us in controls were 0.66, 0.49, and 0.50, and in studied cases 0.55, 0.41 and 0.51, respectively. A total increase in allele I frequency could be observed in Japanese population. Therefore, a comparison of the I/D ACE polymorphism allelic frequency between the Japanese and European population is not possible. The frequency of allele I in our controls is almost the same as in the controls in the French population (Amouyel *et al.* 1996). After differentiation of the control group according to the sex, one can observe an increase of allele I frequency in males (0.57) when compared with females (0.43). This difference was significant ($p<0.03$). An opposite tendency was found in the metamphetamine dependent group. The frequency of allele I in metamphetamine-dependent males was 0.48 when compared with metamphetamine-dependent females (0.56). The disequilibrium in metamphetamine-dependent males with dominant ID genotype was observed. The disequilibrium is obviously caused by the selection effect on the choice of control subjects that do not use any drugs. This selection effect can be explained by the relationship between I/D ACE polymorphism and the type of personality found by Hollá *et al.* (1999). The type of personality influences the dispositions to the drug dependence. Amouyel *et al.* (1996) reported about the more pronounced association ($p<0.006$) between the I/D ACE polymorphism and cognitive impairment in males. We did not find any association between I/D ACE polymorphism and metamphetamine dependence in the population in the Czech Republic. Differences in the frequency of allele I between male and female controls in our study and in above mentioned studies suggest that the I/D ACE polymorphism could play a role in some brain functions and should be investigated further.

Polymorphism M235T of the angiotensinogen gene is not only associated with hypertension (Vašků *et al.* 1998, 1999) but also with the plasma concentration of angiotensinogen. The homozygotes M235 had an increased concentration of angiotensinogen in plasma by about 20 %, the heterozygotes by 10 % in comparison

with homozygotes T235 (Jeunemaitre *et al.* 1992). We firstly investigated the association between this polymorphism and drug dependence. Only one paper concerning the relationship between this polymorphism and brain function has been published up to now (Hollá *et al.* 1999). We found the only disequilibrium in the metamphetamine-dependent males with dominant MT genotype, however, no association between this polymorphism and metamphetamine dependence was found in the Czech population.

Our results did not prove any association of TaqI A polymorphism of the DRD2 gene, I/D polymorphism of ACE gene and M235T polymorphism of AGT gene with the metamphetamine dependence in Caucasians of Czech origin.

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