RESEARCH ARTICLE

Association between smoking behaviour and genetic variants of glial cell line-derived neurotrophic factor

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) promotes development and differentiation of dopaminergic neurons, thus it has an important role in dopamine-related neuropsychiatric disorders. Since the role of dopamine system in smoking is well established, we hypothesized that *GDNF* gene variants may affect smoking behaviour. Self-reported data on smoking behaviour (never smoked, quit, occasional, or regular smokers) and level of nicotine addiction (Hooked on Nicotine Checklist and Fagerstrom Nicotine Addiction Scale), anxiety, as well as buccal samples were obtained from 930 Hungarian young adults (18–35 years). Genetic analysis involved eight *GDNF* single-nucleotide polymorphisms (SNP) (rs1981844, rs3812047, rs3096140, rs2973041, rs2910702, rs1549250, rs2973050 and rs11111). Allele-wise association analyses of the eight *GDNF* SNPs provided a significant association between smoking behaviour and rs3096140 ($P = 0.0039$). The minor allele (C) was more frequent in those groups who smoked in some form (quit, occasional or regular smokers) as compared to those who never smoked ($P = 0.0046$). This result remained significant after Bonferroni correction for multiple testing. In the ever smoking group, no significant differences were found in the level of nicotine addiction by the alleles of these polymorphisms. Also, no significant interaction of rs3096140 and smoking categories were observed on anxiety mean scores. Although previous data demonstrated an association between *GDNF* rs2910704 and severity of methamphetamine use to the best of our knowledge, this is the first study on the role of *GDNF* genetic variations in smoking behaviour. Our results suggest that *GDNF* rs3096140 might be involved in the genetic background of smoking, independent of anxiety characteristics.

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Introduction

Recent genomewide association studies successfully identified some genetic markers of smoking. In a meta-analyses of genomewide association studies of smoking behaviour, the Tobacco and Genetics Consortium has found a gene variant in the chromosome-15 nicotinic acetylcholine receptor gene (CHRNA3 rs1051730) to be associated with the number of cigarettes smoked per day (Tobacco and Genetics 2010), replicating earlier results (Berrettini *et al.* 2008; Thorgeirsson *et al.* 2008). They found some further SNPs associated at a genomewide significance level with cigarettes per day on this large sample, and a BDNF gene polymorphism (rs6265) associated with smoking initiation. These associations seem to be reliable and replicable, however, they only explain a small per cent of the highly heritable smoking behaviour phenotypes (Sullivan and Kendler 1999; Li *et al.* 2003). Thus, further targeted genetic association analyses based on earlier genetic association studies and results from animal models suggesting a reliable association are needed. According to a well-established psychopharmacological hypothesis (Dani and Heinemann 1996) reward mechanisms have crucial role in reinforcement of addictions. Neurobiological studies demonstrated that nicotine intake during smoking effects primarily the mesolimbic dopamine neurons by activation and desensitization of mesolimbic nicotine receptors (Pidoplichko *et al.* 1997). Moreover, various dysfunctions in dopamine neurotransmission were shown to increase the

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risk of nicotine addiction (Pontieri *et al.* 1996). Based on these studies, the dopamine system has a key role in various addictions, including smoking.

GDNF gene codes the glial cell line-derived neurotrophic factor (GDNF) (Lin *et al.* 1993), a trophic factor for dopaminergic neurons. The main function of GDNF is to promote the development and differentiation of dopaminergic neurons (Hudson *et al.* 1995; Granholm *et al.* 2000), and it is an important neuroprotective element of midbrain dopaminergic neurons (Nitta *et al.* 2004). Dopamine dysfunctions are present in Parkinson disease and the early pharmacological studies of GDNF provides promising results about application of GDNF in the treatment of the disease (Gill *et al.* 2003; Lang *et al.* 2006).

It has been demonstrated that mice lacking one of the *GDNF* alleles (*GDNF*+/−) can be characterized by a lower striatal GDNF protein level than their wild-type littermates (Airavaara *et al.* 2004) and had an augmented reward, because increased GDNF levels attenuated the reward processes (Griffin *et al.* 2006). It has also been shown that reduction in the expression of *GDNF* gene potentiated methamphetamine self-administration, enhanced motivation to take methamphetamine, increased vulnerability to drug-primed reinstatement, and prolonged cue-induced reinstatement of extinguished methamphetamine seeking behaviour (Yan *et al.* 2007). Moreover, it has been shown that GDNF infusion into the ventral tegmental area (VTA) blocks both the adaptation for chronic cocaine or morphine intake and the rewarding effects of cocaine. In line with these findings, the intraVTA infusion of antiGDNF antibody enhanced responses to cocaine in rats with heterozygous null mutation in *GDNF* gene (Messer *et al.* 2000). Based on these observations, GDNF pathways of the VTA must be involved in the mechanism of drug abuse. There are promising results on possible application of GDNF for the treatment of drug dependence. In an animal study, hydrophobic dipeptide Leu-Ile was administered to the animals; Leu-Ile protects against neuronal death by inducing brain-derived neurotrophic factor and GDNF. They found that pretreatment with Leu-Ile blocked the acquisition of methamphetamine induced place preference and sensitization, while posttreatment with Leu-Ile attenuated them even after the development of methamphetamine-induced rewarding effects and sensitization (Niwa *et al.* 2007). Further, association analyses of *GDNF* polymorphisms have revealed an effect of *GDNF* polymorphism on methamphetamine use (Yoshimura *et al.* 2011). It has also been proposed that the high comorbidity of mood disorders and addictions may be due to overlapping neurological or genetic factors (Kendler *et al.* 1993; Lyons *et al.* 2008). In line with this hypothesis, we previously demonstrated an association between *GDNF* polymorphisms and mood characteristics on healthy sample (Kotyuk *et al.* 2013a) and on patients with bipolar or major depression (Kotyuk *et al.* 2013b). In the present study, we raised the question if *GDNF* polymorphisms play a role in smoking behaviour and nicotine addiction assessed by the Hooked on Nicotine Checklist (HONC) and the Fagerstrom Nicotine Dependence (FTND) scale in a sample of healthy young adults.

Materials and methods

Sample

Thousand two hundred and twenty-two independent Caucasian (Hungarian) young adults from several Hungarian education facilities were involved in the study on a voluntary basis. The study protocol was designed in accordance with the guidelines of the Declaration of Helsinki, and was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB). The participants signed a written informed consent, provided buccal samples and filled questionnaire on their smoking habits. The selection criteria included no previous history of depression, diabetes or any psychiatric illness, age range between 18 and 35 years, and nonrelated participants (all based on self-report). Participants with invalid self-report data for the smoking habit questionnaire were also excluded. Of the 1066 potential participants, 42 had previous psychiatric illness or were from high risk populations (e.g. reformatory facilities), 15 were excluded because their family members have already participated in the study, five provided invalid answers for the smoking habit questionnaire and 74 participants were excluded because data was missing on their smoking habits. Finally, 930 participants' data were analysed (44.2% males, 55.8% females; mean age: 21.3 ± 2.9 years). Genotypic and phenotypic data of the present study is publicly available through the NCBI dbGaP data repository: http://www.ncbi.nlm.nih.gov/gap.

Phenotype measures

The questions contained in the questionnaire were about age, gender, previous psychiatric illness and smoking habits. The smoking habit questionnaire contained 27 questions. The first four questions were about the frequency of smoking and previous attempts of quitting. Further questions contained the HONC (DiFranza *et al.* 2002; Urbán *et al.* 2004) questionnaire and the FTND (Heatherton *et al.* 1991; Urbán *et al.* 2004). The nicotine dependence scales (both HONC and FTND scales) were filled only by regular and occasional smokers. To increase reliability, we only calculated the values of these scales if the answers for the previous four questions were coherent, therefore, we excluded controversial answers. Further, values of the scales were only calculated if at least 70% of the scale's items were answered. This criteria narrowed the regular and occasional smokers sample from 348 to 334 for the HONC and 212 for the FTND scales.

Level of anxiety and depression have been assessed by the Hungarian version (Muszbek *et al.* 2006) of hospital anxiety and depression scale (Zigmond and Snaith 1983), as described elsewhere (Kotyuk *et al.* 2013a).

Sample preparation and SNP genotyping

The SNP selection criteria, the sample preparation and SNP genotyping are described in details elsewhere (Kotyuk *et al.* 2013a). Briefly, a proper coverage of *GDNF* gene was ensured by selection of tagging SNPs from HapMap data using a pairwise tagging method. SNPs referred in previous association studies were prioritized. Concentration of isolated DNA was measured by an intercalation assay. Genotyping was carried out by the TaqMan OpenArray genotyping system of Applied Biosystems using sequence-specific, fluorescent TaqMan probes and a miniaturized PCR system working with nanoliter scale sample volume. Raw data obtained by end-point (postPCR) detection were evaluated by the TaqMan Genotyper v1.2 software. DNA samples, 2% were measured in duplicates, showing higher than 98% reproducibility. Linkage disequilibrium of the studied *GDNF* polymorphisms is described elsewhere (Kotyuk *et al.* 2013a). Overall, the studied SNPs are in low to high linkage in the present sample. Based on the Lewontin's D' measure, one linkage block has been identified among rs1549250, rs2910702 and rs2973041. Although, no functional data is available on the SNPs studied here, it is important to note that rs3096140 SNP is in the close proximity of critical sites of alternative splicing.

Statistical analysis

Statistical analyses were carried out in SPSS 20.0 for Windows to test the gender differences in nicotine addiction independent samples. Pearson correlations were carried out to test the relationship between age and nicotine addiction. To analyse the relationship between HONC and FTND, Pearson's correlation analysis was used. Possible gender differences in the genotype distributions and Hardy–Weinberg equilibrium (HWE) of genotype frequencies were tested with chi-square (χ^2) analyses. Chi-square analyses were also used for the case–control association analyses testing allele distribution differences in the four smoking categories. One-way analyses of covariance (ANCOVA) were used for the dimensional analyses testing the differences in the mean dependence scores of HONC and FTND of carriers of the different alleles (with gender as a covariant variable). All the association analyses were carried out in an allele-wise model. The possible false positive associations were ruled out by correcting for multiple testing. Bonferroni correction (Bonferroni 1936; Miller 1981) has been applied with a corrected level of significance of 0.00625 (0.05/8 = 0.00625) as the nominal P (value 0.05) was divided by the number of SNPs tested (eight).

Results

Descriptive data and reliability of the measured phenotypes and genotypes

Based on the first four self-report questions about the participants' smoking habits, we created four categories of smoking behaviour: never smoked, those who already quit smoking, occasional smokers and regular smokers. From the 930 participants, 494 were never smoked (53.1%), 88 participants have already quit smoking (9.5%), 163 participants were occasional smokers (17.5%), and 185 regular smokers (19.9%). These seem to be in line with the results from other studies (Bogdanovica *et al.* 2011) indicating that the present sample is in agreement with the Hungarian population's smoking habits. Based on our questionnaire data, on average, participants of the present sample started to smoke at the age of 16 and have been smoking for 4–5 years. Cronbach's alpha values were calculated for HONC and FTND to test the internal consistency on the subsamples of smokers (either regular or occasional). The Cronbach's alpha values were satisfactory for both HONC (0.843) and FTND (0.646). The HONC scale had a mean value of 4.624 (±3.2) with individual scores ranging from 0 to 13 (HONC mean value was 2.64 ± 2.6 for occasional smokers, and 6.31 ± 2.8 for regular smokers). The item means were between 0.05 and 0.72 based on 638 participants' answers. The FTND scale had a mean value of 1.188 (±1.4) and the individual scores ranged between 0 and 6 (FTND mean value was 0.43 ± 0.9 for occasional smokers and 1.36 ± 1.6 for regular smokers). The item means were between 0.08 and 0.31 based on the 426 participants' answers. The intercorrelation of the two scales was tested with Pearson's correlation. The two scales were in a

Table 1. Genotype distribution of the studied GDNF polymorphisms.

dbSNP number	Genotype	*N*	Per cent	HWE	Call rate (%)
rs1981844	GG	402	54.6	$P = 0.988$	75
	CG	285	38.7		
	CC	49	6.7		
rs3812047	GG	673	76.5	$P = 0.401$	87
	GA	188	21.4		
	AA	19	2.2		
rs3096140	TT	435	47.6	$P = 0.999$	91
	TC	390	42.7		
	CC	88	9.6		
rs2973041	AA	512	70.6	$P = 0.721$	72
	AG	191	26.3		
	GG	22	3.0		
rs2910702	AA	400	54.2	$P = 0.947$	73
	GA	289	39.2		
	GG	49	6.6		
rs1549250	TT	243	32.6	$P = 0.996$	74
	TG	366	49.1		
	GG	136	18.3		
rs2973050	CC	249	40.8	$P = 0.495$	61
	TC	292	47.9		
	TT	69	11.3		
rs11111	AA	669	75.8	$P = 0.718$	88
	AG	196	22.2		
	GG	18	2.0		

dbSNP number, polymorphism identification number in an open-access archive made by the National Center for Biotechnology Information (NCBI) and National Human Genome Research Institute (NHGRI); HWE, Hardy–Weinberg equilibrium.

significant moderate correlation: $r = 0.449$ ($P < 0.001$). HWE was tested to check if there were any significant difference between the distributions of observed and calculated genotype frequencies. As shown in table 1, genotypes of all the eight SNPs were in HWE.

Age and sex as possible confounds

The possible age and gender effects on the phenotypes were tested. Age and gender differences by the four smoking categories were tested first. There were no significant difference in the frequency of males and females in the four smoking categories, however, there was a significant age difference in smoking habits ($F(3, 926) = 4.084$; $P = 0.007$). The results showed that 88 participants who quit smoking have the highest mean age (22.16 ± 3.5), followed by the regular smokers (21.62 ± 2.6), the never smokers (21.20 ± 3.0) and the occasional smokers (20.99 ± 2.6). We also tested the gender differences on both scales with independent samples *t*-tests. No significant differences were observed by genders in case of the HONC scale ($t(628) = -0.278$; $P = 0.981$). However, in case of FTND scale, we found a significant gender effect: $t(422) = 3.006$; $P = 0.003$. In average, the males had higher nicotine dependence (1.41 ± 0.1) measured by the FTND than female participants (0.99 ± 1.4). We tested with Pearson's correlation the relationship between the age and both nicotine addiction scales. There was no significant correlation between age and HONC ($r = 0.049$; $P = 0.206$), nor between age and FTND ($r = -0.018$; $P = 0.708$). The possible gender differences in the genotype distribution of the polymorphisms were also tested: no significant differences were found based on χ^2 analyses. Analysis of the possible age difference in genotypic distribution has been tested by using one sample variance analysis. A significant mean age difference was found in the genotypes of rs11111 ($F(2, 818) = 3.055$; $P = 0.048$). There was also a significant difference by mean age in case of rs3096140 ($F(2, 846) = 3.996$; $P = 0.019$). The other polymorphisms did not show significant associations with age. Based on these results, we used age and gender as covariates in the genetic association analyses.

Case–control analyses of smoking behaviour and GDNF polymorphisms

First, distributions of allele frequencies among never smokers and ever smokers have been tested in accordance with the standard genetic-epidemiological form of addictions in terms of lifetime occurrence of smoking behaviour (Madden *et al.* 1999, 2004; Maes *et al.* 2004). In these 2×2 χ^2 analyses,

Table 2. Case–control analysis: allele distribution in various smoking categories.

dbSNP number	Allele	2*N	MAF	Never (2*N = 988)	Ever smoker-lifetime vulnerability		
					Quit (2*N = 176)	Occasional (2*N = 326)	Regular (2*N = 370)
rs1981844	C	366	0.264	27.7%	25.0%	24.8%	24.8%
	G	1020		72.3%	75.0%	75.2%	75.2%
					$\chi^2 = 1.495$ df=1 $P = 0.221$; $\chi^2 = 1.497$ df = 3 $P = 0.683$		
rs3812047	A	204	0.126	12.8%	15.2%	11.6%	11.6%
	G	1418		87.2%	84.8%	88.4%	88.4%
					$\chi^2 = 0.076$ df = 1 $P = 0.783$; $\chi^2 = 1.558$ df = 3 $P = 0.669$		
rs3096140	C	530	0.312	28.2%	42.0%	33.3%	32.1%
	T	1168		71.8%	58.0%	66.7%	67.9%
					$\chi^2 = 8.048$ df = 1 $P = 0.005$; $\chi^2 = 13.326$ df = 3 $P = 0.003982$		
rs2973041	G	217	0.163	18.1%	11.7%	14.6%	14.9%
	A	1117		81.9%	88.3%	84.4%	85.1%
					$\chi^2 = 3.861$ df = 1 $P = 0.049$; $\chi^2 = 4.554$ df = 3 $P = 0.208$		
rs2910702	G	355	0.262	24.5%	32.0%	27.8%	26.9%
	A	999		75.5%	68.0%	72.2%	73.1%
					$\chi^2 = 2.403$ df = 1 $P = 0.121$; $\chi^2 = 3.538$ df = 3 $P = 0.316$		
rs1549250	G	585	0.428	42.9%	44.4%	43.2%	41.3%
	T	783		57.1%	55.6%	56.8%	58.7%
					$\chi^2 = 0.009$ df = 1 $P = 0.926$; $\chi^2 = 0.399$ df = 3 $P = 0.940$		
rs2973050	T	401	0.352	34.6%	41.3%	34.4%	35.3%
	C	737		65.4%	58.7%	65.6%	64.7%
					$\chi^2 = 0.248$ df = 1 $P = 0.618$; $\chi^2 = 1.658$ df = 3 $P = 0.646$		
rs11111	G	216	0.132	14.2%	12.8%	13.7%	9.9%
	A	1426		85.8%	87.2%	86.3%	90.1%
					$\chi^2 = 1.830$ df = 1 $P = 0.176$; $\chi^2 = 3.864$ df = 3 $P = 0.277$		

Significant after Bonferroni correction for multiple testing ($P < 0.00625$) are in bold; dbSNP number, polymorphism identification number in an open-access archive made by the National Center for Biotechnology Information (NCBI) and National Human Genome Research Institute (NHGRI); MAF, minor allele frequencies; the first χ^2 test statistics represent analyses between the never smoker and ever smoker groups; the second χ^2 test statistics represent comparison of allele distributions in all four smoking groups.

the rs3096140 showed a significant association. No significant associations were observed with the other polymorphisms. The results are summarized in table 2 which shows that the minor (C) allele of rs3096140 might be a risk factor of lifetime occurrence of smoking behaviour ($\chi^2 = 8.048$, df = 1, $P = 0.0046$).

Further analyses have been carried out to specify these associations. Allele frequencies for each of the eight SNPs were tested among all four categories (never smoked, quit smoking, occasional smokers and regular smokers) (see table 2). The rs3096140 SNP showed a significant association with smoking behaviour ($P = 0.003982$) which remained significant even after the stringent Bonferroni correction for multiple testing (Bonferroni 1936; Miller 1981). As summarized in table 2, the rs3096140 minor (C) allele was more frequent in all three groups who ever smoked: ratio of C allele were 32.1% among regular smokers, 33.3% among occasional smokers, and as high as 42.0% among those who quit smoking. In those who never smoked, frequency of the C allele was as low as 28.2%. These results show that the biggest difference in the allele frequency of rs3096140 is among the never smoker and quitter groups. This result was supported by 2 × 2 post hoc analyses in the never smoker versus quit, never smoker versus occasional, never smoker versus regular smoker groups (figure 1).

Dimensional association analysis of nicotine addiction and GDNF polymorphisms

The mean score values of the HONC, as well as that of the FTND were compared between major and minor allele carriers of *GDNF* SNPs by ANCOVA. These questionnaires were filled by the occasional and regular smokers only, thus only these two groups were included in the analyses. Results are presented in table 3. We observed a nominally significant association between *GDNF* rs11111 and the level of nicotine addiction measured by HONC ($P = 0.039$). The association showed that the minor allele (G) of rs11111 is a protective factor against high nicotine addiction level. However, this result did not remain significant after Bonferroni correction for multiple testing. No significant association was found between the analysed *GDNF* polymorphisms and nicotine addiction phenotype measured by FTND.

Smoking, rs3096140 and anxiety

Hospital anxiety and depression scale (HADS) data are available for most of the participants of the present sample ($N = 800$), for details, see Kotyuk *et al.* (2013a). Possible interaction effects of smoking behaviour and rs3096140 on anxiety have been tested (figure 2). HADS anxiety mean scores of participants possessing any of the rs3096140 alleles showed a similar pattern in case of the never smoker, occasional and regular smoker groups. In these three cases, anxiety in C allele was somewhat higher as compared to the T allele. On the other hand, C carriers of the quitter group showed an opposite pattern. However, two-way ANOVA did not show any significant effects: neither the main effect of smoking categories ($P = 0.071$), or the main effect of rs3096140 ($P = 0.170$), nor the interaction effect ($P = 0.251$) was significant. These results suggest that association between smoking behaviour and rs3096140, and association between anxiety and rs3096140 are independent. HADS depression scores have not been analysed due to the floor effect characterizing this heathy young adult sample.

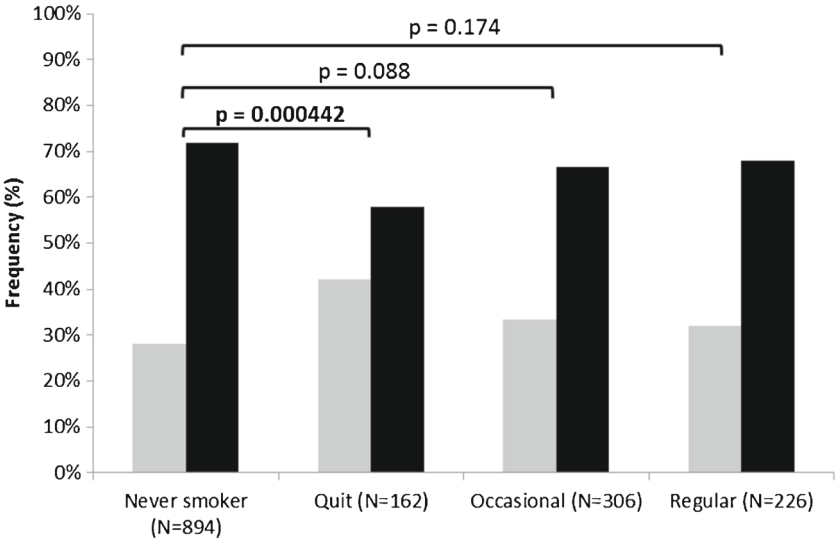

Figure 1. Testing the association for smoking initiation: allele frequency of rs3096140 in four groups. 2 × 2 χ^2 tests were used to define allele distribution differences in the nonconsumer versus quit, nonconsumer versus occasional, nonconsumer versus regular groups. Rs3096140 T allele frequencies are represented by gray bars, and bars in black represent the frequencies of rs3096140 C allele.

Table 3. Dimensional analysis: mean scores of HONC and FTND by the alleles of the analysed SNPs.

dbSNP number		2*N	Mean score of HONC		2*N	Mean score of FTND	
rs1981844	C	122	4.60 (±3.2)	F(1, 494) = 0.1	79	1.25 (±1.6)	F(1, 320) = 1.4
	G	376	4.52 (±3.3)	$P = 0.864$	245	1.08 (±1.4)	$P = 0.240$
rs3812047	A	67	4.48 (±2.9)	F(1, 572) = 0.4	44	1.25 (±1.4)	F(1, 372) = 0.4
	G	509	4.72 (±3.3)	$P = 0.550$	332	1.16 (±1.5)	$P = 0.534$
rs3096140	C	206	4.66 (±3.0)	F(1, 616) = 0.1	120	1.27 (±1.4)	F(1, 384) = 0.2
	T	414	4.60 (±3.3)	$P = 0.865$	268	1.19 (±1.5)	$P = 0.698$
rs2973041	G	69	4.17 (±3.2)	F(1, 466) = 1.1	45	1.11 (±1.4)	F(1, 304) = 0.1
	A	401	4.60 (±3.2)	$P = 0.302$	236	1.07 (±1.3)	$P = 0.859$
rs2910702	G	132	4.56 (±3.0)	F(1, 472) = 0.1	82	1.16 (±1.4)	F(1, 308) = 0.5
	A	344	4.56 (±3.3)	$P = 0.990$	230	1.02 (±1.2)	$P = 0.480$
rs1549250	G	202	4.48 (±3.1)	F(1, 474) = 0.2	128	1.16 (±1.4)	F(1, 312) = 0.9
	T	276	4.60 (±3.4)	$P = 0.672$	188	1.01 (±1.2)	$P = 0.337$
rs2973050	T	141	4.73 (±3.2)	F(1, 396) = 1.2	95	1.09 (±1.4)	F(1, 272) = 0.6
	C	259	4.34 (±3.3)	$P = 0.275$	181	0.96 (±1.2)	$P = 0.448$
rs11111	G	67	3.91 (±3.1)	F(1, 576) = 4.3	36	0.83 (±1.0)	F(1, 370) = 2.6
	A	513	4.74 (±3.3)	$\mathbf{P = 0.039}$	338	1.15 (±1.3)	$P = 0.110$

Nominally significant result is in bold.

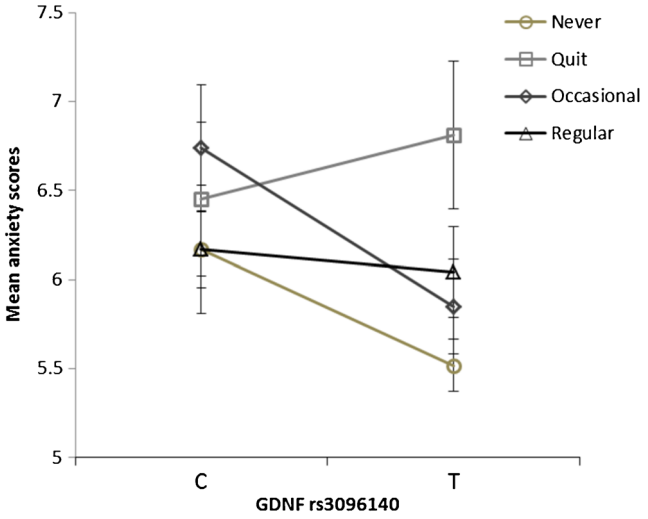

Figure 2. Effects of smoking behaviour and rs3096140 on anxiety. Mean HADS anxiety scores in the never (closed circles), quit (open squares), occasional (open rhombus) and regular groups (open triangles) as a function of rs3096140 C and T alleles. Error bars represent standard errors of the mean.

Discussion

The presented genetic association analyses were carried out on a sample of 930 healthy young adults. First, we compared the allele frequencies of the analysed eight *GDNF* polymorphisms (see table 2) in the four smoking categories. According to the results, there was a significant difference in the allele frequencies in case of rs3096140 ($P = 0.0046$): the C allele was more frequent in the smoker groups as compared to the nonsmokers (see figure 1), suggesting that the C allele is a risk factor for smoking initiation. Interestingly, the frequency of the C allele was the highest among smokers who quit (42%) and somewhat lower in the group of occasional smokers (33.3%) and regular smokers (32.1%). Even though the difference is more between the never smokers and quitter groups, it is clear, that the quitter, regular and occasional smoker groups show the same allele frequency pattern. Thus, we followed the standard genetic-epidemiological approach in terms of lifetime occurrence of a behaviour and analysed the allele frequencies between never and ever smokers. We suggest that although there were no significant difference in the analysis of never and regular smokers, the result between never users and lifetime occurrence of smoking behaviour (created by combining the quitter, occasional and regular categories) could still have an important impact on the literature or the genetic background of smoking behaviour, focussing on lifetime vulnerability of smoking.

We propose that the lack of association between the scales of nicotine addiction and GDNF rs3096140 also supports the importance of lifetime occurrence of the behaviour in genetic association analysis. The lack of association between rs3096140 and, never and regular smokers, and the lack of association between rs3096140 and nicotine addiction suggests that the association between rs3096140 and smoking is not based on the heaviness or 'regularness' of this behaviour, rather based on the vulnerability for smoking (based on the association between never and ever smoker categories). This supports the idea, that the association analysis of never–regular smokers and never–ever smokers are different, imply different phenotypes.

It seems that this association might not be driven by high nicotine addiction, rather by some kind of personality traits which predicts lifetime occurrence of the behaviour, e.g. trying out smoking and quitting. Further investigation of *GDNF* rs3096140 and, e.g. novelty seeking or impulsivity would be needed to clarify this association. We also tested if there is any association between *GDNF* polymorphisms and level of nicotine addiction, but no significant results were observed (see table 3). It is well known that smoking is a very complex phenotype and even though there are some overlapping genetic factors of the different aspects, phenotype specific genetic risk factors are also plausible. This is in line with the fact that different aspects of smoking behaviour have different level of heritability (e.g. Madden *et al.* 1999). For

example, it has been demonstrated that the heritability level of smoking initiation is 56% while the heritability level of nicotine addiction is 67% suggesting that the genetic variations that predispose to smoking initiation and to nicotine dependence have both common and specific factors (Sullivan and Kendler 1999).

GDNF is a novel candidate gene in psychiatric genetics, and there are only a few published association studies available on schizophrenia (Lee *et al.* 2001; Michelato *et al.* 2004; Williams *et al.* 2007), on attention deficit hyperactivity disorder (Syed *et al.* 2007; Laurin *et al.* 2008), on methamphetamine use (Yoshimura *et al.* 2011) and on mood characteristics (Kotyuk *et al.* 2013a, b). To the best of our knowledge, no genetic association study has been published between *GDNF* polymorphisms and smoking behaviour. On the other hand, in a Japanese study (Yoshimura *et al.* 2011) of *GDNF* alleles and the severity of methamphetamine use eight *GDNF* polymorphisms were analysed. The minor (C) allele of the rs2910704 SNP was significantly more frequent in those who used only methamphetamine compared to the multisubstance users. Our results are in line with these findings suggesting that variations in the *GDNF* gene might be related not only to methamphetamine use but also to smoking.

Regarding smoking and mood disorders, comorbidity is well supported (Covey *et al.* 1990; Balfour and Ridley 2000; Audrain-McGovern *et al.* 2009). Previously, we observed an association between *GDNF* rs3812047, rs3096140 and anxiety on healthy young adults (Kotyuk *et al.* 2013a) and we replicated the effect of rs3812047 on anxiety on a sample of bipolar depressive patients (Kotyuk *et al.* 2013b). On the sample of healthy young adults we demonstrated that the C allele of rs3096140 is a risk factor for higher anxiety level. The very same genetic factor was shown here as a risk factor for smoking. However, we did not find a significant interaction effect of rs3096140 and smoking categories on anxiety. Taken together, these results suggest that *GDNF* polymorphisms may have a crucial role in numerous neuropsychological diseases. Further investigation of *GDNF* gene variants and psychiatric diseases are needed to clarify these associations.

One of the limitations of the presented study is the relatively low sample size, especially in some of the smoking categories. It would also be desirable to repeat the present findings on a sample that is age matched and have same number of participants in each smoking group. Also, as in any self-report study, self-classification could lead to some limitations. For example, the similarities between rs3096140 allele distributions by the occasional and regular smokers might be unexpected. However, it is probably due to sample characteristics. The present sample mainly contains young healthy adults recruited at different Hungarian educational institutions, and the selection criteria included age range between 18 and 35 years. In this age population, it is possible that participants report themselves as occasional smokers at the time of data collection, even though they will not stay occasional smokers, and probably continue their smoking career and become regular smokers in time. This is also supported by the number of occasional smokers in the present sample ($N = 163$). It would be really hard to find this many true occasional smokers. However, in case of the standard genetic-epidemiological analysis this did not cause any distortions. In any case, repetition of the present analyses on independent samples is necessary. Further, there is a possibility for false positive results, although Bonferroni correction for multiple testing was used to avoid this.

In sum, the present study demonstrated an association between rs3096140 and smoking behaviour, suggesting that the T allele might be a risk factor for lifetime occurrence of smoking. However, further analysis is needed to clarify the possible relationship between *GDNF* polymorphisms and addictions, addictions and the possible moderating factors, such as anxiety.

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