1-1-2012

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10.1089/gtmb.2012.0016

GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 16, Number 9, 2012

Pp. 1044–1050
DOI: 10.1089/gtmb.2012.0016

Influence of Brain-Derived Neurotrophic Factor Genetic Polymorphisms on the Ages of Onset for Heroin Dependence in a Chinese Population

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Aim: The study aims at evaluating the association between brain-derived neurotrophic factor (BDNF) gene polymorphisms and heroin-dependent patients in the Chinese population. Three polymorphisms of the BDNF-gene (rs10835210, rs16917234, and rs6265) in 486 heroin-dependent patients and in 226 healthy controls were genotyped for analyzing the association of these polymorphisms with age of onset of heroin dependence. We defined the healthy cases as "unknown phenotype" and used the endophenotype (behavior traits) to stratify the heroin dependents group on the basis of self-reporting traits for examining the association between BDNF polymorphisms (rs10835210, rs16917234, and rs6265) and heroin dependence. *Results:* Allelic distributions of BDNF gene polymorphisms did not differ significantly between heroin-dependent patients and controls. However, we found that the AA carriers of BDNF rs6265 had an earlier onset of heroin dependence and a clearer tendency of family history of heroin-dependent than GG carriers after controlling behavior characteristics across rs6265 genotypes. *Conclusions:* Our findings suggested that the BDNF genetic polymorphism (rs6265) may have effects on the age of onset of heroin dependence among the Chinese population. The BDNF gene could contribute to vulnerabilities to heroin dependence.

Introduction

Heroin dependence is a problem of public health importance. The inducement of the disease is complicated. Twin and family studies have showed that genetic factors may interact with environmental factors, promoting heroin dependence and relapse after withdrawal (Kreek *et al.*, 2005; Li and Burmeister, 2009).

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin superfamily (Matsuo *et al.*, 2009), promotes neuronal survival, and regulates the proliferation and differentiation of nerve cells in the peripheral and central nervous systems (Hartmann *et al.*, 2001). BDNF serves an important role during brain development and in synaptic plasticity (Yang *et al.*, 2009). Data derived from animal studies have demonstrated that BDNF modulates dopaminergic and serotonergic functions that are heavily linked to substance abuse (Dluzen *et al.*, 1999; Lyons *et al.*, 1999). BDNF plays a role in hippocampal function and episodic memory in humans, because a common single-nucleotide polymorphism (SNP) (G196A or Val66Met. dbSNP: rs6265) resulting in a valine (Val) to methionine (Met) substitution in the prodomain has shown to effect intracellular trafficking and activity-dependent secretion of BDNF (Egan *et al.*, 2003; Chen *et al.*, 2004).

Recently, many studies have been carried out on investigating the genetic association of BDNF and drug dependence, including heroin, alcoholism, smoking, and methamphetamine. Although there are some positive results (Beuten *et al.*, 2005; Cheng *et al.*, 2005; Lang *et al.*, 2007; Zhang *et al.*, 2006; Wojnar *et al.*, 2009), opposite or null effects have also been reported (Tsai *et al.*, 2005; Matsushita *et al.*, 2006; Itoh *et al.*, 2005).

The chromosomal markers whose alleles distinguish drug dependents from controls were identified on the basis of pooled-sample microarray, suggesting that a dinucleotide-repeat polymorphism at the 5′ end of the main coding exon of BDNF was associated with vulnerability to poly-substance abuse (Uhl *et al.*, 2001). For heroin dependence, the first evidence was a study that compared the genotypes of 200 heroin-dependent cases with 122 controls of Han Chinese men (Cheng *et al.*, 2005). Significant differences in the BDNF

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Val66Met genotype distribution were found between the heroin-dependent subjects ($p = 0.045$) and controls. Meanwhile, in the heroin-dependent group, the Val/Val homozygote had a later onset of substance abuse compared with the Met allele carriers, suggesting that the BDNF Val66Met polymorphism or a nearby locus might be involved in the pathogenesis of heroin dependence (Cheng *et al.*, 2005). The findings just mentioned suggest that BDNF may represent an appropriate candidate gene that confers risk for substance abuse and dependence.

The investigation of endophenotypes is an emerging approach in genetic association studies (Hejjas *et al.*, 2007), as it holds promise for discovering the complex genetic background of multifactorial phenotypes such as psychiatric disorders. Gottesman and Shields (1973) described "endophenotypes" as internal phenotypes discoverable by a "biochemical test or microscopic examination". The theories and research patterns of endophenotypes, including cue-elicited heroin craving, the ages of onset for heroin dependence, certain behavior traits, and so on, were introduced into the field of heroin research in established studies (Franke *et al.*, 2001; Li *et al.*, 2006; Shao *et al.*, 2006). As one of the most complex diseases, phenotypes of the heroin dependence can be analyzed using a self-reported questionnaire, including the heroin-induced subjective responses and the process involved in dependence.

Therefore, we hypothesized that the BDNF gene polymorphisms would be associated with a certain process in heroin dependence or some heroin-induced subjective responses. The healthy volunteers were defined as "unknown phenotype" (the people who have no chance to get heroin), and the endophenotype was used to stratify the case group in this study. The investigation of association between BDNF genes (rs6265, rs16917234, and rs10835210) and the endophenotype could be helpful in understanding the mechanism of genetic susceptibility of heroin dependence.

Materials and Methods

Recruitment and selection of participants

The heroin dependents in the Shanghai Voluntary Drug Dependence Treatment Center in China were recruited. Eligibility criteria included age 18 years or older; diagnosed as "opioid dependence" according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Healthy volunteers were recruited as healthy controls from a medical examination center. All controls had neither past history, nor a family history of drug dependence and psychiatric diseases. The entire samples were of Han Chinese origin. The study was approved by both ethnic committees of the Shanghai Mental Health Center and the Chinese Academy of Sciences.

Phenotype

The traits were measured by a self-reported questionnaire, including a comfortable response on the first use of heroin, age at onset, times of heroin dependence preceding the first occurrence of pleasant feeling, the frequency and amount of heroin used per day, the duration when a person transformed the first use to dependence, and the occurrence of tolerance. Moreover, we recorded whether a heroin-dependent had been poisoned due to an overdose of heroin. The endophenotypes were derived on the basis of self-reporting behavioral traits. In addition, the healthy cases were defined as an "unknown phenotype."

Genotype

The tSNPs were selected with the tagging algorithm with criteria of $r^2 > 0.8$ and a minor allele frequency ≥ 0.1 by the Haploview program (version 4.2).

Genomic DNA was extracted from 200 μL venous blood with the QIAamp DNA Blood Mini Kit (Qiagen, Beijing, China) according to the manual instructions. We genotyped the DNA sequence variants of the BDNF gene using the MassARRAY system (Sequenom, Inc., San Diego, CA). The primer sequences are shown in Table 1. PCRs were performed in a total volume of 5 μL containing 1 μL DNA, 0.2 μL HotStar Taq polymerase (5 U/μL), 0.4 μL $MgCl_2$ (25 mM), 0.5 μL polymerase chain reaction (PCR) buffer (10×), 0.1 μL dNTPs (2.5 mM), 1.8 μL H_2O, and 1 μL of the designed primers. The PCR conditions were as follows: 95°C for 2 min followed by 45 cycles of 95°C for 20 s, 56°C for 30 s and 72°C for 1 min, and then an extension at 72°C for 5 min. The samples were kept at 4°C.

The products were treated with shrimp alkaline phosphatase to remove excess dNTPs after PCR. The PCR products were used as templates for the primer extension reactions using the iPlex1 Gold reagent kit (Sequenom, Inc.). The products were spotted onto the MassARRAY Spectro CHIP with an auto-spot arm and air dried. The target plate was then inserted into the MALDI-TOF mass spectrometer of the MassARRAY Compact System (Sequenom, Inc.). Genotypes were automatically identified by the MassARRAY TYPER software (version 3.4, Sequenom, Inc.).

Statistical analysis

Allelic frequencies between different phenotypes of the case group and "unknown phenotype" of the health controls were compared by the chi-square test or the Fisher's exact test where necessary. The onset age between different phenotypes was compared by the *t*-test or one-way analysis of variance test. The Student–Newman–Kuls (SNK) test was used for multiple comparisons. The significance for the results was set at $p < 0.05$.

Results

Sample characteristics

A total of 486 heroin-dependents and 226 healthy volunteers (composed of control group) were enrolled in the study (Table 2). The mean age of controls (37.47 ± 17.85) was older than that of cases (33.30 ± 7.82). Men were 46.91% (228) and 46.46% (105) in cases and controls, respectively. In addiction, the mean of onset age of drug dependence was 23.56 ± 7.89 years, and the average duration of dependence to heroin was 9.73 ± 6.87 years.

Heroin-induced behaviors

The heroin-induced behaviors were interviewed face to face, including reason for the first use of heroin, type of dependence, subjective response to early opiate use, magnitude of euphoria, feeling of rush, intention to abstain from dependence, and other details (Table 3).

Table 1. Primer Sequences Used for Genotyping of the Single-Nucleotide Polymorphisms

SNPs	*Forward primers*	*Reverse primers*	*Extension primers*
rs10835210	ACGTTGGATGACATCTCTTGAACTCAGTCC	ACGTTGGATGCAACACACTGTGTATAAGGC	GAACTCAGTCCTGAAAATAA
rs16917234	ACGTTGGATGGCATCCCACTCTATAAATTTC	ACGTTGGATGCTCCTGCATTAAGCTACAAG	TGCATTAAGCTACAAGTAATGA
rs6265	ACGTTGGATGCATCATTGGCTGACACTTTC	ACGTTGGATGTTTTCTTCATTGGGCCGAAC	CCAACAGCTCTTCTATCA

SNP, single-nucleotide polymorphism.

Table 2. Demographic Characteristics of the Heroin Dependents and Controls in Han Chinese

	Case (n = 486)	*Control (n = 226)*	*Statistical index*	p
Age (years)	33.30 ± 7.82	37.47 ± 17.85	$t = 3.36$	0.0009
Men (%)	228 (46.91)	105 (46.46)	$\chi^2 = 0.0127$	0.9101

Genotyping quality control

Genotypes for the three BDNF SNPs were carried out by duplicates. A deviation from the Hardy–Weinberg equilibrium was not observed in any locus except for rs16917234 ($p = 0.016986$). The genotype data at this locus were re-examined carefully, and the possibility of genotyping error was excluded.

Association of BDNF SNPs and heroin dependence

No significant differences were found in the frequencies of the genotype or allele in the three BDNF SNPs between the heroin-dependent group and controls (rs10835210 genotype, $p = 0.9822$, allele, $p = 0.85956$; rs16917234, genotype $p = 0.0705$, allele, $p = 0.42989$; rs6265, genotype $p = 0.4408$, and allele $p = 0.19538$) (Table 4).

The effect of BDNF genetic polymorphisms on the age of onset of heroin dependence

The mean age of onset (in years) for the bearer groups were 22.32 ± 6.22 for BDNF (rs6265) AA ($n = 117$), 24.19 ± 6.38 for BDNF (rs6265) GG ($n = 130$), and 22.77 ± 6.19 for BDNF (rs6265) AG ($n = 230$). The onset age was significantly different among the three genotypic groups ($p = 0.0423$). The AA carriers had an earlier onset of heroin dependence than did GG carriers by a further analysis with the SNK test (Table 5).

In addiction, the CC and CT carriers of BDNF (rs16917234) had a later onset compared with the TT carriers, although these values did not reach the power of statistical significance, equaling to 25.09 ± 4.91, 22.65 ± 6.13 and 23.92 ± 6.66, respectively. Meanwhile, the mean ages of onset for the C- and T-allele bearer groups were 24.01 ± 6.53 and 23.00 ± 6.30, respectively.

Association with heroin-induced behaviors

The associations between different heroin-induced behaviors and the allelic distributions of BDNF SNPs were not statistically significant except for "peer" behavior. A significant difference was found in the frequencies of the genotypes in rs6265 between the "alone" group and the "with family" group ($p = 0.0489$), and the carriers of GG had a less common family history of drug dependence than the carriers of AA did (Table 6).

Discussion

The results of our present study demonstrated that different genotype carriers of BDNF had a different onset age of heroin dependence. Although no significant differences were found in the frequency of the genotype and allele between heroin dependents and controls (rs10835210 genotype: $p = 0.9822$, allele, $p = 0.85956$; rs16917234 genotype: $p = 0.0705$,

Table 3. Behavior and Clinical Characteristics of 486 Heroin-Dependent Subjects

	1	*2*	*3*	*4*	*Missing values*	
Type of dependence	66 (14.44)	362 (79.21)	29 (6.34)		29	1: smoking 2: injection 3: both
Reason for first use of heroin	30 (6.29)	337 (70.65)	23 (4.82)	87 (18.24)	9	1: physical 2: psychologic 3: environment 4: combined
Subjective response to early opiate use	340 (69.96)	73 (15.02)	73 (15.02)		0	1: uncomfortable 2: euphoria 3: unclear
Magnitude of euphoria	89 (18.31)	118 (24.28)	279 (57.41)		0	1: intense 2: moderate 3: mild
Feeling of rush	330 (67.90)	156 (32.10)			0	1: yes 2: no
Peer	328 (67.63)	137 (28.25)	20 (4.12)		1	1: alone 2: friends 3: family
Intention to abstain from dependence	148 (30.64)	327 (67.7)	8 (1.66)		3	1: yes 2: unclear 3: no
Toxic from overdose	187 (38.48)	299 (61.52)			0	1: yes 2: none

allele, $p=0.42989$; rs6265. genotype: $p=0.4408$. allele, $p=0.19538$), we observed that AA carriers of BDNF rs6265 had an earlier onset of heroin dependence and more family history than GG carriers ($p=0.0423$ and $p=0.0489$ respectively). In addition, TT carriers of BDNF rs16917234 had an earlier onset of heroin dependence compared with CC carriers, although the values did not reach statistical significance ($p=0.0798$). Our study showed that the BDNF genetic polymorphism may have effects on the age of onset of heroin dependence among the Chinese population. Our findings are consistent with Cheng's study (Cheng *et al.*, 2005), suggesting that the BDNF genetic polymorphism may affect the age of onset of substance dependence, and the Met allele may be a risk factor for early-onset substance dependence. However, there was controversy among reports. Regarding the early onset of heroin dependence, no significant difference was observed between GA carriers and GG or AA carriers at the BDNF rs6265 locus (Hou *et al.*, 2010); whereas Jia *et al.* (2011) reported that the onset of dependence was significantly earlier in individuals with GG or GA genotypes compared with AA individuals

Table 4. Allelic and Genotype Frequencies for Heroin-Dependent Patients and Controls for Single-Nucleotide Polymorphisms rs10835210, rs16917234, and rs6265

			No. (%)			
			Control	*Case*	*Chi-square*	p *value*
rs10835210	Genotype	AA	18 (7.96)	38 (7.82)	0.0359	0.9822
		CC	115 (50.88)	251 (51.65)		
		CA	93 (41.15)	197 (40.53)		
	Allele	A	129 (28.54)	273 (28.09)	0.0300	0.85956
		C	323 (71.46)	699 (71.91)		
rs16917234	Genotype	CC	13 (5.75)	12 (2.47)	5.3032	0.0705
		TT	156 (69.03)	336 (69.14)		
		CT	57 (25.22)	138 (28.40)		
	Allele	C	83 (18.36)	162 (16.67)	0.6200	0.42989
		T	369 (81.64)	810 (83.33)		
rs6265	Genotype	AA	46 (20.35)	117 (24.07)	1.6385	0.4408
		GG	71 (31.42)	135 (27.78)		
		AG	109 (48.23)	234 (48.15)		
	Allele	A	201 (44.47)	468 (48.15)	1.6800	0.19538
		G	251 (55.53)	504 (51.85)		

Table 5. Different Genotypes and Alleles on Onset Age of Heroin Dependence

			N	*Mean*	*Standard deviation*	*F/t*	p *value*
rs10835210	Genotype	AA	38	23.87	6.06	0.35	0.7021
		CC	246	23.00	6.48		
		CA	193	22.95	6.08		
	Allele	A	231	23.10	6.07	0.24	0.8124
		C	439	22.98	6.30		
rs16917234	Genotype	CC	11	25.09	4.91	2.54	0.0798
		TT	335	22.65	6.13		
		CT	131	23.92	6.66		
	Allele	C	142	24.01	6.53	1.65	0.0996
		T	466	23.00	6.30		
rs6265	Genotype	AA	117	22.32[a]	6.22	3.18	0.0423
		GG	130	24.19[a]	6.38		
		AG	230	22.77	6.19		
	Allele	A	347	22.62	6.19	1.41	0.1580
		G	360	23.29	6.29		

[a]Further analysis with SNK test revealed that onset age of AA carriers was earlier than that of GG carriers.
SNK, Student–Newman–Keuls.

among heroin-dependent individuals. These differences in the reported studies might be influenced by sample size and genetic heterogeneity. The subjects in Hou's study were 96 heroin-dependent individuals, and such a small sample size of the study may limit the generalizability of the results. The study outcome might be as a result of the stratification effect in the sample collection as well. In Jia's report, the large sample size (case number 487; control number 492) provided sufficient data that clarify the relevance of the polymorphisms in the populations from the northwest part of China. The cases and controls in our study were chosen from Shanghai, residents in the south-eastern part of China. In addition, the disparity in findings between our study and the other investigations may reflect the differences in the other considerable factors associated with the onset age of heroin dependence, such as socioeconomic status, perceived peer use, and accessibility of substances. The existence of other susceptibility genes to heron dependence could not be excluded either.

The investigation of the genetic basis for the onset of drug dependence is very important on account of the early age at the first use, which is a marker of elevated risk for developing drug use disorders (Sartor *et al.*, 2009). There were conflicting heterogeneities among studies that probed the relationship between the BDNF polymorphism and heroin dependence. If the polymorphism does not have a direct effect, but is rather in linkage disequilibrium with the true associated allele to varying degrees in different populations, inconsistent associations with the polymorphism could be the result due to population differences in linkage disequilibrium patterns (Petryshen *et al.*, 2010).

BDNF has important roles in neural development, cell survival, and synaptic plasticity. The lower-activity allele (Met66) was associated with reduced cognitive performance (Cheng *et al.*, 2005). However, the higher-activity BDNF allele (Val66) was associated with BPD (Neves-Pereira *et al.*, 2002) and substance abuse (Cheng *et al.*, 2005; Sim *et al.*, 2010). Chronic drug exposure increased BDNF levels in the ventral tegmental area neurons (Vargas-Perez *et al.*, 2009). The 66Met allele of the Val66Met polymorphism of the BDNF gene was associated with lower BDNF secretion in response to neuronal stimulation compared with the 66Val allele in human beings (Tsai, 2007). Thus, subjects carrying the Val allele may have higher intracranial BDNF activity than people who are Met/Met homozygous (Egan *et al.*, 2003). Therefore, 66Val carriers have an increased euphoric effect after drug abuse, which makes them more vulnerable to have drug abuse. Previous studies found that the Met allele might be a risk allele in the development of obsessive–compulsive disorder (OCD) and was associated with an earlier age at onset of OCD in men (Hemmings *et al.*, 2008; Rocha *et al.*, 2010). These findings suggested that the BDNF Val66Met polymorphism may have genetic pleiotropy, which is the ability of a mutation in a single gene to give rise to multiple phenotypic outcomes, and, thus, simultaneously convey separate advantageous traits and disadvantageous traits in the same organism (Hong *et al.*, 2011). One research indicated the lack of a significant frequency difference of the SNPs between heroin dependents and control subjects in Japan (Itoh *et al.*, 2005), whereas the results were opposite in the study of a Taiwan population (Cheng *et al.*, 2005). In addition, there was a trend toward higher BDNF 66Val homozygote frequency in both European- and African-American polysubstance abusers than in controls, although these values did not reach statistical significance (Liu *et al.*, 2005). The inconsistent findings apparently may be attributable to the lack of proper control over multi-factors influencing heroin dependence in real-world settings.

Table 6. Genotype Frequencies for the Brain-Derived Neurotrophic Factor Genetic Polymorphism in Different Peers

		No. (frequency %)				
SNPs	*Genotype/allele*	Alone	With family	Control	*Chi-square*	p *value*
rs10835210	AA	28 (8.54)	1 (5.00)	18 (7.96)	1.5986	0.8090
	CC	169 (51.52)	13 (65.00)	115 (50.88)		
	CA	131 (39.94)	6 (30.00)	93 (41.15)		
rs16917234	CC	10 (3.05)	0 (0)	13 (5.75)	4.4758	0.3454
	TT	225 (68.6)	16 (80.00)	156 (69.03)		
	CT	93 (28.35)	4 (20.00)	57 (25.22)		
rs6265	AA	81 (24.70)	8 (40.00)	46 (20.35)	6.0349	0.0489
	GG	96 (29.27)	1 (5.00)	71 (31.42)		
	AG	151 (46.04)	11 (55.00)	109 (48.23)		

The definition of phenotypes is a major problem for any genetic study of dependence. For most genetic association studies, a control group is drawn from the majority of a population that has not been exposed to the drug. The power to detect the genetic effect on drug dependence is lowered by the use of unscreened controls. A suitable control group for a genetic associate study would not be the general population, but rather individuals with the same environmental background in order to control for this major effect that may otherwise swamp the genetic effect (Buckland, 2008). Given this background, it is even less easy to find the samples who have been sufficiently exposed to heroin but are clearly not addicted.

As for the "peer" behavior, we also observed that the frequencies of the genotype in rs6265 between the "alone" group and "with family" group ($p = 0.0489$) were significantly different. Our results suggested that the carriers of AA of BDNF rs6265 had a more common family history of drug dependence than the carriers of GG did.

In the present study, we, therefore, defined the healthy cases as "unknown phenotype"; and used "endophenotype" to stratify the heroin dependents group; and aimed at looking for the exact association between the BDNF gene (rs10835210, rs16917234, and rs6265) and heroin dependents for discovering the complex genetic background of multi-factorial phenotypes. We used the endophenotype, classified by the self-reported behaviors or objective responses on heroin dependence, to split heroin dependents into two groups. For instance, those who mainly reported rush responses on the use of heroin were classified as being endophenotype positive; while others who reported no rush responses were classified as being endophenotype negative. Most associations between different heroin-induced behaviors with the genotype and allele distribution of BDNF SNPs were not significant statistically except for "peer" and onset age of heroin dependence.

There are limitations in this study. Heroin-induced behavioral traits were measured by a self-reported questionnaire. The recall bias might have been inevitably involved when the information was collected based on the recall of subjective reactions that occurred in a distant past. Other suitable types of endophenotypes for heroin dependence still need to be found, such as P300 event-related potential (Iacono *et al.*, 2000), personal traits (Ebstein *et al.*, 1996), and withdrawal symptoms (Sander *et al.*, 2006). The imbalance of sample size would impact the results of between-group analysis. However, it does not impact the association between the SNP site and phenotype.

In conclusion, the BDNF gene could contribute to vulnerabilities to heroin dependence. AA carriers (BDNF rs6265) had an earlier onset of heroin dependence than did GG carriers ($p = 0.0423$). The CC carriers of BDNF rs16917234 had a later onset of heroin dependence compared with the TT carriers, although these values did not reach statistical significance ($p = 0.0798$). Our findings suggested that BDNF genetic polymorphisms (rs6265 and rs16917234) may have effects on the age of onset of heroin dependence among the Chinese Han population. To our knowledge, this is the first attempt in mainland China that simultaneously examines the impact of BDNF genetic polymorphisms rs6265 and rs16917234 on the onset age of heroin dependence. In addition, we did not find any association between SNP rs10835210 and heroin dependence, and, therefore, this polymorphism unlikely plays a major role in the pathogenesis of heroin dependence in our Chinese Han sample. A further study conducted on independent samples is needed to confirm these findings.

Acknowledgments

This research was supported by the National Twelfth Five-Year Plan for Science and Technology Support (2012BAI37B03), the Major State Basic Research Program-973 of China (2005CB522801; 2011CB503806), the National Natural Science Foundation of China (30670889, 30901238, 31070727, and 81001281), the Funding Project for Academic Human Resources Development in Institutions of Higher Learning Under the Jurisdiction of Beijing Municipality (PHR201008393), and the National High Technology Research and Development Program-863 of China (2006AA02Z434). The authors thank the patients and volunteers for their support and participation.

Author Disclosure Statement

No competing financial interests exist.

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