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An Association Between Epac-1 Gene Variants and Anxiety and Depression in Two Independent Samples

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Deficiency in signal transduction might play a role in the development of anxiety and depression, as suggested by a study on the involvement of the PKA-independent Epac pathway. We investigated the association between Epac-1 gene variants, also known as RapGEF-3, and measures of anxiety and depression in a Dutch twin-family sample. Replication was sought in a USA sample consisting of unrelated individuals. Genotype and phenotype data were available for 910 Dutch and 684 USA individuals. Longitudinal self-report measures of neuroticism, anxiety and depression and genetic factor scores (GFS-NL), based on these measures, were analyzed in the Dutch sample. In the USA sample, neuroticism and Genetic Factor Scores (GFS-USA), based on neuroticism and diagnoses of anxiety disorders and depression, were analyzed. Three intronic SNPs were genotyped. Analyses were performed in QTDT. Genotype and haplotype frequencies differed significantly between the samples. In the Dutch sample, rs2072115 showed a significant dominant effect for anxiety and depression. Subjects with haplotype G-C-C (ordered rs2072115-rs757281-2074533) had significantly lower anxiety, neuroticism and GFS-NL scores. In the USA sample, a significant additive effect of rs2074533 on GFS-USA was found. Subjects with haplotypes G-C-C and A-C-T had significantly higher and lower GFS-USA scores, respectively. Both samples showed an association between Epac-1 gene variants and anxiety and depression, but for different variants or in opposite directions. The divergent results could be due to differences in linkage disequilibrium between the investigated SNPs and a functional polymorphism in the Dutch and USA sample.

Key words: RapGEF-3; association study; anxiety; depression

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INTRODUCTION

Despite efforts, the success of gene finding studies for anxiety and depression is still rather limited [Levinson, 2006; Stoppel et al., 2006]. One of the many possible reasons for the lack of success is that until now association studies have mostly focused on a small group of candidate genes, that is mainly involved in monoaminergic neurotransmission [Levinson, 2006]. But the etiology of these disorders might lie elsewhere. One plausible pathway would be genes involved in signal transduction [Shelton, 2007].

This signal transduction hypothesis is supported by studies that found associations between polymorphisms in the Regulator of G Protein Signaling 2 (RGS-2) gene and the apoptosis protease activating factor-1 (APAF-1) gene with, respectively, anxiety and depression [Harlan et al., 2006; Leygraf et al., 2006]. Furthermore, a decreased activation and expression of Rap-1 was shown in the prefrontal cortex and hippocampus of depressed suicide victims [Dwivedi et al., 2006]. Rap-1, in its activated form, is involved in several important physiologic functions: cell proliferation and survival, cell adhesion and differentiation, as well as plasticity [Bos et al., 2001; Zhu et al., 2002]. Rap-1 is activated by Protein Kinase A (PKA) ánd by cAMP through a protein called Epac

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(derived from exchange protein directly activated by cAMP) [de Rooij et al., 1998; Kawasaki et al., 1998]. The Epac-family consists of two genes: Epac-1 and Epac-2, also known as Rap Guanine nucleotide Exchange Factors 3 and 4 (RapGEF-3 and RapGEF-4). Epac-1 is ubiquitously expressed and Epac-2 is predominantly expressed in the brain and adrenal glands [Kawasaki et al., 1998]. By activating the PKA-independent Epac pathway, cAMP facilitates neurotransmission [Kaneko and Takahashi, 2004]. The same study that found decreased activation and expression of Rap-1 showed increased protein levels of Epac-2, but not Epac-1 in the prefrontal cortex and hippocampus of depressed suicide victims while the mRNA levels of Epac-1 and 2 together in these brain regions were normal [Dwivedi et al., 2006]. They had no hypothesis about the difference between the results for Epac-1 and Epac-2.

The importance of Epac for brain function has been recently confirmed by a study showing that Epac signaling is required for hippocampus-dependent memory retrieval [Ouyang et al., 2008]. In this study, no distinction was made between Epac-1 and 2. Given these results, it seems timely to investigate the role of genes involved in cellular processes in the development of anxiety and depression. As Epac-1 and 2 are both expressed in brain regions associated with depression, such as the hippocampus and the amygdala, both seem equally likely to be involved in the development of these symptoms. We present a study investigating the association between three polymorphisms in the Epac-1 gene and several indicators for anxiety and depression in a Dutch sample. Replication was sought in an independent sample from the USA. In both samples, haplotype analyses were also performed.

MATERIALS AND METHODS

Subjects

The Dutch sample consisted of twins and their family members selected from the Netherlands Twin Register. Families were selected with sibling pairs scoring concordant (high–high) or discordant (high–low) for nicotine dependence. Subjects who scored low smoked but were not nicotine dependent. Once a sibling pair was identified, all registered family members, regardless of their scores, were approached to provide a DNA sample. A thousand and eight subjects returned their DNA. The current study was restricted to individuals with phenotypic data and aged between 16 and 65 years at the time of assessment, resulting in a sample of 914 individuals from 301 families. The sample included 42 men and 94 women from a complete monozygotic twin pair, 203 brothers and 356 sisters (including dizygotic twins) and 100 fathers and 119 mothers. Additionally, genotypic, but not phenotypic data were available for 14 fathers and 27 mothers. These data were used to estimate the haplotypes.

The USA replication sample, further referred to as the USA sample, was drawn from two large population-based twin studies of the Mid-Atlantic Twin Registry (MATR). The sampling and ascertainment procedures for this study have been described elsewhere [Kendler and Prescott, 2006]. In this study, all subjects were unrelated. The sample was originally used for studying nicotine dependence using a three-group design: non-smokers (n = 244, 164 men and 80 women), defined as those who never smoked a cigarette up to the time of the assessment; regular smokers with low nicotine dependence (n = 215, 151 men and 64 women) and regular smokers with high nicotine dependence (n = 229, 150 men and 79 women).

Table I shows the descriptives of the samples used for the association analysis. The Dutch and USA samples only differed in their sex distribution. The Dutch sample consisted for 62% of women, while the USA sample consisted for 32% of women.

Instruments

The Netherlands. Association analyses were performed on self-report anxiety, neuroticism and depression scales measured as part of a longitudinal survey and on genetic factor scores (GFS-NL) based on these self-report data. In 1991, 1993, 1997, 2000, and 2002, anxiety was measured with the Spielberger State Trait Anxiety Inventory-Trait version (STAI) [Spielberger et al., 1970; Van der Ploeg et al., 1979] and neuroticism with the Amsterdamse Biografische Vragenlijst (ABV) [Wilde, 1970]. The 30-item neuroticism scale of the ABV is modeled after the neuroticism scale of the Eysenck Personality Questionnaire [Eysenck and Eysenck, 1964]. In 1991, 1995, 1997, 2000, and 2002, anxious depression was measured with the Young Adult Self Report (YASR) [Achenbach, 1990; Verhulst et al., 1997). In 1993 and 1997, depression was assessed with the Beck Depression Inventory [Beck et al., 1974]. The scores were transformed following earlier analyses of these data [Boomsma et al., 2000]. Log transformations were used for the anxiety, neuroticism and anxious depression scales. An arcsin transformation was used for depression measured with the BDI. This did not result in a normal distribution, but significantly reduced kurtosis.

The formula to calculate GFS-NL was derived from a multivariate genetic analysis on self-report anxiety, depression, neuroticism and somatic-anxiety data collected in twins and their siblings. Somatic anxiety was measured with the ABV [Wilde, 1970]. This analysis revealed that covariances for these traits could be fully attributed to a common genetic factor [Boomsma et al., 2000]. The value on this common genetic factor can be estimated for each individual using the individual scores on the traits and a weight matrix that depends on the factor loadings on the common genetic factor. Since the factor loadings on the common genetic factor were different for males and females, the formulae to estimate the genetic factor score were different for males and females. More detailed information on the factor scores is described elsewhere [Boomsma et al., 2000].

Cross-sectional correlations between the scores on the neuroticism, anxiety, anxious depression and depression scales varied from 0.48 to 0.75 [Middeldorp et al., 2006]. In a subsample of subjects (N = 1,255), lifetime diagnoses were assessed for major depression,

TABLE I. Descriptives of the Dutch and USA Sample

	Dutch	USA
N men/N women	345/569	462/222
Mean age (SD)	41 (12.1)[a]	37 (8.5)
Ancestry	European	European

[a]Age was averaged across occasions for subjects with more than one measurement.

panic disorder and/or agoraphobia, social phobia and generalized anxiety disorder using the Composite International Diagnostic Interview (CIDI) [World Health Organization, 1992]. Subjects with any diagnosis scored significantly higher on anxiety, neuroticism and depression than subjects without a diagnosis [Middeldorp et al., 2006]. The correlation between GFS-NL and neuroticism, anxiety and depression scores varied between 0.67 and 0.92 with the highest correlation with neuroticism.

Analyses were carried out on the mean scores over the occasions, which have the advantage of using all the available data while minimizing measurement error and the complexity of the statistical analysis.

USA. The replication study was carried out on neuroticism measured with the 12 items from the short form of the Eysenck Personality Questionnaire [Eysenck and Eysenck, 1975] and on Genetic Factor Scores (GFS-USA) reflecting an individual's shared genetic susceptibility across major depressive disorder, generalized anxiety disorder, panic disorder, agoraphobia, social phobia and neuroticism. Calculation of GFS-USA scores is described in detail in Hettema et al. [2006a,b]. In brief, a multivariate genetic analysis [Kendler et al., 1992; Neale and Cardon, 1992] was used to identify a latent phenotype reflecting the shared genetic susceptibility across these disorders and neuroticism. Two common genetic factors were included in the model. The first factor, the relevant one for this study, represented the portion of genetic covariation among the psychiatric disorders due to the genes for neuroticism while the second factor accounted for genetic influences that increase the covariation independent of neuroticism. The correlation between neuroticism and GFS-USA is 0.79.

Genotyping and Statistical Analyses

Genomic DNA was isolated from buccal swabs collected from the subjects by a protocol reported previously [Meulenbelt et al., 1995]. The Epac-1 (or RapGEF3) gene is located on chromosome 12q12-12q13.12, is about 20.5 kb, and contains 28 exons. Single nucleotide polymorphism (SNP) markers were selected from SNP database (dbSNP) at the National Center for Biotechnology and Information (http://www.ncbi.nlm.nih.gov/SNP/index.html). There are about 30 SNPs listed in the dbSNP for the gene. For an earlier association study on smoking and nicotine dependence in the USA sample, 12 SNPs were selected (roughly 2 kb/SNP in the gene itself plus 1 SNP in the promoter and 1 SNP downstream of the gene) to test with the FP-TDI protocol [Chen et al., 1999; Chen, 2003]. Seven of these SNPs were not used in the analysis, as two failed PCR, two significantly deviated from Hardy–Weinberg Equilibrium (HWE), and three had genotype failure rate >35%. Three of the five remaining SNPs, rs2072115, rs757281, and rs2074533 showed a significant association with smoking and were also genotyped in the Dutch sample. SNPs rs2072115, rs757281, and rs2074533 cover a segment of 10,673 base pairs of the gene (2,950 bp between rs 2072115 and rs757291 and 7732 base pairs between rs757281 and rs 2974553. SNPs are listed in order of transcription), from introns 3 to 20. All three SNPs are intronic.

For the Dutch sample, genotyping was conducted with pyrosequencing. A two-step PCR was used to amplify the samples. The primary PCR primers had common tails at their 5′ ends. The secondary primers were the common tails of the primary primers. To facilitate purification, one of the tails was labeled with biotin at the 5′ end. The sequences for the two primary PCR primers and one pyrosequencing primer for rs2072115 were cggtgcgcgtcgctcaggtgg-cagggagcagcaggaactatgc; tccgatatcccgggtcgtgcctccttgcccagcctcact and agggggatggaggaactatg, respectively. The sequences for rs757281 were cggtgcgcgtcgctcaggtaaggtgggcagcggctggctaat; tccgatatcccggg-tcgtcatggaccacccaatgagtcagaa and tgggatggggctggctaa. The sequences for rs2074533 were cggtgcgcgtcgctcaggagcctcttcggatgtatccacca; tccgatatcccgggtcgtgcccagcacatagtggatcagctc and ttcggatgtatccaccagg. The sequences of the common tails were biotin-tccgatatcccgggtcgt and cggtgcgcgtcgctcagg. PCRs were conducted in 10 μl of volume containing 10 ng of genomic DNA, 50 nM of each primer, 200 μM dNTPs, 2.5 mM $MgCl_2$ and 0.5 unit DNA polymerase and thermocycled for 10 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec. The reaction was paused to add 10 μl of reaction mixture containing 0.25 unit DNA polymerase, 200 nM tail primers and 200 μM dNTPs and resumed for 25 more cycles. Manufacture's protocols were used for template cleanup and pyrosequencing reaction. The genotypes were scored by the company's software and checked for Mendelian errors and Hardy–Weinberg equilibrium. On average 90% of the 1008 subjects were genotyped for the three SNPs.

For the USA sample, genotyping was carried out using FP-TDI protocol [Chen et al., 1999; Chen, 2003] with minor modification in PCR amplification. PCRs were performed in 384-well plate, with a reaction volume of 12 ml. The first reaction was of 10 ml, containing 5 ml of genomic DNA solution (the amount of DNAs varied between 2.0 and 2.5 ng from sample to sample), 100 nM of each PCR primer, 1_ HotMaster Taq Buffer, 25 mM dNTPs, 0.55Uof HotMaster Taq DNA Polymerase (Eppendorf Corp., Westbury, NY). PCR primers for rs2072115 were ttctagcacagggacgaacc and ggaaggtagaaggggacagg. FP-TDI primer was ctcagagggtgcctttctaa. For rs757281 and rs2074533, PCR and FP-TDI primers were ccctcctttcatttcccaat, acacctgggcagacatcaat, gtgggacagggctggctaat; ggacctggcaggccagctga, tgagtccagggagagcaggc and agaggctgactcag-taagagtcattt, cagtgctattcttatgaccaccaag, ttttagagtgatttagccatgcgctc, respectively.

Statistical power analyses were performed in Quanto [Gauderman and Morrison, 2006]. Differences between genotype and haplotype frequencies in the Dutch and USA sample were analyzed using χ^2 tests. Associations between the three SNPs and the traits were investigated in QTDT using the test that models total association with sex included as a fixed effect [Abecasis et al., 2000]. QTDT has an option to account for the dependency among individuals from the same pedigree as a function of their genetic relatedness in a covariance structure model.

Haplotypes were constructed with SIMWALK2 [Sobel and Lange, 1996] and HAPLOTYPER [Niu et al., 2002] for the Dutch and USA sample respectively. Single haplotype associations with the phenotypes were investigated in QTDT, again with sex included as a fixed effect.

RESULTS

In both samples, means and standard deviations of the anxiety and depression related phenotypes were similar in the genotyped

TABLE II. N (%) Subjects and Mean Transformed Scores per Genotype for Neuroticism (Neur) (SD = 2.7), Anxiety (Anx) (SD = 2.5), Anxious Depression (Anx Dep) (SD = 9.4), Depression (Dep) (SD = 1.8) and the Genetic Factor Score (GFS-NL) (SD = 0.80) in the Dutch Sample (columns 3–8) and N (%) Subjects and Mean Score per Genotype for Neur (SD = 3.1) and the Genetic Factor Score GFS-USA (SD = 0.68) in the USA Replication Sample (columns 9–11)

SNP (Total N Dutch/USA)		N (%) Dutch	Neur	Anx	Anx Dep	Dep	GFS-NL	N (%) USA	Neur	GFS-USA
rs2072115 (828/663)	AA	516 (62.3%)	18.7	34.9**	20.2	1.9**	0.02	371 (54.2%)	3.11	0.02
	AG	262 (31.6%)	18.9	35.1**	20.7	2.4**	0.11	246 (36.0%)	3.29	0.08
	GG	50 (6.0%)	17.8	34.1**	18.3	1.8**	−0.19	46 (6.7%)	3.24	0.15
rs757281 (849/661)	CC	576 (67.8%)	18.7	34.9	19.9	2.1	0.01	431 (63.0%)	3.28	0.04
	CG	252 (29.7%)	18.7	35.0	20.2	2.1	0.04	206 (30.1%)	3.06	0.07
	GG	21 (2.5%)	19.2	35.1	21.4	1.4	0.10	24 (3.5%)	3.33	0.27
rs2074533 (842/660)	CC	263 (31.2%)	18.4	34.8	19.1	2.1	−0.01	208 (30.4%)	3.37	0.14*
	CT	420 (49.9%)	18.9	35.0	20.8	2.0	0.05	318 (46.5%)	3.18	0.05*
	TT	159 (18.9%)	18.6	34.8	19.4	1.8	−0.02	134 (19.6%)	2.85	−0.09*

[a] $P < 0.005$ testing an additive allele effect.
[b] $P < 0.05$ testing a dominant effect.

samples compared to the total samples from which these samples were drawn. Because of the selection based on nicotine dependence, the association between nicotine dependence and the factor scores was also investigated in the genotyped and total samples. The correlation between the maximal score for the Fagerstrom test for nicotine dependence [Fagerstrom, 1978; Fagerstrom and Schneider, 1989] and the factor scores were 0.13 and 0.10 in the total Dutch and US sample respectively and 0.12 and 0.29 in the genotyped samples. Thus, overall, the genotyped samples seemed representative for the total samples.

Table II shows the genotype frequencies and the mean scores per genotype for the three SNPs and the different measures in the Dutch and USA samples. Minor allele frequencies were 0.18, 0.22, and 0.44 respectively for rs757281, rs2072115, and rs 2074533 in the Dutch sample and 0.19, 0.25, and 0.44 in the USA sample. The three SNPs were in Hardy–Weinberg equilibrium in both samples. The genotype frequencies differed significantly between the Dutch and the USA sample for rs2072115 ($P = 0.03$), but not for the other two SNPs.

A power analysis showed that the Dutch sample had a power of 81%, 99% and virtually 100% to find an effect that explained respectively 1.0%, 2.5%, or 5.0% of the variance with an alpha of 0.05. For the USA sample, these figures were 67%, 97%, and 99%.

In the Dutch sample, the additive models did not yield any significant association. Given the pattern of the mean scores per genotype, dominant models were also tested. A significant dominant effect of SNP rs2072115 for the anxiety and depression scales was found with the major allele (A) being the risk allele (P-values are 0.02 and 0.01, respectively). For the other measures, the effect, although not significant, was in the same direction.

In the USA sample, there was a significant additive effect of SNP rs2074533 on GFS-USA with the minor allele (T) being protective ($P = 0.004$). A significant dominant effect was also found for this marker, but the fit of the dominant model was worse than of the additive model. For neuroticism, the effect, although not significant, was in the same direction. The other SNPs did not yield significant additive or dominant effects.

Linkage disequilibrium between the three SNPs differed somewhat in the two samples (Table III) and the differences in haplotype frequencies were significant ($P < 0.0001$; Table IV). In the Dutch sample, the G-C-C haplotype (order rs2072115-rs757281-rs2074533) had a significantly negative effect on anxiety, neuroticism and GFS-NL (P-values are 0.02, 0.03, and 0.04, respectively). No other significant haplotype effects were found. In the USA sample, the G-C-C haplotype was again significantly associated with GFS-USA ($P = 0.03$), but the effect was in the opposite direction, that is, a positive effect on the mean. In addition, the A-C-T haplotype had a significant negative effect on GFS-USA ($P = 0.01$).

DISCUSSION

This study investigated the association between three SNPs in the Epac-1 gene and several indicators of anxiety and depression in two samples. First, in a Dutch twin-family sample, SNP rs2072115 showed a significant dominant effect for anxiety and depression. Further, haplotype G-C-C (ordered rs2072115-rs757281-2074533) had a significant negative effect on anxiety, neuroticism and GFS-NL. Next, replication was sought in a USA sample of unrelated individuals. A significant additive effect of rs2074533 was found for GFS-USA. Haplotypes G-C-C- and A-C-T had a significant positive

TABLE III. Linkage Disequilibrium in the Dutch and USA Sample Expressed in D′ and r^2

	Dutch		USA	
	D′	r^2	D′	r^2
rs2072115-rs757281	0.937	0.050	0.806	0.052
rs2072115-rs2074533	0.664	0.101	0.556	0.085
rs757281-rs2074533	0.406	0.026	0.308	0.018

TABLE IV. Frequencies of the Haplotypes (Order rs2072115-rs757281-2074533) and Associations With the Phenotypes

	% Dutch	% USA
ACC	29.0	19.5
GGT	0.1	0.0
AGT	5.5	4.4
GCC	14.7*	21.7**
ACT	33.1	34.8***
GGC	1.1	0.6
AGC	10.6	14.9
GCT	6.0	4.2

[a] $P < 0.05$ for anxiety, neuroticism and GFS-NL with a negative effect on the mean score.
[b] $P < 0.05$ for GFS-USA with a positive effect on the mean score.
[c] $P < 0.01$ for GFS-USA with a negative effect on the mean score.

and negative effect on GFS-USA respectively. To summarize, both samples showed an association between the EPAC-1 gene and measures assessing a general vulnerability for anxiety and depression, but for different variants or in opposite direction.

It becomes clear that the effects of the SNPs are very small, not even half an SD. This could explain that SNP rs2072115 in the Dutch sample and SNP rs2074533 in the USA sample did not reach significance for all measures, although the scores showed similar patterns. This is in agreement with the results of recent genome wide association studies that did not identify any common variants of very large effect [Welcome Trust Case Control Consortium, 2007; Shifman et al., 2008; Terracciano et al., 2008].

Sullivan argued that precise replication, that is, a significant effect in the same SNP in the same direction for the same phenotype, is required for association studies [Sullivan, 2007]. That would mean that we failed to replicate our findings and that the significant effects found in the Dutch and the USA sample could be due to chance. Neale and Sham [2004], on the other hand, argued that inconsistencies arising from population differences can lead to non-replication when testing association in SNPs or haplotypes with differences in LD between the SNPs under study and the causal variant yielding divergent results. Congruent with this explanation are the different LD patterns and different genotype and haplotype frequencies in the Dutch and USA sample (Tables II–IV). Also, we used different measures, although correlations among different instruments that assess these traits are generally high.

We used phenotypic measures that were averaged over time to reduce the number of tests that were carried out and to decrease measurement error. Five correlated phenotypes were analyzed in the Dutch sample and two in the USA sample. Given the dependency among traits and among SNPs, the significance level of the *P*-value was not corrected for multiple testing. The majority of the *P*-values were around 0.02. In case of a genuine effect, these *P*-values are what would be expected given the sample sizes and the expected effect sizes. However, chance cannot be ruled out as an explanation for our findings. Still, these findings warrant further research into the involvement of Epac in the development of anxiety and depression considering the findings in other research areas [Dwivedi et al., 2006; Ouyang et al., 2008]. It seems especially important to examine genetic variation in the Epac-1 and Epac-2 proteins in concert, as it is possible that a loss of function of one protein can be compensated by another.

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