The Corticotropin-Releasing Hormone Gene and Behavioral Inhibition in Children at Risk for Panic Disorder


Background: Behavioral inhibition to the unfamiliar (BI) is a heritable temperamental phenotype involving the tendency to display fearful, avoidant, or shy behavior in novel situations. BI is a familial and developmental risk factor for panic and phobic anxiety disorders. We previously observed an association between BI and a microsatellite marker linked to the corticotropin releasing hormone (CRH) gene in children at risk for panic disorder. To evaluate this further, we genotyped additional families for this marker and a panel of markers encompassing the CRH locus.

Methods: Sixty-two families that included parents with panic disorder and children who underwent laboratory-based behavioral observations were studied. Family-based association tests and haplotype analysis were used to evaluate the association between BI and polymorphisms spanning the CRH locus.

Results: We examined a set of markers which we found to reside in a block of strong linkage disequilibrium encompassing the CRH locus. The BI phenotype was associated with the microsatellite marker ($p = .0016$) and three single nucleotide polymorphisms (SNPs), including a SNP in the coding sequence of the gene ($p = .023$). Haplotype-specific tests revealed association with a haplotype comprising all of the markers ($p = .015$).

Conclusions: These results suggest that the CRH gene influences inhibited temperament, a risk factor for panic and phobic anxiety disorders. Genetic studies of anxiety-related temperament represent an important strategy for identifying the genetic basis of anxiety disorders.

Key Words: CRH, behavioral inhibition, temperament, anxiety, panic disorder, haplotype, genetic association

Panic and phobic anxiety disorders are common, heritable disorders associated with substantial suffering, disability and economic costs (Hettema et al 2001; Lepine 2002). Efforts to identify specific susceptibility genes must confront the genetic and phenotypic complexity of the anxiety disorders. It is likely that multiple genes of modest effect interacting with environmental factors contribute to the development of panic disorder and social phobia, making the identification of the relevant genes a challenging endeavor. In addition, it is unclear whether the constellations of symptoms used as diagnostic criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) reflect the optimal phenotype definitions for genetic analyses (Smoller and Tsuang 1998).

Heritable traits that underlie or predispose to anxiety disorders may represent “intermediate phenotypes” more amenable to genetic analysis than are the disorders themselves. Behavioral inhibition to the unfamiliar (BI), a stable, heritable temperamental profile characterized by a tendency to be shy, avoidant and behaviorally-restrained in situations that are novel or unfamiliar (Kagan and Snidman 1999, 2004). We have argued that BI offers several advantages for the genetic dissection of panic and phobic disorders (Smoller et al 2001, 2003). These include the fact that BI is a familial and developmental risk factor for panic disorder and social phobia (Battaglia et al 1997; Biederman et al 2001; Mannasis et al 1995; Rosenbaum et al 2000; Schwartz et al 1999) and has an estimated heritability exceeding that of the anxiety disorders themselves (DiLalla et al 1994; Hettema et al 2001; Robinson et al 1992). Physiologic studies suggest that BI reflects a distinct neurobiologic substrate (Kagan and Snidman 1999; Kagan et al 1988; Schwartz et al 2003a), and preclinical studies suggest that BI may represent an evolutionarily-conserved phenotype with behavioral and physiologic analogues in a variety of animal species (Kalin et al 1998a; Takahashi 1995).

Corticotropin releasing hormone (CRH), its receptors, and related peptides have been among the most strongly and consistently implicated neurobiologic systems in animal models of BI-like behavior (Bakshi and Kalin 2000). Central administration of CRH increases fear-related behaviors in rodents and primates (Smagin et al 2001; Strome et al 2002) while CRH antagonists have anxiolytic effects (Habib et al 2000). Temporarily fearful rhesus monkeys, who exhibit a phenotype analogous to BI, display stable increases in right frontal EEG activation, basal plasma cortisol levels, and cerebrospinal fluid CRH (Kalin et al 1998a, 1998b, 2000). These findings resemble the observation of right frontal EEG activation and elevated salivary cortisol in inhibited children (Kagan et al 1988; Schmidt et al 1999). Transgenic overexpression of CRH results in a BI-like phenotype that includes inhibition of activity in novel environments (Stenzel-
Poore et al 1994; van Gaalen et al 2002), although this has not been seen in all studies (Groenink et al 2003).

We have been examining the possibility that genetic variation at the CRH locus is associated with BI. We recently reported an association between BI and a microsatellite marker tightly linked to the CRH gene (Smoller et al 2003) that was particularly marked in children at risk for panic disorder (p = .0009). To further evaluate this preliminary association between CRH and BI in children at risk for panic disorder, we have now typed a panel of SNPs in and around CRH in an expanded sample of children at risk for panic. Here we report that individual markers and a multimarker haplotype spanning the CRH locus show association with BI.

Methods and Materials

Participants

Families were recruited from a sample of 231 families who had participated in a study of children at risk for anxiety disorders conducted at Massachusetts General Hospital (MGH). Details of the study sample and behavioral assessments are provided elsewhere (Rosenbaum et al 2000, Smoller et al 2001). In the children-at-risk study, the proband was a parent who either had a history of treatment for panic disorder or major depression or who was a control with no history of major anxiety syndromes or mood disorders. Children from these families were classified as “inhibited” or “not inhibited” on the basis of a behavioral assessment conducted at age 21 months, 4 years, or 6 years (described below). Families were invited to participate in this genetic study of BI through either a mailing describing the study or at the time of a second wave follow-up of the study cohort. A total of 203 individuals from 62 families that included a parent with panic disorder and at least one child who had undergone behavioral assessments were available for these analyses. Forty-nine of these families were included in our prior analysis of association with the CRH.PCR1 microsatellite (Smoller et al 2003). The ethnicity of all but four families was Caucasian.

The protocol was approved by the Massachusetts General Hospital Institutional Review Board. After complete description of the genetic study, parents provided written informed consent for themselves and their children, who also provided oral or written assent.

Phenotypic Assessments

As described previously (Rosenbaum et al 2000; Smoller et al 2001), behavioral assessments were performed at the Harvard Infant Study Laboratory (under the direction of JK and NS). Children were studied at one of three ages—21 months, 4 years, or 6 years—using age-specific measurement protocols. In brief, the evaluation consists of behavioral protocols designed to assess the child’s reaction to unfamiliar persons and events over a 60 minute battery. The assessments were videotaped and scored by raters who were blind to the assessment of psychopathology in the children and their parents and blind to genotype status. The relevant dependent variables were behavioral signs of uncertainty, including fretting and crying, cessation of vocalization or activity, retreat or withdrawal from an unfamiliar event, and frequency of smiles and spontaneous comments. Studies conducted over the past 20 years have established that these variables differentiate inhibited from uninhibited children between age 1 and age 8 (Kagan et al 1998; Reznick et al 1986). As in our previous genetic analyses (Smoller et al 2001, 2003), children classified as inhibited for this study met at least one of three pre-established definitions from prior studies of BI (Rosenbaum et al 2000). A total of 48 children with BI and 31 children without BI from 62 families with a parent with panic disorder were studied.

DNA Collection and Extraction

Buccal cells were collected from participants using buccal brushing and mouth swishes. DNA was extracted using either the NaOH method (Richards et al 1993) or a commercial kit (Genra Systems, Minneapolis, Minnesota).

Selection of Single Nucleotide Polymorphisms

To examine the linkage disequilibrium (LD) structure in and around CRH, SNPs were selected to cover a region comprising the gene and approximately 120 kb of flanking sequence on either side. To maximize the informativeness of the selected SNPs, we prioritized SNPs that had been identified independently in two databases: dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and Celera (http://www.celera-discovery-system.com). To examine the quality of the assays and determine LD structure, the markers were genotyped in a panel of 93 individuals from 12 multigenerational CEPH pedigrees. The microsatellite marker CRH.PCR1 and 12 SNPs in a block of LD that comprised the CRH gene were genotyped in the study sample as described below.

Genotyping Methods

The microsatellite marker CRH.PCR1 (GDB:196438) (Gu et al 1993) was genotyped using polymerase chain reaction (PCR) amplification and gel electrophoresis as previously described (Smoller et al 2003). Genotyping of SNPs was performed by mass spectrometry as follows. Primers were designed using SpectroDESIGNER software (Sequenom, San Diego, California) to have a Tm above 56°C with a mass range between 5000 and 8000 Da as described by Buetow and colleagues (Buetow et al 2001). Each PCR reaction contained AmpliTaq Gold (.2U, Perkin Elmer, Boston, Massachusetts), dNTPs (.2 mM), MgCl2 (2.5 mM), genomic DNA (5 ng), locus specific primers (.12 μM final concentration of each primer), in the supplied buffer for a final volume of 6 μl using the following PCR conditions: 92°C x 15 min, 46 cycles of 94°C x 20 sec, 56°C x 30 sec, and 72°C x 60 sec followed by a final extension of 72°C x 5 min. Following the PCR reaction, dNTPs were removed by shrimp alkaline phosphatase (SAP) by adding 2 μl of SAP (.3 U) in Thermosequenase buffer and incubating at 37°C x 20 min, followed by inactivation at 85°C x 5 min. The homogeneous MassEXTEND reaction was performed by adding to the SAP-treated product 2 μl of a solution containing d/dNTPs (50 μM each), MassEXTEND primers (1200 nM), Thermosequenase buffer (Amersham, Piscataway, New Jersey), and Thermosequenase (.126 U/μl). The termination mix of ddNTPs and dNTPs was predicted by the SpectroDESIGNER software and was specific for each SNP genotyped. The reaction was thermocycled under the following conditions: 94°C x 2 min, 55 cycles of 94°C x 5 sec, 52°C x 5 sec, 72°C x 5 sec, then 72°C x 5 min. To remove salt, 3 μg of SpectroCLEAN, a proprietary ion-exchange resin, was added. The sample plate was rotated for 6 min at 32 rpm and then centrifuged for 4 min at 2000 rpm. Using a 24-pin SpectroPOINT, 7 nl of each reaction was then loaded onto each position of a single well SpectroCHIP preloaded with 7 nl of matrix (3-hydroxypropionic acid). SpectroCHIPs were analyzed in automated mode by a MassARRAY RT mass spectrometer (Sequenom, San Diego, California) (Buetow et al 2001). The resulting spectra were analyzed by SPECTROTYPER soft-
ware (Sequenom) after baseline correction and peak identification.

**Statistical Methods**

The LD structure of the **CRH** locus and flanking sequence was determined by genotyping markers in a panel of 93 individuals from 12 multigenerational CEPH pedigrees. These analyses were conducted using the Haploviev program (Barrett et al 2005) according to the 4-gamete rule (Wang et al 2002).

Family-based association analyses were performed using the Family Based Association Test (FBAT) Program 1.5.5 (http://www.biostat.harvard.edu/H11011/fbat/default.html). FBAT is based on a unified approach to family-based association analysis that allows valid testing of association with any phenotype, sampling structure, and pattern of missing marker allele information (Laird et al 2000; Rabinowitz and Laird 2000). When parental genotypes are missing, this method computes a test statistic by conditioning on genotypes of any observed parents and offspring, adjusting for admixture (Laird et al 2000). The FBAT statistic reduces to the more familiar transmission/disequilibrium test (TDT) statistic when the dataset consists of parent-affected child trios, and like the TDT, FBAT is immune to confounding by admixture. As shown by Horvath and colleagues (Horvath et al 2001), when there are missing parents, FBAT is similar to, but more powerful than the RC-TDT (Knapp 1999) and considerably more powerful than the S-TDT (Spielman and Ewens 1998). FBAT can also incorporate unaffected offspring and recent analyses have demonstrated that for phenotypes with a relatively high population prevalence (such as BD), this inclusion of unaffected offspring provides a substantial power advantage over the TDT (Lange and Laird 2002; Whittaker and Lewis 1998). Therefore, to maximize power, the offset option of FBAT was used to incorporate all offspring who were phenotyped and genotyped. Association tests were performed for markers for which there were a minimum of 10 informative families (the default minimum for FBAT). Of the 62 families included in the association analyses, there were 14 in which genotypes were available for only one parent; additional sibling genotypes were available in 5 of these families.

Haplotype analyses were conducted using the haploFBAT extension of FBAT 1.5.5 (http://www.biostat.harvard.edu/~fbat/default.html). HaploFBAT uses a weighted conditional approach to test for haplotype association, is robust to population admixture, and can handle missing parental genotypes and missing phase information (Horvath et al 2004). Its power is comparable to that of TRANSMIT under most circumstances (Horvath et al 2004). The program estimates haplotype frequencies using the expectation maximization (EM) algorithm. Both haplotype-specific and a multiallelic (global) test were calculated for haplotypes for which there were a minimum of 10 informative families. All testing was performed with a type I error rate of .05.

**Results**

**LD Structure of the CRH Locus**

In order to determine the LD relationships among SNPs in and around the **CRH** gene, markers were genotyped in a panel of 93 individuals from 12 CEPH pedigrees, as described in Methods. Fifty-five SNPs spanning a region of more than 200 kb encompassing the **CRH** gene were typed, including approximately 155 kb centromeric and 114 kb telomeric of the gene. Figure 1 displays the inter-marker LD (using the D' statistic) for the 25

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**Figure 1.** LD Structure of Markers Encompassing the **CRH** Gene in CEPH pedigrees. LD blocks were identified using the 4-gamete rule as implemented in Haploviev. The magnitude of linkage disequilibrium indexed by the D' statistic are also shown. Red squares without numbers indicate complete LD (D' = 1, LOD > 2). D' values are given in the squares for values < 1.0. Pale blue squares indicate D' = 1 but inter-marker LOD = 2. The locations of genes and the **CRH.PCR1** microsatellite are shown at top. Interblock LD (D') is shown in the right hand corner. Gene symbols: **CRH**, Corticotropin Releasing Hormone; **RNF29**, Ring Finger Protein 29; **MGC26226**, also known as DNAJC5B; **CRH.PCR1**, microsatellite marker.
SNPs that passed quality control metrics and had a minor allele frequency of at least 5%. As shown in Figure 1, the CRH gene lies within a single haplotype block (Block 3) spanning 26 kb. Analysis of the LD structure in the panic family sample (not shown) identified the same LD block (Block 3) seen in the CEPH sample. This block extends into the adjacent gene, MURF2 (Centner et al. 2001). An additional 5 SNPs (rs6984398, rs1870392, rs2004424, hCV1139588, and rs749328) with minor allele frequencies ≤5% were located in Block 3; we included these SNPs in the association analyses described below to allow for the possibility that less common SNPs might be associated with BI in our larger family sample.

**Association Analysis of CRH Markers**

Based on the LD structure described above, we selected all 12 SNPs within Block 3 for association analysis with BI, and included the microsatellite CRH.PCR1 which we have previously shown is in strong LD with SNPs in this block (Smoller et al. 2003) (Figure 1). One SNP was excluded from the analyses because it was monomorphic (hCV1139588) and another because it was not in Hardy-Weinberg equilibrium (rs749328).

Table 1 shows the allele frequencies and FBAT results for the CRH.PCR1 microsatellite and the 8 SNPs for which there were at least 10 informative families. As we had previously found in the smaller sample (Smoller et al. 2003), the 173bp allele of the microsatellite was significantly associated with BI in this extended sample of children at risk for panic (p = .0016). The overall multiallelic test for this marker also showed significant evidence of association (p = .041). Three of the eight SNPs tested were individually associated with BI: rs6999100 (p = .0078), rs6159 (p = .023), and rs1870393 (p = .046).

In order to determine whether haplotype analyses would support the individual marker data, we examined haplotypes composed of the 11 markers listed in Table 1. Eight haplotypes were detected with a frequency of >2% (Figure 2). Of these, two haplotypes (H1 and H2) accounted for 61% of the haplotype diversity and exceeded the informative family threshold for analysis. As shown in Table 2, the global multiallelic haplotype test revealed significant evidence of association ($\chi^2 = 6.14$, df = 2, p = .046). Haplotype-specific analyses revealed an association between BI and haplotype H1 ($Z = -2.42$, p = .015). The negative sign of the test statistic indicates that this haplotype was significantly under-transmitted to affected offspring. This haplotype included all of the alleles that were significantly undertransmitted in the single marker analyses (i.e. rs6999100-T; rs6159- T; rs1870393-A). The other common haplotype differed only at the microsatellite marker. A third haplotype (H5, with a frequency of 6%), contained only alleles that were overtransmitted; however, as a result of its low frequency, there too few informative families to permit haplotype-specific analysis of H5. To determine whether CRH SNPs were associated with BI in the absence of the microsatellite, we examined a haplotype formed only by the SNPs in block 3. However, the 10-SNP haplotype did not provide sufficient informative families to permit global testing. We therefore examined a haplotype comprising the three SNPs that most tightly encompass the gene (rs11997416, rs6159 and rs6984398) and found evidence of association (global test $\chi^2 = 8.79$, df = 2, p = .012). Haplotype-specific tests also revealed significant association for the most common haplotype (rs11997416-G rs6159-T rs6984398-T): $Z = -2.58$, p = .0099.

**Discussion**

Although one must always be cautious when interpreting a single study, our results are compelling for several reasons. CRH has been implicated in the neurobiology of anxiety-related behavior and anxious temperament by numerous physiological and genetic studies of experimental animal models (Bakshi and Kalin 2000). The temperamental phenotype BI has been identified as a familial and developmental risk factor for panic and phobic anxiety disorders. We observed associations between markers in and around the CRH gene with BI in children at risk for panic disorder. We examined markers residing within an LD block encompassing the gene. In single marker analyses, all three markers with a minor allele frequency of at least 15% were associated with BI. In addition, we have demonstrated an association between a haplotype comprising these CRH markers.

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### Table 1. Single Marker FBAT Association Analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>Alleles</th>
<th>Allele Frequency</th>
<th>Test Statistic</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH.PCR1</td>
<td>171 bp</td>
<td>0.08</td>
<td>1.094</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>173 bp</td>
<td>0.50</td>
<td>-3.15</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>175 bp</td>
<td>0.24</td>
<td>0.748</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>177 bp</td>
<td>0.09</td>
<td>1.883</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>179 bp</td>
<td>0.06</td>
<td>1.129</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Multiallelic</td>
<td></td>
<td>$\chi^2 = 11.6$, df = 5</td>
<td>0.041</td>
</tr>
<tr>
<td>rs6999100</td>
<td>C/T</td>
<td>0.17/0.83</td>
<td>2.66</td>
<td>0.0078</td>
</tr>
<tr>
<td>rs11997416</td>
<td>A/G</td>
<td>0.12/0.88</td>
<td>1.44</td>
<td>0.15</td>
</tr>
<tr>
<td>rs6159</td>
<td>G/T</td>
<td>0.18/0.82</td>
<td>2.28</td>
<td>0.023</td>
</tr>
<tr>
<td>rs6984398</td>
<td>C/T</td>
<td>0.07/0.93</td>
<td>1.054</td>
<td>0.29</td>
</tr>
<tr>
<td>rs1870392</td>
<td>C/G</td>
<td>0.96/0.04</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>rs1870393</td>
<td>C/A</td>
<td>0.17/0.83</td>
<td>1.99</td>
<td>0.046</td>
</tr>
<tr>
<td>rs3210836</td>
<td>T/C</td>
<td>0.93/0.07</td>
<td>0.943</td>
<td>0.35</td>
</tr>
<tr>
<td>rs2004424</td>
<td>C/A</td>
<td>0.93/0.07</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>rs1814583</td>
<td>G/A</td>
<td>0.12/0.88</td>
<td>1.54</td>
<td>0.14</td>
</tr>
<tr>
<td>rs1044938</td>
<td>C/T</td>
<td>0.12/0.88</td>
<td>1.63</td>
<td>0.10</td>
</tr>
</tbody>
</table>

FBAT, Family Based Association Test; CRH, corticotropin releasing hormone; SNP, single nucleotide polymorphism.

*For the microsatellite, alleles shown are those for which there were ≥10 informative families. For the SNPs, the first allele in each pair is the over-transmitted allele.

Not tested due to <10 informative families.
and BI. To our knowledge, this represents the first study to demonstrate association between an anxiety-related trait and both individual markers and multimarker haplotypes of a candidate gene as well as the first demonstration of association between the CRH gene and a human anxiety-related phenotype. A specific haplotype was negatively associated with BI, suggesting that it is less commonly transmitted to inhibited children (a “protective” effect). As noted, another haplotype (H5) consisting only of over-transmitted alleles was observed but was not sufficiently common to permit haplotype-specific testing.

If the CRH gene is related to inhibited temperament, it may act through effects on the hypothalamic-pituitary-adrenal (HPA) axis and/or limbic brain systems. CRH synthesized in the paraventricular nucleus of the hypothalamus is the principal trigger for activation of the HPA axis stress response (Smagin et al. 2001). Activity of the HPA axis has been associated with BI and related phenotypes in animal and human studies. For example, glucocorticoids appear to be crucial for the development of behavioral inhibition and fear-associated freezing in rats (Takahashi 1996) and elevated cortisol levels have been observed in behaviorally inhibited primates (Kalin et al. 1998a, 1998b) and children (Goldsmith and Lemery 2000; Kagan et al. 1988). However, CRH effects at extra-hypothalamic sites may be more relevant to anxiety-related behavior. Using a conditional knockout mouse, in which the CRHR1 receptor was inactivated postnatally in forebrain and limbic structures but not the pituitary, Muller and colleagues (Muller et al. 2003) have shown that limbic CRH receptor function mediates anxiety-related behavior independent of the HPA axis. CRH and CRH receptors are abundant in the amygdala, which is known to be a key mediator of anxiety and fear behavior (Davis 1999) and is thought to play a central role in the neurobiology of panic disorder (Gorman et al. 2000). Infusion of CRH into the amygdala produces anxiety-like behaviors in animal models (Sajdyk et al. 1999) and antagonists of the CRHRI receptor, which is highly expressed in the amygdala (as well as the neocortex, pituitary, brain stem and cerebellum) have anxiolytic effects (Takahashi 2001). The amygdala appears to be an important center for response to both fearful and novel stimuli (Schwartz et al. 2003b). Importantly, neuroimaging research suggests that BI reflects a stable, heightened amygdalar response to novelty. Schwartz and colleagues (Schwartz et al. 2003a), using a functional MRI probe (Schwartz et al. 2003b), found that adults who had been categorized as behaviorally inhibited in the second year of life displayed greater activation of the amygdala in response to novel stimuli than did adults who had been categorized as uninhibited in the second year of life. On the other hand, data from a study of primate fear behavior suggest a more modest role for the amygdala in mediating anxious temperament (Kalin et al. 2001) and it is likely that other brain structures are involved. For example, the hippocampus also appears to mediate anxiety/fear behavior and response to novelty (Belzung 1992; Kjelstrup et al. 2002) and CRH may produce anxiety in part by altering hippocampal activity of serotonin (Kagamiishi et al. 2003), a neurotransmitter strongly implicated in anxiety disorders.

The association of CRH and BI was observed in families with parental loading for panic disorder. Inhibited children in these families may represent a genetically loaded subgroup in which the effect of CRH variants might be more influential. Alternatively, this finding could reflect a gene-environment interaction in which exposure to parental anxiety may modify an effect of CRH on BI. There is accumulating evidence that early exposure to parental stress, including parental psychopathology, can produce dysregulation of CRH and HPA axis systems and increased vulnerability to anxiety and mood symptoms in offspring (Essex et al. 2002; Newport et al. 2002). Infants of mothers with panic disorder have elevated salivary cortisol levels compared to infants of unaffected mothers (Warren et al. 2003). Future studies might address the role of parental panic disorder in the CRH/BI association by comparing families in which parental panic disorder was active during the child’s development to families in which parental panic disorder was remitted or not yet symptomatic during this period.

We have hypothesized that BI may be an intermediate phenotype or endophenotype for panic and phobic anxiety disorders (Smoller et al. 2001, 2003). Genetic linkage and association studies of anxiety disorders have implicated several genes

### Table 2. Haplotype-Specific and Multiallelic Haplotype Association Analysis (HaploFBAT)

<table>
<thead>
<tr>
<th>Haplotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Test Statistic</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype-Specific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>Z = −2.42</td>
<td>.015</td>
</tr>
<tr>
<td>H2</td>
<td>Z = .79</td>
<td>.43</td>
</tr>
<tr>
<td>Multiallelic (global)</td>
<td>χ² = 6.14, df = 2</td>
<td>.046</td>
</tr>
</tbody>
</table>

FBAT, Family Based Association Test.

<sup>a</sup>Haplotypes tested were those with ≥10 informative families.

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and chromosomal regions, although to our knowledge, none has implicated the CRH locus or this region of chromosome 8q. However, the rationale for studying a temperamental antecedent such as BI is that, to the extent that BI represents an intermediate phenotype for these disorders, susceptibility loci may be more readily identifiable by examining the temperament phenotype than by examining clinical diagnoses (Gottesman and Gould 2003; Leboyer 2003). The question of whether CRH variants are also associated with clinically diagnosed panic disorder or social phobia should be the subject of future studies. To our knowledge, no other studies examining CRH as a candidate gene for anxiety-related traits or disorders have been reported.

The etiology of anxiety-related traits is likely to be complex and involve epistatic and gene-environment interactions of multiple quantitative trait loci. If CRH is related to BI, it is likely one of several genes involved. For example, the extensively studied insertion/deletion promoter polymorphism in the serotonin transporter gene has been associated with phenotypes such as shyness (Arbelle et al 2003), harm avoidance (Lesch et al 1996), and amygdalar response to fearful stimuli (Hariri et al 2002) that may be related to BI. Defining the set of genes that contribute to anxiety-related traits is an important project for future studies; such efforts may provide unprecedented insights into the biologic pathways and gene-environment interactions that underlie anxiety disorders and may suggest novel approaches for the treatment of these serious disorders.

Our results should be interpreted in light of several limitations. First, as in most association studies of complex traits, the possibility of spurious association must be considered. However, two features of this study reduce the likelihood that our findings are falsely positive: A) the use of family-based association methods, which exclude the possibility of confounding due to population admixture; and B) the finding of statistically significant association with multiple linked markers and a multimarker haplotype, which supports the inference that a true signal is being detected. Second, the sample size available for this analysis was relatively modest. The low minor allele frequency of several of the SNPs tested further limits the power of our analyses. Of note, the SNPs that did not achieve nominal statistical significance had the lowest minor allele frequencies (<15%), and it is possible that one or more of these SNPs might be shown to be associated in a larger sample. Thus, the principal concern here would be type II error, so that our demonstration of association with multiple polymorphisms and the haplotype suggests a robust finding. Third, it is unclear which, if any, of the SNPs tested may be causally related to development of the BI phenotype. Of the SNPs we examined, only one (rs6159) is located in the coding sequence of the CRH gene. This SNP, which represents a synonymous change in amino acid position 96 (glycine), was associated with BI in our analysis. While the relevance of this variant is unclear, synonymous exonic SNPs are known to be capable of producing phenotypic variation and disease by effects on alternative splicing (Caceres and Kornblith 2002). In addition, one of the associated SNPs (rs1870393) maps to the promoter region of the CRH locus (as identified in the SNPper database; Riva and Kohane 2004). However, because all of the markers tested lie within a region of strong LD, our ability to resolve the contribution of individual markers is limited. Several sequences within the CRH promoter (Nicholson et al 2004) and one in the intron (Seth and Majzoub 2001) appear to exert regulatory effects on CRH expression. All of these sequences are located within the haplotype block we found to be associated with BI. However, as of July 2004, there are no SNPs in these regulatory elements that have been identified in dbSNP (www.ncbi.nlm.nih.gov/SNP), the HapMap database (www.hapmap.org/) or the Celera database (www.celeradiscovery-system.com). Approaches to identifying the causal variant(s) could include: A) resequencing studies to search for functional variants that distinguish inhibited and uninhibited children; or B) a return to experimental animal models—e.g. using site-specific mutagenesis to examine whether the introduction of specific SNP alleles affects BI-like phenotypes in mice. In addition, our analyses (Figure 1) suggest that there is substantial LD between haplotype Block 3 (which comprises the markers we tested) and Block 2 (markers 4 - 8 in Figure 1). We cannot therefore exclude that the association we observed might be attributable to a locus centromeric to CRH. The adjacent gene RNF29 codes for a muscle-specific ring finger protein that is a microtubule-binding protein involved in myofibrillogenesis and sarcomere assembly, making it an unlikely candidate (Centner et al 2001; Pizon et al 2002). The centromeric boundary of haplotype Block 2 also contains the 3' end of the gene MGC26226 (markers 4 - 6, Figure 1). The function of this gene, also known as DNAJC5B, is not yet known; expression data (as compiled at http://source.stanford.edu) indicate that it is primarily expressed in testis, although it may be expressed at lower levels in brain. Therefore, although CRH is the most plausible source of the association we observed, we cannot exclude the possibility that one of these adjacent genes is responsible. To address this without substantially increasing the number of statistical tests performed, we genotyped the sample for four of the block 2 markers (rs4398895, rs7824630, rs884839, hCV7683382) shown in Figure 1 and performed a global haplotype test. We observed no evidence of association with this haplotype block, supporting the inference that block 3 is the source of the association signal. Finally, p values were not corrected for multiple testing. A Bonferroni correction would seem to be overly conservative given that this study was conducted as an extension and partial replication of our association finding with the microsatellite marker and that the markers examined are correlated due to LD. Nevertheless, the possibility of type I error cannot be excluded.

In summary, we have observed an association between the CRH gene and behavioral inhibition, a form of anxious temperament, in offspring of parents with panic disorder. Future studies will be needed to clarify the specific CRH variants that influence BI and risk for panic disorder. The strategy used in this study brings together elements that we believe can greatly facilitate gene identification for anxiety disorders: the examination of a heritable, biologically-based phenotype that has analogues in experimental animal models and that is a precursor of the clinical disorders of interest, and the selection of a candidate biologic system that has been strongly implicated by prior genetic and physiologic studies in animals and humans. We are very grateful to the families who participated in this work.

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