An Association Analysis of Murine Anxiety Genes in Humans Implicates Novel Candidate Genes for Anxiety Disorders

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Background: Human anxiety disorders are complex diseases with largely unknown etiology. We have taken a cross-species approach to identify genes that regulate anxiety-like behavior with inbred mouse strains that differ in their innate anxiety levels as a model. We previously identified 17 genes with expression levels that correlate with anxiety behavior across the studied strains. In the present study, we tested their 13 known human homologues as candidate genes for human anxiety disorders with a genetic association study.

Methods: We describe an anxiety disorder study sample derived from a Finnish population-based cohort and consisting of 321 patients and 653 carefully matched control subjects, all interviewed to obtain DSM-IV diagnoses. We genotyped altogether 208 single nucleotide polymorphisms (SNPs) (all non-synonymous SNPs, SNPs that alter potential microRNA binding sites, and gap-filling SNPs selected on the basis of HapMap information) from the investigated anxiety candidate genes.

Results: Specific alleles and haplotypes of six of the examined genes revealed some evidence for association (p ≤ .01). The most significant evidence for association with different anxiety disorder subtypes were: p = .0009 with ALAD (δ-aminolevulinate dehydratase) in social phobia, p = .009 with DYNNL2 (dynein light chain 2) in generalized anxiety disorder, and p = .004 with PSAP (prosaposin) in panic disorder.

Conclusions: Our findings suggest that variants in these genes might predispose to specific human anxiety disorders. These results illustrate the potential utility of cross-species approaches in identification of candidate genes for psychiatric disorders.

Key Words: Anxiety disorders, association study, genetic isolate, SNP

Fear and anxiety are normal emotional responses to threatening situations. However, when exaggerated and causing distress, disability, and loss of quality of life, they manifest as anxiety disorders as classified in the DSM-IV (1) and ICD-10 (2). In DSM-IV, anxiety disorders include panic disorder (with or without agoraphobia), obsessive-compulsive disorder, generalized anxiety disorder, post-traumatic stress disorder, social phobia, agoraphobia, and specific phobias. The combined lifetime prevalence of these disorders in the general population has been estimated to be 16.6% on average (3). Anxiety disorders are also highly comorbid with each other and other psychiatric disorders such as depression and substance abuse (4).

Anxiety disorders are complex diseases, influenced by a combination of both genetic and environmental predisposing factors. For example, the heritability of panic disorder and generalized anxiety disorder has been estimated to be 46% and 32%, respectively (5). Models suggest that the genetic architecture of anxiety disorders can be best explained by one shared set of genetic factors mainly influencing predisposition to panic disorder, generalized anxiety disorder, and agoraphobia and another set primarily underlying specific phobias (6). Furthermore, there is evidence for considerable overlap between the genetic factors influencing anxiety disorders and those influencing depression and the personality traits neuroticism and introversion (7,8).

Because the pursuit of human anxiety disorder susceptibility genes by conventional linkage and association mapping has proved to be demanding (reviewed in 9), there is a need to consider the aid of alternative approaches, such as ones relying on animal models for identification of genes and loci with relevance for complex traits (reviewed in 9,10). Several paradigms for the measurement of anxiety in mice have been developed and pharmacologically validated, and they are considered to model aspects of human anxiety (e.g., 11–13). We have previously combined behavioral testing with gene expression profiling of specific brain regions to identify genes whose expression levels might regulate anxiety-related behavior in mice (14). This approach takes advantage of the different innate levels of anxiety-like behavior of well-characterized inbred mouse strains. Briefly, by combining behavioral analysis of six inbred strains with gene expression profiling of seven brain regions involved in the regulation of anxiety, we identified 17 genes with expression levels that correlated significantly with the anxiety phenotype across all strains in at least one brain region. We further showed functionally that 2 of the identified genes, glyoxalase 1 (Glo1) and glutathione reductase 1 (Gsr), regulate anxiety behavior in mice in vivo. Although the 15 remaining genes were not tested functionally, they remain strong candidate genes for anxiety. In the present study, we tested these genes as candidate genes for human anxiety disorders with a genetic association analysis in a Finnish population-based study sample.
Methods and Materials

Study Sample

The individuals in this study derive from the Finnish population-based Health 2000 Study carried out in 2000–2001. This work was approved by the institutional ethics committee of the National Public Health Institute, and written consent was obtained. Identification of individuals with anxiety disorders has been described in detail by Pirkola et al. (15,16). Briefly, the 12-month prevalence of DSM-IV mental disorders was estimated from a representative sample (n = 6005) of the Finnish general adult (≥30 years of age) population with the Composite International Diagnostic Interview (CIDI). Finland represents a well-characterized genetic isolate (17), with approximately 2% of individuals with foreign descent. No ethnic groups were excluded during recruitment to obtain a representative epidemiological cohort, but the interview was conducted in Finnish, excluding all non-Finnish foreigners. A computer-aided version of the mental health interview (M-CIDI) was administered by trained non-psychiatric healthcare professionals and designed to determine the 12-month prevalence of a set of clinically and epidemiologically relevant mental disorders. These included major depressive disorder, dysthymia, generalized anxiety disorder, panic disorder with or without agoraphobia, agoraphobia, social phobia, and alcohol abuse and dependence. The total number of reliably performed mental health interviews was 6005, amounting to 75% of the original sample. Compared with CIDI participants, the dropouts (n = 981) had somewhat higher scores in the Beck Depression Inventory (BDI), indicating depressive symptoms, and General Health Questionnaire-12 (GHQ-12), indicating psychic distress. They also had slightly older age, lower education, and were more frequently single. Although this might bias the final results toward underestimation of prevalence of mental disorders in the general population, we believe that the most common anxiety disorders are well-represented in our cohort. Due to the structure of the interview, we were not able to reliably diagnose obsessive-compulsive disorder and post-traumatic stress disorder and chose to focus on a subset of the most clinically relevant anxiety disorders in the Finnish population.

From this cohort, we initially selected all individuals with a DSM-IV anxiety disorder diagnosis for analysis (core diagnostic group). In addition, we broadened our definition of anxiety disorder subjects to include individuals with sub-threshold diagnoses as defined by the CIDI (extended diagnostic group). For each case, we selected two control subjects who lacked diagnosed anxiety or major mental disorders and were matched according to gender, age (±1 year), and hospital catchment area. The DNA was not available from 34 subjects (15 cases and 19 control subjects), and therefore, the final analyzed study sample consisted of 321 cases and 653 control subjects (Table 1).

| Table 1. Demographic Characteristics of the Health 2000 Anxiety Disorder Study Sample | DSM-IV Cases | DSM-IV Sub-Threshold Cases | Cases Total | Controls | Total | Men | Women | Mean | Age ± SD |
|---|---|---|---|---|---|---|---|---|---|
| Panic Disorder | 108 | 0 | 108 | 218 | 326 | 106 (32.5%) | 220 (67.5%) | 46.7 ± 11.3 |
| Generalized Anxiety Disorder | 73 | 30 | 103 | 206 | 309 | 122 (39.5%) | 187 (60.5%) | 50.6 ± 12.6 |
| Social Phobia | 58 | 7 | 65 | 133 | 198 | 99 (50.0%) | 99 (50.0%) | 45.4 ± 10.2 |
| Agoraphobia | 31 | 15 | 46 | 94 | 140 | 60 (42.9%) | 80 (57.1%) | 52.8 ± 13.0 |
| Phobia, not otherwise specified | 58 | 0 | 58 | 121 | 179 | 54 (30.2%) | 125 (69.8%) | 54.6 ± 13.3 |
| All Studied Anxiety Disorders | 282 | 39 | 321 | 653 | 974 | 357 (36.7%) | 617 (63.3%) | 49.8 ± 12.7 |

*Core diagnostic group.

*Extended diagnostic group.

*Includes both panic disorder with and without agoraphobia.

Single Nucleotide Polymorphism Selection

The association study was carried out in two stages. In stage I, we examined the known human homologues of 13 anxiety candidate genes identified in the mouse (14) with a set of 139 carefully selected single nucleotide polymorphisms (SNPs; Table 2). In stage II, we followed up on positive findings by genotyping 69 additional markers from genes showing evidence for association after stage I. The 208 markers selected for genotyping represented three different variation groups: non-synonymous SNPs, haplotype-tagging SNPs, and putative polymorphic microRNA (miRNA) binding sites.

First, we selected all known validated and unvalidated non-synonymous SNPs with SNPper (18; http://snpper.chip.org), with the aim to identify SNPs that potentially alter the function of the gene products. Second, we retrieved HapMap (19; http://www.hapmap.org) phase I genotype information for the European-derived Caucasian/European ancestry (CEU) population and used the Tagger algorithm as implemented in Haploview (20; http://www.broad.mit.edu/mpg/haploview) to identify complementary tagSNPs for genotyping. TagSNPs were required to have minor allele frequencies ≥.05, when possible and to capture the variation of other SNPs with minor allele frequencies ≥.05 by a minimum r² = .8. For 11 genes, we selected tagging SNPs every 5 kb, on average. For the two largest genes, CDH2 and EPB41L4A, we selected one tagging SNP/exon. In stage II of the study, we selected supplementary tagSNPs with HapMap phase II data. Third, to select SNPs that potentially alter gene expression, we used the Patrocles database (http://www.patrocles.org) to identify validated and unvalidated SNPs resulting in putative polymorphic miRNA binding sites within the 3’-untranslated regions (UTRs) of our candidate genes and genotyped them in stage II.

Genotyping and Quality Control

The DNA was extracted from whole blood at the DNA extraction core facility of the National Public Health Institute with the Puregene manual DNA extraction kit (Gentra Systems, Minneapolis, Minnesota). The DNA quality control (QC) measures consisted of gender-specific polymerase chain reaction to verify that observed gender matched database information and genotyping of five polymorphic microsatellites to verify that samples were not contaminated (21).
Genotyping was done with Sequenom MassARRAY genotyping technology (Sequenom, San Diego, California) with iPLEX chemistry in stage I and iPLEX Gold chemistry in stage II. Assay conditions were as recommended by the manufacturer, and assay information including primer sequences is available from the authors upon request. Genotyping QC measures consisted of checking no-template control samples for allele peaks; reviewing all marker data and verifying that genotype clusters were well distinguished and outliers removed; verifying consistencies in genotype calls of randomly selected duplicate samples (approximately 2% of samples across, and 2% of samples within sample plates); disregarding marker assays with low success rates (<85%); and discarding all genotype information for individual samples with genotype calls for <75% of the analyzed markers. Furthermore, Hardy-Weinberg equilibrium was evaluated for each marker in the control sample with a \( \chi^2 \) test in addition to confirming Mendelian inheritance of marker alleles by genotyping an additional sample of 60 anonymous parent-offspring trios.

### Statistical Analysis

We estimated the magnitude of genotypic relative risk possible to detect with the studied sample size (321 cases and 653 control subjects) with the Genetic Power Calculator tool (22; http://pngu.mgh.harvard.edu/~purcell/gpc). Disease prevalence was set at 5%, corresponding to the Finnish annual anxiety disorder prevalence (15), inheritance to follow a dominant model, and the frequencies of disease and marker alleles were defined as equal with \( D' = 1 \). The simulation was run with a range of marker and disease allele frequencies (.01–.6) to illustrate how they influence power to detect association.

Statistical analyses were carried out for core and extended diagnostic groups for all anxiety disorders combined as well as separately for the subgroups panic disorder, generalized anxiety disorder, and social phobia. Two subgroups, agoraphobia and phobia not otherwise specified, were included in the analysis of all anxiety disorders combined but not analyzed separately due to small sample size and phenotypic heterogeneity, respectively.

A conventional 2 \( \times \) 2 contingency table likelihood-ratio test (LRT) of independence of the SNP allele counts in cases and control subjects (23), with \( p \) values determined from permutation testing, was performed to test individual SNP alleles for association with anxiety disorders. We analyzed 10,000 permutations of the dataset to obtain empirical \( p \) values. In addition, we performed haplotype analyses on 2- and 3-marker “sliding windows” with the UNPHASED software (24; http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased) that estimates haplotype frequencies from unphased genotype data with an expectation-maximization algorithm. Haplotypes were analyzed with the default full model implemented by the software that tests the null hypothesis that there is no difference in odds ratios between haplotypes using a likelihood-ratio test. A zero frequency threshold of .01 was used to exclude haplotypes with estimated frequencies below 1% in both cases and control subjects. Empirical \( p \) values were calculated with 10,000 permutations.

To order the genes according to their significance with regard to anxiety disorders, a supplementary analysis was performed to examine whether significant trends in associations could be detected across individual genes. The obtained pointwise \( p \) values for all SNPs were ranked from most to least significant (1–1008), after which they were divided into four classes representing the top and bottom 10%, second decile, and second quintile, and bottom 60%). For each gene, we then determined the distribution of findings across these classes. A similar analysis was done in which the \( p \) value for each of the 144 markers was minimized over all tests performed to eliminate redundancy.

### Results

#### Power Calculations

With marker and disease allele frequencies of 1%–60%, the power calculations implied that our entire study sample would provide > 80% power to reject the null hypothesis of no association at \( p < .05 \) when a genotypic relative risk was higher than 1.48–2.62, with the exact value depending on the allele frequency (Supplement 1).

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**Table 2. Investigated Candidate Genes and Number of Analyzed SNPs/Gene**

| Symbol | Name | Position | Size (kb) | Stage I | Stage II |
|--------|------|----------|----------|---------|----------|
|        |      |          |          | Non-Synonymous | tagSNPs | miRNA Sites* | tagSNPs | Total |
| ALAD   | Aminolevulinate, \( \delta \)-, dehydratase | 9q33.1 | 15.0 | 1 (1) | 7 | 2 (3) | 4 | 14 (4) |
| CDH2   | Cadherin-2, type 1, N-cadherin (neuronal) | 18q11.2 | 226.3 | 1 (2) | 10 | — | 10 | 21 (2) |
| CPSF4  | Cleavage and polyadenylation specificity factor 4, 30kDa | 7q22.1 | 18.3 | — | 4 | — | — | 4 |
| DYNL2  | Dynamin light chain, LC8-type 2 | 17q22 | 6.0 | — | 4 | — | 2 (1) | 6 (1) |
| EBP41L4A | Erythrocyte membrane protein band 4.1 like 4A | 5q22.2 | 256.7 | 3 | 10 (1) | — | 14 | 27 (1) |
| EPXK1  | Epoxide hydrolase 1, microsomal (xenobiotic) | 1q42.1 | 20.3 | 2 (5) | 6 | — | — | 8 (5) |
| GLO1   | Glyoxalase I | 6p21.3-p21.1 | 27.2 | 1 (2) | 6 | — | 1 (1) | 5 (1) |
| GSR    | Glutathione reductase | 8p21.1 | 49.0 | 2 (6) | 7 | — | 7 | 16 (6) |
| PSAP   | Proprotein convertase subtilisin/kexin type 5 | 10q21-q22 | 35.0 | — | 9 | 1 (1) | 5 | 15 (4) |
| PTGDS  | Prostaglandin D2 synthase 21kDa (brain) | 9q34.2-q34.3 | 4.2 | — | 4 | — | 2 (1) | 6 (4) |
| S100A10 | S100 calcium binding protein A10 | 1q21 | 21.0 | — | 2 (3) | — | — | 3 (2) |
| SCN1B  | Sodium channel, voltage-gated, type I \( \beta \) | 19q13.1 | 9.8 | — | 2 (3) | — | — | 3 (2) |
| SLC15A2 | Solute carrier family 15 (H+/peptide transporter), member 2 | 3q13.33 | 47.2 | 2 (3) | 7 | — | — | 9 (3) |

| Total | 12 (29) | 80 (1) | 3 (5) | 49 (2) | 144 (37) |

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*Putative polymorphic microRNA (miRNA) binding sites selected with the Patrocles database.
Genotyping QC

Out of the total 208 SNPs selected for genotyping, 181 marker assays (87%) met our QC criteria. Thirty-seven markers, typically unvalidated or rare non-synonymous polymorphisms or unvalidated putative polymorphic miRNA binding sites, were monomorphic, making the total number of statistically analyzed SNPs 144 (92 in stage I, and 52 in stage II; Table 2). The average genotyping success rate for the analyzed markers was 98.7% (median 99.1%), and it varied from 86.1% (rs16933168) to 99.9% (13 markers). The average genotyping success rate in individual samples for the analyzed markers was 98.7% (median 100%), and it varied from 78.1% to 100%. Eight markers included in the analysis exhibited deviation from Hardy-Weinberg equilibrium in the control sample at p < .05, with the most significant p value being p = .015 for marker rs16933168. Detailed results and allele frequencies for all analyzed markers are available in Supplement 2.

Association Analysis, Stage I

Statistical analysis of single-marker associations for 92 SNPs in stage I identified markers from several genes exhibiting empirical pointwise p values of ≤ .05 (Supplement 2). The following criteria were used to select genes for further investigation in stage II: 1) at least two markers in the gene showing p ≤ .05 in allele-based association tests in any diagnostic group, or 2) a haplotype window associated at p ≤ .05 in any diagnostic group. On the basis of these criteria, eight genes were studied further: ALAD (δ-aminolevulinate dehydratase), CDH2 (cadherin 2; N-cadherin), DYNLL2 (dynin light chain 2), EPB41L4A (erythrocyte membrane protein band 4.1 like 4a), GLO1 (glutathione reductase), GSR (glutathione reductase), PSAP (prosapin), and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase). We did not find any association with SNPs in CPSF4 (cleavage and polyadenylation specificity factor 4), EPB41L4A, and PSAP (prosapin) and PTGDS (prostaglandin D2 synthase).

Table 3. SNPs with p Value ≤ .01 in the LRT for Allelic Association

| Gene   | SNP rs # | Alleles [A1/A2] | Group | Allele Frequencies | Alleric LRT p | Diagnostic Group |
|--------|----------|-----------------|-------|-------------------|---------------|------------------|
| ALAD   | rs818702 | A/G             | Cases | .884 .116         | .008          | SOCPH<sup>a</sup> |
|        |          |                 | Controls | .761 .239         |               |                  |
|        | rs7240351<sup>b</sup> | A/G | Cases | .333 .667 | .006 | SOCPH<sup>c</sup> |
|        |          |                 | Controls | .488 .513 |               |                  |
| CDH2   | rs7719346 | A/G             | Cases | .140 .860 | .008 | GAD<sup>a</sup> |
|        | rs1464766 | A/G             | Cases | .255 .745 | .010 | PD |
|        | rs12657885<sup>b</sup> | C/T | Cases | .918 .083 | .010 | GAD<sup>c</sup> |
|        |          |                 | Controls | .849 .151 |               |                  |
| PSAP   | rs4746097 | C/T             | Cases | .729 .271 | .004 | PD |
|        | rs11597008<sup>b</sup> | C/T | Cases | .615 .385 | .008 | PD |
|        |          |                 | Controls | .502 .498 |               |                  |
| PTGDS  | rs4880179 | A/G             | Cases | .053 .947 | .010 | GAD<sup>c</sup> |
|        |          |                 | Controls | .015 .985 |               |                  |

Empirical p values from 10,000 permutations are shown. SNP, single nucleotide polymorphism; LRT, likelihood-ratio test; SOCPH, social phobia; GAD, generalized anxiety disorder; PD, panic disorder; refer to Table 2 for gene names.

<sup>a</sup>DSM-IV core diagnosis.
<sup>b</sup>Rs-numbers of markers genotyped in stage II.
<sup>c</sup>DSM-IV extended diagnosis.

Association Analysis, Stage II

Genotype data for 52 additional stage II SNPs was added to the stage I information. Upon final analysis, we chose to use pointwise and global haplotype p values of p ≤ .01 (empirically evaluated) as criteria for evidence for association. Five genes (ALAD, CDH2, EPB41L4A, PSAP, and PTGDS) showed evidence for association at this level in single-point analyses (Table 3), whereas associations to GLO1 and GSR remained suggestive (.01 < p < .05; Supplement 2).

Examination of two- and three-marker haplotypes supported the single-point findings in ALAD, CDH2, and PSAP (Table 4). In addition, DYNLL2 was implicated by the haplotype-based analysis, even though SNPs within this gene did not show evidence for association at p ≤ .01 in the single marker analysis. Haplotypes in ALAD and PSAP were associated not only with social phobia and panic disorder, respectively, but also with all anxiety disorders combined. We did not find haplotype associations with empirical p values of < .01 for any combination of adjacent markers in the genes PTGDS and EPB41L4A in analysis of sliding windows. However, when performing a combined analysis of two non-adjacent EPB41L4A markers showing single-point associations (rs7719346 and rs146476, 83 kb apart), we identified haplotypes associated with either increased or decreased risk for social phobia. All genes with SNPs and/or haplotypes associated to anxiety disorders with p ≤ .01 are depicted in Figure 1.

Trend Analysis

Examination of trends in pointwise association across individual genes with a 3-df LRT revealed most noticeably that EPB41L4A was overrepresented among the top findings, with a clear trend toward lower representation as one descends in ranked p values (all ranked p values = .0003; p minimized p values = .008; Supplement 3). We further explored the significance of this finding, focusing on the degree of overrep-
Discussion

Adaptive responses related to anxiety, fear, and stress seem evolutionarily conserved in mammals, and genes influencing anxiety-like behavior in mice can therefore be considered excellent candidate genes also for human anxiety disorders (see e.g., 9). Several quantitative trait loci for anxiety-like behavior have been identified in mouse crosses (e.g., 25, 26). Syntenic regions in humans have been tested as candidate regions for panic disorder (27) and personality trait behavioral inhibition (28). In this study, we tested whether variation in 13 genes previously shown to be differentially expressed between non-anxious and anxious inbred mouse strains predisposes humans to anxiety disorders. Six of the genes (ALAD, CDH2, DYNLL2, EPB41L4A, PSAP, and PTGDS) showed empirical p values of ≤ .01 in either single marker, haplotype, or both analyses. To our knowledge, this is the first study showing evidence for association between anxiety disorders and variants in these genes. Neither have any conclusive functional connections between them and anxiety been established so far, although they all seem important for proper functioning of the central nervous system.

Delta-aminolevulinate dehydratase catalyzes conversion of δ-aminolevulinate into porphobilinogen within the heme biosynthetic pathway (29). It is markedly inhibited by lead (30), and a functional polymorphism in ALAD might modulate the adverse effects of lead burden on phobic anxiety symptoms (31). Our most significant association for ALAD also is for a phobic phenotype but not for the same polymorphism (rs1800435). This difference might be explained by differences in study population, cohort demography, and analyzed phenotype. We found a rare risk haplotype (rs818702, rs11789221, and rs8177822), enriched among social phobia subjects. Intriguingly, the rare A allele of rs817782 creates a putative miRNA binding site for miR-204 and miR-211 (www.patrocles.org), within the ALAD 3'-UTR. We previously found Alad expressed at a higher level in the hippocampus and periaqueductual grey of anxious mice (14).

The CDH2 gene encodes a cell-cell adhesion molecule (32) with several functions, including establishment of embryonic left-right asymmetry (33), synaptic adhesion (34), and dendritic spine (35) and cortical morphology (36). We have shown that increased expression of Cdh2 in the pituitary gland correlates with increased anxiety-related behavior in mice (14). This study

Table 4. Two- and Three-Marker Haplotypes with Global p Value ≤ .01 in Sliding Window Analysis

| Gene   | Markers                           | Haplotype        | Cases  | Controls | Global p | Diagnostic Group |
|--------|-----------------------------------|------------------|--------|----------|----------|------------------|
| ALAD   | rs11789221 - rs8177822 - rs818708 | C-A-C            | .014   | .002     | .006     | ANX<sup>a</sup>  |
|        | C-C-C                             | .578             | .607   |          |          |                  |
|        | C-C-T                             | .408             | .391   |          |          |                  |
|        | rs818702 - rs11789221 - rs8177822 | A-C-A            | .036   | .000     | .0009    | SOCPH<sup>b</sup>|
|        | A-C-C                             | .848             | .766   |          |          |                  |
|        | G-C-C                             | .116             | .234   |          |          |                  |
| CDH2   | rs1041985 - rs7240351             | C-A              | .333   | .487     | .001     | SOCPH<sup>b</sup>|
|        | C-G                               | .317             | .160   |          |          |                  |
|        | T-G                               | .350             | .353   |          |          |                  |
| DYNLL2 | rs10132 - rs9900038 - rs9902118   | A-G-C            | .131   | .061     | .009     | GAD<sup>b</sup>  |
|        | A-G-T                             | .621             | .717   |          |          |                  |
|        | G-A-C                             | .248             | .222   |          |          |                  |
| EPB41L4A| rs7719346 - rs1464766            | A-A              | .053   | .197     | .006     | SOCPH<sup>b</sup>|
|        | A-G                               | .174             | .124   |          |          |                  |
|        | G-A                               | .201             | .159   |          |          |                  |
|        | G-G                               | .572             | .521   |          |          |                  |
| PSAP   | rs7092990 - rs4746097             | C-C              | .729   | .616     | .008     | PD               |
|        | C-T                               | .196             | .310   |          |          |                  |
|        | T-T                               | .075             | .074   |          |          |                  |
|        | rs7092990 - rs4746097 - rs11597008| C-C-C            | .612   | .552     | .008     | ANX<sup>b</sup>  |
|        | C-C-T                             | .100             | .116   |          |          |                  |
|        | C-T-T                             | .197             | .259   |          |          |                  |
|        | T-T-T                             | .091             | .073   |          |          |                  |

Empirical p values from 10,000 permutations are shown. ANX, all studied anxiety disorders; SOCPH, social phobia; GAD, generalized anxiety disorder; PD, panic disorder; refer to Table 2 for gene names.

<sup>a</sup>DSM-IV core diagnosis.

<sup>b</sup>DSM-IV extended diagnosis.
Figure 1. Genomic structure, relative single nucleotide polymorphism (SNP) positions, and linkage disequilibrium (LD) plots for the six genes showing evidence for association. The SNP haplotype windows with global p values ≤ .01 are indicated above the genomic structure of the gene. The shown haplotype-based p value for EPB41L4A is from an analysis of two non-adjacent SNPs. The rs-numbers of SNPs showing p ≤ .01 in the single-point analysis are highlighted in green. The triangular plots represent pairwise comparisons of intermarker LD, measured in D'. The span of the coding sequence of each gene is depicted with a green line. Figure modified from Locusview2.0 output (T. Petryshen, A. Kirby, and M. Ainscow, unpublished software).
implicates the CDH2 intron 14 as a region influencing risk for social phobia.

The DYNLL (dynein light chain LC8) proteins are components of the motor protein complexes of dynein and myosin Va (37,38). In neurons, the dynein complex is involved in axonal retrograde transport (39). The DYNLL2 binds multiple proteins in the brain, including synaptic scaffolding proteins and enzymes of gluamat metabolism (40). Interestingly, DISC1, a susceptibility factor for schizophrenia, schizoaffective disorder, bipolar disorder, depression, and autism spectrum disorders (41), is also part of the dynein complex (42). Our results suggest that haplotypes of DYNLL2, which is expressed at a higher level in the periaqueductal grey of anxious mice (14), modulate susceptibility to generalized anxiety disorder.

The functions of EPB41L4A are largely unknown, but it might regulate interactions between the plasma membrane and the cytoskeleton (43). The EPB41L4A is upregulated in vitro by activation of β-catenin (44). Beta-catenin and the Wnt signaling pathway have been implicated in the pathophysiology and treatment of mood disorders, and recent characterization of forebrain-specific conditional β-catenin knock-out mice revealed some evidence for a depression-like phenotype (45). The Epb41l4a is expressed at a higher level in the pituitary gland of anxious mice (14). In this study, we found that genetic variants within EPB41L4A might influence susceptibility to several anxiety disorders. Our most significant association was to a common His→Tyr polymorphism (rs7719346) in generalized anxiety disorder.

The PSAP gene encodes a glycoprotein precursor that is cleaved into four soluble saposins A–D, which stimulate the hydrolysis of sphingolipids (46). Unprocessed PSAP exists also as an integral surface membrane protein in neuronal cells (47). Prosaposin has been suggested to function as a neurotrophic factor (48,49) and to be a neural injury-repair protein (50). Consistently, Psap knockout mice show neurodegeneration (51). Suggestive evidence for association between PSAP and schizophrenia was recently reported (52). In this study, we found two SNPs upstream of PSAP associating with panic disorder, in a region containing putative regulatory elements (53). We previously observed increased expression of Psap in periaqueductal grey of anxious mice (14).

Prostaglandin D2 synthase is the enzyme responsible for the biosynthesis of brain prostaglandin D2, which functions as a neuromodulator and/or trophic factor (54) and participates in the regulation of sleep (55). Changes in cerebrospinal fluid PTGDS quantity have been observed in multiple sclerosis, schizophrenia, and Parkinson’s disease (56). Anxious mice show decreased expression of Ptgds in the bed nucleus of the stria terminalis and periaqueductal grey (14). We found here association of rs4880179 with generalized anxiety disorder.

The results of this study should be interpreted in the context of certain limitations including: 1) limited diagnostic subgroup sample size, 2) problems related to multiple testing correction when a large number of SNPs have been investigated, and 3) differences between mouse and human studies. Each of these is briefly discussed in the following text.

There is a disadvantage in studying a complex phenotype with a limited sample size. Nevertheless, we feel that both combined and separate analyses of specific anxiety disorders are warranted, because these diseases seem to be influenced by both shared and disorder-specific genes (6). Our restricted sample size is exemplified by some fluctuation in p values in the analyses of core and extended diagnostic groups (e.g., rs7719346 and rs4880179). However, our cohort is comparatively well-characterized. It is derived from a population-based study and represents the general Finnish population, which has reduced genetic and environmental heterogeneity (17,57). Both cases and control subjects were interviewed, and because we carefully matched control subjects we do not consider population stratification to be a source of false positives.

Due to the large number of SNPs studied (n = 144) in several diagnostic subgroups (n = 7), we have carried out 1008 statistical tests. Therefore, none of our results would survive correction for multiple testing across all tests with a conventional Bonferroni correction. For this reason, replication in other cohorts is needed to assess the significance of these genes to anxiety disorders in general. Nevertheless, a potential role for particularly EPB41L4A in susceptibility to anxiety disorders was suggested by an analysis of trends in pointwise association between genes.

Although mice can be used to model some aspects of human anxiety fairly well (e.g., 9), it is possible that cognitive differences account for discrepancies between our mouse and human study, besides the type of variation (gene expression vs. DNA polymorphisms) investigated. Although Glo1 and Gsr were functionally shown to regulate anxiety in mice (14), it seems unlikely on the basis of this study that common genetic variants within these genes play a major role in the etiology of human anxiety disorders. Others examining a functional Ala/Glu polymorphism (rs2736654) within GLO1 similarly failed to establish a convincing association with panic disorder (58). However, the genes for which no associations were discovered might nevertheless be important modulators of anxiety, because the role of genetic variation in their hypothetical trans-acting regulatory elements remains to be assessed. In fact, considering that the investigated genes were identified on the basis of differential expression, it is appealing that two of our strongest associations, in ALAD and PSAP, are within potential regulatory regions that might regulate gene expression. Because functional characterization of candidate genes in animal models is laborious and time-consuming, we have not shown causality for all genes studied in this work. However, we have now potentially identified the subset of our candidate genes that is most relevant for human anxiety disorders. This information will enable us to prioritize genes for future functional testing in mice.

In conclusion, we report here that six candidate genes first identified as being differentially expressed between anxious and non-anxious mouse strains are also implicated as possible susceptibility genes for human anxiety disorders. Future replication of our results in independent study samples as well as functional studies are needed to shed light on the role of these genes in the regulation of normal and pathological anxiety. Nevertheless, our results illustrate the potential utility of cross-species approaches in the identification of susceptibility genes for human psychiatric disorders.

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