nature genetics

Genetic dissection of a behavioral quantitative trait locus shows that *Rgs2* modulates anxiety in mice

Binnaz Yalcin[1,3], Saffron A G Willis-Owen[1,3], Jan Fullerton[1], Anjela Meesaq[1], Robert M Deacon[2], J Nicholas P Rawlins[2], Richard R Copley[1], Andrew P Morris[1], Jonathan Flint[1] & Richard Mott[1]

Here we present a strategy to determine the genetic basis of variance in complex phenotypes that arise from natural, as opposed to induced, genetic variation in mice. We show that a commercially available strain of outbred mice, MF1, can be treated as an ultrafine mosaic of standard inbred strains and accordingly used to dissect a known quantitative trait locus influencing anxiety. We also show that this locus can be subdivided into three regions, one of which contains *Rgs2*, which encodes a regulator of G protein signaling. We then use quantitative complementation to show that *Rgs2* is a quantitative trait gene. This combined genetic and functional approach should be applicable to the analysis of any quantitative trait.

Crosses between inbred strains of mice are frequently used to map quantitative trait loci (QTLs) that give rise to the genetic component of quantitative variation in traits of biomedical interest, such as those underlying susceptibility to depression and anxiety[1]. It has proven difficult to identify genes underlying behavioral QTLs: although 94 such QTLs have been reported to exceed a genome-wide significance threshold, in no case has the responsible gene, or genes, been identified[2]. One problem is that each QTL individually makes only a modest contribution to the phenotype; on average, a detectable behavioral QTL accounts for ~5% of the total phenotypic variance[2].

Over the past ten years, anxiety-related QTLs in mice have been identified on 13 chromosomes[3–5]. Although the individual effect of each QTL is small, their detection can be replicated[6], and one QTL has been mapped to a small interval of ~1 cM on chromosome 1 (near 145 Mb on the National Center for Biotechnology Information mouse genome build 30) using a genetically heterogeneous stock of mice[7–9]. Despite extensive analysis of the genes and variants at this locus[10], however, the molecular nature of QTLs that influence anxiety-like behavior in mice remains obscure.

Positional cloning of small-effect QTLs by purely genetic means is extremely difficult because many recombinants are needed to isolate a single gene. Genetic mapping has the additional problem that it locates a functional variant (or variants) rather than a gene. The positions of genes and sequence variants that affect gene expression do not always coincide. Functionally important elements have been discovered far from their cognate genes[11], and regulatory elements for expression of one gene may lie in an intron of another, functionally unrelated, gene[12,13].

Alternative strategies to obtain functional evidence that a gene contributes to behavioral variation can also be extremely challenging. In a few cases, the molecular basis of large-effect QTLs (those explaining 40% or more of the phenotypic variance in an intercross) has been identified by the analysis of gene expression differences[14,15], but the method has so far not been successful when applied to the much more common small-effect QTLs that are responsible for individual differences in behavior. Moreover, where cellular processes are causally remote from the phenotype, as is the case for behavior, expression differences or altered protein function provide only circumstantial evidence to implicate a gene as a QTL. Variation in gene expression is not necessarily translated into behavioral differences, and a gene's effect may depend on where and when it is expressed in the brain[16].

Two approaches might overcome these problems. First, high-resolution mapping in outbred populations, taking advantage of recombination between loci accumulating over many generations, has been successfully applied to mapping small-effect QTLs in fruit flies[17,18] and humans[19–22]. We reasoned that a similar strategy might work in outbred mice.

Second, a method called quantitative complementation testing has been used to investigate the role of candidate genes in QTL mapping experiments in fruit flies[18,23], and a similar method was used in a study of a QTL in yeast (reciprocal hemizygosity analysis[24]). It has not yet been used in mammals. The method requires no information about the nature of responsible sequence variants, their mode of action or their location with respect to the candidate gene, but it does rely on access to deficiency stocks or recessive mutants. These resources are now becoming available for mouse genetics.

Here we describe the application of both methods to characterize the chromosome 1 QTL, and we show that the gene *Rgs2*, encoding a regulator of G protein signaling, is a candidate in this region that modulates variation in anxiety-like behavior.

[1]Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, UK. [2]Department of Experimental Psychology, University of Oxford, Oxford, UK. [3]These authors contributed equally to this work. Correspondence should be addressed to J.F. (jf@well.ox.ac.uk) or R.M. (rmott@well.ox.ac.uk).

Published online 17 October 2004; doi:10.1038/ng1450

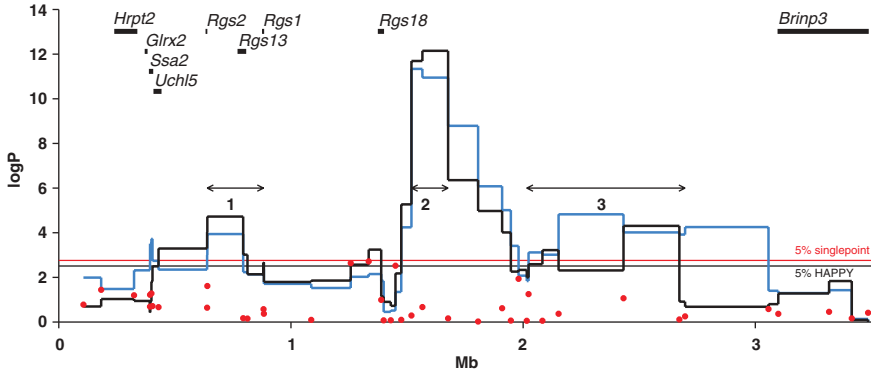


Figure 1 Single-marker and multipoint HAPPY QTL mapping in MF1 mice. Genetic mapping of two kinds in MF1 mice (single-point analysis of variance results, red dots; HAPPY mapping, black and blue lines) with the physical map of gene positions, shown as horizontal black bars at the top of the figure. HAPPY analysis was carried out in two ways, assuming either four (black line) or eight (blue line) progenitor strains. The horizontal black and red lines indicate 5% significance threshold for HAPPY and single point analyses, respectively. The 95% c.i. for the three QTL peaks are shown as three numbered lines with arrowheads.

RESULTS

Single-marker mapping using MF1 outbred mice

We mapped more precisely the region previously shown to contain a QTL influencing anxiety on chromosome 1 (ref. 9). We measured anxiety in 729 outbred MF1 mice using an open-field arena, a brightly lit white arena that is an unwelcome and potentially threatening environment for the animal. Open-field activity (OFA) and open-field defecation (OFD) are indices of rodent fearfulness or 'emotionality', which has many parallels with human anxiety. We previously showed that an analysis that combines OFA and OFD increases power to detect an effect[9]. We define a new composite phenotype, 'emotionality' (EMO), constructed by taking the difference between the standardized scores for OFA and OFD and rescaling the scores to a standard normal distribution.

We obtained genotypes for 42 single-nucleotide polymorphisms (SNPs) over the 3.5-Mb region[10] of the 729 mice and analyzed the EMO scores using single-marker analysis of variance (**Fig. 1**). We determined the significance threshold by permutation, and the 5% threshold, expressed as logP, a negative logarithm of the *P* value, is 2.76 (slightly less than a Bonferroni-corrected 5% threshold of 2.93), equaled by a single marker at 1.3 Mb with a logP value of 2.76. Consequently, single-marker analysis provides only weak evidence of genetic association to this locus.

MF1 haplotypes are derived from inbred strains

We next used a more powerful mapping method. We previously showed that a multipoint method, HAPPY, performs substantially better than single-marker analysis in detecting QTLs[8], but to apply this technique we need to establish that the MF1 mice can be treated as if they were descended from a small number of known progenitor strains, that is, as a heterogeneous stock[8]. Each allele in a heterogeneous stock can theoretically be traced back to one of eight progenitor strains. But we do not know the ancestry of the MF1 mice, which were created in the early 1970s by crossing the LACA line, a standard prolific outbred mouse line, with another outbred albino line called CF. Both LACA and CF mice are related to Swiss mice but are not known to share an ancestor with any of the common inbred strains[25].

We investigated whether the haplotype structure of the MF1 mice was related to that of the progenitor strains in the heterogeneous stock (C57BL/6J, BALB/cJ, RIII, AKR, DBA/2, I, A/J and C3H) that we had originally used to map the QTLs[9]. In 12 MF1 mice we sequenced a total of 62 kb surrounding the nine genes in the region and found only four differences with the inbred strain sequences[10]. All 42 genotyped SNPs were polymorphic and had the same alleles as the heterogeneous stock mice. These data suggested that MF1 haplotypes were very similar to those found in inbred strains.

To test this idea further, we reconstructed the haplotypes of the 729 MF1 mice over the 42 SNPs, using PHASE2 (refs. 26,27) and treating the mice as unrelated. We then devised a dynamic programming algorithm to reconstruct these haplotypes as a mosaic of inbred strains using the least number of chromosomal breakpoints. The mosaics for the 14 most common MF1 haplotypes together account for more than 95% of the chromosome complement (**Fig. 2**). All haplotypes can be derived from just four inbred strain haplotypes (C3H, AKR, C57BL/6J and I; because there are no sequence differences between C3H and A/J over the region of interest[10], these two strains are interchangeable).

If this mosaic is meaningful then we would expect it to have far fewer breakpoints than a mosaic reconstructed from random progenitor strains. We tested whether the number of breakpoints in the mosaic was statistically unlikely by permuting the alleles of the inbred strains at each marker position, reconstructing the optimal mosaic and counting the number of breakpoints. The number of breakpoints using the bona-fide strain haplotypes was less than that observed in each of 10,000 permutations. Consequently, the MF1 haplotypes can be modeled as a mosaic and therefore analyzed like a heterogeneous stock descended from these inbred progenitor strains.

HAPPY analysis of MF1 mice

To avoid assuming a particular mosaic is correct, we searched for QTLs using HAPPY[8], which estimates the probability of descent from each inbred strain. HAPPY models the MF1 haplotype mosaics using a hidden Markov model, integrating over all possible mosaic reconstructions weighted according to their relative probabilities[8]. HAPPY uses unphased genotypes rather than the haplotypes determined by PHASE2 (refs. 26,27) and so does not introduce bias resulting from incorrect specification of the haplotype phase assignment. Hypothesis testing for QTL detection is based on a test for differences between the estimated phenotypic effects attributable to each progenitor strain at the locus of interest.

We carried out the HAPPY analysis using the four strains identified in the mosaic as plausible progenitors of MF1 mice. The method detects genetic effects with much more power than single-marker analysis (**Fig. 1**). The 5% threshold for region-wide significance for HAPPY logP scores is 2.51; this was exceeded at three places: peak 1 at 0.7 Mb (logP = 5.0, 95% confidence interval (c.i.) = 0.43–0.81); peak 2 at 1.7 Mb (logP = 10.9, 95% c.i. = 1.43–1.67 Mb); and peak 3 at 2.5 Mb (logP = 4.2, 95% c.i. = 2.01–2.69 Mb; confidence intervals are for an additive four-strain QTL model, using a bootstrap procedure[8]). As expected from previous mapping data, the size of the effect attributable to the locus is small, with each peak contributing less than 5% of the total phenotypic variance. Together the three peaks account for 12% of the variance.

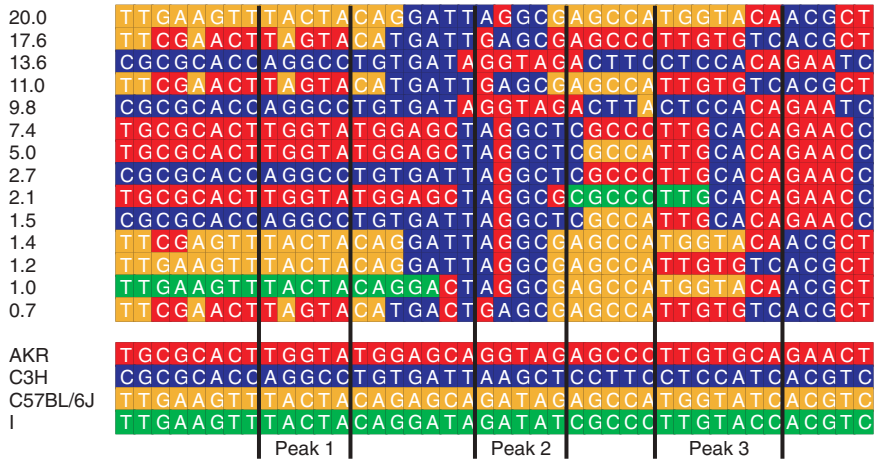


Figure 2 Reconstruction of MF1 haplotypes as inbred strain mosaics. The top part of the figure shows haplotypes that account for 95% of the MF1 chromosomal complement. To the left of each haplotype is its frequency in the population, expressed as a percentage. Each haplotype is represented horizontally as a string of sequence variants at the 42 SNPs used for mapping in the MF1. The bottom part of the figure shows the haplotypes of four inbred strains (C3H, AKR, C57BL/6J and I). The origin of each MF1 haplotype from these inbred strains, as determined by a dynamic programming algorithm, is indicated by color coding of each nucleotide (red for C3H, blue for AKR, yellow for C57BL/6J and green for I). Blocks of contiguous color in the MF1 represent unrecombined haplotypes. The labeled black vertical lines demarcate the 95% c.i. for the three QTL peaks.

To test whether our choice of strains was skewing the result, we also analyzed the MF1 mice by using all eight heterogeneous stock progenitors. Both analyses identified the same three peaks (**Fig. 1**). The much lower significance levels of single-marker association mapping compared with HAPPY reflect the fact that the strain distribution patterns (SDPs) of the SNPs need not coincide with the QTL allele effects, as noted in previous analyses[8,28]. For example, if the SDP at the functional variant is different from the SDPs of nearby SNP markers (*e.g.*, because it is not diallelic), then no marker is a good surrogate for it. This problem is avoided by multipoint methods such as HAPPY, which consider combinations of markers that induce new SDPs and therefore might coincide with the SDP of the functional variant.

The QTL region has three independent effects

We next asked whether the three peaks were truly independent, as linkage disequilibrium between markers might contribute to interdependence between the peaks. We used our reconstruction of MF1 haplotypes from putative progenitor strains as an index of historical recombination (**Fig. 2**). On average, 8.4 recombinants separate the MF1 haplotypes from the progenitor haplotypes. The position of the 95% c.i. containing each QTL peak is shown in **Figure 2**, superimposed on the derivation of the common haplotypes. The haplotypes cannot be reconstructed in such a way that an ancestral haplotype spans all the peaks and no two peaks lie on the same progenitor strain haplotype (**Fig. 2**), indicating that the peaks are probably independent. We may not have correctly ascertained the founders, however, and so our recombination estimates may be biased. Therefore, we investigated the independence of the three effects by fitting them simultaneously, testing the significance of each QTL peak in the presence of the other two using partial F-tests. All three peaks remained significant (logP = 2.5, 11.9 and 3.3), suggesting that they are independent and real effects, although the significance levels of the first and third peaks were lower and the location estimated for the third peak shifted slightly.

Figure 1 shows the relationship between the QTL peaks and known genes in the region. The second and third QTL peaks are located in a region devoid of known genes, although there are several expressed sequences. Neither the human nor the mouse region was predicted to encode any known microRNA sequences. The 95% c.i. of the second peak, at 0.7 Mb, contains just two genes, *Rgs2* and *Rgs13* (regulator of G-protein signaling 2 and 13). Only *Rgs2* lies completely within a 95% c.i.

Quantitative complementation of *Rgs2*

On the basis of the MF1 fine-mapping data, *Rgs2* is a strong candidate gene. Therefore, we used quantitative complementation to test whether *Rgs2* interacts with a functional variant. The test uses four strains: two that bear different QTL alleles (referred to here as high and low lines), a strain bearing a recessive mutation of *Rgs2* (m); and a wild-type strain (+) that is ideally coisogenic with the mutant. We determined phenotypes of mice with the four genotypes high/m, low/m, high/+ and low/+ and analyzed them in an experiment with two factors: 'Cross', representing the presence or absence of the mutation, and 'Line', representing natural allelic variation at the QTL. We suppose that the QTL exerts its effect by altering the expression of the gene, as might be the case if it lies in the promoter of the gene or in a more distant enhancer element. In this case, the two effects, one due to the gene and one to the QTL, will not be independent and their joint effect (a failure to complement) will be detected as a significant interaction between Line (high or low) and Cross (m or +) in the analysis of variance. The interaction coefficient between Line and Cross is identical to the contrast (high/m – low/m) – (high/+ – low/+) and measures the failure for the wild type to complement the mutation on different backgrounds (low versus high).

We obtained a recessive mutation of *Rgs2* suitable for the quantitative complementation test, but because the *Rgs2* mutant was made on a 129/P2 strain and backcrossed to C57BL/6J[29], obtaining a wild type on a coisogenic background was difficult. But the genomes of inbred strains of laboratory mice are closely related and can be described as a mosaic structure of alternating segments of sequence similarity and difference[30–32]. We reasoned that the problem of mixed strain background might be overcome if we could show that the genetic effect of any sequence variant in the mutant strain was identical to its effect in C57BL/6J; in other words, even though the sequence might not be identical, the two strains would carry the same QTLs.

We used genotyping data and sequence comparisons to determine whether we could use C57BL/6J as the wild-type control for the complementation test. Analysis of 98 microsatellite markers showed that the genome of the mutant mouse is C57BL/6J, apart from a 37-Mb region on chromosome 1 (between 113 Mb to 150 Mb on the National Center for Biotechnology Information mouse genome build #30). Mapping in the heterogeneous stock indicated that this region contains only the QTLs analyzed here, due to a contrast between two strains (A/J and C3H: low EMO) on one hand and the other six strains on the other (C57BL/6J, DBA/2, I, AKR, RIII and BALB/cJ: high EMO)[8,9,28,33].

Table 1 Analysis of variance for quantitative complementation of the *Rgs2* mutant

Phenotype	Line	Mutant	Wild-type	Interaction coefficient	*P* value
EMO	High	0.987	0.162	0.838	0.009
	Low	−0.462	−0.448		
OFA	High	0.645	−0.147	0.803	0.029
	Low	−0.183	−0.172		
OFD	High	−0.632	−0.238	−0.566	0.062
	Low	0.483	0.311		
EPM open-arm entries	High	0.277	−0.363	0.900	0.017
	Low	−0.097	0.162		
EPM open-arm time	High	0.765	−0.218	0.700	0.049
	Low	−0.009	−0.291		
Latency to eat new food	High	0.064	0.021	−1.041	0.003
	Low	0.653	−0.430		
Home-cage activity	High	0.746	0.487	0.259	0.964
	Low	−0.191	−0.703		

For each phenotype, the mean trait values for the four combinations of Line (high C57BL/6J versus low C3H/HeJ) and Cross (*Rgs2* mutant versus C57BL/6J wild-type), the Cross × Line interaction coefficient and its *P* value from the analysis of variance are shown. EPM, elevated plus maze.

We investigated the region containing the three QTL peaks, sequencing, in the mutant, amplimers of ~1.2 kb at an average interval of 8.5 kb across the region. We found no polymorphisms unique to the *Rgs2* mutant; the *Rgs2* mutant sequence was identical to that of C57BL/6J from 0.5 to 2.95 Mb and identical to that of DBA/2 from 2.95 Mb onward. Mapping experiments identified no QTLs segregating between C57BL/6J and DBA/2 in the region of sequence difference[8,28,33,34].

We tested for an interaction between Line and Cross at the *Rgs2* locus by quantitative complementation, again using the EMO phenotype[9] measured in 117 mice. On the basis of our mapping in the heterogeneous stock and from sequence analysis of the QTL region, we knew that C57BL/6J carried the high QTL allele and that either A/J or C3H carried the low QTL allele[8,10]. We used C57BL/6J and C3H for quantitative complementation, crossing both with the *Rgs2* mutant and with the wild type (C57BL/6J). The interaction between Line and Cross was significant ($P = 0.009$), implicating *Rgs2* as a gene involved in the QTL (**Table 1**).

Rgs2 modulates anxiety

If *Rgs2* is the quantitative trait gene, then it should have a specific pattern of action[35] that affects both OFA and OFD, but in opposite directions as increased anxiety is associated with lower activity and higher defecation. The interaction coefficient should be positive for OFA and negative for OFD. Furthermore, the interaction coefficient for EMO should be larger than those for either OFA or OFD. The gene should also affect other measures of anxiety. In the elevated plus maze, we expected the interaction coefficient to be positive for number of entries and time spent in the open arms of the maze. In another test of novelty, the latency (or amount of time taken) to try a new food, the interaction coefficient should be negative. Last, *Rgs2* should not affect activity measured in a nonthreatening environment, such as the distance traveled in 30 min in a home cage (home cage activity). Quantitative complementation of *Rgs2* produced the expected pattern of results (**Table 1**).

Because the control strain used in the complementation test is not identical to the mutant strain, we needed to show that the results were not due to unknown QTL next to *Rgs2* that might have been segregating between the DBA/2 and C57BL/6J haplotypes. We directly tested this possibility with another quantitative complementation test using DBA/2, rather than C3H, as the contrasting strain to C57BL/6J. If a QTL segregates between these two strains at the *Rgs2* locus, then there should be a failure to complement. We found that the interaction between strain and background was not significant: $P = 0.3$ for OFA, $P = 0.97$ for OFD and $P = 0.48$ for EMO. Furthermore, we did not uncover any functional effect attributable to differences between DBA/2 and C57BL/6J sequence variants in MF1 mice by comparing a model in which a different genetic effect is allowed in each strain with a model in which it is constrained by the strain distribution pattern of the variant, so that strains sharing the same allele must have the same genetic effect. These results indicate that the quantitative complementation result is not compromised by the use of C57BL/6J as a control and that the effect is indeed specific to a small-effect QTL segregating between C3H and C57BL/6J.

DISCUSSION

We report here the identification of a gene, *Rgs2*, underlying a small-effect QTL that contributes to behavioral variation in the mouse. The variance due to this QTL in the segregating cross is ~5%, which is typical for behavioral QTLs. Other information about the function of *Rgs2* is consistent with this finding. *Rgs2* is widely expressed in the brain[36], and the *Rgs2* mutation has an effect on behavior[29]. Comparing the behavior of the homozygous *Rgs2* mutant with that of C57BL/6J mice indicates that the mutation makes mice more anxious (see **Supplementary Table 1** online). Regulators of G-protein signaling are known to have a role in rapid behavioral changes[37,38]; their involvement in modulating activity levels in the tests used here to measure anxiety in rodents is consistent with these observations. In common with other *Rgs* genes, *Rgs2* affects a wide range of phenotypes including hypertension[39], immune response[29] and implantation in the womb[40].

Although *Rgs2* modulates anxiety in the mouse, the genetic data indicate that it is only one component of the QTL. The position of one QTL peak over *Rgs2* (**Fig. 1**) suggests that the functional variant interacting with *Rgs2* is close to, or inside, the gene. The positions of the other QTL peaks suggest that *Rgs18* and *Brinp3* are good candidates for other components (**Fig. 1**), but confirmation is needed because these peaks lie in intergenic regions, more than 100 kb from the nearest known expressed sequence. We cannot rule out the possibility that these peaks interact with *Rgs2* as well. Although several expressed sequence tags align to the genome sequence under the second QTL peak, they probably do not represent protein-coding genes because they have no homology to known protein-coding genes, are not spliced and often contain long and short interspersed element repeats. This observation is important, as it indicates that concentrating solely on known expressed sequences may result in missing important loci.

The complexity of the architecture of the QTL is similar to that reported elsewhere. Studies that isolate genetic effects in congenic and recombinant inbred mouse lines often report that one relatively large effect comprises several loci with much smaller effects[41–44]. In *Drosophila melanogaster*, four different fine-mapping QTL studies reported a similar phenomenon[45]. Similar complexity will probably be found at other QTLs.

This study establishes two new approaches to genetic mapping in mice. First, we showed that it is possible to use commercially available outbred mice to map small-effect QTLs with a high degree of precision (to within a few hundred kilobases). This success was due to the

unexpected finding that MF1 mice can be treated as mosaics of standard inbred strains and analyzed accordingly using probabilistic ancestral reconstruction. It will be of interest to determine whether the genomes of other outbred lines can be treated similarly.

Second, we used a quantitative complementation test to show that *Rgs2* modulates anxiety in mice. Whereas genetic fine-mapping locates the functional sequence variants, quantitative complementation identifies the candidate genes. A significant failure to complement implies either allelism (the gene contains the functional variant) or epistasis (the gene interacts with the functional variant, which may be elsewhere in the genome). We cannot exclude the possibility of an interaction between *Rgs2* and loci on other chromosomes, but this explanation is unlikely for two reasons. First, we have been unable to detect epistasis between any open-field behavior QTL so far detected in the heterogeneous stock; second, we found no evidence of epistasis acting on open-field or elevated plus maze measures of anxiety in two F_2 intercrosses (C75BL/6J × BALB/cJ and DBA/2 × C57BL/6J)[28,46].

The combined genetic and functional approaches described here provide a general method for identifying small-effect genes underlying QTLs. Using the analytical techniques we developed, together with information about the sequence structure of inbred strains and available mutants, the entire experiment could be carried out within a year. It should therefore be possible to detect the genes underlying other QTLs in the same way.

METHODS

Mice and crosses. We acquired outbred F_2 generation MF1 mice and inbred C3H/HeJ and C57BL/6J mice at 5–6 weeks of age from Harlan UK. We obtained the *Rgs2* mutant from J. Penninger (Amgen Institute, University of Toronto, Canada)[29]. For the quantitative complementation experiment, we made F_1 hybrids by crossing C3H/HeJ (low EMO) and C57BL/6J (high EMO) mice to the homozygous *Rgs2* mutant (23 and 30 F_1 mice, respectively) and crossing C3H/HeJ with C57BL/6J mice (40 mice, low × control). For the remaining component of the cross, we phenotyped 24 C57BL/6J inbred mice (high × control). For the complementation using DBA/2, we used an equivalent number of DBA/C57BL and DBA/*Rgs2* mutant F_1 hybrids. We phenotyped 729 MF1 mice in 21 families with a mean size of 30 (s.d. = 12). We maintained mice in a vivarium with controlled temperature, light and humidity on a 12-h light-dark cycle. We carried out all behavioral tests during the daylight phase of the cycle. We housed mice in single-sex littermate groups with free access to food and water. All mice were tested when they were between 6 and 8 weeks of age.

Phenotyping. We measured OFA in a brightly lit, 60-cm-diameter, enclosed white arena with no background noise. We placed mice in the apparatus and monitored them for 5 min by video camera; movements were analyzed using an image analyzer (Videotrack (version NT4.0)) from Viewpoint. At the end of each trial, we recorded the number of fecal boli deposited. We also monitored behavior in the elevated plus maze (apparatus described in ref. 47) using an automated tracking system. We measured the number of entries, time in seconds and the distance traveled in the open and closed arms. We measured food neophobia as the latency to eat a new food (a solution of one-third full-cream sweetened condensed milk and two-thirds water). Mice were restricted to 1 g of food overnight. Mice were given three trials of 2 min each. We stopped the trial when the mouse first tasted the food. The test apparatus has been described[47]. We measured baseline activity in a home-cage environment using a photo activity system from San Diego Instruments. We measured the number of beam breaks during a 30-min test period.

DNA extraction and genotyping. We extracted DNA from 0.5-cm tail snips using a phenol-chloroform method[48] and separated it into 96-well plates at a concentration of 10 ng μl^{-1} for genotyping. We designed extension and amplification primers for SNP genotyping using SpectroDESIGNER. Oligonucleotides were synthesized at Metabion. We carried out PCR with Hotstar *Taq* obtained from Qiagen. Each 5-µl PCR contained 2.5 ng of genomic DNA, 0.2 U of HotStar *Taq*, 5 pmol of forward and reverse primers, 2 mM of each dNTP, 1× HotStar *Taq* PCR buffer as supplied by the enzyme manufacturer (contains 1.5 mM $MgCl_2$, Tris-Cl, KCl and $(NH_4)_2SO_4$, pH 8.7) and 25 mM $MgCl_2$ (Qiagen). The temperature profile consisted of an initial enzyme activation at 95 °C for 15 min, followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 60 s, and a final incubation at 72 °C for 3 min. We treated PCR products with shrimp alkaline phosphatase (Sequenom) for 20 min at 37 °C first to remove excess dNTPs. We used a thermosequenase (Sequenom) for the base extension reactions. The base extension conditions were 94 °C for 2 min, followed by 55 cycles of 94 °C for 5 s, 52 °C for 5 s, and 72 °C for 5 s. We removed unincorporated nucleotides from extension products using SpectroCLEAN resin. A few nanoliters of each sample were arrayed onto a 384 SpectroCHIP by a SpectroPOINT robot. The chip was read in the Bruker Biflex III Mass Spectrometer system and data analyzed on SpectroTYPER; the resulting genotypes were then automatically uploaded into an Integrated Genotyping System. To determine the relationship between the *Rgs2* mutant and C57BL/6J mice, we amplified 98 microsatellite markers, distributed across the genome, that distinguish C57BL/6J from 129/P2 and compared the allele sizes with those present in the *Rgs2* mutant. After PCR amplification, we separated products by electrophoresis through 4% agarose gels and scored marker sizes with reference to a size standard.

DNA sequencing. We used Primer3 to design oligonucleotide primers. We amplified genomic DNA segments in a 50-µl PCR reaction using oligonucleotides synthesized at MWG: 100 ng of DNA, 0.2 U of Gold *Taq*, 10 pmol of forward and reverse primers, 8 mM of each dNTP, 1× PCR buffer and 25 mM $MgCl_2$. The PCR conditions were 95 °C for 15 min; 13 cycles of 95 °C for 30 s, 62 °C for 30 s (−0.5 °C per cycle) and 72 °C for 60 s; 29 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 55 s; and 72 °C for 7 min. We purified PCR products on a 96-well Millipore purification plate and resuspended them in 30 µl of water. We prepared two sequencing reactions for each DNA sample, one with the forward primer and one with the reverse primer. We removed the PCR reagents from solution by an ethanol precipitation in the presence of sodium acetate. All sequencing reactions were carried out on an ABI3700 sequencer.

Haplotype mosaic generation. We determined haplotypes of MF1 mice using PHASE2, using the program's default options[26,27]. All mice were analyzed together, ignoring family information. The derivation of the haplotype mosaic from inbred strains was reconstructed using the following dynamic programming algorithm that finds a mosaic that minimizes the number of breakpoints required. Suppose there are ordered N markers. Let a_{ij} be the allele at marker position j in the ith haplotype obtained from PHASE. Let s_{kj} be the allele at marker position j in the kth inbred strain. Let x_{ikj} equal 0 if $a_{ij} = s_{kj}$ or equal 1 otherwise. An optimal mosaic is a sequence $k(ij)$ of inbred strains such that the allele $a_{ij} = s_{k(ik)}$ and the number of breakpoints where $k(ij)$ differs from $k(i\,j–1)$ is minimal. Let R_{ijk} be the score of an optimal partial mosaic of the ith haplotype for marker positions 1..j, constrained so that the final jth position is assigned to strain k. Then $R_{1jk} = -a x_{ik1}$ and $R_{ijk} = \max_n \{R_{i\,j-1n} - a\, x_{ijn} + \delta_{nk}\}$ for $j > 1$. δ_{nk} is the delta function, and a is a negative weight parameter chosen such that a breakpoint always occurs in preference to a mismatched allele. Let M_{ijk} be the strain n that maximizes R_{ijk} in this recursion, and M_{iN} the strain k that maximizes R_{ijk}. Then an optimal mosaic is given by the sequence defined as $S(iN) = M_{iN}$; $S(ij) = M_{ijS(ij+1)}$; $j < N$. We carried out permutation analysis by shuffling the alleles at each marker position in the inbred strains and reconstructing the mosaic 10,000 times.

QTL mapping. We transformed phenotypes into Gaussian deviates by first ranking them and then replacing each rank with its corresponding quantile in the standard normal distribution. We carried out QTL mapping using the HAPPY software package, implemented in C and R 1.9.0. We determined the presence of a QTL at an interval between two adjacent genotyped markers as described[8]. As progenitors for HAPPY mapping analysis, we used the eight inbred strains that founded the HS strain as well as the four strains identified by the strain reconstruction. We estimated region-wide significance levels by permuting the transformed phenotype values, repeating the single point or HAPPY analysis, recording the maximal logP value and ranking the results of

1,000 analyses to determine significance thresholds. The 5% thresholds for single-point (2.96) and HAPPY (2.51) were close to the Bonferroni approximation assuming independent tests (2.95). We tested the independence of genetic effects by comparing a model with all three QTL peaks fitted simultaneously with three submodels in which each peak was omitted in turn, evaluating significance by a partial F-test. We determined a 95% c.i. for each QTL location by bootstrapping, where the subjects were resampled with replacement 1,000 times and the most significant marker interval was recorded. We estimated the probability that the QTL was in a given marker interval as the frequency with which the interval was most significant in the bootstrapped analyses.

Quantitative complementation testing. We analyzed quantitative complementation results as a linear model in the R statistical analysis package version 1.9.0 of the form $E(y) = \mu + C + L + C \times L$. Here, y is the trait and μ is the intercept, equal to the expected effect for a mouse of genotype high/+, C is the difference between the main effects of Cross (low versus high), L the difference between the main effect of Line (mutant versus wild-type), and C×L the interaction between Cross and Line. Failure to complement is indicated by a significant interaction coefficient in the analysis of variance.

URLs. An annotated interactive version of the sequence is available at http://bioinformatics.well.ox.ac.uk/project-anxiety/. The database of micro RNA sequences used to search the sequence is available at http://www.sanger.ac.uk/Software/Rfam/mirna/. The HAPPY package is available at http://www.well.ox.ac.uk/happy/, and R software is available from http://www.r-project.org/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank J. Penninger for providing the *Rgs2*-mutant mice and S. McCormick for comments on the manuscript. This work was funded by the Wellcome Trust.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 25 June; accepted 13 September 2004
Published online at http://www.nature.com/naturegenetics/

1. Flint, J. & Mott, R. Finding the molecular basis of quantitative traits: successes and pitfalls. *Nat. Rev. Genet.* **2**, 438–445 (2001).
2. Flint, J. Analysis of quantitative trait loci that influence animal behavior. *J. Neurobiol.* **54**, 46–77 (2003).
3. Gershenfeld, H.K. & Paul, S.M. Mapping quantitative trait loci for fear-like behaviors in mice. *Genomics* **46**, 1–8 (1997).
4. Turri, M.G., De Fries, J.C., Henderson, N.D. & Flint, J. Multivariate analysis of quantitative trait loci influencing variation in anxiety-related behavior in laboratory mice. *Mamm. Genome* **15**, 69–76 (2004).
5. Henderson, N.D., Turri, M.G., DeFries, J.C. & Flint, J. QTL analysis of multiple behavioral measures of anxiety in mice. *Behav. Genet.* **34**, 267–293 (2004).
6. Turri, M.G., Henderson, N.D., DeFries, J.C. & Flint, J. Quantitative trait locus mapping in laboratory mice derived from a replicated selection experiment for open-field activity. *Genetics* **158**, 1217–1226 (2001).
7. McClearn, G.E., Wilson, J.R. & Meredith, W. The use of isogenic and heterogenic mouse stocks in behavioral research. in *Contributions to Behavior-Genetic Analysis: The Mouse as a Prototype* (eds. Lindzey, G. & Thiessen, D.) 3–22 (Appleton Century Crofts, New York, 1970).
8. Mott, R., Talbot, C.J., Turri, M.G., Collins, A.C. & Flint, J. A method for fine mapping quantitative trait loci in outbred animal stocks. *Proc. Natl. Acad. Sci. USA* **97**, 12649–12654 (2000).
9. Talbot, C.J. *et al.* High-resolution mapping of quantitative trait loci in outbred mice. *Nat. Genet.* **21**, 305–308 (1999).
10. Yalcin, B. *et al.* Unexpected complexity in the haplotypes of commonly used inbred strains of laboratory mice. *Proc. Natl. Acad. Sci. USA* **101**, 9734–9739 (2004).
11. Nobrega, M.A., Ovcharenko, I., Afzal, V. & Rubin, E.M. Scanning human gene deserts for long-range enhancers. *Science* **302**, 413 (2003).
12. Higgs, D.R. *et al.* A major positive regulatory region located far upstream of the human α-globin gene locus. *Genes Dev.* **4**, 1588–1601 (1990).
13. Lettice, L.A. *et al.* Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly. *Proc. Natl. Acad. Sci. USA* **99**, 7548–7553 (2002).
14. Aitman, T.J. *et al.* Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat. Genet.* **16**, 197–201 (1997).
15. Tafti, M. *et al.* Deficiency in short-chain fatty acid β-oxidation affects τ oscillations during sleep. *Nat. Genet.* **34**, 320–325 (2003).
16. Gross, C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416**, 396–400 (2002).
17. Lai, C., Lyman, R.F., Long, A.D., Langley, C.H. & Mackay, T.F. Naturally occurring variation in bristle number and DNA polymorphisms at the scabrous locus of *Drosophila melanogaster*. *Science* **266**, 1697–1702 (1994).
18. De Luca, M. *et al.* Dopa decarboxylase (Ddc) affects variation in *Drosophila* longevity. *Nat. Genet.* **34**, 429–433 (2003).
19. Zhang, Y. *et al.* Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. *Nat. Genet.* **34**, 181–186 (2003).
20. Helms, C. *et al.* A putative RUNX1 binding site variant between *SLC9A3R1* and *NAT9* is associated with susceptibility to psoriasis. *Nat. Genet.* **35**, 349–356 (2003).
21. Ueda, H. *et al.* Association of the T-cell regulatory gene *CTLA4* with susceptibility to autoimmune disease. *Nature* **423**, 506–511 (2003).
22. Tokuhiro, S. *et al.* An intronic SNP in a RUNX1 binding site of *SLC22A4*, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.* **35**, 341–348 (2003).
23. Long, A.D., Mullaney, S.L., Mackay, T.F.C. & Langley, C.H. Genetic interactions between naturally occurring alleles at quantitative trait loci and mutant alleles at candidate loci affecting bristle number in *Drosophila melanogaster*. *Genetics* **144**, 1497–1510 (1996).
24. Steinmetz, L.M. *et al.* Dissecting the architecture of a quantitative trait locus in yeast. *Nature* **416**, 326–330 (2002).
25. Beck, J.A. *et al.* Genealogies of mouse inbred strains. *Nat. Genet.* **24**, 23–25 (2000).
26. Stephens, M. & Donnelly, P. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *Am. J. Hum. Genet.* **73**, 1162–1169 (2003).
27. Stephens, M., Smith, N.J. & Donnelly, P. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.* **68**, 978–989 (2001).
28. Talbot, C.J. *et al.* Fine scale mapping of a genetic locus for conditioned fear. *Mamm. Genome* **14**, 223–230 (2003).
29. Oliveira-Dos-Santos, A.J. *et al.* Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc. Natl. Acad. Sci. USA* **97**, 12272–12277 (2000).
30. Wade, C.M. *et al.* The mosaic structure of variation in the laboratory mouse genome. *Nature* **420**, 574–578 (2002).
31. Lindblad-Toh, K. *et al.* Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. *Nat. Genet.* **24**, 381–386 (2000).
32. Wiltshire, T. *et al.* Genome-wide single-nucleotide polymorphism analysis defines haplotype patterns in mouse. *Proc. Natl. Acad. Sci. USA* **100**, 3380–3385 (2003).
33. Hitzemann, R. *et al.* Multiple cross mapping (MCM) markedly improves the localization of a QTL for ethanol-induced activation. *Genes Brain Behav.* **1**, 214–222 (2002).
34. Plomin, R., McClearn, G.E., Gora-Maslak, G. & Neiderhiser, J.M. Use of recombinant inbred strains to detect quantitative trait loci associated with behavior. *Behav. Genet.* **21**, 99–116 (1991).
35. Turri, M.G., Datta, S.R., DeFries, J., Henderson, N.D. & Flint, J. QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice. *Curr. Biol.* **11**, 725–734 (2001).
36. Grafstein-Dunn, E., Young, K.H., Cockett, M.I. & Khawaja, X.Z. Regional distribution of regulators of G-protein signaling (RGS) 1, 2, 13, 14, 16, and GAIP messenger ribonucleic acids by in situ hybridization in rat brain. *Brain Res. Mol. Brain Res.* **88**, 113–123 (2001).
37. Dong, M.Q., Chase, D., Patikoglou, G.A. & Koelle, M.R. Multiple RGS proteins alter neural G protein signaling to allow *C. elegans* to rapidly change behavior when fed. *Genes Dev.* **14**, 2003–2014 (2000).
38. Rahman, Z. *et al.* RGS9 modulates dopamine signaling in the basal ganglia. *Neuron* **38**, 941–952 (2003).
39. Heximer, S.P. *et al.* Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J. Clin. Invest.* **111**, 445–452 (2003).
40. Huang, Z.P. *et al.* Expression of regulator of G-protein signalling protein 2 (RGS2) in the mouse uterus at implantation sites. *Reproduction* **126**, 309–316 (2003).
41. Legare, M.E., Bartlett, F.S. & Frankel, W.N. A major effect QTL determined by multiple genes in epileptic EL mice. *Genome Res.* **10**, 42–48 (2000).
42. Wanstrat, A. & Wakeland, E. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nat. Immunol.* **2**, 802–809 (2001).
43. Fijneman, R.J., de Vries, S.S., Jansen, R.C. & Demant, P. Complex interactions of new quantitative trait loci, Sluc1, Sluc2, Sluc3, and Sluc4, that influence the susceptibility to lung cancer in the mouse. *Nat. Genet.* **14**, 465–467 (1996).
44. van Wezel, T., Ruivenkamp, C.A., Stassen, A.P., Moen, C.J. & Demant, P. Four new colon cancer susceptibility loci, Scc6 to Scc9 in the mouse. *Cancer Res.* **59**, 4216–4218 (1999).
45. Mackay, T.F. The genetic architecture of quantitative traits: lessons from *Drosophila*. *Curr. Opin. Genet. Dev.* **14**, 253–257 (2004).
46. Flint, J., De Fries, J.C. & Henderson, N.D. Little epistasis for anxiety-related measures in the DeFries strains of laboratory mice. *Mamm. Genome* **15**, 77–82 (2004).
47. Contet, C., Rawlins, J.N. & Deacon, R.M. A comparison of 129S2/SvHsd and C57BL/6JOlaHsd mice on a test battery assessing sensorimotor, affective and cognitive behaviours: implications for the study of genetically modified mice. *Behav. Brain. Res.* **124**, 33–46 (2001).
48. Sambrook, J., Fritsch, E.F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989).

ORIGINAL ARTICLE

Influence of *RGS2* on Anxiety-Related Temperament, Personality, and Brain Function

Jordan W. Smoller, MD, ScD; Martin P. Paulus, MD; Jesen A. Fagerness, BA; Shaun Purcell, PhD; Lesley H. Yamaki, BS; Dina Hirshfeld-Becker, PhD; Joseph Biederman, MD; Jerrold F. Rosenbaum, MD; Joel Gelernter, MD; Murray B. Stein, MD, MPH

Context: Although anxiety disorders are heritable, their genetic and phenotypic complexity has made the identification of susceptibility genes difficult. Well-validated animal models and intermediate phenotypes provide crucial tools for genetic dissection of anxiety. The gene encoding regulator of G protein signaling 2 (*Rgs2*) is a quantitative trait gene that influences mouse anxiety behavior, making its human ortholog (*RGS2*) a compelling candidate gene for human anxiety phenotypes.

Objective: To examine whether variation in *RGS2* is associated with intermediate phenotypes for human anxiety disorders.

Design: Family-based and case-control association analysis of single-nucleotide polymorphisms at the *RGS2* locus in 3 independent samples.

Setting: Massachusetts General Hospital, University of California, San Diego, and San Diego State University.

Participants: Study participants included a family-based sample (n=119 families) of children who underwent laboratory-based assessments of temperament (behavioral inhibition), a sample of 744 unrelated adults who completed assessments of extraversion and introversion, and 55 unrelated adults who underwent functional magnetic resonance imaging measures of response to emotional faces.

Main Outcome Measures: Laboratory-based behavioral measures of childhood temperament, self-report measure of personality, and functional magnetic resonance imaging response to emotion processing.

Results: Markers spanning *RGS2* were associated with childhood behavioral inhibition, a temperamental precursor of social anxiety disorder (haplotype $P=3\times10^{-5}$; odds ratio, 2.99 in complete trios). In independent samples, *RGS2* markers, including rs4606, which has previously been associated with *RGS2* expression, were also associated with introversion (a core personality trait in social anxiety disorder) and with increased limbic activation (insular cortex and amygdala) during emotion processing (brain phenotypes correlated with social anxiety). The genotype at rs4606 explained 10% to 15% of the variance in amygdala and insular cortex activation to emotional faces.

Conclusions: These results provide the first evidence that a gene that influences anxiety in mice is associated with intermediate phenotypes for human anxiety disorders across multiple levels of assessment, including childhood temperament, adult personality, and brain function. This translational research suggests that some genetic influences on anxiety are evolutionarily conserved and that pharmacologic modulation of *RGS2* function may provide a novel therapeutic approach for anxiety disorders.

Arch Gen Psychiatry. 2008;65(3):298-308

ANXIETY DISORDERS ARE THE most common class of psychiatric disorders and are associated with a substantial burden of illness and more than $80 billion in annual costs to society. Anxiety disorders are known to be familial and heritable,[1] but the identification of susceptibility genes has been difficult to accomplish for several reasons. The etiology of these disorders is thought to reflect the effects of multiple genes of individually modest effect interacting with environmental factors. Limited understanding of the underlying neurobiological mechanism means that many genes are plausible risk candidates, but few are compelling. Beyond this genetic and biological complexity, considerable phenotypic complexity exists for anxiety disorders. Although the constellations of symptoms used as diagnostic criteria in the *DSM-IV* have been useful for clinical practice, it is unlikely that they are the optimal phenotype definitions for genetic analyses.[1] Family, twin, and linkage studies[1-3] suggest that genes confer susceptibility to anxiety proneness in a manner that cuts across clinical diagnostic labels. Although identifying anxiety susceptibility genes is a formidable challenge, well-validated animal models and intermediate phenotypes, including anxious temperament and functional neuroimaging phenotypes, provide crucial tools.

A quantitative trait locus on mouse chromosome 1 has been the most widely

Author Affiliations are listed at the end of this article.

replicated locus linked to anxious temperament phenotypes in mice.[4] Yalcin et al[5] fine-mapped this locus and identified the gene encoding regulator of G protein signaling 2 (*Rgs2*) as a quantitative trait gene underlying this linkage signal. *Rgs2* knockout mice exhibit increased anxiety and fear behavior, altered hippocampal synaptic plasticity, and elevated sympathetic tone. The Rgs2 protein, which is expressed in cortical and limbic brain regions, is part of a family of proteins that accelerate deactivation of G proteins to reduce G protein–coupled receptor (GPCR) signaling.[6] Neurotransmitters strongly implicated in the biological basis of anxiety, including serotonin and norepinephrine, act at GPCRs. We previously observed modest evidence of linkage between markers that encompass *RGS2* and a phenotype of anxiety disorder proneness in a targeted genome screen.[3] Thus, convergent evidence implicates *RGS2* as a compelling candidate locus underlying anxiety proneness in humans. We examined whether variation at the *RGS2* locus influences intermediate phenotypes for anxiety disorder at the level of behavior and brain function. We first examined the association of *RGS2* markers with behavioral inhibition to the unfamiliar (BI), an anxiety-related form of temperament characterized by a tendency to be shy, avoidant, and behaviorally restrained in situations that are novel or unfamiliar.[7] Mouse models of unconditioned and novelty-induced fear responses closely parallel behavioral and biological features of human BI, including inhibition of behavior and increased sympathetic nervous system reactivity.[8] We next examined the BI-related adult personality trait of introversion (low extraversion), which is also characterized by inhibition and avoidant behavior and, finally, limbic responses to emotional faces on functional magnetic resonance imaging (fMRI), a neuroimaging phenotype linked to BI,[9] anxiety proneness,[10] and social anxiety disorder (SAD).[11,12]

METHODS

SAMPLE

Behavioral Inhibition Sample

Participants were recruited from a sample of families who had participated in a study of children at risk for anxiety disorders conducted at Massachusetts General Hospital. Details of the study sample and behavioral assessments are provided elsewhere.[13] Children from these families were classified as "inhibited" or "not inhibited" on the basis of a behavioral assessment conducted at the ages of 21 months, 4 years, or 6 years. A total of 119 families that included at least 1 child who had undergone behavioral assessments were available at the time of these analyses. The self-reported race of all but 9 families was white. The protocol was approved by the Massachusetts General Hospital institutional review board. After complete description of the study, parents provided written informed consent for themselves and their children, who also provided oral or written assent.

Personality Sample

Participants (n=744; 516 female) were recruited from among undergraduate psychology students at San Diego State University (SDSU). Participants had blood drawn for genetic studies and completed questionnaires. Participants gave informed written consent to participate in this part of the study, which was approved by the Human Research Protection Programs at both the SDSU and University of California, San Diego. Participants received $25 for providing the blood sample. Power estimates indicate that the sample provides more than 85% power to detect an association at $\alpha < .05$ with a quantitative trait locus explaining as little as 1.5% of the trait variance.

Neuroimaging Sample

Participants. Initially, approximately 3000 SDSU undergraduate students participated in screening for a behavioral experiment in return for course credits. Of those individuals who participated in the behavioral study, approximately 1 of 3 expressed a willingness to participate in an fMRI study; an estimated 1 of 2 provided consent to be contacted for further assessment, and 1 of 2 of these proved eligible. We obtained 2 different samples, which had been collected during another ongoing project. During this time, our imaging studies shifted from a 1.5-T scanner (Siemens, Erlangen, Germany) to a 3.0-T scanner (Signa EXCITE; GE Healthcare, Milwaukee, Wisconsin). Sample 1 (1.5-T scanner) consisted of 29 healthy, right-handed individuals (17 women) with a mean (SD) age of 18.2 (0.62) years with a mean (SD) educational level of 12.6 (0.60) years. Sample 2 (3.0-T scanner) consisted of 26 healthy, right-handed individuals (24 women) with a mean (SD) age of 21.0 (2.6) years with a mean (SD) educational level of 14.5 (1.4) years. All study participants underwent the Structured Clinical Interview for *DSM-IV* to identify anxiety and mood disorders and were excluded if they were currently seeking, or had ever sought, treatment for their anxiety or mood symptoms. None of the participants had taken any psychotropic medications in the prior 12 months. Participants consumed less than 400 mg of caffeine daily. This study was approved by the SDSU and University of California, San Diego, institutional review boards. All participants gave their informed, written consent to participate and perform the emotion face-processing task during fMRI.

Behavioral Temperament Assessment. As described previously,[13] children underwent laboratory-based temperament assessments at 1 of 3 ages (21 months, 4 years, or 6 years) using age-specific measurement protocols. In brief, the evaluation consisted of behavioral protocols designed to assess the child's reaction to unfamiliar persons and events during a 90-minute battery. In these protocols, the child, with the mother present, encountered a variety of unfamiliar procedures and tasks, including physiologic measurements and cognitively challenging tasks administered by an unfamiliar female examiner, and their behavioral responses were observed and quantified. The assessments were videotaped and scored by raters who were blind to the assessment of psychopathologic conditions 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 (see Rosenbaum et al[13] for full description of coded variables). Studies conducted during the past 20 years have established that these variables differentiate inhibited from uninhibited children between the ages of 1 year and 8 years. As in our previous genetic studies of BI, children were classified as inhibited if they met at least 1 of 3 prespecified categorical definitions of BI[14]; children who met none of these definitions were considered unaffected. The genotyped families included 73 children with BI and 89 children without BI (total, 162 children). The sample included 77

two-parent families that comprised 114 trios, 41 single-parent families (including 17 with more than 1 genotyped offspring and 6 with phenotyped sibling pairs), and 1 sibling pair with no parental genotypes.

Self-report Personality Assessment. The NEO-Personality Inventory–Revised is a widely used, 240-item (plus 3 validity items) self-report measure of personality, grouped into 5 major domains: neuroticism (N), extraversion (E), openness to experience (O), conscientiousness (C), and agreeableness (A).[15] Some individuals completed a shorter (60-item) version of the NEO, the NEO-Five Factor Inventory,[15] which provides domain scores (expressed as T scores) that are highly correlated with those obtained from the full instrument. T scores were calculated directly from college-age, sex-specific norms. The phenotype of interest was extraversion T scores.

FUNCTIONAL MRI

Task

During fMRI, each participant was tested with a slightly modified[16] version of the emotion face assessment task (see Hariri et al[17]). During each 5-second trial, a participant is presented with a target face (on the top of the computer screen) and 2 probe faces (on the bottom of the screen) and is instructed to match the probe with the same emotional expression to the target by pressing the left or right key on a button box. A block consists of 6 consecutive trials during which the target face is angry, fearful, or happy. During the sensorimotor control task, individuals were presented with 5-second trials of either wide or tall ovals or circles in an analogous configuration and instructed to match the shape of the probe to the target. We did not use neutral faces as a comparator condition because there is mounting evidence that neutral faces are not actually processed as neutral.[18] Each block of faces and of the sensorimotor control task was presented 3 times in a pseudorandomized order. A fixation cross that lasted 8 seconds was interspersed between each block presented at the beginning and end of the task (resulting in 14 fixation periods). For each trial, response accuracy and reaction time data were obtained. There were 18 trials (3 blocks of 6 trials) for each face set and for shapes. The whole task lasted 512 seconds (matching the scan length).

Image Acquisition (1.5 T)

During the task, 1 blood oxygenation level–dependent (BOLD) fMRI run was collected for each study participant using a 1.5-T scanner (Siemens; T2-weighted echo planar imaging; repetition time [TR], 2000 milliseconds; echo time [TE], 40 milliseconds; 64×64 matrix; 20 4-mm axial sections; 256 repetitions). During the same experimental session, a T1-weighted image (MPRAGE; TR, 11.4 milliseconds; TE, 4.4 milliseconds; flip angle, 10°; field of vision [FOV], 256×256; 1-mm^3 voxels) was obtained for anatomical reference. For preprocessing, voxel time series were interpolated to correct for nonsimultaneous section acquisition within each volume and corrected for 3-dimensional motion.

Image Acquisition (3 T)

During the task, an fMRI run sensitive to BOLD contrast was collected for each participant using a 3.0-T scanner (Signa EXCITE) (T2-weighted echo planar imaging; TR, 2000 milliseconds; TE, 32 milliseconds; FOV, 250×250 mm^3; 64×64 matrix; 30 2.6-mm axial sections with a 1.4-mm gap; 290 scans). The fMRI acquisitions were time-locked to the onset of each trial. During the same experimental session, a high-resolution T1-weighted image (spoiled gradient recalled; T1 relaxation time, 450; TR, 8 milliseconds; TE, 4 milliseconds; flip angle, 12°; FOV, 256×256; 1-mm^3 voxels) was obtained for anatomical reference.

Genetic Methods

Selection of Single-Nucleotide Polymorphisms. For the analysis of BI temperament, single-nucleotide polymorphisms (SNPs) were selected to capture genetic variation across the *RGS2* locus. The SNPs were selected from a genomic region that comprised the *RGS2* gene, 25 kb of the 5′ flanking sequence, and 10 kb of the 3′ flanking sequence as defined in genome build hg17 using the phase 1 International HapMap Web site (http://www.hapmap.org). Ten tagging SNPs were selected from this region using Tagger (http://www.broad.mit.edu/mpg/tagger/) with $r^2 > 0.8$ and the "aggressive" tagging algorithm. We also included 5 SNPs in the gene (rs2746071, rs2746073, rs17647363, rs4606, and rs3767488) that were not available in HapMap at the time of selection but were found in dbSNP (http://www.ncbi.nlm.nih.gov/SNP). Linkage disequilibrium relationships within the SNP set were calculated using the Gabriel criteria as implemented in Haploview (http://www.broad.mit.edu/mpg/haploview/index.php). A single haplotype block encompassed the gene and all markers with the exception of the 5′ marker rs3856223, which nevertheless was in strong linkage disequilibrium with markers in the block and was thus included in the haplotype analysis. The final set of 15 SNPs achieved an average density of 1 SNP per 2 kb across a 32-kb region and an average r^2 of 0.90 with untyped HapMap markers that have minor allele frequency of 10% or more, indicating excellent coverage of variation across the locus. Because our marker set included 5 SNPs not included in HapMap, this average r^2 is an underestimate of the true variation captured by our set. For the NEO-E analyses, we selected a subset of the 15 markers based on the results of the BI analysis, and for the fMRI analyses, we selected rs4606 because of the recent report of its functional significance.[19]

Genotyping Method. Genotyping of SNPs in the BI family sample was performed by mass spectrometry (Sequenom, San Diego, California). Markers were retained for analysis if they met the following criteria: (1) no significant deviation from Hardy Weinberg equilibrium ($P > .01$) and (2) minimum call rate of 85% (average call rate, 96.4%). For 1 SNP (rs4606), genotypes were repeated and the resulting genotypes combined (overall call rate, 98%). All 15 markers were thus retained. Affected and unaffected individuals were spread across genotyping plates to avoid bias due to plate-specific genotyping error.

Genotyping for the NEO-E and fMRI Samples. The SNPs were genotyped with a fluorogenic 5′ nuclease assay method (ie, the TaqMan technique) using the ABI PRISM 7900 Sequence Detection System (ABI, Foster City, California). All genotypes were assayed in duplicate, and discordant genotypes (which ranged from 0% to 0.3%, depending on the marker) were discarded. All markers were in Hardy-Weinberg equilibrium.

Ancestral Proportion Scores. The ancestries of the study participants were estimated using a set of unlinked genetic markers by Bayesian cluster analysis, using STRUCTURE software (http://pritch.bsd.uchicago.edu/software.html). The markers were the set of short tandem repeats selected for ancestry information and described previously.[20] STRUCTURE implements Bayesian cluster modeling that can infer population genetic patterns without prior information of population origins.

The model was specified as "admixture" and "allele frequencies correlated," with 100 000 burn-in and 100 000 Markov chain Monte Carlo iterations. Analysis with STRUCTURE indicated adequate fit for a 3-class solution, which was used in the NEO-E and fMRI analyses.

STATISTICAL ANALYSES

Family-Based Association Analysis of Temperament

Family-based association analyses of the BI sample were performed using the Family Based Association Test (FBAT) Program 1.7.1 (http://www.biostat.harvard.edu/~fbat/default.html). The offset option of FBAT (using an offset equal to the sample prevalence) was used to incorporate all offspring who were phenotyped and genotyped. Haplotype-specific and global haplotype tests were performed using permutation (N = 100 000 cycles) by the hbat-p option. The min-p test was used to calculate a global haplotype test. This test evaluates the statistical significance of the smallest observed *P* value among all the individual haplotypes and estimates a *P* value by permutation. The odds ratio associated with the risk haplotype was calculated from complete trios using WHAP (http://pngu.mgh.harvard.edu/purcell//whap/).

NEO-E (Introversion) Association

Single-marker and haplotype-based analyses of the NEO-E quantitative trait were performed using WHAP, with empirical *P* values determined by permutation testing. Sample mean and variance were fixed to optimize model stability in single-marker and haplotype analyses. Analyses incorporated a 3-class population solution derived from STRUCTURE to avoid confounding due to population stratification.

Our analytic strategy was based on sequentially maximizing the prior probability of association and reducing multiple testing by first testing the full set of markers for the BI phenotype, retaining SNPs with the strongest signals for the introversion analysis, and then focusing on the most relevant markers for the fMRI analyses. The fMRI analysis focused on rs4606 in particular because it is the marker that has been associated with gene expression levels. To further support these results and confirm they were not a function of genotyping error, we also examined rs10801152 (the SNP that showed the strongest statistical evidence across the BI and introversion analyses).

fMRI Analysis

All structural and functional image processing was performed with the Analysis of Functional Neuroimages (AFNI) software package.[21] Echoplanar intensity images were coregistered to the 128th image using a 3-dimensional coregistration algorithm. The time series of the alignments in the x, y, z and roll, pitch, yaw direction was used to obtain motion regressors for each study participant. Because small motion corrections are similar in angle (eg, roll) and displacement (eg, x), we used only 3 motion parameters (roll, pitch, yaw) as nuisance regressors to account for motion artifacts. The 4 orthogonal regressors of interest were (1) happy, (2) angry, (3) fearful, and (4) circle or oval (ie, shape) sensorimotor condition. These regressors were convolved with a modified γ variate function to account for the delay and the dispersion brain response of the BOLD-fMRI signal due to hemodynamics.[22,23] Additional regressors were used to model residual motion in the roll, pitch, and yaw directions and baseline and linear trends. The AFNI program 3dDeconvolve was used to calculate the estimated voxel-wise response amplitude. A gaussian filter with a full width at half maximum of 4 mm was applied to the voxelwise percentage of signal change data to account for individual variations in the anatomical landmarks.

Data from each participant were normalized to Talairach coordinates. Whole-brain analyses were followed by a priori analysis of regions of interest using masks (defined by the Talairach demon atlas)[24] in the bilateral amygdala, insular cortices, ventromedial prefrontal cortex, and primary visual cortex. On the basis of these areas of interest, it was determined via simulations that a voxel-wise a priori probability of .05 would result in a corrected clusterwise activation probability of .05 if a minimum volume of 128 µL and 2 connected voxels (in the amygdala, which is a small structure) or 512 µL and 8 connected voxels (in all other regions of interest) was considered. The areas of interest were superimposed on each individual's voxelwise percentage of signal change brain image. Only activations within the areas of interest, which also satisfied the volume and voxel connection criteria, were extracted and used for further analysis. The corrected voxelwise probabilities are as follows: amygdala, $P < .012$; insular cortex, $P < .000069$; medial prefrontal cortex, $P < .00014$; and visual cortex, $P < .000070$. These corrected voxel probabilities are based on Monte Carlo simulations using AFNI's program AlphaSim using the filtered data and the a priori defined regions of interest.

To maximize power by increasing the number of individuals homozygous for the putative rs4606 risk (G) allele, we pooled the 1.5-T and 3-T data sets rather than considering them separately. Specifically, for these analyses we used a regression approach with magnet type (1.5 T or 3 T) and ancestral informative markers (AIM 1 and AIM 2) coefficients as covariates and genotype for rs4606 (CC = 0, CG = 1, GG = 2) as the variable of interest. Areas with a significant gene effect (ie, voxelwise partial correlation coefficient with $P < .05$) that also fulfilled the volume-threshold cluster condition within the regions of interest were extracted, and additional statistical analyses were conducted using SPSS statistical software, version 15.0 (SPSS Inc, Chicago, Illinois). Results of the regression analyses are presented in eTable 1 (available at http://www.archgenpsychiatry.com).

RESULTS

ASSOCIATION OF *RGS2* WITH CHILDHOOD ANXIOUS TEMPERAMENT

We first examined whether *RGS2* influences behavioral and neurobiological phenotypes underlying human anxiety by examining a form of human anxious temperament that shares core phenotypic and biologic features with mouse models of unconditioned and novelty-induced fear behavior. Behavioral inhibition to the unfamiliar is a heritable temperamental profile characterized by a tendency to be shy, avoidant, and behaviorally restrained in situations that are novel or unfamiliar.[25] Biological features of BI include evidence of increased sympathetic tone and limbic hyperreactivity to novel stimuli.[9,25] In addition, BI is a familial and developmental risk factor for anxiety disorders (and, in particular, SAD)[26] but has greater estimated heritability than the diagnostic categories.[27] The sample comprised 119 families in which children underwent standardized laboratory-based behavioral assessments of BI, as previously described.[13] To capture genetic variation across the *RGS2* locus, we genotyped a set of 15 SNPs with an average density of 1 SNP per 2 kb across a 32-kb region spanning

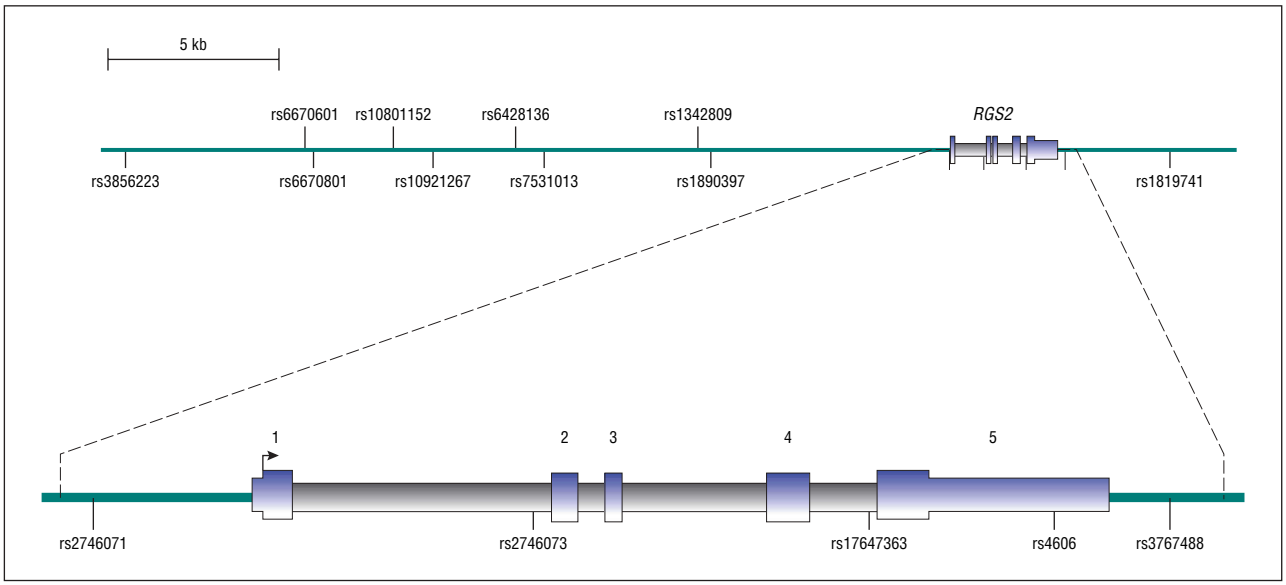


Figure 1. Schematic of the gene encoding regulator of G protein signaling 2 (*RGS2*) locus, depicting positions of genotyped single-nucleotide polymorphisms. The lower portion of the figure shows a magnification of the *RGS2* locus. Kb indicates kilobase.

Table 1. Association of Behavioral Inhibition to the Unfamiliar With Markers Spanning the *RGS2* Locus: Single Marker Results

Marker	Position (hg17)	Major/Minor Alleles	MAF	*z* (Allele)[a]	*P* Value
rs3856223	189484723	C/T	0.35	1.90 (T)	.057
rs6670601	189490272	A/C	0.47	0.54 (C)	.59
rs6670801	189490498	A/G	0.36	1.51 (G)	.13
rs10801152	189492961	A/T	0.31	2.99 (T)	.0028[b]
rs10921267	189494228	C/T	0.27	2.43 (T)	.015
rs6428136	189496545	T/G	0.27	2.92 (G)	.0036
rs7531013	189497600	G/A	0.47	0.26 (G)	.79
rs1342809	189502209	G/T	0.17	2.05 (G)	.040
rs1890397	189502590	G/A	0.49	0.04 (A)	.97
rs2746071	189509221	A/G	0.29	1.98 (G)	.047
rs2746073	189510884	T/A	0.25	2.57 (A)	.010
rs17647363	189512155	A/G	0.16	1.53 (A)	.13
rs4606	189512829	C/G	0.27	3.01 (G)	.0026[b]
rs3767488	189513296	A/G	0.25	2.78 (G)	.0055
rs1819741	189516495	T/C	0.26	3.07 (C)	.0021[b]

Abbreviations: MAF, minor allele frequency; *RGS2*, gene encoding regulator of G protein signaling 2.
[a] *z* Statistic and overtransmitted allele.
[b] *P* values are significant after Bonferroni correction for 15 single-marker tests.

RGS2 (**Figure 1**). As indicated in **Table 1**, **Table 2**, and **Figure 2**, 9 of the 15 SNPs tested were associated with BI, including the G allele of the 3′ UTR SNP rs4606 ($P = .0026$), which has been shown to be associated with reduced *RGS2* expression in vitro.[19] A haplotype that comprised all 15 markers was also associated with BI (permuted $P = 3.0 \times 10^{-5}$). The odds ratio for BI associated with the risk haplotype, calculated from complete family trios, was 2.99 (95% confidence interval, 1.31-6.84).

ASSOCIATION OF *RGS2* WITH SOCIAL ANXIETY–RELATED PERSONALITY IN ADULTS

In adults, social inhibition can be indexed by the personality trait of introversion (low extraversion), which, like BI, is a heritable trait[28] characterized by low levels of sociability and aversion to large groups. Longitudinal data suggest that childhood BI is a developmental precursor of introversion (but not neuroticism).[29] Like BI, introversion is associated with risk for anxiety disorders, including SAD.[30] If variants in *RGS2* are associated with temperamental shyness, we hypothesized that these variants would also be associated with introversion (low extraversion). We genotyped the 4 markers that showed the strongest signal in the BI sample in an independent sample of 744 college undergraduates (228 men and 516 women) who completed the NEO-Personality Inventory–Revised, from which the extraversion scale (NEO-E) can be derived.[15] Consistent with our prediction, we observed an association between NEO-E and the same alleles of these 4 markers that were associated with BI (**Table 3**). A haplotype of these 4 alleles was also associated with

Table 2. Association of Behavioral Inhibition to the Unfamiliar With Markers Spanning the *RGS2* Locus: Haplotype Results (>5% Frequency) With Permuted *P* Values

Haplotype	Frequency	*P* Value
C-A-A-A-C-T-A-G-A-A-T-A-C-A-T	0.48	.68
T-C-G-T-T-G-G-G-G-G-A-A-G-G-C	0.18	2×10^{-5}
C-C-A-A-C-T-G-T-G-A-T-G-C-A-T	0.08	.75
C-A-A-A-C-T-G-G-G-A-T-A-C-A-T	0.07	.59
T-C-G-A-C-T-G-T-G-A-T-G-C-A-T	0.07	.11
Global minimal *P* permutation test		3×10^{-5}

Abbreviation: *RGS2*, gene encoding regulator of G protein signaling 2.

introversion (global and haplotype-specific $P = .04$). Although our primary hypothesis was that *RGS2* would be associated with introversion, we performed secondary analyses to determine whether the effect was specific to this trait. In those analyses, we observed no association between *RGS2* markers and the other NEO subscales (neuroticism, openness, conscientiousness, or agreeableness).

ASSOCIATION OF *RGS2* WITH SOCIAL ANXIETY–RELATED BRAIN FUNCTION

In light of previous studies that suggest that BI and social anxiety are mediated by hyperreactivity of brain structures (especially amygdala and insular cortices) thought to underlie anxiety proneness,[9-12] we hypothesized that *RGS2* variants associated with BI and introversion would also show association with functional reactivity of these structures during emotion processing. To investigate this, we examined genotype effects on limbic brain reactivity to emotional faces, a neuroimaging assay of anxious temperament. Previous fMRI studies[31] have shown that limbic brain circuits involved in anxiety are activated when individuals view novel or emotional faces. In particular, increased amygdala activation to novel or emotional faces has been associated with inhibited temperament,[9] social anxiety traits,[32] and SAD,[12] although other areas, including the anterior cingulate cortex and the insular cortex, have also been implicated.[31,33] By directly indexing brain function, anxiety-related fMRI phenotypes may provide more proximal and therefore more powerful measures of gene action.

For these analyses, we selected the 3′ UTR SNP rs4606, which has been associated with variation in *RGS2* messenger RNA expression.[19] We genotyped rs4606 in 2 independent groups (29 tested in a 1.5-T magnet and 26 tested in a 3-T magnet) of healthy volunteers drawn from an ongoing study with college-age individuals. To maximize power for analyses of the putative risk allele (rs4606-G), we pooled the samples and included a covariate for the magnets. Individuals in both magnets were tested during fMRI using the same version of a slightly modified emotion face assessment task that has been shown to be sensitive to genetic influence.[17] For each 5-second trial, an individual is presented with a target face (on the top of the computer screen) and 2 probe faces

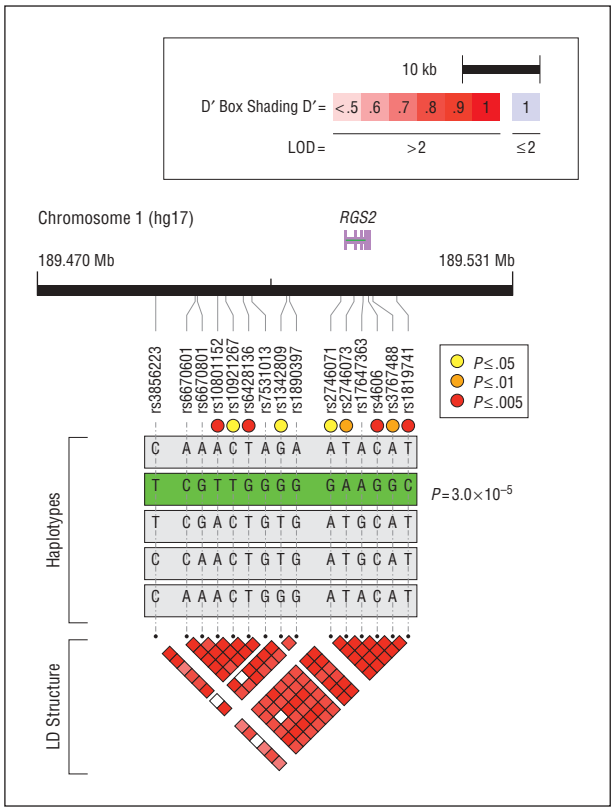


Figure 2. Linkage disequilibrium (LD) and association analyses for the gene encoding regulator of G protein signaling 2 (*RGS2*) in the behavioral inhibition to the unfamiliar family sample. Gene position for the *RGS2* locus is shown above the chromosome bar. Relative positions of genotyped single-nucleotide polymorphism markers are shown, and significant single-marker association results are depicted with colored circles. Haplotypes with frequencies greater than 5% are shown below the markers, and the associated haplotype is shaded in green. Pairwise marker LD using the D′ statistic is indicated by the matrix at bottom (red indicates strong LD; white, weak LD). LOD indicates logarithm of the odds. Figure was drawn with Locusview (T. Petryshen, A. Kirby, and M. Ainscow, unpublished software; http://www.broad.mit.edu/mpg/locusview). Kb indicates kilobase; Mb, megabus.

(on the bottom of the screen) and is instructed to match the probe with the same emotional expression (happy, sad, or angry) to the target by pressing the left or right key on a button box. During the sensorimotor control task, study participants were presented with 5-second trials of either wide or tall ovals or circles in an analogous configuration and instructed to match the shape of the probe to the target. Several investigators have used this task to show significant activations in the amygdala during the presentation of faces vs the sensorimotor control condition.[17,34] Moreover, we have found previously that the degree of insular cortex activation during this task was modulated by both short-term administration of an anxiolytic and by the degree of anxiety proneness.[10,16] The rs4606 G allele, which showed association with BI and introversion in the analyses described herein, was significantly associated with the degree of left amygdala and bilateral insular cortex activation (**Figure 3**, **Figure 4**, and eTable 1). Specifically, in models that control for magnet (1.5 T vs 3 T) and ancestry-informative marker clusters,[20] the rs4606 G allele was independently associated with the extracted average activation

Table 3. Single Marker and Haplotype Association of 4 *RGS2* Markers With Introversion

Marker	Risk Alleles[a]	LRT	*P* Value
rs10801152	T	7.18	.0074
rs6428136	G	5.54	.019
rs4606	G	4.11	.043
rs1819741	C	3.81	.051
4-Marker haplotype[b]	T-G-G-C	4.32	.038

Abbreviations: LRT, likelihood ratio test statistic (1 *df*); *RGS2*, gene encoding regulator of G protein signaling 2.
[a] Allele associated with introversion.
[b] Haplotype-specific test with a minimum haplotype frequency of 5%. Analysis of ancestry-informative markers indicated no effect of population stratification.

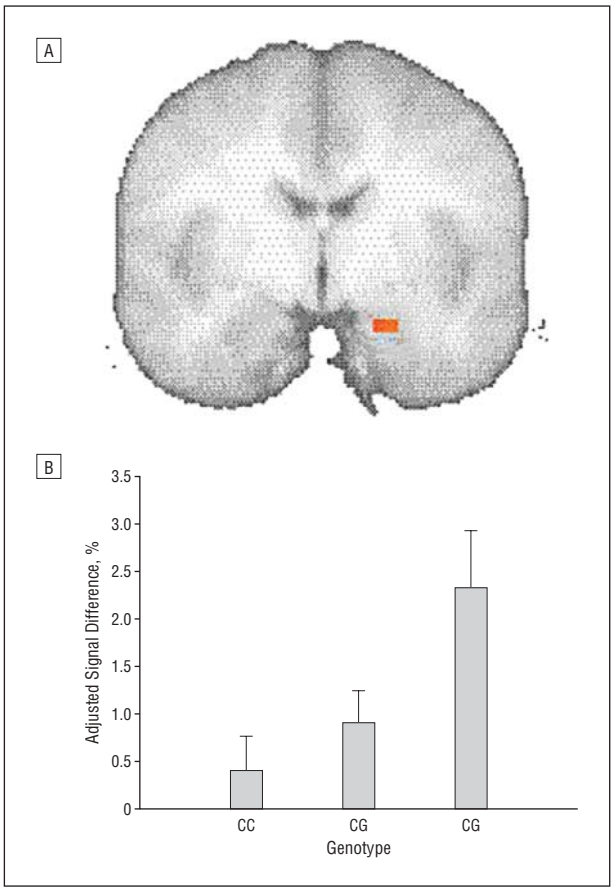


Figure 3. Activation differences associated with the rs4606 genotype in the left amygdala for the combined 1.5-T and 3-T samples. The marginal means are obtained relative to the covaried ancestral proportion weights and field strength indicators. A, Active voxels are volume thresholded at an a posteriori $P<.05$. Red area indicates significant difference across genotype. B, Associated bar graphs show the signal difference between emotion face processing and the sensorimotor control condition (adjusted for ancestral proportion weights and field strength indicators). Error bars indicate SD.

of 2 clusters in the left insular cortex ($P=.009$ and $P=.007$, respectively) and a cluster in the right insular cortex ($P=.005$) and left amygdala ($P=.02$). The rs4606 SNP accounted for approximately 15% of the variance in amygdala activation and approximately 10% to 15% of the variance in the insular cortex activation. To corroborate the finding with rs4606, we also examined rs1081152, which in our analyses showed a strong association with both inhibited temperament and introversion. As with rs4606, we observed significant association of the rs1081152 risk allele (T) with left insular cortex ($P=.009$) and amygdala ($P=.03$) activation (**Figure 5** and eTable 2). On the basis of the combined volume and voxelwise *P* value threshold, we did not find any other clusters in the brain associated with the *RGS2* SNPs. Finally, in a secondary analysis, we confirmed that the rs4606 genotype effect on insula-amygdala activation was similar in each magnet (1.5 T and 3 T) considered separately (eFigure; available at http://www.archgenpsychiatry.com).

COMMENT

On the basis of consistent results derived from a set of different but interrelated anxiety paradigms in independent samples, we observed compelling evidence that *RGS2*, the ortholog of a mouse anxiety quantitative trait gene, is also associated with anxiety-related phenotypes in humans. A particularly strong effect was seen for childhood BI, which closely parallels behavioral and biologic features of mouse phenotypes influenced by murine *Rgs2*. Our findings are the first, to our knowledge, to document association of a specific gene with social anxiety across 3 levels of phenotypic analysis: a laboratory-based behavioral measure of childhood temperament, a self-report measure of adult personality, and a neuroimaging measure of functional brain activity.

The finding of *RGS2*-related activation in the amygdala is analogous to similar findings with the serotonin transporter promoter polymorphism[17] and the catechol O-methyltransferase val-met variant.[34] In addition, our results are the first, to our knowledge, to demonstrate an association between the insular cortex, a limbic brain region involved in emotional processing,[33] and a gene implicated in anxiety. The insular cortex is part of a neural system involved in homeostatic processing of autonomic arousal and visceral changes, signaling executive areas to initiate avoidant behavior and altering self-awareness.[33] The insular cortex, medial prefrontal cortex, and amygdala play crucial roles in linking internal physiologic states to external cues or events. Although some investigators have proposed that the connectivity between the amygdala and the medial prefrontal cortex or anterior cingulate is a critical genetically determined factor, dysfunction of which predisposes individuals to anxiety or depression,[35] the limited number of high-risk allele individuals in our sample prevented a rigorous test of this hypothesis using functional connectivity measures. Clearly, future investigation will need to examine genetic determinants of functional connectivity between amygdala or insular cortex and other areas that are important for emotion regulation.[36]

RGS2 is one of a family of regulators of G protein signaling that function as guanosine triphosphatase (GTPase) accelerating proteins, terminating G protein signaling by binding to activated Gα subunits and accelerating the rate of guanosine triphosphate (GTP) hydrolysis.[6] *RGS2* regulates $G_{i/o}$ and Gqα and is expressed in brain regions

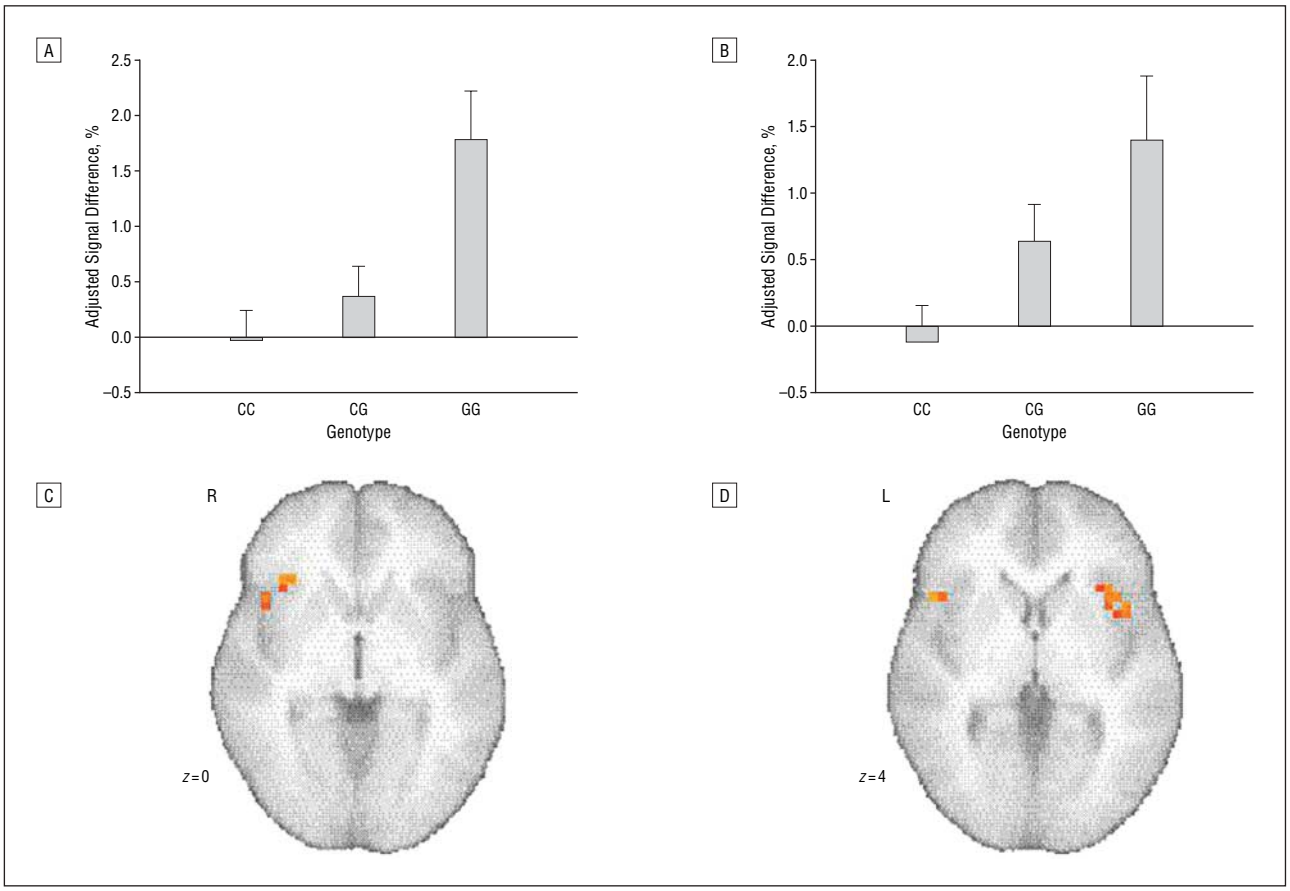


Figure 4. Activation differences associated with the rs4606 genotype in bilateral insular cortices for the combined 1.5-T and 3-T samples. The marginal means are obtained relative to the covaried ancestral proportion weights and field strength indicators. For the statistical effect, see eTable 1 (available at http://www.archgenpsychiatry.com). A and B, The bar graphs show the larger of the 2 areas within the right anterior insular cortex (A) and the left anterior insular cortex (B). Error bars indicate SD. C and D, Axial sections in Talairach coordinates at z=0 and z=4 show 2 regions on the left (D) and 1 on the right (C) (in red).

thought to underlie anxiety, including the hippocampus, amygdala, cerebral cortex, hypothalamus, and dorsal raphe nuclei.[6,37,38] Neurotransmitters implicated in anxiety, including serotonin, norepinephrine, and dopamine, act at GPCRs. *RGS2* has been shown to markedly decrease Gqα signaling by serotonin 2A receptors,[39] which, in turn, play a key role in anxiety and stress responses, as well as response to serotonergic antidepressants. *RGS2* has also been shown to regulate hippocampal synaptic plasticity by increasing neurotransmitter release via presynaptic $G_{i/o}$-mediated Ca^{2+} channel inhibition.[40] Neuronal *RGS2* transcription is modulated by plasticity-inducing synaptic stimuli and by agents known to affect anxiety and mood symptoms,[6,38] and *RGS2* expression has been implicated in experience-dependent development of neural circuits.[37] *Rgs2*-deficient mice exhibit increased anxiety behavior,[41] increased sympathetic tone, reduced heart rate variability, altered blood pressure response to a novel environment, and increased urinary norepinephrine excretion[42]—features also reported in human BI. Our results suggest that at least some genetic influences on fear responses to novelty are evolutionarily conserved. The identity of the specific phenotype-influencing variant(s) mediating *RGS2* effects on human anxiety phenotypes cannot be determined from these data, although the dense map of SNPs examined in the analysis of BI captures a minimum of 90% of the genetic variation in the region and is likely to have directly or indirectly assayed the relevant variants. Resequencing of the gene in previous studies[19,43] has not revealed common coding sequence variants. However, the G allele of rs4606, which was associated with anxiety phenotypes in our study, has been associated with reduced *RGS2* expression in both peripheral blood mononuclear cells and fibroblasts in hypertensive patients.[19] Reduced *RGS2* expression is expected to be associated with anxiety given that deletion of the gene is associated with anxious temperament in mice.[5,41]

Taken together, our results suggest a model in which genetic variation associated with reduced expression of *RGS2* contributes to increased reactivity of limbic brain structures modulating anxious temperament and social anxiety. At a behavioral level, this genetic effect is most evident in direct measurements of inhibited temperament (which itself has been shown to be associated with amygdala reactivity in previous research[9]), with a weaker effect detectable on adult social anxiety–related personality. This model rests in part on the premise that our measures of temperament, personality, and brain function are phenotypically convergent. One way to verify this would be to measure all 3 phenotypes in the same individuals and examine their relationship; this was not possible because BI is based on laboratory-based behavioral temperament observations in young children (who could

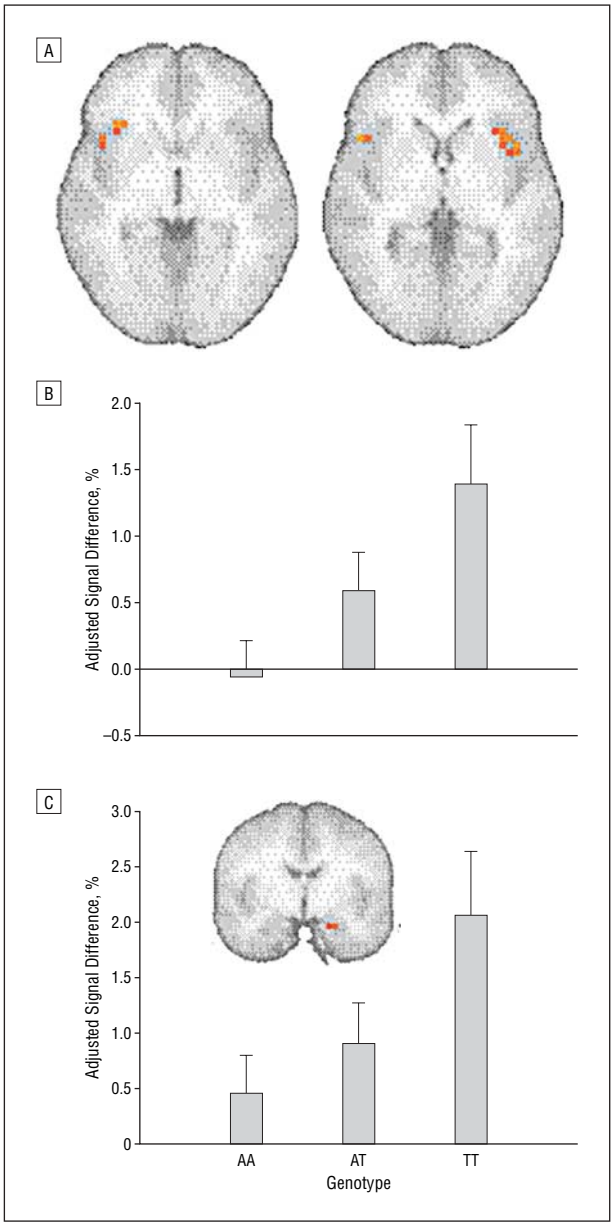


Figure 5. The rs10801152 genotype effect—marginal signal difference (face shape)—as a function of genotype adjusted for magnet strength and ancestral proportion. A, The axial section and bar graphs show activation differences (in red) associated with the rs10801152 genotype in the left anterior insular cortex (circle). B, The axial section and bar graphs show activation differences associated with the rs10801152 genotype in the left amygdala. Red areas indicate the voxels within the amygdala and insular cortex that differ by genotype; error bars, SD.

not complete the NEO or undergo functional imaging). However, substantial evidence indicates that BI, introversion, and limbic reactivity are, in fact, convergent phenotypes: prior studies have demonstrated an association between BI measured and introversion,[29,44] between BI and limbic reactivity to emotional faces,[9,45] and among all 3 traits (BI, introversion, limbic reactivity to emotional faces) and SAD.[11,12,30,46-51]

In secondary analyses that examined the specificity of *RGS2* effects on personality, we did not observe association with neuroticism, which has been reported to be linked to the 1q region syntenic with mouse chromosome 1.[52] Our data do not support the hypothesis that *RGS2* underlies this linkage signal because our adult sample was powered to detect loci explaining as little as 1.5% of the variance in neuroticism. However, the linked region contains many genes, and it may be that 1 or more of these genes contribute to neuroticism. Of note, however, a recent whole genome association study of neuroticism failed to detect any loci at this region.[53] To our knowledge, no linkage or association studies that include loci on 1q have examined the phenotype of introversion. Prior studies suggest that introversion is more specifically related to BI and social anxiety,[44,50,54,55] whereas neuroticism appears to be a nonspecific risk factor for depression and anxiety disorders (especially generalized anxiety disorder).[56,57] Although neuroticism mainly captures negative emotionality and worry, introversion is more directly related to social inhibition and shyness, core features of BI. Caspi et al[29] examined temperament in 3-year-old children and followed up these children to adulthood. Inhibited temperament at the age of 3 years was associated with introversion at the age of 26 years but was unrelated to adult neuroticism. Gladstone and Parker[44] found that an adult measure of BI was strongly and similarly correlated with introversion ($r=0.75$) and social anxiety ($r=0.77$). In a subsequent study,[47] retrospective childhood BI was significantly associated with social phobia but not panic disorder, generalized anxiety disorder, or agoraphobia. Numerous other studies,[26,48,49] including longitudinal studies, have confirmed the specific relationship between BI and social anxiety, and we have previously shown in a college sample that introversion is highly and significantly correlated with measures of shyness and social anxiety.[58]

Further studies will be needed to determine which, if any, anxiety disorder phenotypes are most tightly related to *RGS2*. Given the association with BI and introversion, 2 traits that are risk factors for SAD, we would predict that SAD is the most likely anxiety disorder to be associated with *RGS2*. A recent study by Leygraf et al[59] reported nominally significant evidence of association between *RGS2* markers and panic disorder, although these results would not survive correction for multiple testing. To the extent that genetic effects on *DSM-IV* anxiety disorders may be smaller than effects on the intermediate phenotypes examined herein, much larger samples may be needed for studies of the clinical disorders.

In conjunction with studies in mouse models, our findings suggest that *RGS2* modulators could provide a novel therapeutic approach for the treatment of anxiety disorders. For example, agents that facilitate *RGS2* would be expected to inhibit GPCR signaling in response to neurotransmitters targeted by antidepressants that effectively treat anxiety disorders. The hypothesis that anxiety proneness is related to reduced *RGS2* expression implies that agents that enhance *RGS2* activity would be anxiolytic. The regional expression of *RGS2* in limbic and paralimbic brain areas coupled with its selectivity for Gqα-mediated and $G_{i/o}$ signaling might enhance the therapeutic action of GPCR-based treatments of anxiety and mood disorders.[6]

Submitted for Publication: June 25, 2007; final revision received October 10, 2007; accepted October 10, 2007.

Author Affiliations: Psychiatric Genetics Program in Mood and Anxiety Disorders (Dr Smoller and Ms Yamaki), Psychiatric and Neurodevelopmental Genetics Unit, Center for Human Genetic Research (Drs Smoller and Purcell, Mr Fagerness, and Ms Yamaki), and Department of Psychiatry (Drs Smoller, Purcell, Hirshfeld-Becker, Biederman, and Rosenbaum, Mr Fagerness, and Ms Yamaki), Massachusetts General Hospital, Harvard Medical School, Boston; Departments of Psychiatry (Drs Paulus and Stein) and Family and Preventive Medicine (Dr Stein), University of California, San Diego, and VA San Diego Healthcare System; and Departments of Psychiatry, Genetics, and Neurobiology, Division of Human Genetics in Psychiatry, Yale University School of Medicine, New Haven, Connecticut (Dr Gelernter).
Correspondence: Jordan W. Smoller, MD, ScD, Center for Human Genetic Research, Simches Research Bldg, 185 Cambridge St, Boston, MA 02114 (jsmoller@hms.harvard.edu).
Author Contributions: Drs Gelernter and Stein contributed equally to this work.
Financial Disclosure: Dr Smoller has served as a consultant for Eli Lilly and Company; has received honoraria from Hoffmann-La Roche, Inc, Enterprise Analysis Corp, and MPM Capital; and has served on an advisory board for Roche Diagnostics Corporation. A patent application related to work described in this article is pending. Dr Paulus has been a paid consultant for GlaxoSmithKline Pharmaceuticals; has received research support from Forest Laboratories, GlaxoSmithKline, Roche, Sepracor, the National Institute on Drug Abuse (NIDA), the National Institute of Mental Health (NIMH), and the VA Merit System; and has received speaking honoraria from Pfizer. Dr Biederman currently receives research support from Bristol-Myers Squibb, Eli Lilly and Company, Janssen Pharmaceuticals Inc, McNeil, Otsuka, Shire, the NIMH, and the National Institute of Child Health and Human Development; is currently a consultant/advisory board member for Janssen, McNeil, Novartis, and Shire; and currently serves on the speakers bureaus of Janssen, McNeil, Novartis, Shire, and UCB Pharma, Inc. In addition, he has received research support, consultation fees, or speaker's fees from Abbott, AstraZeneca, Celltech, Cephalon, Eli Lilly and Company, Esai, Forest, Gliatech, NARSAD, Neurosearch, New River, the NIDA, Novartis, Noven, Pfizer, Pharmacia, the Prechter Foundation, the Stanley Foundation, and Wyeth. Dr Rosenbaum has served on one or more advisory boards for AstraZeneca, Bristol-Myers Squibb, Cephalon, Cyberonics, Eli Lilly and Company, Forest, GlaxoSmithKline, Janssen, MedAvante, Novartis, Orexigen, Pfizer, Roche Diagnostics, Sanofi, Shire, Sepracor, Somerset, and Wyeth; has offered consultation to Compellis, EPIX, Neuronetics, Organon, Somaxon, and Supernus; and has received one or more honoraria for lectureship from Boehringer-Ingelheim, Bristol-Myers Squibb, Cyberonics, Eli Lilly and Company, Forest, and Schwartz. He also attended the Massachusetts General Hospital Psychiatry Academy, sponsored by AstraZeneca, Bristol-Myers Squibb, Cephalon, Eli Lilly and Company, GlaxoSmithKline, Janssen, and Wyeth. He has equity holdings in Compellis, MedAvante, and Somaxon. Dr Stein has been a paid consultant for AstraZeneca, Avera Pharmaceuticals, BrainCells Inc, Bristol-Myers Squibb, Cephalon, Eli Lilly and Company, EPI-Q Inc, Forest Laboratories, GlaxoSmithKline Pharmaceuticals, Hoffmann-La Roche Pharmaceuticals, Integral Health Decisions Inc, Jazz Pharmaceuticals, Johnson & Johnson, Mindsite, Pfizer, Sanofi-Aventis, Solvay Pharmaceuticals, Transcept Pharmaceuticals Inc, UCB Pharma, Virtual Reality Medical Center, and Wyeth and has received research support from Eli Lilly and Company, Forest Laboratories, GlaxoSmithKline, and UCB Pharma.
Funding/Support: This study was supported in part by NIMH grant K-08 MH01770 (Dr Smoller); a NARSAD Young Investigator Award (Dr Smoller); NIMH grant R01MH47077-11(Dr Rosenbaum) and a gift from the Rose and Eugene Kleiner Family Foundation; funds from the US Department of Veterans Affairs (the VA Medical Research Program [Merit Review to Dr Gelernter]); NIDA grants DA12690, DA12849, and DA15105; and NIMH grants K24 MH64122 and R01 MH65413 (Dr Stein).
Additional Information: The e-Tables and e-Figure are available at http://www.archgenpsychiatry.com.
Additional Contributions: The following assisted in this work: Pamela Sklar, MD, PhD, Lisa Watras, BA, Brian Galloway, BA, Priya Moorjani, MS, Alan Simmons, PhD, Jerome Kagan, PhD, and Nancy Snidman, PhD. We are grateful to the individuals and families who participated in this work.

REFERENCES

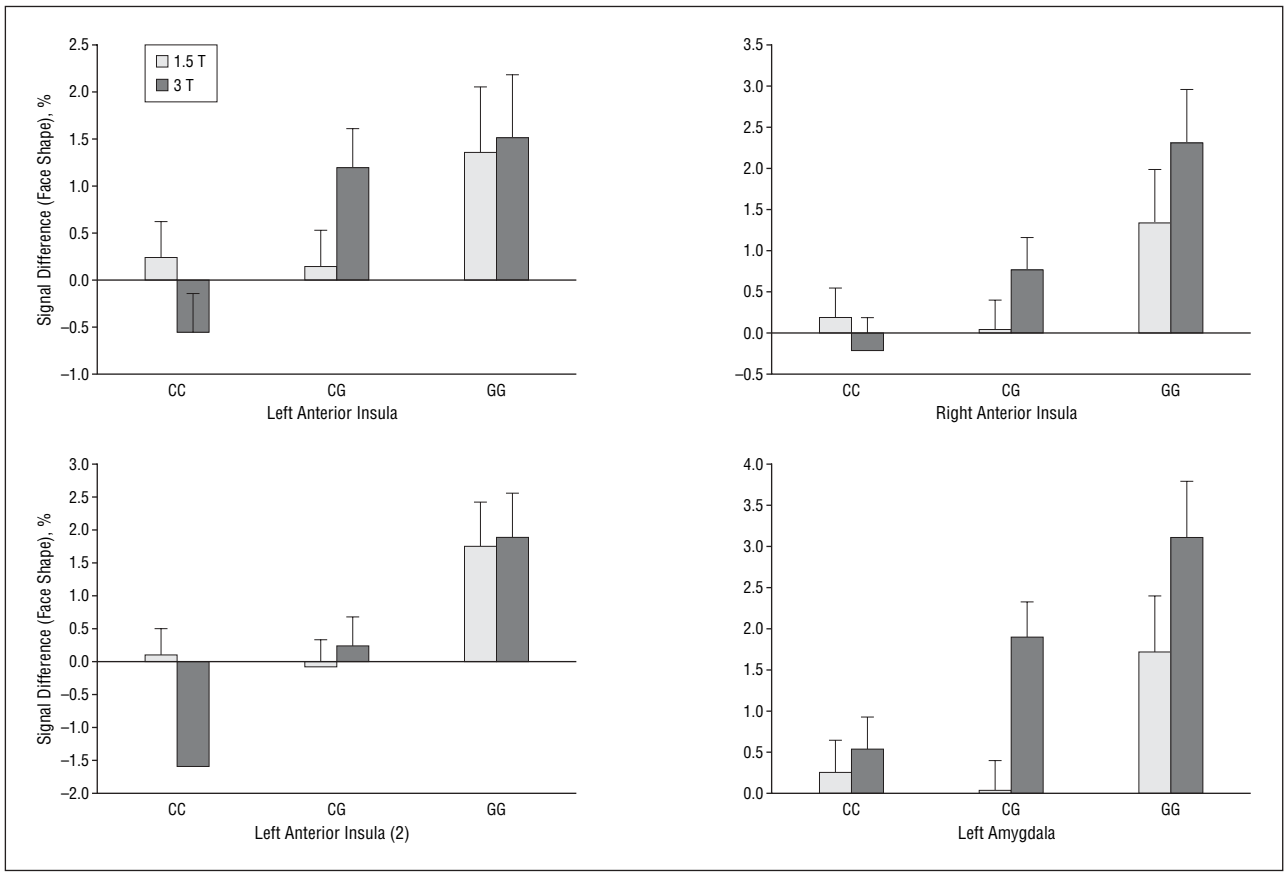
1. Smoller JW, Tsuang M. Panic and phobic anxiety: defining phenotypes for genetic studies. *Am J Psychiatry*. 1998;155(9):1152-1162.
2. Kaabi B, Gelernter J, Woods SW, Goddard A, Page GP, Elston RC. Genome scan for loci predisposing to anxiety disorders using a novel multivariate approach: strong evidence for a chromosome 4 risk locus. *Am J Hum Genet*. 2006;78(4):543-553.
3. Smoller JW, Acierno JS Jr, Rosenbaum JF, Biederman J, Pollack MH, Meminger S, Pava JA, Chadwick LH, White C, Bulzacchelli M, Slaugenhaupt SA. Targeted genome screen of panic disorder and anxiety disorder proneness using homology to murine QTL regions. *Am J Med Genet*. 2001;105(2):195-206.
4. Flint J. Analysis of quantitative trait loci that influence animal behavior. *J Neurobiol*. 2003;54(1):46-77.
5. Yalcin B, Willis-Owen SA, Fullerton J, Meesaq A, Deacon RM, Rawlins JN, Copley RR, Morris AP, Flint J, Mott R. Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice. *Nat Genet*. 2004;36(11):1197-1202.
6. Neubig RR, Siderovski DP. Regulators of G-protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov*. 2002;1(3):187-197.
7. Kagan J. *Galen's Prophecy*. New York, NY: BasicBooks; 1994.
8. Flint J, Corley R, DeFries JC, Fulker DW, Gray JA, Miller S, Collins AC. A simple genetic basis for a complex psychological trait in laboratory mice. *Science*. 1995;269(5229):1432-1435.
9. Schwartz CE, Wright CI, Shin LM, Kagan J, Rauch SL. Inhibited and uninhibited infants "grown up": adult amygdalar response to novelty. *Science*. 2003;300(5627):1952-1953.
10. Stein MB, Simmons AN, Feinstein JS, Paulus MP. Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. *Am J Psychiatry*. 2007;164(2):318-327.
11. Phan KL, Fitzgerald DA, Nathan PJ, Tancer ME. Association between amygdala hyperactivity to harsh faces and severity of social anxiety in generalized social phobia. *Biol Psychiatry*. 2006;59(5):424-429.
12. Stein MB, Goldin PR, Sareen J, Zorrilla LT, Brown GG. Increased amygdala activation to angry and contemptuous faces in generalized social phobia. *Arch Gen Psychiatry*. 2002;59(11):1027-1034.
13. Rosenbaum JF, Biederman J, Hirshfeld-Becker DR, Kagan J. A controlled study of behavioral inhibition in children of parents with panic disorder and depression. *Am J Psychiatry*. 2000;157(12):2002-2010.
14. Smoller JW, Rosenbaum J, Biederman J, Kennedy J, Dai D, Racette SR, Laird NM,

Kagan N, Snidman N, Hirshfeld-Becker D, Tsuang MT, Sklar PB, Slaugenhaupt SA. Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. *Biol Psychiatry*. 2003;54(12):1376-1381.
15. Costa PT, McCrae RR. *NEO-PI-R Professional Manual*. Odessa, FL: Psychological Assessment Resources; 1992.
16. Paulus MP, Feinstein JS, Castillo G, Simmons AN, Stein MB. Dose-dependent decrease of activation in bilateral amygdala and insula by lorazepam during emotion processing. *Arch Gen Psychiatry*. 2005;62(3):282-288.
17. Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, Egan MF, Weinberger DR. Serotonin transporter genetic variation and the response of the human amygdala. *Science*. 2002;297(5580):400-403.
18. Cooney RE, Atlas LY, Joormann J, Eugene F, Gotlib IH. Amygdala activation in the processing of neutral faces in social anxiety disorder: is neutral really neutral? *Psychiatry Res*. 2006;148(1):55-59.
19. Semplicini A, Lenzini L, Sartori M, Papparella I, Calo LA, Pagnin E, Strapazzon G, Benna C, Costa R, Avogaro A, Ceolotto G, Pessina AC. Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *J Hypertens*. 2006;24(6):1115-1124.
20. Yang BZ, Zhao H, Kranzler HR, Gelernter J. Practical population group assignment with selected informative markers: characteristics and properties of Bayesian clustering via STRUCTURE. *Genet Epidemiol*. 2005;28(4):302-312.
21. Cox RW. AFNI: software for analysis and visualization of functional magnetic resonance neuroimages. *Comput Biomed Res*. 1996;29(3):162-173.
22. Cohen MS. Parametric analysis of fMRI data using linear systems methods. *Neuroimage*. 1997;6(2):93-103.
23. Friston KJ, Frith CD, Turner R, Frackowiak RS. Characterizing evoked hemodynamics with fMRI. *Neuroimage*. 1995;2(2):157-165.
24. Lancaster JL, Woldorff MG, Parsons LM, Liotti M, Freitas CS, Rainey L, Kochunov PV, Nickerson D, Mikiten SA, Fox PT. Automated Talairach atlas labels for functional brain mapping. *Hum Brain Mapp*. 2000;10(3):120-131.
25. Kagan J, Reznick S, Snidman N. Biological bases of childhood shyness. *Science*. 1988;240(4849):167-171.
26. Biederman J, Hirshfeld-Becker DR, Rosenbaum JF, Hérot C, Friedman D, Snidman N, Kagan J, Faraone SV. Further evidence of association between behavioral inhibition and social anxiety in children. *Am J Psychiatry*. 2001;158(10):1673-1679.
27. Robinson JL, Kagan J, Reznick JS, Corley R. The heritability of inhibited and uninhibited behavior: a twin study. *Dev Psychol*. 1992;28(6):1030-1037.
28. Jang KL, Livesley WJ, Vernon PA. Heritability of the big five personality dimensions and their facets: a twin study. *J Pers*. 1996;64(3):577-591.
29. Caspi A, Harrington H, Milne B, Amell JW, Theodore RF, Moffitt TE. Children's behavioral styles at age 3 are linked to their adult personality traits at age 26. *J Pers*. 2003;71(4):495-513.
30. Bienvenu OJ, Nestadt G, Samuels JF, Costa PT, Howard WT, Eaton WW. Phobic, panic, and major depressive disorders and the five-factor model of personality. *J Nerv Ment Dis*. 2001;189(3):154-161.
31. Phan KL, Wager TD, Taylor SF, Liberzon I. Functional neuroimaging studies of human emotions. *CNS Spectr*. 2004;9(4):258-266.
32. Killgore WD, Yurgelun-Todd DA. Social anxiety predicts amygdala activation in adolescents viewing fearful faces. *Neuroreport*. 2005;16(15):1671-1675.
33. Paulus MP, Stein MB. An insular view of anxiety. *Biol Psychiatry*. 2006;60(4):383-387.
34. Drabant EM, Hariri AR, Meyer-Lindenberg A, Munoz KE, Mattay VS, Kolachana BS, Egan MF, Weinberger DR. Catechol O-methyltransferase val158met genotype and neural mechanisms related to affective arousal and regulation. *Arch Gen Psychiatry*. 2006;63(12):1396-1406.
35. Pezawas L, Meyer-Lindenberg A, Drabant EM, Verchinski BA, Munoz KE, Kolachana BS, Egan MF, Mattay VS, Hariri AR, Weinberger DR. 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. *Nat Neurosci*. 2005;8(6):828-834.
36. Meyer-Lindenberg A, Weinberger DR. Intermediate phenotypes and genetic mechanisms of psychiatric disorders. *Nat Rev Neurosci*. 2006;7(10):818-827.
37. Ingi T, Aoki Y. Expression of RGS2, RGS4 and RGS7 in the developing postnatal brain. *Eur J Neurosci*. 2002;15(5):929-936.
38. Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, Barnes CA, Lanahan AA, Siderovski DP, Ross EM, Gilman AG. Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J Neurosci*. 1998;18(18):7178-7188.
39. Ghavami A, Hunt RA, Olsen MA, Zhang J, Smith DL, Kalgaonkar S, Rahman Z, Young KH. Differential effects of regulator of G protein signaling (RGS) proteins on serotonin 5-HT1A, 5-HT2A, and dopamine D2 receptor-mediated signaling and adenylyl cyclase activity. *Cell Signal*. 2004;16(6):711-721.
40. Han J, Mark MD, Li X, Xie M, Waka S, Rettig J, Herlitze S. RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating G(i/o)-mediated inhibition of presynaptic Ca(2+) channels. *Neuron*. 2006;51(5):575-586.
41. Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, Mariathasan S, Saski T, Wakeham A, Ohaski PS, Roder JC, Barnes CA, Siderovski DP, Penninger JM. Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc Natl Acad Sci U S A*. 2000;97(22):12272-12277.
42. Gross V, Tank J, Obst M, et al. Autonomic nervous system and blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol*. 2005;288(5):R1134-R1142.
43. Riddle EL, Rana BK, Murthy KK, Plehm R, Blumer KJ, Diedrich A, Jordan J, Luft FC. Polymorphisms and haplotypes of the regulator of G protein signaling-2 gene in normotensives and hypertensives. *Hypertension*. 2006;47(3):415-420.
44. Gladstone G, Parker G. Measuring a behaviorally inhibited temperament style: development and initial validation of new self-report measures. *Psychiatry Res*. 2005;135(2):133-143.
45. Pérez-Edgar K, Roberson-Nay R, Hardin MG, Poeth K, Guyer AE, Nelson EE, McClure EB, Henderson HA, Fox NA, Pine DS, Ernst M. Attention alters neural responses to evocative faces in behaviorally inhibited adolescents. *Neuroimage*. 2007;35(4):1538-1546.
46. Hayward C, Killen J, Kraemer H, Taylor C. Linking self-reported childhood behavioral inhibition to adolescent social phobia. *J Am Acad Child Adolesc Psychiatry*. 1998;37(12):1308-1316.
47. Gladstone GL, Parker GB, Mitchell PB, Wilhelm KA, Malhi GS. Relationship between self-reported childhood behavioral inhibition and lifetime anxiety disorders in a clinical sample. *Depress Anxiety*. 2005;22(3):103-113.
48. Hirshfeld-Becker DR, Biederman J, Henin A, Faraone SV, Davis S, Harrington K, Rosenbaum JF. Behavioral inhibition in preschool children at risk is a specific predictor of middle childhood social anxiety: a five-year follow-up. *J Dev Behav Pediatr*. 2007;28(3):225-233.
49. Schwartz CE, Snidman N, Kagan J. Adolescent social anxiety as an outcome of inhibited temperament in childhood. *J Am Acad Child Adolesc Psychiatry*. 1999;38(8):1008-1015.
50. Bienvenu OJ, Samuels JF, Costa PT, Reti IM, Eaton WW, Nestadt G. Anxiety and depressive disorders and the five-factor model of personality: a higher- and lower-order personality trait investigation in a community sample. *Depress Anxiety*. 2004;20(2):92-97.
51. Lorberbaum JP, Kose S, Johnson MR, Arana GW, Sullivan LK, Hamner MB, Ballenger JC, Lydiard RB, Brodrick PS, Bohning DE, George MS. Neural correlates of speech anticipatory anxiety in generalized social phobia. *Neuroreport*. 2004;15(18):2701-2705.
52. Fullerton J, Cubin M, Tiwari H, Wang C, Bomhra A, Davidson S, Miller S, Fairburn C, Goodwin G, Neale MC, Fiddy S, Mott R, Allison DB, Flint J. Linkage analysis of extremely discordant and concordant sibling pairs identifies quantitative-trait loci that influence variation in the human personality trait neuroticism. *Am J Hum Genet*. 2003;72(4):879-890.
53. Shifman S, Bhomra A, Smiley S, Wray NR, James MR, Martin NG, Hettema JM, An SS, Neale MC, van den Oord EF, Kendler KS, Chen X, Boomsma DI, Middeldorp CM, Hottenga JJ, Slagboom PE, Flint J. A whole genome association study of neuroticism using DNA pooling [published ahead of print July 21, 2007]. *Mol Psychiatry*. doi:10.1038/sj.mp.4002048.
54. Bienvenu OJ, Brown C, Samuels JF, Liang KY, Costa PT, Eaton WW, Nestadt G. Normal personality traits and comorbidity among phobic, panic and major depressive disorders. *Psychiatry Res*. 2001;102(1):73-85.
55. Hummelen B, Wilberg T, Pedersen G, Karterud S. The relationship between avoidant personality disorder and social phobia. *Compr Psychiatry*. 2007;48(4):348-356.
56. Hettema JM, Prescott CA, Kendler KS. Genetic and environmental sources of covariation between generalized anxiety disorder and neuroticism. *Am J Psychiatry*. 2004;161(9):1581-1587.
57. Kendler KS, Gardner CO, Gatz M, Pedersen NL. The sources of co-morbidity between major depression and generalized anxiety disorder in a Swedish national twin sample. *Psychol Med*. 2007;37(3):453-462.
58. Stein MB, Schork NJ, Gelernter J. A polymorphism of the beta1-adrenergic receptor is associated with low extraversion. *Biol Psychiatry*. 2004;56(4):217-224.
59. Leygraf A, Hohoff C, Freitag C, Willis-Owen SA, Krakowitzky P, Fritze J, Franke P, Bandelow B, Fimmers R, Flint J, Deckert J. Rgs 2 gene polymorphisms as modulators of anxiety in humans? *J Neural Transm*. 2006;113(12):1921-1925.

Web-only material for YOA70086

Jordan W. Smoller, MD

eFigure.



eFigure. Results of analysis of covariance with magnet and rs4606 genotype as the main variables and ancestral-informative markers 1 and 2 as covariates. All values for the bar graphs are corrected for the covariates. The risk allele rs4606-G was associated with increased activation during the faces relative to the sensorimotor control condition in all areas identified in the pooled magnet analyses described in the text. Error bars indicate SD.

eTable 1. Multiple Regression Analyses of the rs4606 Genotype for the Constrained Regions of Interest in the Amygdala and Left and Right Insular Cortex[a]

Region	Tesla	AIM 1	AIM 2	rs4606
Left insula (1)[b]				
Coefficient	0.17	−0.75	−1.29	0.74
t	0.68	−1.32	−1.80	2.73
P value	.498	.19	.08	.009
Left insula (2)[b]				
Coefficient	−0.10	0.01	−0.62	1.14
t	−0.27	0.01	−0.58	2.84
P value	.79	.99	.56	.007
Right insula				
Coefficient	0.39	0.41	−0.41	0.78
t	1.64	0.74	−0.60	2.97
P value	.12	.46	.55	.005
Left amygdala				
Coefficient	0.71	−1.62	−2.26	0.84
t	2.28	−2.22	−2.46	2.40
P value	.03	.03	.02	.02

[a] Tesla indicates the 1.5-T vs the 3-T magnet as a covariate, and AIM 1 and AIM 2 refer to ancestry-informative marker covariates derived from STRUCTURE (http://pritch.bsd.uchicago.edu/software.html). As indicated, the association with the rs4606 genotype explains a significant proportion of the variance in activation, even after adjusting for these covariates.

[b] There were 2 areas of significant activation within the left insula.

e-Table 2. Multiple Regression Analyses of the rs10801152 Genotype for the Constrained Regions of Interest in the Amygdala and the Left and Right Insular Cortex Defined by the rs4606 Results[a]

Region	Tesla	AIM 1	AIM 2	rs10801152
Left insula (1)[b]				
Coefficient	0.19	−0.66	−1.21	0.70
t	0.78	−1.18	−1.72	2.74
P value	.44	.24	.09	.009
Left insula (2)[b]				
Coefficient	−0.13	0.08	−0.14	0.63
t	−0.34	0.10	−0.12	1.56
P value	.74	.92	.90	.12
Right insula				
Coefficient	0.37	0.47	−0.02	0.36
t	1.49	0.81	−0.03	1.34
P value	.14	.42	.98	.19
Left amygdala				
Coefficient	0.72	−1.54	−2.13	0.74
t	2.34	−2.14	−2.35	2.21
P value	.02	.04	.02	.03

[a] The rs10801152 T allele (which was also associated with behavioral inhibition to the unfamiliar and introversion) was associated with left insula and amygdala activation. Tesla indicates the 1.5-T vs the 3-T magnet as a covariate, and AIM 1 and AIM 2 refer to ancestry-informative marker covariates derived from STRUCTURE (http://pritch.bsd.uchicago.edu/software.html).

[b] There were 2 areas of significant activation within the left insula.

Development and Psychopathology **24** (2012), 1179–1193

doi:10.1017/S0954579412000636

Bringing a developmental perspective to anxiety genetics

LAUREN M. McGRATH,[a] SYDNEY WEILL,[a] ELISE B. ROBINSON,[a] REBECCA MacRAE,[b] AND JORDAN W. SMOLLER[a]

[a]*Massachusetts General Hospital; and* [b]*Massachusetts Institute of Technology*

Abstract

Despite substantial recent advancements in psychiatric genetic research, progress in identifying the genetic basis of anxiety disorders has been limited. We review the candidate gene and genome-wide literatures in anxiety, which have made limited progress to date. We discuss several reasons for this hindered progress, including small samples sizes, heterogeneity, complicated comorbidity profiles, and blurred lines between normative and pathological anxiety. To address many of these challenges, we suggest a developmental, multivariate framework that can inform and enhance anxiety phenotypes for genetic research. We review the psychiatric and genetic epidemiological evidence that supports such a framework, including the early onset and chronic course of anxiety disorders, shared genetic risk factors among disorders both within and across time, and developmentally dynamic genetic influences. We propose three strategies for developmentally sensitive phenotyping: examination of early temperamental risk factors, use of latent factors to model underlying anxiety liability, and use of developmental trajectories as phenotypes. Expanding the range of phenotypic approaches will be important for advancing studies of the genetic architecture of anxiety disorders.

Many excellent papers in this Special Issue address the ways in which genetic and genomic sciences are informing our understanding of developmental psychopathology. Here, we take a complementary approach in which we explore the ways that developmental science can contribute to more informative genetic studies. We focus specifically on anxiety disorders and argue that a developmental approach to the phenotype will be particularly important, given the early onset and high prevalence of anxiety disorders in children.

We cover four main topics: the current state of the candidate gene and genome-wide association literatures in anxiety disorders; current obstacles to gene finding in anxiety genetics; the psychiatric and genetic epidemiology of anxiety disorders with a specific focus on data that can guide developmental, multivariate approaches to phenotype definition; and strategies for developmentally sensitive phenotyping that could be used in existing samples and in future study designs.

We note here a clarification of our use of the broad term *anxiety disorders*. *DSM-IV-TR* lists 13 separate anxiety disorders, but we will focus on the most commonly diagnosed, idiopathic anxiety disorders: generalized anxiety disorder (GAD), panic disorder (PD) with and without agoraphobia, agoraphobia without a history of PD, separation anxiety disorder, social phobia, and specific phobia. Although the *DSM-IV-TR* includes obsessive–compulsive disorder (OCD) and posttraumatic stress disorder (PTSD) in the anxiety category, there is also evidence that these disorders have partly distinct etiologic and neurobiological underpinnings (Eley et al., 2003; Graybiel & Rauch, 2000; Heim & Nemeroff, 2009; Tambs et al., 2009). For this reason, this paper will not include OCD and PTSD, an approach that is consistent with the new *DSM-5* proposal for the anxiety disorders category. We also acknowledge the strong phenotypic and genetic overlap between depression and many of the anxiety disorders (Brady & Kendall, 1992; Franic, Middeldorp, Dolan, Ligthart, & Boomsma, 2010; Kendler, Neale, Kessler, Heath, & Eaves, 1992; Middeldorp, Cath, Van Dyck, & Boomsma, 2005; Weissman et al., 2005), but for simplicity we focus here on the anxiety disorders themselves.

Anxiety disorders are among the most common forms of child, adolescent, and adult psychopathology (lifetime prevalence of 28.8%; Kessler, Berglund, et al., 2005; Merikangas, He, Brody, et al., 2010; Merikangas, He, Burstein, et al., 2010). These disorders not only affect a large number of individuals but also are chronic and disabling conditions resulting in considerable individual and societal cost. Anxiety disorders as a group had the largest burden of role disability among the common mental health conditions, exceeding the burden for mood disorders and substance abuse/dependence disorders in a large, national representative sample of adults (Merikangas et al., 2007). In addition, anxiety disorders typically emerge in childhood (Kessler, Berglund, et al., 2005; Merikangas, He, Burstein, et al., 2010) and can be impairing across development through disruption to family, peer, and academic functioning (Essau, Conradt, & Petermann, 2000; Ezpeleta, Keeler, Erkanli, Costello, & Angold, 2001). The economic burden of anxiety disorders is substantial (estimated at $63 billion in

The authors were supported by T32-MH16259 (to L.M.M.), T32-MH017119 (to E.B.R.), and K24-MH094614 (to J.W.S.). Special thanks to Alysa Doyle, Laramie Duncan, Erin Dunn, and Alisha Pollastri for helpful comments on the manuscript.

Address correspondence and reprint requests to: Lauren M. McGrath, Massachusetts General Hospital, Simches Research Building 6th floor, 185 Cambridge Street, Boston, MA 02114; E-mail: mcgrath@pngu.mgh.harvard.edu.

1998 dollars; Greenberg et al., 1999). Beyond the costs for psychiatric treatment, there are also additional directs costs, such as repeated use of health care services for physical symptoms, and indirect costs, such as lost productivity at work (Greenberg et al., 1999). Improved prevention and treatment for anxiety disorders would be impactful both for individual quality of life and for societal productivity.

As with most psychiatric disorders, familial and genetic influences are among the best substantiated risk factors for anxiety disorders. Given this, the hope is that clarifying the genetic basis of these syndromes will point to more effective and specific interventions. Anxiety disorders are arguably in one of the strongest positions among the psychiatric disorders to execute successful translational work from genetic risk factors to novel treatments because of the uniquely strong neurobiological (Etkin, 2010; Johansen, Cain, Ostroff, & LeDoux, 2011; Pine, 2007; Shin & Liberzon, 2010) and animal modeling literature (Flint, Shifman, Munafo, & Mott, 2008) in fear and anxiety.

Review of Anxiety Genetics

Heritability

There is now accumulated evidence that anxiety disorders are familial and heritable (Hettema, Neale, & Kendler, 2001). The heritability estimates across each anxiety disorder and anxious traits are generally comparable in children, adolescents, and adults, with estimates clustering in the range of h^2 values from ~0.25 to 0.60 (for reviews, see Franic et al., 2010; Gregory & Eley, 2007; Hettema et al., 2001). The variability across studies could be due to many potential factors, including parent report versus self-report, age of assessment, categorical versus dimensional categorizations, and specific anxiety disorder analyzed (Franic et al., 2010; Gregory & Eley, 2007). Despite these factors contributing to variability, there is also good consistency across studies that anxious traits and anxiety disorders are moderately heritable.

Candidate gene approaches

Like the psychiatric genetics field more generally, the candidate gene literature in anxiety genetics is large and complex, with few replicable associations (Duncan & Keller, 2011; Hewitt, 2012; Sullivan, 2007). To assess the current state of the literature, we conducted a systematic review of published candidate gene studies for the anxiety disorders or anxiety symptom measures (not including temperament or personality traits). Through Pubmed searches and manual inspection of meta-analyses and reviews, we identified over 350 anxiety candidate gene studies. Restricting the search to studies with a sample size of at least 200 cases (or nuclear families) yielded only 65 reports from which main effects could be extracted. Although this sample size threshold is quite low given current standards in complex disease genetics (Sullivan, 2010), it was chosen because of the small pool of well-powered anxiety genetic studies. We focus on the main effects because studies of Gene × Environment and Gene × Gene interactions have been largely underpowered (Duncan & Keller, 2011). We also focus on single nucleotide polymorphisms (SNPs) rather than haplotypes, which are difficult to compare across studies. An enumeration of the 65 studies is available from the first author upon request.

Our review of these 65 studies revealed that PD has been the most frequently studied single disorder ($N = 30$ studies), compared to GAD ($N = 4$), social anxiety disorder ($N = 1$), specific phobia ($N = 1$), and separation anxiety ($N = 0$). Anxiety symptom measures and/or a combined phenotype across anxiety disorders have also been frequently studied ($N = 29$ studies). Fifty-six different genes were investigated across the studies with many studies including more than one gene. The three most commonly studied genes were catechol-*O*-methyltransferase (*COMT*, 8 studies), solute carrier family 6, member 4 (*SLC6A4*, also known as serotonin transporter [*5-HTT*], 7 studies), and brain-derived neurotrophic factor (*BDNF*, 4 studies), each of which has been the focus of at least one meta-analysis for anxiety phenotypes, which will be discussed further below. Only 13 of these 56 genes (23%) had been studied in two independent reports with sample sizes above the 200 case (or family) minimum. Keeping in mind this lower bound, the average case sample size in this truncated sample was 387 cases ($SD = 185$, $Mdn = 376$) and 416 controls ($SD = 224$, $Mdn = 351$). Across studies, the largest case-control analysis examined *SLC6A4* in 1,161 cases diagnosed with an anxiety disorder and/or depression and 1,051 controls (Wray et al., 2009). It is worth nothing that this study found no consistent evidence of association with the commonly investigated 5-HTT linked polymorphic region (*5-HTTLPR*) variant (Wray et al., 2009).

Given the high rate of false positives in genetic association studies, particularly when power is limited, we investigated whether there was any evidence for independently replicated association of a variant with a specific anxiety disorder at $p < .05$ (uncorrected). Studies considering a cross-disorder anxiety phenotype (e.g., case = GAD or PD) or anxiety symptoms were considered together. There was no restriction on the measures used to obtain these diagnoses or quantitative traits. Even when applying these liberal statistical and definitional criteria, there was *not a single instance of replication*. Although it is possible we have overlooked an instance of replication, our search makes clear that replicated association between a genetic variant and any anxiety disorder is at least rare when a sample size restriction of 200 cases is imposed. This observation is consistent with candidate gene findings in the larger psychiatric genetics literature (Duncan & Keller, 2011; Hewitt, 2012; Sullivan, 2007).

Three genetic variants have been studied frequently enough to be the subject of meta-analyses: the *5-HTTLPR* polymorphism of *SLC6A4* with panic (Blaya, Salum, Lima, Leistner-Segal, & Manfro, 2007), the Val/Met polymorphism of *COMT* with panic (Domschke, Deckert, O'Donovan M, & Glatt, 2007; Zintzaras & Sakelaridis, 2007), and the Val/Met polymorphism of *BDNF* with a cross-disorder anxiety phenotype (including phobias, GAD, PD, OCD, and PTSD; Frustaci,

Pozzi, Gianfagna, Manzoli, & Boccia, 2008). Only one of these four meta-analyses reported a qualified positive result for *COMT* with panic (Domschke et al., 2007). Although the overall meta-analysis showed no significant effect, there was significant heterogeneity in the analysis, which was attributed to a female-specific effect and differential effects in Caucasian and Asian populations (Domschke et al., 2007). This meta-analysis included 6 case-control samples, each of which had fewer than 200 cases (total combined $N = 557$ cases and 763 controls; Domschke et al., 2007), which is why we did not observe the same effects in our review of the *COMT* literature. All of these meta-analyses have noted the tentative nature of the conclusions that can be drawn, whether positive or negative, because of the small number of case-control studies with small samples. Publication bias is also a crucial concern for the psychiatric genetics literature (Duncan & Keller, 2011).

In summary, keeping in mind the sample size requirements we imposed on the literature review, we did not identify any replication of candidate gene variants, even considering our liberal statistical criteria. This result is supported by four meta-analyses, none of which have reported a clear main effect. Our findings are also consistent with the observation that a priori candidate genes have not generally emerged as significant in genome-wide scans (e.g., Collins, Kim, Sklar, O'Donovan, & Sullivan, 2012). For this reason, we believe that efforts to assemble large anxiety samples for genome-wide investigations will be fruitful in focusing the field on novel, replicable genetic risk variants.

Genome-wide approaches

An alternative to the candidate gene approach is to utilize genome-wide scans, which examine genetic loci throughout the genome. Both linkage and association designs can be used for genome-wide scans. We focus here on genome-wide association results, which have been successful in identify risk loci for other psychiatric disorders. (For a review of linkage studies in anxiety, see Domschke & Reif, 2012; Maron, Hettema, & Shlik, 2010; Webb et al., 2012.)

Genome-wide association studies (GWAS). To date, there have been two small-scale GWAS for anxiety disorders, both focusing on PD (Erhardt et al., 2011; Otowa et al., 2009). The study by Otowa et al. (2009) in 200 patients and 200 controls reported two genome-wide significant SNPs in the genes transmembrane protein 16B (*TMEM16B*) and plakophilin 1. However, a subsequent replication attempt in 558 cases and 566 controls did not support these findings (Otowa et al., 2010).

Erhardt et al. (2011) used a discovery sample of 216 cases and 222 controls along with several replication samples. The authors reported two SNPs in transmembrane (TMEM) protein 132D (*TMEM132D*) that were nominally associated with PD in three independent samples (combined sample = 909 cases and 915 controls), although the joint analysis p values did not exceed a genome-wide significance threshold ($ps = 10^{-6}$). The authors went on to examine the biological relevance of these SNPs, demonstrating that the risk genotype was associated with higher *TMEM132D* mRNA expression in human postmortem frontal cortex. These results were supported by findings in a mouse model where anxiety-related behaviors were associated with a SNP in *Tmem132d* and with mRNA expression of *Tmem132d* in the anterior cingulate (Erhardt et al., 2011). The convergence of results indicating genetic association across samples, biological plausibility, and support from animal models is a compelling picture that would be further strengthened by genome-wide significant results in larger, independent samples.

In the broader psychiatric genetics literature, GWAS has been a key strategy for identifying replicable common genetic risk variants (Ripke et al., 2011; Sklar et al., 2011; Sullivan, 2012). This tangible progress has been invaluable for psychiatric genetics, but GWAS designs can have limitations that are important to consider. Because the sample size must be large, the phenotyping is often less precise than in smaller studies, sometimes relying on a few items from quantitative trait scales. In addition, GWAS studies typically have limited information on environmental exposures, which constrains testing of Gene × Environment interactions. Finally, by design, most GWAS studies only assay common genetic variants, which typically have modest effects and likely do not capture the full genetic architecture of complex disorders.

Genome-wide copy number variation (CNV) studies. CNVs are another form of genetic variation that can be investigated in genome-wide studies. CNVs are segments of DNA that range from 1 kilobase (kb) to millions of base pairs that are either deleted or duplicated. In autism and schizophrenia, large, rare CNVs have been shown to be etiologically important in a subset of cases (for a review, see Malhotra & Sebat, 2012).

To date, there has been only one genome-wide CNV study of anxiety disorders (Kawamura et al., 2011). This study focused on PD and included 535 cases and 1,520 controls of Japanese ancestry. The study did not detect an excess burden of rare CNVs, but the authors reported a Bonferroni-corrected significant association ($p < .05$) with common duplications in the 16p11.2 region. CNV detection is difficult in this pericentromeric region, so replication of the finding using multiple methods for CNV calling and laboratory validations will be important. The region is approximately 2 megabases away from a large, rare CNV in 16p11.2 that has been associated with autism and other neurodevelopmental disorders (Malhotra & Sebat, 2012).

In summary, genome-wide studies of anxiety disorders have been limited to date. However, given the successes achieved with these methods in other complex disorders, there is reason to be hopeful that larger studies will provide novel clues to the genetic basis of pathologic anxiety. We turn now to the major challenges facing the field of anxiety genetics and our recommendations to address some of these challenges.

Challenges in Gene Finding for Anxiety

There are four primary challenges that have hindered progress in gene finding for anxiety disorders or traits: (a) small sam-

ple sizes (b) etiological heterogeneity, (c) a complicated comorbidity profile, and (d) blurred lines between normative and pathological anxiety.

Small sample sizes

A major catalyst for psychiatric genetics has been the Psychiatric GWAS Consortium (PGC), a collaborative effort to assemble large samples for GWAS in major depressive disorder (MDD), bipolar disorder, schizophrenia, attention-deficit/hyperactivity disorder, and autism (Sullivan, 2010). The anxiety disorders have not been part of the primary PGC efforts to date. A major lesson from this and other large-scale genome-wide studies has been the realization that sample sizes on the order of tens of thousands of individuals are necessary to identify and replicate genetic variants of modest effect size in complex, neurobehavioral phenotypes (i.e., Ripke et al., 2011; Sklar et al., 2011).

Even for smaller-scale candidate gene studies, it is clear that the typical sample sizes that have been examined in genetic studies of anxiety disorders (on average, a few hundred cases and controls) have been underpowered by at least an order of magnitude. If we take as an optimistic effect size, the largest odds ratios for genome-wide significant results observed in recent GWAS studies of schizophrenia and bipolar disorder (odds ratio = ~1.2; Ripke et al., 2011; Sklar et al., 2011), a liberal significance threshold of $p < .05$ and the most favorable minor allele frequency (MAF = 0.5), approximately 1,000 cases and 1,000 controls would be needed to achieve 80% power under an additive model (Gauderman & Morrison, 2006). Even under this most optimistic scenario, we identified *only one* of the hundreds of published case-control candidate gene studies (Wray et al., 2009) that was adequately powered. In the context of these power limitations, it is difficult to exclude Type I or Type II error in the existing candidate gene literature.

GWAS of anxiety have been similarly underpowered (~200 cases and 200 controls). Using the same optimistic effect size (odds ratio = ~1.2) and MAF (0.5) and a genome-wide significant threshold of $p = 5 \times 10^{-8}$, power analyses indicate that a minimum of 5,000 cases and 5,000 controls would be necessary to obtain 80% power. This estimate extends upward as the risk allele becomes less common (i.e., 7,000 cases/7,000 controls MAF = 0.2; 12,000 cases/12,000 controls MAF = 0.1; Gauderman & Morrison, 2006). As Figure 1 indicates, sample sizes of the magnitude necessary for successful genome-wide studies have not yet been reported for the anxiety disorders, although sample collection efforts are ongoing. The next generation of anxiety genetic studies will need to carefully consider power in light of plausible effect size estimates.

Etiologic heterogeneity

The PGC analyses, especially those for MDD, can also serve as a cautionary tale for anxiety genetics. The PGC mega-analysis of MDD (>18,000 cases/controls) yielded no genome-wide significant signals (PGC, 2012) in contrast to bipolar disorder and schizophrenia, which were more successful (Ripke et al., 2011; Sklar et al., 2011). One explanation for this disappointing result is that etiological heterogeneity is particularly important for depression and other disorders with high population prevalence and moderate heritability (PGC, 2012). This heterogeneity, which may include nongenetic phenocopies and etiologically distinct subtypes, can make gene finding particularly challenging. For example, consider the standard case-control genetic association study, which selects cases based on a cross-sectional assessment of lifetime diagnosis of an anxiety disorder in adulthood. Cases may show substantial etiologic heterogeneity owing to normative and transient peaks in anxiety over the lifespan and different developmental trajectories to the same anxiety disorder outcome (i.e., early vs. later onset). Such heterogeneity could substantially reduce power in a genetic association study.

Figure 1 compares the genetic landscape of the anxiety disorders to the disorder groups included in the PGC, as defined by approximate heritability and prevalence estimates. Thus far, replicable genetic findings have been identified only in highly heritable, lower prevalence disorders with the largest sample sizes. Anxiety disorders and MDD share a genetic landscape that may be particularly difficult for gene finding. In addition to large samples, the field may also need new approaches to phenotyping that can increase power for genetic association studies. For the anxiety disorders, a developmental, multivariate approach to phenotyping may be important for reducing heterogeneity.

Complicated comorbidity profile

Anxiety disorders are highly comorbid with each other in both child and adult samples (Costello, Egger, & Angold, 2005; Kessler, Chiu, Demler, Merikangas, & Walters, 2005). Although comorbidity is a general issue in psychiatry, it is compounded in anxiety, where there are 13 different disorders within the same anxiety class, in addition to cross-class comorbidities. Standard case-control genetic association studies typically focus on individuals who meet and do not meet criteria for one specific disorder. The complex comorbidity of anxiety disorders raises challenging questions about the design and interpretation of studies. For example, should controls be screened only for the specific anxiety disorder being investigated or for all the anxiety disorders? Evidence that the various anxiety disorders share genetic underpinnings (discussed below) would argue for selecting controls free of any disorder in the class; however, the high prevalence of anxiety disorders may limit the feasibility of such a strategy. Moreover, if a significant association is found between a genetic variant and the disorder of interest, how does one ensure that the association is with the primary disorder and not a secondary, highly comorbid disorder? (Smoller, Lunetta, & Robins, 2000). As we discuss later, latent modeling approaches that extract the common phenotypic variance

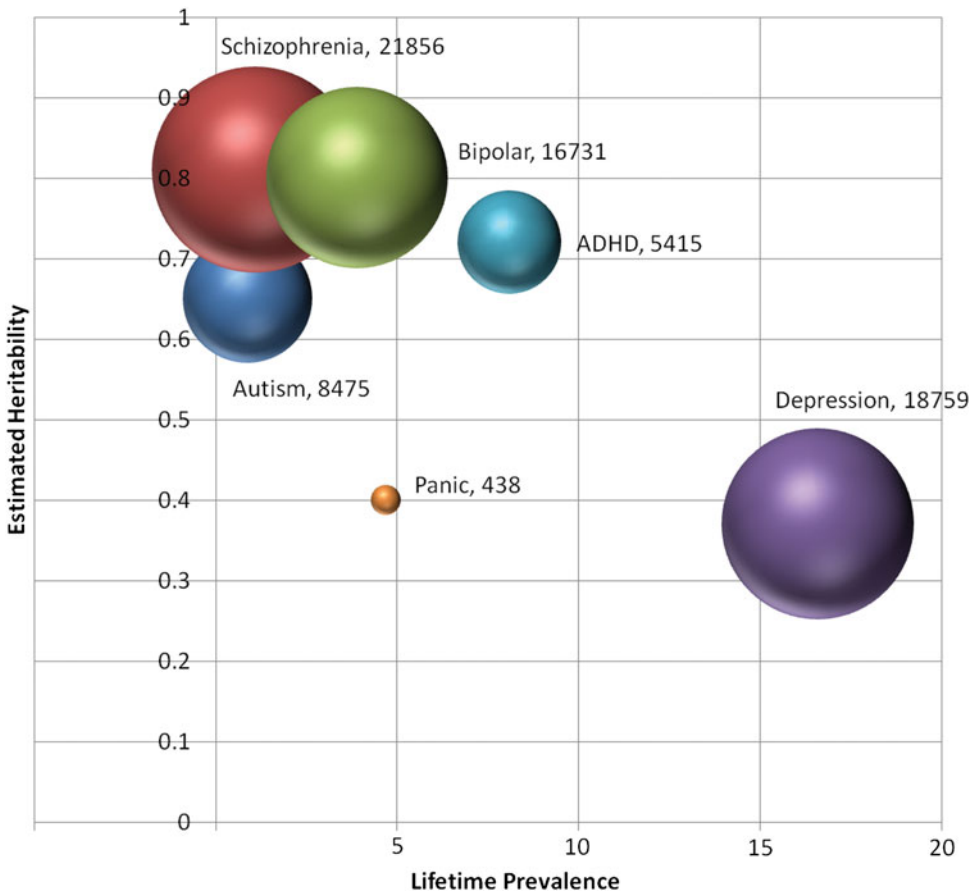


Figure 1. The estimated heritability versus prevalence for disorders included in the Psychiatric GWAS Consortium (PGC) and panic disorder. Each bubble is centered at a point estimate for the lifetime prevalence and heritability of the disorder. The area of the bubbles is proportional to the largest discovery genome-wide association studies sample (cases and controls) reported to date (panic disorder: Erhardt et al., 2011; attention-deficit/hyperactivity disorder [ADHD]: Neale et al., 2010; depression: PGC, 2012; schizophrenia: Ripke et al., 2011; bipolar disorder: Sklar et al., 2011; autism: Wang et al., 2009). Total sample size is also given in the labels. (As an approximation, trios were counted as equivalent to a case.) [A color version of this figure can be viewed online at http://journals.cambridge.org/dpp]

among anxiety disorders may be useful for modeling the multidimensional nature of the phenotype (see the Latent Modeling of the Multidimensional Anxiety Phenotype Section).

Blurred lines between normative and pathological anxiety

Anxiety is a universal human experience, and the line between pathological and normal anxiety is unclear. For case-control genetic association studies, an arbitrary diagnostic boundary must be drawn, creating the dilemma of how to handle individuals just above or below the threshold. Alternatively, quantitative trait approaches, which assume a continuous liability distribution (Plomin, Haworth, & Davis, 2009), can be used for constructing optimally informative latent phenotypes. If genetic variations that influence anxiety-related traits in nonclinical samples are continuous with those affecting pathologic anxiety, genetic studies could take advantage of existing population-based cohorts that have longitudinal anxiety information.

In summary, dissecting the genetic and phenotypic complexity of anxiety disorders will likely require larger, genome-wide study designs and innovative phenotypic techniques. In the rest of this paper, we will focus on new phenotypic approaches that are guided by the psychiatric and genetic epidemiology of anxiety disorders. We propose a developmental, multivariate framework that can incorporate quantitative traits, multidimensional phenotypes, and developmental trajectories, addressing many of the limitations just discussed. We turn now to a discussion of the psychiatric and genetic epidemiology of anxiety disorders, with a focus on implications for optimizing phenotype definition and incorporating a developmental perspective.

Psychiatric and Genetic Epidemiology of Anxiety Supports a Developmental, Multivariate Perspective

Early age of onset and chronic course

Anxiety disorders have an earlier age of onset than many other classes of psychopathology, including mood, psychotic, and substance use disorders. The median age of onset of anx-

iety disorders is reported to be 6 years in an adolescent population-based sample (Merikangas, He, Burstein, et al., 2010) and 11 years in an adult population-based sample (Kessler, Berglund, et al., 2005). The discrepancy is likely due to the inherent difficulties in retrospective reporting of onset.

Within the class of anxiety disorders, there are large variations in the median age of onset for specific types of anxiety. Separation anxiety and specific phobia are the earliest onset in childhood, followed by social phobia in early adolescence, and then PD/agoraphobia and GAD in late adolescence and early adulthood (Kessler, Berglund, et al., 2005). These age-of-onset patterns indicate a developmental shift in the expression of anxiety at different ages (for reviews, see Beesdo, Knappe, & Pine, 2009; Costello et al., 2005; see Figure 2b).

Anxiety disorders also tend to have a chronic course across development (for a review, see Hirshfeld-Becker, Micco, Simoes, & Henin, 2008). In large epidemiological samples, children and adolescents meeting criteria for an anxiety disorder were at high risk for meeting criteria as adults, with odds ratios generally in the range of 2.0–3.0 (Costello, Mustillo, Erkanli, Keeler, & Angold, 2003; Gregory et al., 2007; Kim-Cohen et al., 2003; Newman et al., 1996; Pine, Cohen, Gurley, Brook, & Ma, 1998). There is evidence for both "homotypic continuity" (the future occurrence of the same disorder) and "heterotypic continuity" (the future occurrence of a different anxiety disorder; Gregory et al., 2007; Pine et al., 1998).

These developmental patterns have been largely neglected in anxiety phenotyping for genetic studies. All of the genome-wide studies and most of the candidate gene studies reviewed above have been conducted in cross-sectional, adult samples. Only 13% of the case-control studies have been in child or adolescent samples. Of these, only a handful have explicitly incorporated developmental trajectory information into the phenotype, a method discussed further below (see Ernst et al., 2011; Petersen et al., 2012). By neglecting the developmental nature of the anxiety phenotype, the field may be missing a critical opportunity for gene finding. New phenotypic models could incorporate early developmental time points and allow for changing symptomatology over time (see the Developmentally Sensitive Phenotypes for Anxiety Genetic Studies Section).

Genetic contributions to comorbidity

In the following two sections we highlight evidence that the strong cross-sectional and longitudinal relationship among the anxiety disorders is partially attributable to shared genetic risk factors. These findings can guide multivariate, longitudinal models of anxiety liability for genetic studies.

Genetically informative designs, such as the twin study, can be used to examine the genetic basis of co-occurring disorders or traits by examining the correlation between one disorder or trait in one twin (e.g., GAD) and another disorder or trait in the second twin (e.g., PD) for monozygotic and dizygotic twin pairs. A higher cross-trait correlation for monozygotic compared to dizygotic twin pairs provides evidence of shared genetic contributions to the two disorders or traits.

There have been several multivariate twin studies of childhood anxiety disorders and anxiety-related behaviors (Eley et al., 2003; Eley, Rijsdijk, Perrin, O'Connor, & Bolton, 2008; Hallett, Ronald, Rijsdijk, & Eley, 2009; Ogliari et al., 2010). The common thread uniting most of these studies is that there are common genetic risk factors underlying many of the childhood anxiety disorders and traits, although the magnitude of overlap differs depending on the age of the sample, measures used, and disorders or traits considered (for reviews see Franic et al., 2010; Gregory & Eley, 2007). Two studies have estimated the proportion of the phenotypic correlation that is due to shared genetic factors to be in the range of .30–.50 for anxiety-related behaviors in preschool and middle childhood (Eley et al., 2003; Hallett et al., 2009). One additional study, in a sample of broader age range (8–17 years), reported even higher estimates of .60–.99 for quantitative measures of generalized anxiety, social phobia, separation anxiety, and PD (Ogliari et al., 2010). The highest estimate of .99 was for social phobia and panic symptoms.

Multivariate twin studies in adults support the findings in child samples that the anxiety disorders reflect partly shared genetic influences (Hettema, Prescott, Myers, Neale, & Kendler, 2005; Kendler, Prescott, Myers, & Neale, 2003; Middeldorp et al., 2005; Mosing et al., 2009; Tambs et al., 2009). These studies provide some support for a two-factor internalizing model comprising two partly distinct genetic factors: anxious-misery (with loadings on depression, generalized anxiety, and panic, agoraphobia, social phobia) and fear (with loadings on specific phobias; Hettema et al., 2005; Kendler et al., 2003). At this point, the two-factor genetic model has not been explored in child samples, so its relevance to childhood anxiety remains to be determined.

Overall, these multivariate genetic results suggest that though the anxiety disorders may show phenotypic differentiation, even beginning at early developmental stages (Mian, Godoy, Briggs-Gowan, & Carter, 2011), many of the disorders share genetic influences. We concur with previous proposals that, for gene-finding efforts, it would be reasonable to focus on clusters of disorders with shared genetic risk factors rather than on a single individual anxiety disorder (Kendler et al., 2003). One methodological strategy would be to model a latent anxiety liability factor composed of anxiety disorders with substantial genetic overlap (see Figure 2 and the Latent Modeling of the Multidimensional Anxiety Phenotype Section).

Genetic contributions to the stability of anxiety

Longitudinal twin studies in which the same anxiety phenotypes are measured in both twins on two or more occasions can address questions about genetic and environmental contributions to the stability of anxiety over time. There have been several multivariate, longitudinal twin studies addressing this question in childhood and adolescence (Boomsma, van Beijsterveldt, Bartels, & Hudziak, 2007; Kendler, Gardner, Annas, & Lichtenstein, 2008; Kendler, Gardner, Annas, Neale, et al., 2008; Kendler, Gardner, & Lichtenstein, 2008;

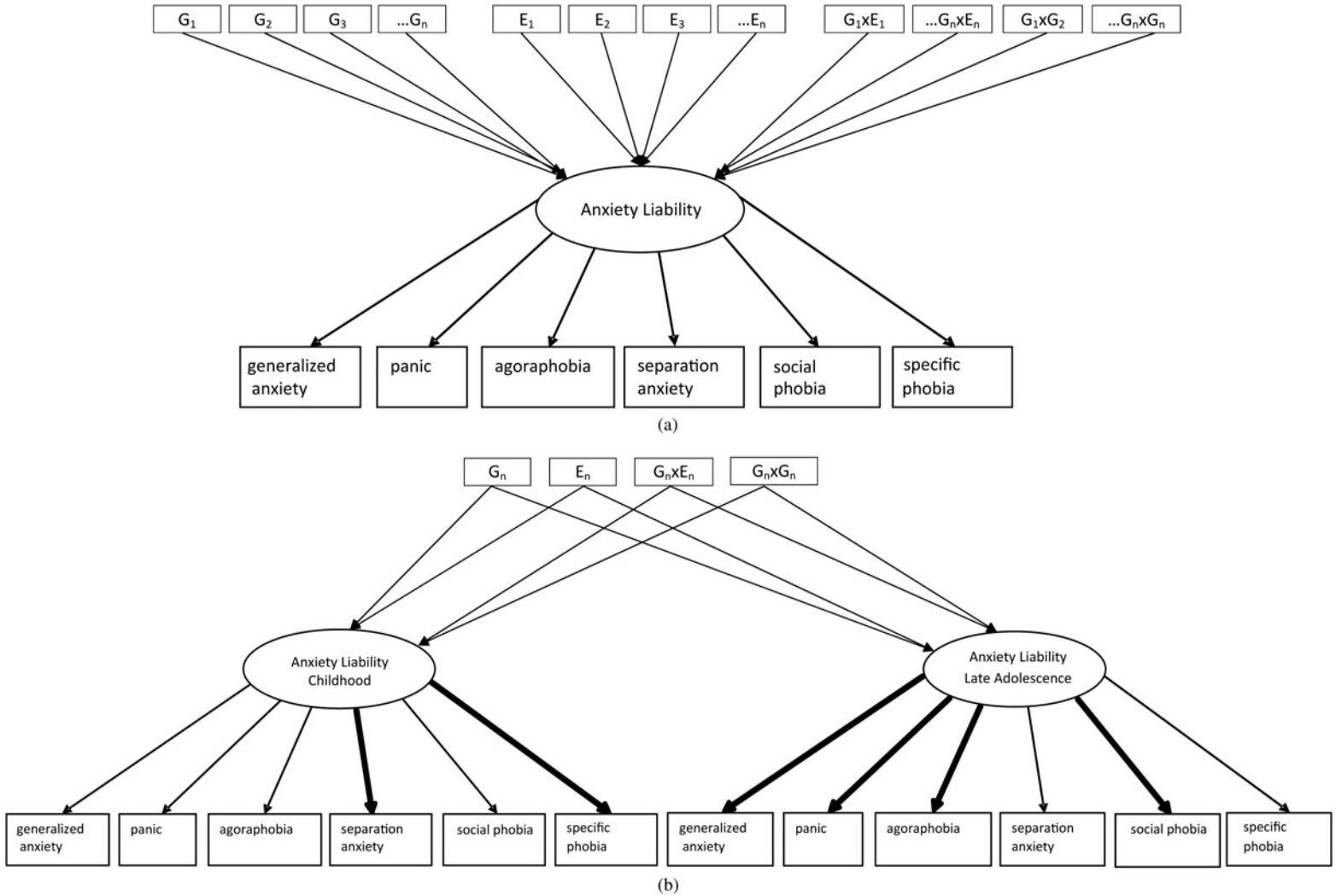


Figure 2. (a) An illustration of a one-factor latent model of anxiety liability influenced by genetic variants (G), environmental factors (E), Gene×Environment interactions (G×E), and Gene ×Gene interactions (G×G). Although multiple predictors are included to reflect the expected etiologic complexity of anxiety, this model could also be used to test single predictors, such as a single nucleotide polymorphism. (b) An illustration of a two-factor latent model of anxiety liability with developmental shifts in the factor loadings from childhood to late adolescence. In childhood, anxiety liability is predicted to be expressed as the earliest onset anxiety disorders: separation anxiety and specific phobia. In late adolescence, anxiety liability is predicted to be expressed as the later onset anxiety disorders: generalized anxiety, panic, agoraphobia, and social phobia. The thickness of the arrows indicates that the weighting of the variable is predicted to be stronger. The multiple genetic, environmental, G×E, and G×G predictors are condensed from (a) to simplify the figure but represent the same degree of etiologic complexity depicted in (a).

Roberson-Nay, Eaves, Hettema, Kendler, & Silberg, 2012; Trzaskowski, Zavos, Haworth, Plomin, & Eley, 2011). Depending on the measures included in the study, these twin studies can address genetic and environmental contributions to both homotypic and heterotypic continuity.

In one study explicitly addressing homotypic versus heterotypic continuity of anxiety-related behaviors (i.e., negative cognition, negative affect, fear, social anxiety) from ages 7 to 9 years, genetic influences on homotypic continuity were generally stronger than heterotopic continuity (Trzaskowski et al., 2011). Estimates of the proportion of homotypic continuity due to stable genetic factors ranged from .57 to .67. Genetic influences on heterotypic continuity were more varied, with estimates ranging from .28 (for fear at age 7 and negative affect at age 9) to .66 (for negative cognitions at age 7 and negative affect at age 9). This variability suggests that specific clusters of anxiety-related behaviors may be more genetically related over time than others, consistent with the two-factor genetic models in adults presented by Kendler et al. (2003) and Hettema et al. (2005). Additional evidence for genetic contributions to heterotypic continuity comes from a recent study reporting a shared genetic diathesis between childhood separation anxiety disorder and adult-onset panic attacks (Roberson-Nay et al., 2012).

Twin studies spanning larger age ranges have addressed mainly homotypic continuity. For example, Kendler, Gardner, and Lichtenstein (2008) examined a large twin sample assessed at ages 8–9, 13–14, 16–17, and 19–20 years with self- and parent-report measures of anxious/depressed symptoms. They found evidence for a "developmentally dynamic" pattern of genetic risk factors involving both "genetic attenuation" and "genetic innovation." Genetic attenuation refers to the finding that genetic risk factors present at age 8–9 continued to contribute to anxious/depression symptoms over time but accounted for less of the variance, starting with 72% at age 8–9 and ending with 12% at age 19–20. Genetic innovation refers to the fact that strong new genetic effects emerged at each time point measured. Thus, both stable and developmentally dynamic genetic risk factors contribute to anxiety symptoms. A similar pattern was observed in the same twin sample using a phobia assessment (Kendler, Gardner, Annas, & Lichtenstein, 2008; Kendler, Gardner, Annas, Neale, et al., 2008). Among the phobias, social phobia showed the lowest degree of genetic continuity. The pattern of genetic effects on social phobia was distinguished by new and substantial genetic influences coming online in adolescence and early adulthood, compared to the pattern in the other specific phobias where the genetic innovations were more modest (Kendler, Gardner, Annas, & Lichtenstein, 2008). Once individuals reach adulthood, there is more evidence for genetic stability over time with only minor genetic innovations compared to childhood and adolescence (Gillespie et al., 2004; Nes, Roysamb, Reichborn-Kjennerud, Harris, & Tambs, 2007; Rijsdijk et al., 2003).

These studies highlight the value of incorporating a developmental perspective for genetic studies of anxiety. For example, the genetic epidemiology of social phobia suggests that genetic contributions to social phobia in adolescence and adulthood are substantially distinct. In this case, studies that collapse across developmental periods (such as the standard practice of assessing lifetime diagnoses in adulthood) will substantially increase the etiologic heterogeneity of the sample and diminish the power to detect genetic effects (see Figure 3). However, specific genetic risk factors may be more easily identified by focusing on a specific developmental period (e.g., adolescent vs. adult-onset social anxiety) or including longitudinal phenotypes that distinguish specific trajectories (see the Developmental Trajectories as Phenotypes Section).

Developmentally Sensitive Phenotypes for Anxiety Genetic Studies

The genetic epidemiology of anxiety disorders has been well studied in sophisticated multivariate and developmental designs, but gene-finding efforts have not generally incorporated these approaches.

Given the evidence for (a) the early onset and chronic course of anxiety disorders, (b) shared genetic etiology of specific clusters of anxiety disorders, and (c) developmentally stable and dynamic genetic contributions to anxiety disorders, we propose three corresponding developmental strategies for phenotypic definition in genetic studies: (a) examination of early temperamental precursors of anxiety, (b) latent modeling of the multidimensional anxiety phenotype, and (c) examination of developmental trajectories. We explain these strategies further and then illustrate the approach with a representative application.

Early temperamental precursors of anxiety

There has been a rich tradition of developmental work characterizing temperamental risk factors for anxiety (e.g., Kagan & Snidman, 2004). Temperament describes a biologically based behavioral profile that is relatively stable across time and context in childhood (Nigg, 2006; Perez-Edgar & Fox, 2005; Rothbart, 2007). Multiple dimensions of temperament have been described in internalizing disorders, but much of the focus has been on behavioral inhibition (BI) to the unfamiliar (e.g., Biederman et al., 2001; Fox, Henderson, Marshall, Nichols, & Ghera, 2005; Hirshfeld-Becker et al., 2007; Kagan, Snidman, Kahn, & Towsley, 2007; Rosenbaum et al., 2000).

BI is a stable, heritable temperamental profile that is associated with increased risk for later anxiety disorders, especially social anxiety (Biederman et al., 2001; Hirshfeld-Becker et al., 2007; Schwartz, Snidman, & Kagan, 1999). It is characterized by withdrawn and wary behaviors to novel situations and is measured with developmentally sensitive observational tasks (Kagan & Snidman, 2004) or parent reports of child temperament and shyness (e.g., Carter, Briggs-Gowan, Jones, & Little, 2003; Eley et al., 2003).

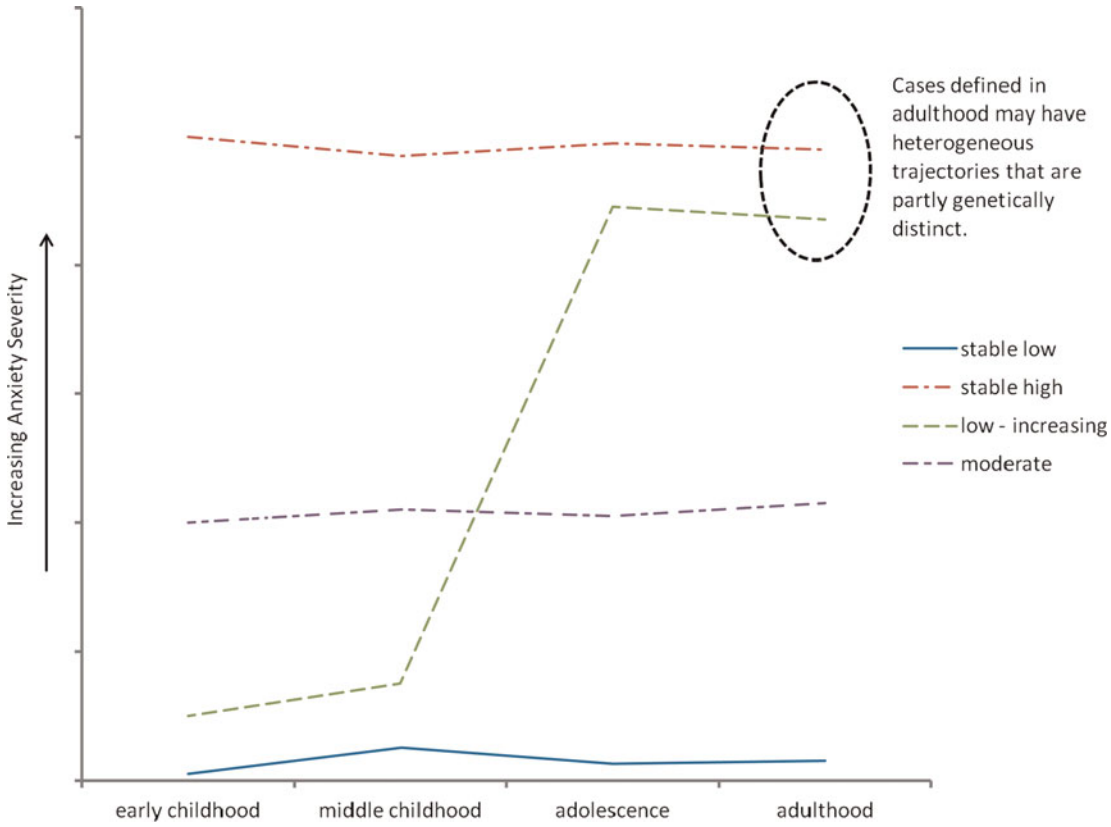


Figure 3. Examples of developmental trajectories that may have etiologic significance. Different pathways to adult disorder may reflect heterogeneous etiologies that are obscured in standard case-control studies of adults. The lines from top to bottom indicate stable high, moderate, low increasing, and stable low, respectively. [A color version of this figure can be viewed online at http://journals.cambridge.org/dpp]

There has been some debate about the extent to which BI is a separate construct from early manifestations of anxiety symptoms (Egger & Angold, 2006); however, there is an emerging consensus that the two concepts are related but distinguishable (Rapee, Schniering, & Hudson, 2009). One piece of evidence in support of this consensus is that even though BI has been associated with increased risk for later anxiety disorders, only about half of children with BI go on to develop an anxiety disorder (Degnan & Fox, 2007). While prior research has identified environmental and neurocognitive predictors of the transition from anxious temperament to anxiety disorder (Degnan, Almas, & Fox, 2010; Degnan & Fox, 2007), there has been much less research on how genetic variation influences this trajectory.

For genetic studies of anxiety susceptibility, BI is a particularly compelling phenotype because estimates of its heritability tend to be higher on average than estimates reported for child and adolescent anxiety disorders (DiLalla, Kagan, & Reznick, 1994; Goldsmith & Lemery, 2000; Plomin et al., 1993; Robinson, Kagan, Reznick, & Corley, 1992). Three twin studies utilizing observational measures of BI in toddlerhood found comparable heritability estimates h^2 ranging from ∼.40 to .55 and extending up to .70 in one study (DiLalla et al., 1994; Plomin et al., 1993; Robinson et al., 1992). Genetic studies also indicate overlap between genetic influences on inhibited temperament and other anxiety-related behaviors (Eley et al., 2003; Goldsmith & Lemery, 2000).

Several groups have pursued the strategy of examining anxiety-related temperament as an intermediate phenotype for anxiety disorders (Fox et al., 2005; Smoller et al., 2003, 2005, 2008). Intermediate phenotypes are traits that capture aspects of the underlying liability for a disorder but may be more closely related to the genetic risk factors than is the disorder itself (Gottesman & Gould, 2003; Kendler & Neale, 2010). The study highlighted below illustrates a strategy of examining multiple intermediate phenotypes, including temperament, personality, and neuroimaging profiles and capitalizing on genetic findings from experimental animal models (Smoller et al., 2008).

There are evolutionarily conserved fear responses in a wide range of species that recapitulate the behavioral and biological features of human fear (Flint, 2003). This cross-species correspondence makes genes implicated in animal models of anxiety compelling candidates for human studies. In 2004, regulator of G protein signaling 2 (*Rgs2*; Yalcin et al., 2004) was identified as a quantitative trait gene influencing anxious temperament through fine mapping of a

well-replicated linkage signal for anxiety phenotypes in mice (Yalcin et al., 2004). The RGS2 protein is expressed in cortical and limbic brain regions and modulates G protein coupled receptor signaling in response to neurotransmitters such as serotonin and norepinephrine (Grafstein-Dunn, Young, Cockett, & Khawaja, 2001; Neubig & Siderovski, 2002). *Rgs2* knockout mice exhibit increased anxiety and fear behavior, altered hippocampal synaptic plasticity, and elevated sympathetic tone (Oliveira-Dos-Santos et al., 2000; Yalcin et al., 2004).

Given the multiple lines of evidence in both human and animal models implicating *Rgs2* in anxiety-related behaviors, Smoller et al. (2008) tested the association of human *RGS2* with BI in a sample of children ages 2–6 years ($N = 119$ nuclear families) who underwent lab-based temperament assessments. Multiple variants in the *RGS2* locus were associated with BI, including a SNP (*rs4606*) that is associated with reduced *RGS2* expression in vitro (Semplicini et al., 2006).

To further explore this association between BI and *RGS2*, the authors also examined a personality phenotype closely related to BI in an independent adult sample ($N = 744$). Developmental studies have suggested that BI is a developmental precursor of introversion in adulthood (Caspi et al., 2003). The four markers showing the strongest association with childhood BI were also found to be associated with introversion among adults.

In a third adult sample ($N = 55$), the authors focused on brain phenotypes thought to mediate anxiety proneness: amygdala and insula reactivity during emotion processing (e.g., Killgore & Yurgelun-Todd, 2005; Schwartz, Wright, Shin, Kagan, & Rauch, 2003; Stein, Goldin, Sareen, Zorrilla, & Brown, 2002; Stein, Simmons, Feinstein, & Paulus, 2007). The alleles previously associated with BI and introverted personality were also associated with increased left amygdala and bilateral insular cortex activation in response to emotional faces.

The relevance of *RGS2* to pathologic anxiety is supported by several studies that have reported association to a variety of anxiety disorders, including GAD, PD, and PTSD (Amstadter et al., 2009; Koenen et al., 2009; Leygraf et al., 2006; Mouri et al., 2009; Otowa et al., 2011), although the associated alleles have not been consistent across studies and the samples examined have been relatively small. Additional indirect evidence comes from a recent report that a SNP in microRNA-22, an epigenetic regulator of *RGS2*, is associated with PD (Muinos-Gimeno et al., 2011).

The *RGS2* story is one illustration of a strategy in which developmental precursors of anxiety, such as heritable temperamental traits, were used as intermediate phenotypes in genetic studies. A crucial next step in this approach is to test the association of identified genetic variants with the anxiety disorder of interest because it is possible that a genetic variant could be associated with temperament but not related anxiety disorders (Kendler & Neale, 2010). Studies attempting to demonstrate an association with the disorder of interest may require larger sample sizes than the original intermediate phenotype study because of the expected increase in phenotypic and etiologic heterogeneity in clinical samples.

Latent modeling of the multidimensional anxiety phenotype

Another approach to investigating complex, multidimensional anxiety phenotypes is to utilize statistical approaches that can model multiple outcomes. This could be done, for example, by constructing a latent phenotypic factor from the anxiety disorders known to share genetic underpinnings in the developmental period being considered (see Figure 2). The latent factor score could then be used in genetic analyses as a quantitative measure of "anxiety liability." Although there is a rich tradition of latent modeling approaches in behavioral research, many of these methods have not been widely integrated into psychiatric genetic research (for examples, see McGrath et al., in press; Medland & Neale, 2010; Middeldorp et al., 2010). Here, we focus specifically on structural equation modeling (SEM) approaches (see Kline, 2005; Loehlin, 2004).

SEM provides several advantages for genetic research, including (a) reduction of measurement error, (b) the ability to test for genetic effects on means *and* covariance, and (c) developmental modeling. First, reduction of measurement error may increase power to detect genetic signals (Schulze & McMahon, 2004). Second, a latent phenotypic model can be used to test for genetic effects on the means of the latent factors as well as on the covariance structure among the anxiety disorders or traits that are modeled. The issue of covariance differences as a function of genotype is of particular relevance to neuropsychiatric phenotypes, where subgroups, or specific clusters of behavior, may be expected as a function of genotype (e.g., Craddock, O'Donovan, & Owen, 2006; Wessman et al., 2009). In other words, SEM models can test the hypothesis that subsets of anxiety disorders are more or less correlated as a function of genotype, allowing for the identification of genetically meaningful subgroups. For example, individuals with a specific genetic variant could be at increased risk for comorbid panic and agoraphobia compared to those without the variant. If this is the case, the correlation between panic and agoraphobia would be stronger among those with the risk variant. This application of SEM to investigate genetic differences in covariance has been vastly underexplored in psychiatry.

Third, SEM can take advantage of longitudinal data to extract stable traits over time and to explicitly model developmental trajectories. Here, we point out that SEM can incorporate developmental information by permitting changing weights on the diagnoses and dimensions contributing to a latent anxiety liability factor at different time points. For example, separation anxiety may be a stronger indicator of anxiety liability in early childhood compared to PD, which has a low prevalence at this development stage. However, in late adolescence, the opposite pattern may be expected. Figure 2b illustrates a potential phenotypic model for anxiety disorders

in childhood and late adolescence, where stronger indicators are bolded. The genetic association analysis can then be conducted with the empirically derived, developmentally sensitive latent factors as the phenotype. This latent model approach diminishes the multiple-testing burden that would result if each anxiety diagnosis were tested individually.

In a study illustrating the incorporation of SEM in genetic analyses, Middeldorp et al. (2010) studied child/adolescent ($N = 1{,}240$) and adult ($N = 1{,}943$) participants who received repeated measures of anxious/depression symptoms. In both samples, a single latent anxious depression factor was modeled that incorporated multiple raters and time points. The heritability of the latent anxious depression factor was found to be higher ($h^2 \sim .60$–$.70$) than the individual anxious depression measures ($h^2 \sim .40$–$.50$), as expected based on the increased reliability of the latent factor. After multiple-testing correction, the authors did not find any significant associations with a set of candidate genes chosen based on previous literature (serotonergic and neurotrophic genes). Nevertheless, we concur with the authors that the latent modeling approach will be quite valuable for future genome-wide analyses because it can harness the reliable variance in anxiety measures across raters and time points (Middeldorp et al., 2010).

Developmental trajectories as phenotypes

Repeated measures can also be used to explicitly model developmental trajectories over time using SEM and other modeling approaches (Grimm, Ram, & Hamagami, 2011; McArdle, Nesselroade, Schinka, & Velicer, 2003; Muthen, 2001). Consistent with a developmental psychopathology orientation (Cicchetti & Toth, 2009), research on childhood internalizing disorders has explored developmental trajectories associated with psychopathology (e.g., Carter et al., 2010; Duchesne, Larose, Vitaro, & Tremblay, 2010; Letcher, Sanson, Smart, & Toumbourou, 2012). The idea of using developmental trajectories as phenotypes for psychiatric genetic studies is more novel (for examples, see McQueen et al., 2007; Petersen et al., 2012; Sakai et al., 2010), and new methods are emerging (Das et al., 2011; Kerner, North, & Fallin, 2009).

By examining phenotypic stability and change over time, trajectories are more informative than cross-sectional assessments that may be sensitive to normative anxiety patterns and transient environmental influences. For example, there are normative developmental peaks in anxiety, such as a period of separation distress and stranger wariness in toddlerhood and a period of increased concern regarding peer rejection in adolescence (Beesdo et al., 2009; Costello et al., 2005). Moreover, through the life span, bouts of anxiety in response to specific triggers can be normative responses. Thus, studies relying only on cross-sectional assessments of anxiety may capture variation that reflects transient factors rather than an underlying genetically influenced diathesis.

There are several approaches to incorporating developmental trajectories into genetic analyses. For example, using linear growth curve models, the rate of change of a phenotype over time (i.e., slope) can be estimated for each individual in an analysis. This slope parameter can then be used as a quantitative phenotype in genetic studies. The question being tested by such an analysis is whether there are genetic variants that are associated with a more accelerated increase in anxiety symptoms over time. Nonlinear growth curve models are more complex than linear models but may map more closely to developmental patterns in the data (Grimm et al., 2011). A different approach is to cluster individuals with similar developmental trajectories. Across phenotypic studies there have been diverse trajectories identified, but there is converging support for low, low-increasing, moderate, and stable high anxiety trajectories (Cote, Tremblay, Nagin, Zoccolillo, & Vitaro, 2002; Duchesne et al., 2010; Duchesne, Vitaro, Larose, & Tremblay, 2008; Feng, Shaw, & Silk, 2008; Letcher et al., 2012; Marmorstein et al., 2010; Figure 3). A variety of methods can be used to derive clusters (e.g., Muthen, 2002; Nagin, 1999), which can then be used as phenotypes for genetic studies.

In a recent illustration of this approach, Ernst et al. (2011) used data from a longitudinal cohort representative of the Quebec general population. Individuals were randomly selected for participation when they were in kindergarten. Annual ratings of anxiety traits from 6 to12 years old were used to cluster 640 individuals into five different developmental trajectories: high, moderately high, decreasing low, low, and very low. In early adulthood (21–23 years), the participants were reassessed with personality measures and a psychiatric diagnostic interview.

The study examined a functional 11-base pair deletion in tropomyosin-related kinase B (*TRKB*, also known as neurotrophic tyrosine kinase receptor type 2 gene), a receptor for *BDNF* that is involved in synaptic modeling, neurodevelopment, and cell signaling (Ernst et al., 2011), and has been implicated in mouse (Bergami et al., 2008) and human anxiety (Ernst et al., 2009). Results showed that children in the high and moderately high trajectory clusters were more likely to carry the deletion (4.1%, 4.0%, respectively) than those in the decreasing low, low, or very low clusters (0%, 0.6%, or 0%, respectively). In early adulthood, individuals carrying the deletion had higher trait anxiety scores and an approximately threefold increased odds of GAD and PD. The results suggest that individuals carrying a deletion in *TRKB* are at increased risk for anxiety pathology from childhood through early adulthood (Ernst et al., 2011). While replication is needed to validate this association, the study illustrates the utility of using developmental trajectories as phenotypes for genetic studies, a strategy that could be scaled to accommodate genome-wide association data.

Conclusions

Progress in anxiety genetics has lagged behind many of the other psychiatric disorders, in part because of a predominant focus on candidate genes and insufficient sample sizes. These

limitations could be addressed by large-scale collaborations to assemble anxiety samples for GWAS and other genome-wide investigations. However, the phenotypic complexity of anxiety disorders or traits also presents real challenges for genetic studies that will not be automatically addressed by collaborative sample collections. Innovative phenotypic techniques may be necessary to maximize the impact of emerging genetic resources for anxiety. Fortunately, there is a rich literature on the psychiatric and genetic epidemiology of anxiety disorders that can guide more sophisticated phenotyping approaches. Data on the early onset and chronic course of anxiety, shared genetic risk factors among specific clusters of disorders, and developmentally dynamic genetic influences have yet to fully inform phenotypic models for genetic studies. These findings support a shift in thinking away from standard, single disorder, case-control studies in adults to a more developmental, multivariate perspective on study design and phenotyping. In this review, we have proposed three developmentally sensitive phenotyping approaches: (a) examination of early temperamental precursors of anxiety, (b) latent modeling of the multidimensional anxiety phenotype, and (c) examination of developmental trajectories. Given that large-scale anxiety genetic studies are currently being pursued, this is an opportune time to consider new phenotypic approaches.

References

Amstadter, A. B., Koenen, K. C., Ruggiero, K. J., Acierno, R., Galea, S., Kilpatrick, D. G., et al. (2009). Variant in RGS2 moderates posttraumatic stress symptoms following potentially traumatic event exposure. *Journal of Anxiety Disorders*, *23*, 369–373.

Beesdo, K., Knappe, S., & Pine, D. S. (2009). Anxiety and anxiety disorders in children and adolescents: Developmental issues and implications for DSM-V. *Psychiatric Clinics of North America*, *32*, 483–524.

Bergami, M., Rimondini, R., Santi, S., Blum, R., Gotz, M., & Canossa, M. (2008). Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. *Proceedings of the National Academy of Sciences*, *105*, 15570–15575.

Biederman, J., Hirshfeld-Becker, D. R., Rosenbaum, J. F., Herot, C., Friedman, D., Snidman, N., et al. (2001). Further evidence of association between behavioral inhibition and social anxiety in children. *American Journal of Psychiatry*, *158*, 1673–1679.

Blaya, C., Salum, G. A., Lima, M. S., Leistner-Segal, S., & Manfro, G. G. (2007). Lack of association between the serotonin transporter promoter polymorphism (*5-HTTLPR*) and panic disorder: A systematic review and meta-analysis. *Behavioral and Brain Functions*, *3*, 41.

Boomsma, D., van Beijsterveldt, C. E., Bartels, M., & Hudziak, J. J. (2007). Genetic and environmental influence on anxious/depression: A longitudinal study in 3- to 12-year-old children. In J. J. Hudziak (Ed.), *Genetic and environmental influences on developmental psychopathology and wellness*. Washington, DC: American Psychiatric Association.

Brady, E. U., & Kendall, P. C. (1992). Comorbidity of anxiety and depression in children and adolescents. *Psychological Bulletin*, *111*, 244–255.

Carter, A. S., Briggs-Gowan, M. J., Jones, S. M., & Little, T. D. (2003). The Infant–Toddler Social and Emotional Assessment (ITSEA): Factor structure, reliability, and validity. *Journal of Abnormal Child Psychology*, *31*, 495–514.

Carter, A. S., Godoy, L., Wagmiller, R. L., Veliz, P., Marakovitz, S., & Briggs-Gowan, M. J. (2010). Internalizing trajectories in young boys and girls: The whole is not a simple sum of its parts. *Journal of Abnormal Child Psychology*, *38*, 19–31.

Caspi, A., Harrington, H., Milne, B., Amell, J. W., Theodore, R. F., & Moffitt, T. E. (2003). Children's behavioral styles at age 3 are linked to their adult personality traits at age 26. *Journal of Personality*, *71*, 495–513.

Cicchetti, D., & Toth, S. L. (2009). The past achievements and future promises of developmental psychopathology: The coming of age of a discipline. *Journal of Child Psychology and Psychiatry*, *50*, 16–25.

Collins, A. L., Kim, Y., Sklar, P., O'Donovan, M. C., & Sullivan, P. F. (2012). Hypothesis-driven candidate genes for schizophrenia compared to genome-wide association results. *Psychological Medicine*, *42*, 607–616.

Costello, E. J., Egger, H. L., & Angold, A. (2005). The developmental epidemiology of anxiety disorders: Phenomenology, prevalence, and comorbidity. *Child and Adolescent Psychiatric Clinics of North America*, *14*, 631–648.

Costello, E. J., Mustillo, S., Erkanli, A., Keeler, G., & Angold, A. (2003). Prevalence and development of psychiatric disorders in childhood and adolescence. *Archives of General Psychiatry*, *60*, 837–844.

Cote, S., Tremblay, R. E., Nagin, D., Zoccolillo, M., & Vitaro, F. (2002). The development of impulsivity, fearfulness, and helpfulness during childhood: Patterns of consistency and change in the trajectories of boys and girls. *Journal of Child Psychology and Psychiatry*, *43*, 609–618.

Craddock, N., O'Donovan, M. C., & Owen, M. J. (2006). Genes for schizophrenia and bipolar disorder? Implications for psychiatric nosology. *Schizophrenia Bulletin*, *32*, 9–16.

Das, K., Li, J., Wang, Z., Tong, C., Fu, G., Li, Y., et al. (2011). A dynamic model for genome-wide association studies. *Human Genetics*, *129*, 629–639.

Degnan, K. A., Almas, A. N., & Fox, N. A. (2010). Temperament and the environment in the etiology of childhood anxiety. *Journal of Child Psychology and Psychiatry*, *51*, 497–517.

Degnan, K. A., & Fox, N. A. (2007). Behavioral inhibition and anxiety disorders: Multiple levels of a resilience process. *Development and Psychopathology*, *19*, 729–746.

DiLalla, L. F., Kagan, J., & Reznick, J. S. (1994). Genetic etiology of behavioral inhibition among 2-year-old children. *Infant Behavior & Development*, *17*, 405–412.

Domschke, K., Deckert, J., O'Donovan, M. C., & Glatt, S. J. (2007). Meta-analysis of *COMT* val158met in panic disorder: Ethnic heterogeneity and gender specificity. *American Journal of Medical Genetics B: Neuropsychiatric Genetics*, *144B*, 667–673.

Domschke, K., & Reif, A. (2012). Behavioral genetics of affective and anxiety disorders. *Current Topics in Behavioral Neurosciences*, *12*, 463–502.

Duchesne, S., Larose, S., Vitaro, F., & Tremblay, R. E. (2010). Trajectories of anxiety in a population sample of children: Clarifying the role of children's behavioral characteristics and maternal parenting. *Development and Psychopathology*, *22*, 361–373.

Duchesne, S., Vitaro, F., Larose, S., & Tremblay, R. E. (2008). Trajectories of anxiety during elementary-school years and the prediction of high school noncompletion. *Journal of Youth and Adolescence*, *37*, 1134–1146.

Duncan, L. E., & Keller, M. C. (2011). A critical review of the first 10 years of candidate gene-by-environment interaction research in psychiatry. *American Journal of Psychiatry*, *168*, 1041–1049.

Egger, H. L., & Angold, A. (2006). Common emotional and behavioral disorders in preschool children: Presentation, nosology, and epidemiology. *Journal of Child Psychology and Psychiatry*, *47*, 313–337.

Eley, T. C., Bolton, D., O'Connor, T. G., Perrin, S., Smith, P., & Plomin, R. (2003). A twin study of anxiety-related behaviours in preschool children. *Journal of Child Psychology and Psychiatry*, *44*, 945–960.

Eley, T. C., Rijsdijk, F. V., Perrin, S., O'Connor, T. G., & Bolton, D. (2008). A multivariate genetic analysis of specific phobia, separation anxiety and social phobia in early childhood. *Journal of Abnormal Child Psychology*, *36*, 839–848.

Erhardt, A., Czibere, L., Roeske, D., Lucae, S., Unschuld, P. G., Ripke, S., et al. (2011). *TMEM132D*, a new candidate for anxiety phenotypes: Evidence from human and mouse studies. *Molecular Psychiatry*, *16*, 647–663.

Ernst, C., Deleva, V., Deng, X., Sequeira, A., Pomarenski, A., Klempan, T., et al. (2009). Alternative splicing, methylation state, and expression profile of tropomyosin-related kinase B in the frontal cortex of suicide completers. *Archives of General Psychiatry*, *66*, 22–32.

Ernst, C., Wanner, B., Brezo, J., Vitaro, F., Tremblay, R., & Turecki, G. (2011). A deletion in tropomyosin-related kinase B and the development of human anxiety. *Biological Psychiatry*, *69*, 604–607.

Essau, C. A., Conradt, J., & Petermann, F. (2000). Frequency, comorbidity, and psychosocial impairment of anxiety disorders in German adolescents. *Journal of Anxiety Disorders*, *14*, 263–279.

Etkin, A. (2010). Functional neuroanatomy of anxiety: A neural circuit perspective. *Current Topics in Behavioral Neurosciences*, *2*, 251–277.

Ezpeleta, L., Keeler, G., Erkanli, A., Costello, E. J., & Angold, A. (2001). Epidemiology of psychiatric disability in childhood and adolescence. *Journal of Child Psychology and Psychiatry*, *42*, 901–914.

Feng, X., Shaw, D. S., & Silk, J. S. (2008). Developmental trajectories of anxiety symptoms among boys across early and middle childhood. *Journal of Abnormal Psychology*, *117*, 32–47.

Flint, J. (2003). Animal models of anxiety and their molecular dissection. *Seminars in Cell and Developmental Biology*, *14*, 37–42.

Flint, J., Shifman, S., Munafo, M., & Mott, R. (2008). Genetic variants in major depression. *Novartis Foundation Symposium*, *289*, 23–32; discussion, 33–42, 87–93.

Fox, N. A., Henderson, H. A., Marshall, P. J., Nichols, K. E., & Ghera, M. M. (2005). Behavioral inhibition: Linking biology and behavior within a developmental framework. *Annual Review of Psychology*, *56*, 235–262.

Franic, S., Middeldorp, C. M., Dolan, C. V., Ligthart, L., & Boomsma, D. I. (2010). Childhood and adolescent anxiety and depression: Beyond heritability. *Journal of the American Academy of Child & Adolescent Psychiatry*, *49*, 820–829.

Frustaci, A., Pozzi, G., Gianfagna, F., Manzoli, L., & Boccia, S. (2008). Meta-analysis of the brain-derived neurotrophic factor gene (*BDNF*) Val66Met polymorphism in anxiety disorders and anxiety-related personality traits. *Neuropsychobiology*, *58*, 163–170.

Gauderman, W. J., & Morrison, J. M. (2006). *QUANTO 1.2.4: A computer program for power and sample size calculations for genetic-epidemiology studies*. Retrieved from http://hydra.usc.edu/gxe/

Gillespie, N. A., Kirk, K. M., Evans, D. M., Heath, A. C., Hickie, I. B., & Martin, N. G. (2004). Do the genetic or environmental determinants of anxiety and depression change with age? A longitudinal study of Australian twins. *Twin Research*, *7*, 39–53.

Goldsmith, H. H., & Lemery, K. S. (2000). Linking temperamental fearfulness and anxiety symptoms: A behavior–genetic perspective. *Biological Psychiatry*, *48*, 1199–1209.

Gottesman, I. I., & Gould, T. D. (2003). The endophenotype concept in psychiatry: Etymology and strategic intentions. *American Journal of Psychiatry*, *160*, 636–645.

Grafstein-Dunn, E., Young, K. H., Cockett, M. I., & Khawaja, X. Z. (2001). Regional distribution of regulators of G-protein signaling (RGS) 1, 2, 13, 14, 16, and GAIP messenger ribonucleic acids by in situ hybridization in rat brain. *Brain Research. Molecular Brain Research*, *88*, 113–123.

Graybiel, A. M., & Rauch, S. L. (2000). Toward a neurobiology of obsessive–compulsive disorder. *Neuron*, *28*, 343–347.

Greenberg, P. E., Sisitsky, T., Kessler, R. C., Finkelstein, S. N., Berndt, E. R., Davidson, J. R., et al. (1999). The economic burden of anxiety disorders in the 1990s. *Journal of Clinical Psychiatry*, *60*, 427–435.

Gregory, A. M., Caspi, A., Moffitt, T. E., Koenen, K., Eley, T. C., & Poulton, R. (2007). Juvenile mental health histories of adults with anxiety disorders. *American Journal of Psychiatry*, *164*, 301–308.

Gregory, A. M., & Eley, T. C. (2007). Genetic influences on anxiety in children: What we've learned and where we're heading. *Clinical Child & Family Psychology Review*, *10*, 199–212.

Grimm, K. J., Ram, N., & Hamagami, F. (2011). Nonlinear growth curves in developmental research. *Child Development*, *82*, 1357–1371.

Hallett, V., Ronald, A., Rijsdijk, F., & Eley, T. C. (2009). Phenotypic and genetic differentiation of anxiety-related behaviors in middle childhood. *Depression and Anxiety*, *26*, 316–324.

Heim, C., & Nemeroff, C. B. (2009). Neurobiology of posttraumatic stress disorder. *CNS Spectrums*, *14*(Suppl. 1), 13–24.

Hettema, J. M., Neale, M. C., & Kendler, K. S. (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *American Journal of Psychiatry*, *158*, 1568–1578.

Hettema, J. M., Prescott, C. A., Myers, J. M., Neale, M. C., & Kendler, K. S. (2005). The structure of genetic and environmental risk factors for anxiety disorders in men and women. *Archives of General Psychiatry*, *62*, 182–189.

Hewitt, J. K. (2012). Editorial policy on candidate gene association and candidate gene-by-environment interaction studies of complex traits. *Behavior Genetics*, *42*, 1–2.

Hirshfeld-Becker, D. R., Biederman, J., Henin, A., Faraone, S. V., Davis, S., Harrington, K., et al. (2007). Behavioral inhibition in preschool children at risk is a specific predictor of middle childhood social anxiety: A five-year follow-up. *Journal of Developmental and Behavioral Pediatrics*, *28*, 225–233.

Hirshfeld-Becker, D. R., Micco, J. A., Simoes, N. A., & Henin, A. (2008). High-risk studies and developmental antecedents of anxiety disorders. *American Journal of Medical Genetics C: Seminars in Medical Genetics*, *148C*, 99–117.

Johansen, J. P., Cain, C. K., Ostroff, L. E., & LeDoux, J. E. (2011). Molecular mechanisms of fear learning and memory. *Cell*, *147*, 509–524.

Kagan, J., & Snidman, N. (2004). *The long shadow of temperament*. Cambridge, MA: Harvard University Press.

Kagan, J., Snidman, N., Kahn, V., & Towsley, S. (2007). The preservation of two infant temperaments into adolescence. *Monographs of the Society for Research in Child Development*, *72*, 1–75; discussion, 76–91.

Kawamura, Y., Otowa, T., Koike, A., Sugaya, N., Yoshida, E., Yasuda, S., et al. (2011). A genome-wide CNV association study on panic disorder in a Japanese population. *Journal of Human Genetics*, *56*, 852–856.

Kendler, K. S., Gardner, C. O., Annas, P., & Lichtenstein, P. (2008). The development of fears from early adolesence to young adulthood: A multivariate study. *Psychological Medicine*, *38*, 1759–1769.

Kendler, K. S., Gardner, C. O., Annas, P., Neale, M. C., Eaves, L. J., & Lichtenstein, P. (2008). A longitudinal twin study of fears from middle childhood to early adulthood: Evidence for a developmentally dynamic genome. *Archives of General Psychiatry*, *65*, 421–429.

Kendler, K. S., Gardner, C. O., & Lichtenstein, P. (2008). A developmental twin study of symptoms of anxiety and depression: Evidence for genetic innovation and attenuation. *Psychological Medicine*, *38*, 1567–1575.

Kendler, K. S., & Neale, M. C. (2010). Endophenotype: A conceptual analysis. *Molecular Psychiatry*, *15*, 789–797.

Kendler, K. S., Neale, M. C., Kessler, R. C., Heath, A. C., & Eaves, L. J. (1992). Major depression and generalized anxiety disorder: Same genes, (partly) different environments? *Archives of General Psychiatry*, *49*, 716–722.

Kendler, K. S., Prescott, C. A., Myers, J., & Neale, M. C. (2003). The structure of genetic and environmental risk factors for common psychiatric and substance use disorders in men and women. *Archives of General Psychiatry*, *60*, 929–937.

Kerner, B., North, K. E., & Fallin, M. D. (2009). Use of longitudinal data in genetic studies in the genome-wide association studies era: Summary of Group 14. *Genetic Epidemiology*, *33*(Suppl. 1), S93–S98.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of *DSM-IV* disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*, 593–602.

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month *DSM-IV* disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*, 617–627.

Killgore, W. D., & Yurgelun-Todd, D. A. (2005). Social anxiety predicts amygdala activation in adolescents viewing fearful faces. *NeuroReport*, *16*, 1671–1675.

Kim-Cohen, J., Caspi, A., Moffitt, T. E., Harrington, H., Milne, B. J., & Poulton, R. (2003). Prior juvenile diagnoses in adults with mental disorder: Developmental follow-back of a prospective-longitudinal cohort. *Archives of General Psychiatry*, *60*, 709–717.

Kline, R. B. (2005). *Principles and practice of structural equation modeling* (2nd ed.). New York: Guilford Press.

Koenen, K. C., Amstadter, A. B., Ruggiero, K. J., Acierno, R., Galea, S., Kilpatrick, D. G., et al. (2009). *RGS2* and generalized anxiety disorder in an epidemiologic sample of hurricane-exposed adults. *Depression and Anxiety*, *26*, 309–315.

Letcher, P., Sanson, A., Smart, D., & Toumbourou, J. W. (2012). Precursors and correlates of anxiety trajectories from late childhood to late adolescence. *Journal of Clinical Child and Adolescent Psychology*.

Leygraf, A., Hohoff, C., Freitag, C., Willis-Owen, S. A., Krakowitzky, P., Fritze, J., et al. (2006). *Rgs 2* gene polymorphisms as modulators of anxiety in humans? *Journal of Neural Transmission*, *113*, 1921–1925.

Loehlin, J. C. (2004). *Latent variable models: An introduction to factor, path, and structural equation analysis* (4th ed.). Mahwah, NJ: Erlbaum.

Malhotra, D., & Sebat, J. (2012). CNVs: Harbingers of a rare variant revolution in psychiatric genetics. *Cell*, *148*, 1223–1241.

Marmorstein, N. R., White, H., Chung, T., Hipwell, A., Stouthamer-Loeber, M., & Loeber, R. (2010). Associations between first use of substances and change in internalizing symptoms among girls: Differences by symptom trajectory and substance use type. *Journal of Clinical Child and Adolescent Psychology*, *39*, 545–558.

Maron, E., Hettema, J. M., & Shlik, J. (2010). Advances in molecular genetics of panic disorder. *Molecular Psychiatry*, *15*, 681–701.

McArdle, J. J., Nesselroade, J. R., Schinka, J. A., & Velicer, W. F. (2003). *Growth curve analysis in contemporary psychological research*. New York: Wiley.

McGrath, L. M., Mustanski, B., Metzger, A., Pine, D. S., Kistner-Griffin, E., Cook, E. H., et al. (in press). A latent modeling approach to genotype–phenotype relationships: Maternal problem behavior clusters, prenatal smoking, and *MAOA* genotype. *Archives of Women's Mental Health*.

McQueen, M. B., Bertram, L., Lange, C., Becker, K. D., Albert, M. S., Tanzi, R. E., et al. (2007). Exploring candidate gene associations with neuropsychological performance. *American Journal of Medical Genetics B: Neuropsychiatric Genetics*, *144B*, 987–991.

Medland, S. E., & Neale, M. C. (2010). An integrated phenomic approach to multivariate allelic association. *European Journal of Human Genetics*, *18*, 233–239.

Merikangas, K. R., Ames, M., Cui, L., Stang, P. E., Ustun, T. B., Von Korff, M., et al. (2007). The impact of comorbidity of mental and physical conditions on role disability in the US adult household population. *Archives of General Psychiatry*, *64*, 1180–1188.

Merikangas, K. R., He, J. P., Brody, D., Fisher, P. W., Bourdon, K., & Koretz, D. S. (2010). Prevalence and treatment of mental disorders among US children in the 2001–2004 NHANES. *Pediatrics*, *125*, 75–81.

Merikangas, K. R., He, J. P., Burstein, M., Swanson, S. A., Avenevoli, S., Cui, L., et al. (2010). Lifetime prevalence of mental disorders in U.S. adolescents: Results from the National Comorbidity Survey Replication—Adolescent Supplement (NCS-A). *Journal of the American Academy of Child & Adolescent Psychiatry*, *49*, 980–989.

Mian, N. D., Godoy, L., Briggs-Gowan, M. J., & Carter, A. S. (2011). Patterns of anxiety symptoms in toddlers and preschool-age children: Evidence of early differentiation. *Journal of Anxiety Disorders*.

Middeldorp, C. M., Cath, D. C., Van Dyck, R., & Boomsma, D. I. (2005). The comorbidity of anxiety and depression in the perspective of genetic epidemiology: A review of twin and family studies. *Psychological Medicine*, *35*, 611–624.

Middeldorp, C. M., Slof-Op 't Landt, M. C., Medland, S. E., van Beijsterveldt, C. E., Bartels, M., Willemsen, G., et al. (2010). Anxiety and depression in children and adults: Influence of serotonergic and neurotrophic genes? *Genes, Brain and Behavior*, *9*, 808–816.

Mosing, M. A., Gordon, S. D., Medland, S. E., Statham, D. J., Nelson, E. C., Heath, A. C., et al. (2009). Genetic and environmental influences on the comorbidity between depression, panic disorder, agoraphobia, and social phobia: A twin study. *Depression and Anxiety*, *26*, 1004–1011.

Mouri, K., Hishimoto, A., Fukutake, M., Nishiguchi, N., Shirikawa, O., & Maeda, K. (2009). Association study of RGS2 gene polymorphisms with panic disorder in Japanese. *Kobe Journal of Medical Sciences*, *55*, E116–E121.

Muinos-Gimeno, M., Espinosa-Parrilla, Y., Guidi, M., Kagerbauer, B., Sipila, T., Maron, E., et al. (2011). Human microRNAs miR-22, miR-138-2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways. *Biological Psychiatry*, *69*, 526–533.

Muthen, B. O. (2001). Second-generation structural equation modeling with a combination of categorical and continuous latent variables: New opportunities for latent class/latent growth modeling. In L. M. Collins & A. G. Sayer (Eds.), *New methods for the analysis of change* (pp. 291–322). Washington, DC: American Psychological Association.

Muthen, B. O. (2002). Beyond SEM: General latent variable modeling. *Behaviormetrika*, *29*, 81–117.

Nagin, D. S. (1999). Analyzing developmental trajectories: A semiparametric, group-based approach. *Psychological Methods*, *4*, 139–157.

Neale, B. M., Medland, S., Ripke, S., Anney, R. J., Asherson, P., Buitelaar, J., et al. (2010). Case-control genome-wide association study of attention-deficit/hyperactivity disorder. *Journal of the American Academy of Child & Adolescent Psychiatry*, *49*, 906–920.

Nes, R. B., Roysamb, E., Reichborn-Kjennerud, T., Harris, J. R., & Tambs, K. (2007). Symptoms of anxiety and depression in young adults: Genetic and environmental influences on stability and change. *Twin Research and Human Genetics*, *10*, 450–461.

Neubig, R. R., & Siderovski, D. P. (2002). Regulators of G-protein signalling as new central nervous system drug targets. *Nature Reviews Drug Discovery*, *1*, 187–197.

Newman, D. L., Moffitt, T. E., Caspi, A., Magdol, L., Silva, P. A., & Stanton, W. R. (1996). Psychiatric disorder in a birth cohort of young adults: Prevalence, comorbidity, clinical significance, and new case incidence from ages 11 to 21. *Journal of Consulting and Clinical Psychology*, *64*, 552–562.

Nigg, J. T. (2006). Temperament and developmental psychopathology. *Journal of Child Psychology and Psychiatry*, *47*, 395–422.

Ogliari, A., Spatola, C. A., Pesenti-Gritti, P., Medda, E., Penna, L., Stazi, M. A., et al. (2010). The role of genes and environment in shaping co-occurrence of *DSM-IV* defined anxiety dimensions among Italian twins aged 8–17. *Journal of Anxiety Disorders*, *24*, 433–439.

Oliveira-Dos-Santos, A. J., Matsumoto, G., Snow, B. E., Bai, D., Houston, F. P., Whishaw, I. Q., et al. (2000). Regulation of T cell activation, anxiety, and male aggression by *RGS2*. *Proceedings of the National Academy of Sciences*, *97*, 12272–12277.

Otowa, T., Shimada, T., Kawamura, Y., Sugaya, N., Yoshida, E., Inoue, K., et al. (2011). Association of *RGS2* variants with panic disorder in a Japanese population. *American Journal of Medical Genetics B: Neuropsychiatric Genetics*, *156B*, 430–434.

Otowa, T., Tanii, H., Sugaya, N., Yoshida, E., Inoue, K., Yasuda, S., et al. (2010). Replication of a genome-wide association study of panic disorder in a Japanese population. *Journal of Human Genetics*, *55*, 91–96.

Otowa, T., Yoshida, E., Sugaya, N., Yasuda, S., Nishimura, Y., Inoue, K., et al. (2009). Genome-wide association study of panic disorder in the Japanese population. *Journal of Human Genetics*, *54*, 122–126.

Perez-Edgar, K., & Fox, N. A. (2005). Temperament and anxiety disorders. *Child and Adolescent Psychiatric Clinics of North America*, *14*, 681–706.

Petersen, I. T., Bates, J. E., Goodnight, J. A., Dodge, K. A., Lansford, J. E., Pettit, G. S., et al. (2012). Interaction between serotonin transporter polymorphism (*5-HTTLPR*) and stressful life events in adolescents' trajectories of anxious/depressed symptoms. *Developmental Psychology*.

Psychiatric GWAS Consortium. (2012). A mega-analysis of genome-wide association studies for major depressive disorder. *Molecular Psychiatry*. Advance online publication. doi:10.1038/mp.2012.21

Pine, D. S. (2007). Research review: A neuroscience framework for pediatric anxiety disorders. *Journal of Child Psychology and Psychiatry*, *48*, 631–648.

Pine, D. S., Cohen, P., Gurley, D., Brook, J., & Ma, Y. (1998). The risk for early-adulthood anxiety and depressive disorders in adolescents with anxiety and depressive disorders. *Archives of General Psychiatry*, *55*, 56–64.

Plomin, R., Emde, R. N., Braungart, J. M., Campos, J., Corley, R., Fulker, D. W., et al. (1993). Genetic change and continuity from fourteen to twenty months: The MacArthur Longitudinal Twin Study. *Child Development*, *64*, 1354–1376.

Plomin, R., Haworth, C. M., & Davis, O. S. (2009). Common disorders are quantitative traits. *Nature Reviews Genetics*, *10*, 872–878.

Rapee, R. M., Schniering, C. A., & Hudson, J. L. (2009). Anxiety disorders during childhood and adolescence: Origins and treatment. *Annual Review of Clinical Psychology*, *5*, 311–341.

Rijsdijk, F. V., Snieder, H., Ormel, J., Sham, P., Goldberg, D. P., & Spector, T. D. (2003). Genetic and environmental influences on psychological distress in the population: General Health Questionnaire analyses in UK twins. *Psychological Medicine*, *33*, 793–801.

Ripke, S., Sanders, A. R., Kendler, K. S., Levinson, D. F., Sklar, P., Holmans, P. A., et al. (2011). Genome-wide association study identifies five new schizophrenia loci. *Nature Genetics*, *43*, 969–976.

Roberson-Nay, R., Eaves, L. J., Hettema, J. M., Kendler, K. S., & Silberg, J. L. (2012). Childhood separation anxiety disorder and adult-onset panic attacks share a common genetic diathesis. *Depression and Anxiety*, *29*, 320–327.

Robinson, J. L., Kagan, J., Reznick, J. S., & Corley, R. (1992). The heritability of inhibited and uninhibited behavior: A twin study. *Developmental Psychology*, *28*, 1030–1037.

Rosenbaum, J. F., Biederman, J., Hirshfeld-Becker, D. R., Kagan, J., Snidman, N., Friedman, D., et al. (2000). A controlled study of behavioral inhibition in children of parents with panic disorder and depression. *American Journal of Psychiatry*, *157*, 2002–2010.

Rothbart, M. K. (2007). Temperament, development, and personality. *Current Directions in Psychological Science*, *16*, 207–212.

Sakai, J. T., Boardman, J. D., Gelhorn, H. L., Smolen, A., Corley, R. P., Huizinga, D., et al. (2010). Using trajectory analyses to refine phenotype for genetic association: Conduct problems and the serotonin transporter (5HTTLPR). *Psychiatric Genetics*, *20*, 199–206.

Schulze, T. G., & McMahon, F. J. (2004). Defining the phenotype in human genetic studies: Forward genetics and reverse phenotyping. *Human Heredity*, *58*, 131–138.

Schwartz, C. E., Snidman, N., & Kagan, J. (1999). Adolescent social anxiety as an outcome of inhibited temperament in childhood. *Journal of the American Academy of Child & Adolescent Psychiatry*, *38*, 1008–1015.

Schwartz, C. E., Wright, C. I., Shin, L. M., Kagan, J., & Rauch, S. L. (2003). Inhibited and uninhibited infants "grown up": Adult amygdalar response to novelty. *Science*, *300*, 1952–1953.

Semplicini, A., Lenzini, L., Sartori, M., Papparella, I., Calo, L. A., Pagnin, E., et al. (2006). Reduced expression of regulator of G-protein signaling 2 (*RGS2*) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *Journal of Hypertension*, *24*, 1115–1124.

Shin, L. M., & Liberzon, I. (2010). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology*, *35*, 169–191.

Sklar, P., Ripke, S., Scott, L. J., Andreassen, O. A., Cichon, S., Craddock, N., et al. (2011). Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. *Nature Genetics*, *43*, 977–983.

Smoller, J. W., Lunetta, K. L., & Robins, J. (2000). Implications of comorbidity and ascertainment bias for identifying disease genes. *American Journal of Medical Genetics*, *96*, 817–822.

Smoller, J. W., Paulus, M. P., Fagerness, J. A., Purcell, S., Yamaki, L. H., Hirshfeld-Becker, D., et al. (2008). Influence of *RGS2* on anxiety-related temperament, personality, and brain function. *Archives of General Psychiatry*, *65*, 298–308.

Smoller, J. W., Rosenbaum, J. F., Biederman, J., Kennedy, J., Dai, D., Racette, S. R., et al. (2003). Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. *Biological Psychiatry*, *54*, 1376–1381.

Smoller, J. W., Yamaki, L. H., Fagerness, J. A., Biederman, J., Racette, S., Laird, N. M., et al. (2005). The corticotropin-releasing hormone gene and behavioral inhibition in children at risk for panic disorder. *Biological Psychiatry*, *57*, 1485–1492.

Stein, M. B., Goldin, P. R., Sareen, J., Zorrilla, L. T., & Brown, G. G. (2002). Increased amygdala activation to angry and contemptuous faces in generalized social phobia. *Archives of General Psychiatry*, *59*, 1027–1034.

Stein, M. B., Simmons, A. N., Feinstein, J. S., & Paulus, M. P. (2007). Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. *American Journal of Psychiatry*, *164*, 318–327.

Sullivan, P. F. (2007). Spurious genetic associations. *Biological Psychiatry*, *61*, 1121–1126.

Sullivan, P. F. (2010). The psychiatric GWAS consortium: Big science comes to psychiatry. *Neuron*, *68*, 182–186.

Sullivan, P. F. (2012). Don't give up on GWAS. *Molecular Psychiatry*, *17*, 2–3.

Tambs, K., Czajkowsky, N., Roysamb, E., Neale, M. C., Reichborn-Kjennerud, T., Aggen, S. H., et al. (2009). Structure of genetic and environmental risk factors for dimensional representations of *DSM-IV* anxiety disorders. *British Journal of Psychiatry*, *195*, 301–307.

Trzaskowski, M., Zavos, H. M., Haworth, C. M., Plomin, R., & Eley, T. C. (2011). Stable genetic influence on anxiety-related behaviours across middle childhood. *Journal of Abnormal Child Psychology*.

Wang, K., Zhang, H., Ma, D., Bucan, M., Glessner, J. T., Abrahams, B. S., et al. (2009). Common genetic variants on 5p14.1 associate with autism spectrum disorders. *Nature*, *459*, 528–533.

Webb, B. T., Guo, A. Y., Maher, B. S., Zhao, Z., van den Oord, E. J., Kendler, K. S., et al. (2012). Meta-analyses of genome-wide linkage scans of anxiety-related phenotypes. *European Journal of Human Genetics*.

Weissman, M. M., Wickramaratne, P., Nomura, Y., Warner, V., Verdeli, H., Pilowsky, D. J., et al. (2005). Families at high and low risk for depression: A 3-generation study. *Archives of General Psychiatry*, *62*, 29–36.

Wessman, J., Paunio, T., Tuulio-Henriksson, A., Koivisto, M., Partonen, T., Suvisaari, J., et al. (2009). Mixture model clustering of phenotype features reveals evidence for association of *DTNBP1* to a specific subtype of schizophrenia. *Biological Psychiatry*, *66*, 990–996.

Wray, N. R., James, M. R., Gordon, S. D., Dumenil, T., Ryan, L., Coventry, W. L., et al. (2009). Accurate, large-scale genotyping of *5HTTLPR* and flanking single nucleotide polymorphisms in an association study of depression, anxiety, and personality measures. *Biological Psychiatry*, *66*, 468–476.

Yalcin, B., Willis-Owen, S. A., Fullerton, J., Meesaq, A., Deacon, R. M., Rawlins, J. N., et al. (2004). Genetic dissection of a behavioral quantitative trait locus shows that *Rgs2* modulates anxiety in mice. *Nature Genetics*, *36*, 1197–1202.

Zintzaras, E., & Sakelaridis, N. (2007). Is 472G/A catechol-*O*-methyl-transferase gene polymorphism related to panic disorder? *Psychiatric Genetics*, *17*, 267–273.

Biology of Mood & Anxiety Disorders

REVIEW Open Access

Anxiety genetics – findings from cross-species genome-wide approaches

Ewa Sokolowska[1] and Iiris Hovatta[1,2*]

Abstract

Anxiety disorders are complex diseases, which often occur in combination with major depression, alcohol use disorder, or general medical conditions. Anxiety disorders were the most common mental disorders within the EU states in 2010 with 14% prevalence. Anxiety disorders are triggered by environmental factors in genetically susceptible individuals, and therefore genetic research offers a great route to unravel molecular basis of these diseases. As anxiety is an evolutionarily conserved response, mouse models can be used to carry out genome-wide searches for specific genes in a setting that controls for the environmental factors. In this review, we discuss translational approaches that aim to bridge results from unbiased genome-wide screens using mouse models to anxiety disorders in humans. Several methods, such as quantitative trait locus mapping, gene expression profiling, and proteomics, have been used in various mouse models of anxiety to identify genes that regulate anxiety or play a role in maintaining pathological anxiety. We first discuss briefly the evolutionary background of anxiety, which justifies cross-species approaches. We then describe how several genes have been identified through genome-wide methods in mouse models and subsequently investigated in human anxiety disorder samples as candidate genes. These studies have led to the identification of completely novel biological pathways that regulate anxiety in mice and humans, and that can be further investigated as targets for therapy.

Keywords: Anxiety disorders, Anxiety-like behavior, Mouse model, Cross-species approach, Genome-wide association study, Quantitative trait locus, Gene expression, Proteomics, Candidate gene

Review

Anxiety disorders

Anxiety and fear are normal emotional responses to threatening situations. In anxiety disorders these responses are exaggerated or prolonged and disturb daily life. Anxiety disorders, including panic disorder, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), social phobia, specific phobias, and generalized anxiety disorder (GAD), were the most common mental disorders within the EU states in 2010 with 14% prevalence [1]. Anxiety disorders are currently treated with drugs and/or cognitive behavioral therapy or other psychosocial treatments. Current pharmacotherapeutic options including benzodiazepines and selective serotonin reuptake inhibitors are not optimal due to addictive properties, development of tolerance, or poor efficacy in some patients. Therefore, new and better anxiolytics are needed, and their development requires understanding of the molecular mechanisms that regulate anxiety. Genetics offer an ideal route to the molecular background of anxiety as any identified genes can directly be linked to their function within the cell and the neural circuits.

Anxiety disorders are complex diseases caused by a combination of genetic and environmental factors. In recent years, several genes have been associated with anxiety disorders [2]. Replicated associations exist to genes belonging to various neurotransmitter or neuropeptide systems [3]. Recently, the first genome-wide association studies (GWAS) aiming to identify common variants have been published in anxiety-related personality trait neuroticism and panic disorder [4-7]. These studies support involvement of a relatively large number of small effect size common and rare variants in the predisposition to anxiety disorders, a notion shared with other psychiatric diseases, such as schizophrenia and major depression. Therefore, very large sample sizes (several thousands of individuals)

* Correspondence: iiris.hovatta@helsinki.fi
[1]Department of Biosciences, Viikki Biocenter, University of Helsinki, Helsinki, Finland
[2]Mental Health and Substance Abuse Services, National Institute for Health and Welfare, Helsinki, Finland

will be needed to identify variants predisposing to anxiety disorders.

Anxiety is an evolutionarily conserved response and can be reliably measured in mice (Table 1). The advantage of mouse models is that the environmental factors can be controlled for, or specifically administered. In addition, brain tissue can be collected at any time point. To complement human genetic studies several groups have used mouse models of anxiety-like behavior for identification of genes and biological pathways that regulate anxiety. In general two approaches can be taken: i) candidate gene studies have mostly used transgenic models to investigate a role of a specific gene in the regulation of anxiety, and ii) genome-wide approaches do not make any prior assumptions regarding which genes contribute to the phenotype. In this review, we will concentrate on genome-wide approaches in mice, which have resulted in the identification of genes regulating anxiety. We have further restricted our focus to those genes that have subsequently been associated at some level to human anxiety disorders. Therefore, several interesting genes that may regulate anxiety but i) have been identified initially through transgenic mouse models, ii) human candidate gene or GWAS studies, or iii) have been identified in mouse models but not shown any link to human anxiety disorders, are not discussed here [8-10].

Anxiety is an evolutionarily conserved response

Why can we use the mouse to model aspects of human anxiety disorders? Neuroevolutionary studies have shown that anxiety is an adaptive response that has been conserved during evolution [12,13]. From this perspective anxiety is viewed as a behavioral state, which occurs in response to signals of danger. On the physiological level these signals initiate activation of the hypothalamus-pituitary-adrenal (HPA) axis [14] and secretion of adrenal steroids called stress hormones, which are present in almost every vertebrate cell [15]. This leads to increased heart rate, deeper breathing, vigilance, decrease in feeding, and exploration of environment [16]. The genes that code for stress hormones are highly conserved across diverse species: primates, rodents, reptiles, and amphibians [17,18].

Mice represent a good model system for human anxiety disorders for several reasons: i) they have a central nervous system (CNS) that is sufficiently developed to model aspects of human anxiety as compared to lower organisms, ii) hundreds of inbred strains are available, and the whole genome sequence of 17 strains has been determined [19], iii) transgenic techniques to manipulate the genome are well established, and iv) their maintenance is cost-effective. The majority of the anxiety-related behavioral tests utilize approach-avoidance behaviors that appear to mirror rodent's behavioral response to conflict in its natural environment. Both approach behaviors, such as mate searching and foraging, and avoidance behaviors, such as escape from the predator, are evolutionarily conserved in some forms from nematodes to mammals [20]. Furthermore, the neural organization of behaviors underlying fearful, sexual, feeding, and escape motivation is relatively similar across species [21]. Disturbed balance in approach-avoidance behaviors is a symptom of autism [22], PTSD [23], and social phobia [24]. Several paradigms to test anxiety in mice, based on the approach-avoidance behavior, have been developed and pharmacologically validated with drugs that are used to treat human disease and are therefore considered appropriate models for human anxiety [25]. The most commonly used tests include the elevated plus maze, open field, light dark box, and novelty-induced hypophagia tests. In these tests mice have to choose between exploring and staying in a safe environment. However, due to cognitive differences between mouse and human, it is recognized that no animal model can mimic all aspects of human anxiety and anxiety disorders. Nevertheless, genes that regulate anxiety in mice are excellent candidate genes for anxiety disorders (Figure 1).

Table 1 Comparison of human anxiety disorders to anxiety-like behavior in mice

Disorder	Human symptoms	Observed behavior in mice	Behavioral test in mice
Generalized anxiety disorder	Excessive worry about everyday life, leading to difficulties in concentration	Decreased social interaction, impaired sustained attention	OF, L/D, Y-maze
Posttraumatic stress disorder	Repeated re-experiencing traumatic events, leading to avoidance of stimuli associated with trauma	Increased freezing response to fear conditioning, decreased fear extinction, more pronounced spontaneous recovery	Cue and contextual fear conditioning, fear extinction
Obsessive-compulsive disorder	Intrusive thoughts that produce repetitive behavior aimed at reducing anxiety	Increased marble burying and excessive grooming	Burrowing test, nest construction test
Social phobia	avoidance of social contact, emotional discomfort caused by presence of unknown people	Low social interaction	Three-chamber test of sociability, social recognition test
Panic disorder	Intense fearfulness of sudden onset, respiratory distress	Increased escape from an aggressor	Resident intruder test
Agoraphobia	Avoidance of wide-open or crowded space	Avoidance of exposed, bright areas	OF, L/D

Anxiety disorders are classified according to the Diagnostic and Statistical Manual of Mental Disorders of American Psychiatric Association (DSM-IV). *OF*, Open field test; *L/D*, Light/dark box test. Modified from [11].

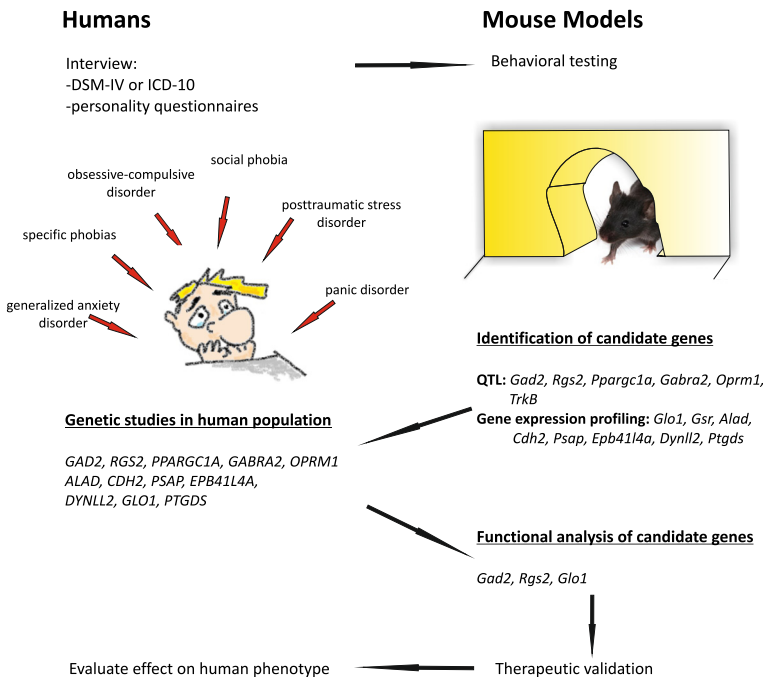


Figure 1 A strategy for a cross-species mouse and human genetic approach to identify susceptibility genes for anxiety disorders. The greatest advantages of using mouse models are the ability to reduce genetic heterogeneity and to control for the environment. The first step is to characterize aspects of a human disease in a mouse model, and to perform a search for candidate genes in a mouse model. It is then important to study these candidates in human populations to determine if they predispose to the disease under study. Mouse models are, however, needed to further characterize the function of the candidate genes, and to carry out potential drug target validation. Examples of candidate genes identified using different approaches in mice and humans are shown. Modified from [2].

Quantitative trait locus (QTL) mapping of anxiety-like behavior

QTL mapping has been used to identify genes that regulate anxiety-like behavior in rodents [26], with the idea that genes in the homologous loci in humans can then be studied as susceptibility genes for the corresponding human phenotype. Traditionally, QTL mapping has been based on genotyping F2 mice using a genome-wide marker panel and measuring the anxiety level of these animals. As a result, loci that likely contain genes affecting the phenotype can be mapped. Due to the low mapping resolution of F2 panels, other sources, such as recombinant inbred strains, heterogeneous stock mice, and outbred animals, have been used for initial and fine mapping [27-29]. It is expected that the Collaborative Cross strains, a collection of recombinant inbred mouse strains derived from eight parental strains, will be an efficient mapping resource in the future to identify both major loci and their modifiers [30]. Although initial enthusiasm for QTL mapping has been suppressed by low efficiency and resolution, and small effect size of individual variants, several anxiety-associated genes have been identified through QTL mapping [31-37]. Here we will discuss those genes that have shown some evidence for association to human anxiety disorders in subsequent studies. These include Gad2, Rgs2, Ppargc1a, Gabra2, Oprm1, and TrkB.

Glutamic acid decarboxylase 2 (Gad2)

One of the earliest cross-species studies investigated behavioral inhibition to the unfamiliar, a heritable temperament character that is considered a risk factor for panic and phobic anxiety [38]. Four genes were selected for genotyping in humans based on their homology to loci previously associated with anxiety or fear behavior in mice. The sample consisted of 72 behaviorally inhibited children and their family members, analyzed in a family-based association analysis. Suggestive evidence for association was found to variants in the *GAD2* gene. GAD2 is an enzyme involved in the gamma-aminobutyric acid (GABA) synthesis, and is therefore an intriguing candidate gene as abnormalities in the GABA system have been observed in anxiety disorders [39]. *GAD2* has been studied as a candidate gene for anxiety disorders in two larger subsequent studies. In the Virginia Adult Twin Study of Psychiatric and Substance Use Disorders 14 SNPs from *GAD2* were first genotyped in 188 cases with internalizing disorders (major depression, GAD, panic disorder, agoraphobia, social phobia, or neuroticism personality trait) and

188 controls. One SNP with $p < 0.1$ and two SNPs within the same haplotype were followed up by genotyping additional 401 cases and 351 controls but the initial finding was not replicated [40]. Another study, consisting of anxiety disorder cases (N = 268), cases with major depression (N = 541), and 541 healthy controls, tested association to 18 SNPs within *GAD2* [41]. One SNP (rs8190646) significantly associated to major depression ($p = 0.00039$). No association to anxiety disorders was found. To mimic the phenotype of the original study [38] association of these SNPs were also tested with behavioral inhibition in 211 anxiety disorder cases, 202 cases with major depression, and 537 controls from the same sample. Significant association to behavioral inhibition was found in the subgroups of anxiety disorder cases and healthy controls, but not to cases with major depression or all groups combined. The contradictory findings in these two larger studies may be explained by several differences, such as phenotype definition and ethnicity of the study participants. The large ongoing GWAS studies should inform whether *GAD2* variants predispose to various anxiety disorders or other psychiatric phenotypes.

Regulator of G-protein signaling 2 (Rgs2)

A more recent successful cross-species study concerns the genetic background of emotionality. Initially, a linkage to chromosome 1 was found by QTL mapping of DeFries mouse strains [42], and the locus was fine mapped in outbred mice [43]. This region contains the *Rgs2* gene, encoding a regulator of G protein signaling. To investigate whether *Rgs2* interacts with the functional variant, quantitative complementation method was applied, and a small-effect QTL contributing to behavioral variation in mice was identified [44]. Furthermore, knock-out mice of *Rgs2* show increased anxiety-like behavior [45]. These results indicate that Rgs2 regulate anxiety-like behavior in mice. To study the involvement of variants in *RGS2* in intermediate phenotypes of human anxiety disorders Smoller et al. studied a family based sample (119 families) of children with behavioral inhibition, 744 unrelated adults who were tested for extraversion and introversion personality traits, and 55 unrelated adults tested with the emotional face assessment during fMRI [46]. *RGS2* SNPs associated with childhood behavioral inhibition (haplotype $p = 0.00003$) and introversion personality trait ($p = 0.007$-0.05 for single SNPs, $p = 0.038$ for a haplotype) as well as increased activation of amygdala and insular cortex in response to watching fearful faces. In another study, four SNPs within *RGS2* showed some association to panic disorder ($p = 0.02$-0.05) in a sample of 173 German cases and 173 controls [47]. Also, one SNP in *RGS2* was associated to GAD in a sample of 607 adults exposed to 2004 Florida hurricane ($p = 0.026$) [48]. However, a recent study of 2661 individuals from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorders aiming to replicate the previous findings failed to find association to three most consistently associated SNPs from these previous studies [49]. Again these discrepant results may be due to differences in the phenotype definitions or ethnic background of the samples. However, twin studies suggest that many of these phenotypes share common risk factors [50], although it is not clear how strongly they are expected to relate to specific risk alleles and their effect size.

Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Ppargc1a)

Hettema et al. [51] combined data from several sources to identify and study 52 novel candidate genes for anxiety-spectrum disorders. They started with using strain distribution pattern analysis in heterogeneous stock mice that differ in anxiety-like behavior [29]. They then ranked these genes according to prior data including 1) extant linkage and knockout studies in mice, 2) a meta-analysis of human linkage scans, and 3) a preliminary human GWAS. Subsequently SNPs covering the nine top-ranked regions containing 14 genes were genotyped in a two-stage association study of subjects from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorders chosen for high or low genetic loading for anxiety-spectrum phenotypes. Several SNPs within the transcriptional co-activator *PPARGC1A* associated with the anxiety phenotype. Initially PPARGC1A was discovered in the muscle cells and brown fat and characterized as a transcriptional co-activator, which stimulates mitochondrial biogenesis by increasing oxidative phosphorylation and by enhancing oxidative respiration [52]. Further studies indicated that PPARGC1A activates nuclear respiratory factor 1 (NRF1) and 2 (NRF2) [53]. These two genes are linked to oxidative stress, and involvement of oxidative stress in anxiety has been suggested by human and rodent studies, as discussed in recent reviews [54,55].

Gabra2, Oprm1 and TrkB in PTSD

Fear conditioning, a form of Pavlovian learning, has been used to model some aspects of PTSD. Parker et al. used an intercross of inbred mouse strains C57BL/6J x DBA/2J to identify, and an F8 advanced intercross line to fine-map, QTL associated with fear conditioning [56]. Subsequently, publicly available DNA sequence information and gene expression data were used to identify candidate genes based on the existence of non-synonymous coding polymorphisms and/or expression QTLs. Several candidate genes previously implicated in PTSD in humans were identified: gamma-aminobutyric acid receptor subunit alpha-2 (*Gabra2*), opioid receptor-mu1 (*Oprm1*), and neurotrophic tyrosine kinase (*TrkB*). GABRA2 modulates

stress response [39] and SNPs within this gene have been associated with PTSD in adult patients previously exposed to child abuse [57]. OPRM1 has been linked with PTSD through different levels of μ-opioid receptor binding potential in a sample consisting of patients with PTSD (N = 16) and controls with (n = 14) or without (n = 15) combat exposure [58]. TRKB is a receptor for brain-derived neurotrophic factor (BDNF). Carriers of the Met allele of the *BDNF* Val66Met polymorphism show impaired fear extinction and disturbed fronto-amygdala activity [10]. In addition to these genes already linked to PTSD, Parker et al. found several other genes associating with fear conditioning in mice, and variants in the homologous human genes should be investigated as candidate genes for PTSD.

Gene expression profiling in brain tissue

Functional genomics experiments represent a data-driven approach for identifying associations between a phenotype and genes or gene networks. Based on the data, specific hypotheses can be formulated and tested *in vitro* and *in vivo*. Inbred mouse strains that differ in their innate anxiety levels have been used to identify gene expression patterns that correlate with behavioral phenotypes across a number of strains [59-61]. Fernandes et al. investigated gene expression in the hippocampus of eight inbred strains, which differ in many behavioral phenotypes, and identified 200 genes showing strain differences. The strongest genetic correlation with a phenotype was found for catechol-O-methyl transferase (*Comt*), a gene previously associated with aggressive behavior [59]. A panel of eight inbred strains was used by Letwin et al. to identify strain and brain region-specific expression differences in five brain regions. They identified several glutamatergic signaling pathway-related genes correlating with anxiety-like behavior [61]. We investigated gene expression differences in seven brain regions of six inbred mouse strains that differ in their innate anxiety levels [60]. We correlated gene expression patterns from seven brain regions, known to regulate some aspects of anxiety, with behavioral anxiety-measures and identified genes with an expression pattern that correlates with anxiety-like behavior. We then functionally verified by lentivirus-mediated gene transfer (overexpression and silencing by RNAi) that two genes, glyoxalase 1 (*Glo1*) and glutathione reductase (*Gsr*) regulate anxiety in mice [60]. Since *Glo1* has been identified by several studies using various approaches, it is discussed further in the next section. The challenge with the translation of the gene expression findings to human anxiety disorders is the poor availability of good quality post mortem brain samples. Another approach is to test if DNA variants in the homologous human genes confer predisposition to anxiety disorders, but since a large number of the gene expression changes are expected to be reactive rather than causal, this approach may work better on a pathway than single gene level.

As a translational step we tested whether genetic variants in 13 genes shown to be differentially expressed between anxious and non-anxious mouse strains predispose humans to anxiety disorders. We carried out a genetic association analysis in a Finnish population-based Health 2000 Cohort consisting of 321 cases and 653 matched controls. Variants in six genes (*CDH2*, *ALAD*, *PSAP*, *EPB41L4A*, *DYNLL2*, and *PTGDS*) showed some evidence ($p < 0.01$) for association to anxiety disorders [62]. Interestingly, *Cdh2* was recently shown to confer susceptibility to compulsive behavior in dogs [63].

Glo1 has been identified through various approaches

Glo1 was one of the genes identified through gene expression profiling in inbred strains having a higher expression level in anxious strains [60]. In the same study, its overexpression in the cingulate cortex by lentivirus-mediated gene transfer resulted in increased anxiety-like behavior, while inhibition by overexpression of an shRNA decreased anxiety-like behavior. *Glo1* was independently identified through a genome-wide search for copy number variants (CNVs) in inbred strains [64]. It was shown that the difference in *Glo1* expression between inbred mouse strains is due to a CNV, the presence of which correlates positively with anxiety-like behavior. To show a causal relationship between the CNV and anxiety-like behavior Distler et al. generated BAC transgenic mice expressing different copy numbers of *Glo1* [65]. The mice with several copies have increased anxiety-like behavior, as expected. GLO1 is a detoxification enzyme, which together with glyoxalase 2 converts cytotoxic methylglyoxal (MG) to non-toxic form [66,67]. When exploring the molecular mechanism of GLO1 underlying anxiety behavior Distler et al. found that overexpression of *Glo1* reduces MG level in the brain. Moreover, they showed that MG is an agonist of GABAA receptors, and therefore reduced levels of MG decrease GABAA receptor activation [65]. This finding conforms well to the known involvement of GABAA receptors in the regulation of anxiety. Interestingly, two proteomics studies have also linked GLO1 with anxiety-like behavior. According to these studies GLO1 is down-regulated in the brain of two separate mouse strains selectively bred for high anxiety behavior compared to their respective low-anxiety strains [68,69], a finding contradictory to the findings in the inbred strains. This surprising difference is likely due to other alleles contributing to the anxiety phenotype in these models and other factors related to the selective breeding of the strains, including differences in initial allelic frequencies, linked alleles, and drift before or during inbreeding [70]. More detailed discussion on the role of GLO1 in behavioral phenotypes is found in an excellent recent review [70].

The role of GLO1 in mental disorders has been studied in humans. Patients with major depression or bipolar disorder show reduced *GLO1* expression when in depressive state, but not during remission [71]. However, cholecystokinin-tetrapeptide (CCK-4), which is used to induce panic attacks, did not have an effect on *GLO1* mRNA levels in peripheral blood cells of 23 healthy volunteers [72]. In schizophrenia patients, rare genetic variants in *GLO1* have been associated with decreased enzyme activity and increased carbonyl stress [73]. Genetic association studies have been carried out in anxiety disorders. A common Ala111Glu substitution in *GLO1*, responsible for conformational change and decreased enzymatic activity, was investigated in 162 panic disorder patients and 288 matched controls from the Italian population [74]. Although there was no evidence of association to the overall diagnosis, some evidence was found for association with panic disorder without agoraphobia (N = 61 patients, p = 0.015). Similarly, Donner et al. failed to find strong evidence for association with this SNP and anxiety disorders in the Finnish population (p = 0.021) [62]. This functional SNP therefore does not seem to play a major role in the predisposition to anxiety disorders. Larger genetic studies are needed to find out whether other common or rare variants within *GLO1* are involved in the etiology of anxiety disorders.

Proteomic studies in mouse models

Altogether three proteomic studies have been carried out in bidirectionally bred mouse strains for high or low levels of anxiety. In the HAB/LAB mouse model several proteins have been identified, including GLO1, discussed already in detail above [69], and another interesting enzyme, enolase-phosphatase [75]. In a different bidirectional mouse model of anxiety-like behavior Szego et al. identified alterations in serotonin receptor-associated proteins [69]. Recent proteomic analysis of rat hippocampus after psychosocial stress revealed 21 differently expressed proteins. They were involved in various cellular functions, including signal transduction, synaptic plasticity, cytoskeleton remodeling and energy metabolism [76].

Since the proteomics-based methods are developing with fast pace, it is expected that they will in the near future reveal biomarker panels to be used in biological diagnostics of psychiatric disorders, in addition to shedding light to the neurobiological mechanisms regulating anxiety.

Conclusions

Because of their high prevalence, anxiety disorders impose high social and economic burden. Integration of data from several approaches is needed to understand the molecular mechanisms that regulate anxiety, and to develop novel pharmacological treatments. Genome-wide approaches to identify regulators of anxiety-like behavior in animal models will greatly complement the ongoing GWAS efforts in human anxiety disorders. There are two major advantages in using mouse models compared to human patient samples. Since environmental factors can be controlled for, or specifically administered in animal models, the power to detect small genetic effects is likely better in animal models compared to human cohorts. Stress, especially in childhood, is a well-established risk factor for anxiety disorders, and several mouse models for childhood stress have been recently developed. These should be investigated in several inbred genetic backgrounds, to identify gene-environment interactions in controlled circumstances. Another benefit of using animal models is the ability to harvest brain tissue at any time point. This allows taking advantage of unbiased genome-wide and proteome-wide identification of genes that regulate anxiety. With mRNA-seq and small RNA-seq it is now possible to identify all expressed genes from a given tissue, at different time points. Bioinformatic integration of this information can then be used to identify dynamic gene regulatory networks, instead of single genes. Optogenetic manipulation of specific cell types, combined with behavioral and gene expression analysis will help to detect yet more specific circuits underlying anxiety behavior. This approach will require development of better methods to dissect specific cell types and to carry out RNA-seq from very small amounts of RNA.

Results from the animal models should be used to formulate and test specific hypotheses in humans, using genetic and imaging approaches. The progress of the translation has been hindered by the relatively small size of well-characterized anxiety disorder cohorts, as can be seen with examples given above. Also, anxiety disorders as a group are phenotypically heterogeneous and it is not expected that all genetic findings replicate across all phenotypes. Integration of results from human genetic and imaging approaches with mouse genetic and functional studies will be essential to understand the neurobiological basis of anxiety disorders, a prerequisite for targeted therapies.

Abbreviations

ALAD: δ-Aminolevulinate dehydratase; BAC: Bacterial artificial chromosome; BDNF: Brain-derived neurotrophic factor; fMRI: Functional magnetic resonance imaging; CCK-4: Cholecystokinin-tetrapeptide; CDH2: Cadherin-2; CNS: Central nervous system; CNV: Copy number variant; Comt: Catechol-O-methyl transferase; DYNLL2: Dynein light chain 2; EPB41L4A: Erythrocyte membrane protein band 4.1 like 4A; GABA: Gamma-aminobutyric acid; GABRA2: Gamma-aminobutyric acid receptor subunit alpha-2; GAD: Generalized anxiety disorder; GAD2: Glutamic acid decarboxylase 2; Glo1: Glyoxalase 1; Gsr: Glutathione reductase; GWAS: Genome-wide association study; HAB/LAB: High anxiety-like behavior/low anxiety-like behavior; HPA: Hypothalamic-pituitary-adrenal axis; MG: Methylglyoxal; NRF: Nuclear respiratory factor; OCD: Obsessive-compulsive disorder; Oprm1: Opioid receptor, mu 1; PPARGC1A: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PSAP: Prosaposin; PTGDS: Prostaglandin D2 synthase; PTSD: Posttraumatic stress disorders; QTL: Quantitative trait locus; Rgs2: Regulator of G-protein signaling 2; SNP: Single nucleotide polymorphism; TrkB: Neurotrophic tyrosine kinase.

Competing interests
The authors declare that they have no competing interest.

Authors' contributions
ES and IH contributed equally to this manuscript. Both authors read and approved the final manuscript.

Acknowledgements
This work was supported by the Academy of Finland, European Research Council Starting Investigator Grant GenAnx, and Sigrid Jusélius Foundation. We thank Hovatta lab members for helpful discussions and Petri Hyytiä for comments on the manuscript.

Received: 9 January 2013 Accepted: 16 April 2013
Published: 9 May 2013

References

1. Wittchen HUJF, Rehm J, Gustavsson A, Svensson M, Jönsson B, Olesen J, Allgulander C, Alonso J, Faravelli C, Fratiglioni L, Jennum P, Lieb R, Maercker A, van Os J, Preisig M, Salvador-Carulla L, Simon R, Steinhausen H: **The size and burden of mental disorders and other disorders of the brain in Europe 2010.** *Eur Neuropsychopharmacol* 2010, **21:**655–679.
2. Hovatta I, Barlow C: **Molecular genetics of anxiety in mice and men.** *Ann Med* 2008, **40:**92–109.
3. Arnold PD, Zai G, Richter MA: **Genetics of anxiety disorders.** *Curr Psychiatry Rep* 2004, **6:**243–254.
4. Calboli FC, Tozzi F, Galwey NW, Antoniades A, Mooser V, Preisig M, Vollenweider P, Waterworth D, Waeber G, Johnson MR, *et al*: **A genome-wide association study of neuroticism in a population-based sample.** *PLoS One* 2010, **5:**e11504.
5. Otowa T, Yoshida E, Sugaya N, Yasuda S, Nishimura Y, Inoue K, Tochigi M, Umekage T, Miyagawa T, Nishida N, *et al*: **Genome-wide association study of panic disorder in the Japanese population.** *J Hum Genet* 2009, **54:**122–126.
6. Shifman S, Bhomra A, Smiley S, Wray NR, James MR, Martin NG, Hettema JM, An SS, Neale MC, van den Oord EJ, *et al*: **A whole genome association study of neuroticism using DNA pooling.** *Mol Psychiatry* 2008, **13:**302–312.
7. Terracciano A, Sanna S, Uda M, Deiana B, Usala G, Busonero F, Maschio A, Scally M, Patriciu N, Chen WM, *et al*: **Genome-wide association scan for five major dimensions of personality.** *Mol Psychiatry* 2010, **15:**647–656.
8. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, *et al*: **Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior.** *Science* 2006, **314:**140–143.
9. Erhardt A, Czibere L, Roeske D, Lucae S, Unschuld PG, Ripke S, Specht M, Kohli MA, Kloiber S, Ising M, *et al*: **TMEM132D, a new candidate for anxiety phenotypes: evidence from human and mouse studies.** *Mol Psychiatry* 2011, **16:**647–663.
10. Soliman F, Glatt CE, Bath KG, Levita L, Jones RM, Pattwell SS, Jing D, Tottenham N, Amso D, Somerville LH, *et al*: **A genetic variant BDNF polymorphism alters extinction learning in both mouse and human.** *Science* 2010, **327:**863–866.
11. Cryan JF, Holmes A: **The ascent of mouse: advances in modelling human depression and anxiety.** *Nat Rev Drug Discov* 2005, **4:**775–790.
12. Nesse R: **Emotional disorders in evolutionary perspective.** *Br J Med Psychol* 1998, **71:**397–415.
13. Stein DJ, Bouwer C: **A neuro-evolutionary approach to the anxiety disorders.** *J Anxiety Disord* 1997, **11:**409–429.
14. Boyce WT, Ellis BJ: **Biological sensitivity to context: I. An evolutionary-developmental theory of the origins and functions of stress reactivity.** *Dev Psychopathol* 2005, **17:**271–301.
15. Korte SM: **Corticosteroids in relation to fear, anxiety and psychopathology.** *Neurosci Biobehav Rev* 2001, **25:**117–142.
16. Cannon WB: *Bodily changes in pain, hunger, fear and rage: Researches into the function of emotional excitement.* New York: Harper & Row; 1915/1929.
17. Lovejoy DA, Balment RJ: **Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates.** *Gen Comp Endocrinol* 1999, **115:**1–22.
18. Lovejoy DA, Jahan S: **Phylogeny of the corticotropin-releasing factor family of peptides in the metazoa.** *Gen Comp Endocrinol* 2006, **146:**1–8.
19. Keane TM, Goodstadt L, Danecek P, White MA, Wong K, Yalcin B, Heger A, Agam A, Slater G, Goodson M, *et al*: **Mouse genomic variation and its effect on phenotypes and gene regulation.** *Nature* 2011, **477:**289–294.
20. O'Connell LA, Hofmann HA: **Genes, hormones, and circuits: an integrative approach to study the evolution of social behavior.** *Front Neuroendocrinol* 2011, **32:**320–335.
21. Elliot AJ, Covington MV: **Approach and avoidance motivation.** *Educ Psychol Rev* 2001, **13:**73–92.
22. Lombardo MV, Ashwin E, Auyeung B, Chakrabarti B, Lai MC, Taylor K, Hackett G, Bullmore ET, Baron-Cohen S: **Fetal programming effects of testosterone on the reward system and behavioral approach tendencies in humans.** *Biol Psychiatry* 2012, **72:**839–847.
23. Stein MB, Paulus MP: **Imbalance of approach and avoidance: the yin and yang of anxiety disorders.** *Biol Psychiatry* 2009, **66:**1072–1074.
24. Dell'Osso L, Rucci P, Ducci F, Ciapparelli A, Vivarelli L, Carlini M, Ramacciotti C, Cassano GB: **Social anxiety spectrum.** *Eur Arch Psychiatry Clin Neurosci* 2003, **253:**286–291.
25. Gordon JA, Hen R: **Genetic approaches to the study of anxiety.** *Annu Rev Neurosci* 2004, **27:**193–222.
26. Ramos A, Moisan MP, Chaouloff F, Mormede C, Mormede P: **Identification of female-specific QTLs affecting an emotionality-related behavior in rats.** *Mol Psychiatry* 1999, **4:**453–462.
27. Mott R, Flint J: **Simultaneous detection and fine mapping of quantitative trait loci in mice using heterogeneous stocks.** *Genetics* 2002, **160:**1609–1618.
28. Yalcin B, Flint J, Mott R: **Using progenitor strain information to identify quantitative trait nucleotides in outbred mice.** *Genetics* 2005, **171:**673–681.
29. Valdar W, Solberg LC, Gauguier D, Burnett S, Klenerman P, Cookson OW, Taylor MS, Rawlins JNP, Mott R, Flint J: **Genome-wide genetic association of complex traits in heterogeneous stock mice.** *Nat Genet* 2006, **38:**879–887.
30. Welsh CE, Miller DR, Manly KF, Wang J, McMillan L, Morahan G, Mott R, Iraqi FA, Threadgill DW, de Villena FP: **Status and access to the collaborative cross population.** *Mamm Genome* 2012, **23:**706–712.
31. Turri MG, DeFries JC, Henderson ND, Flint J: **Multivariate analysis of quantitative trait loci influencing variation in anxiety-related behavior in laboratory mice.** *Mamm Genome* 2004, **15:**69–76.
32. Turri MG, Datta SR, DeFries J, Henderson ND, Flint J: **QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice.** *Curr Biol* 2001, **11:**725–734.
33. Singer JB, Hill AE, Nadeau JH, Lander ES: **Mapping quantitative trait loci for anxiety in chromosome substitution strains of mice.** *Genetics* 2005, **169:**855–862.
34. Ponder CA, Kliethermes CL, Drew MR, Muller J, Das K, Risbrough VB, Crabbe JC, Gilliam TC, Palmer AA: **Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression.** *Genes Brain Behav* 2007, **6:**736–749.
35. Philip VM, Duvvuru S, Gomero B, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, *et al*: **High-throughput behavioral phenotyping in the expanded panel of BXD recombinant inbred strains.** *Genes Brain Behav* 2010, **9:**129–159.
36. Goodson M, Rust MB, Witke W, Bannerman D, Mott R, Ponting CP, Flint J: **Cofilin-1: a modulator of anxiety in mice.** *PLoS Genet* 2012, **8:**1–8.
37. Eisener-Dorman AF, Grabowski-Boase L, Steffy BM, Wiltshire T, Tarantino LM: **Quantitative trait locus and haplotype mapping in closely related inbred strains identifies a locus for open field behavior.** *Mamm Genome* 2010, **21:**231–246.
38. Smoller JW, Rosenbaum JF, Biederman J, Susswein LS, Kennedy J, Kagan J, Snidman N, Laird N, Tsuang MT, Faraone SV, *et al*: **Genetic association analysis of behavioral inhibition using candidate loci from mouse models.** *Am J Med Genet* 2001, **105:**226–235.
39. Lydiard RB: **The role of GABA in anxiety disorders.** *J Clin Psychiatry* 2003, **64**(Suppl 3):21–27.
40. Hettema JM, An SS, Neale MC, Bukszar J, van den Oord EJ, Kendler KS, Chen X: **Association between glutamic acid decarboxylase genes and anxiety disorders, major depression, and neuroticism.** *Mol Psychiatry* 2006, **11:**752–762.
41. Unschuld PG, Ising M, Specht M, Erhardt A, Ripke S, Heck A, Kloiber S, Straub V, Brueckl T, Muller-Myhsok B, *et al*: **Polymorphisms in the GAD2 gene-region are associated with susceptibility for unipolar depression and with a risk factor for anxiety disorders.** *Am J Med Genet B Neuropsychiatr Genet* 2009, **150B:**1100–1109.
42. Flint J, Valdar W, Shifman S, Mott R: **Strategies for mapping and cloning quantitative trait genes in rodents.** *Nat Rev Genet* 2005, **6:**271–286.
43. Talbot CJ, Nicod A, Cherny SS, Fulker DW, Collins AC, Flint J: **High-resolution mapping of quantitative trait loci in outbred mice.** *Nat Genet* 1999, **21:**305–308.

44. Yalcin B, Willis-Owen SA, Fullerton J, Meesaq A, Deacon RM, Rawlins JN, Copley RR, Morris AP, Flint J, Mott R: **Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice.** *Nat Genet* 2004, **36:**1197–1202.
45. Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, *et al*: **Regulation of T cell activation, anxiety, and male aggression by RGS2.** *Proc Natl Acad Sci U S A* 2000, **97:**12272–12277.
46. Smoller JW, Paulus MP, Fagerness JA, Purcell S, Yamaki LH, Hirshfeld-Becker D, Biederman J, Rosenbaum JF, Gelernter J, Stein MB: **Influence of RGS2 on anxiety-related temperament, personality, and brain function.** *Arch Gen Psychiatry* 2008, **65:**298–308.
47. Leygraf A, Hohoff C, Freitag C, Willis-Owen SA, Krakowitzky P, Fritze J, Franke P, Bandelow B, Fimmers R, Flint J, Deckert J: **Rgs 2 gene polymorphisms as modulators of anxiety in humans?** *J Neural Transm* 2006, **113:**1921–1925.
48. Koenen KC, Amstadter AB, Ruggiero KJ, Acierno R, Galea S, Kilpatrick DG, Gelernter J: **RGS2 and generalized anxiety disorder in an epidemiologic sample of hurricane-exposed adults.** *Depress Anxiety* 2009, **26:**309–315.
49. Hettema JM, Sun C, Chen X, Kendler KS: **Genetic association study between RGS2 and anxiety-related phenotypes.** *Psychiatr Genet* 2013, **23:**92.
50. Hettema JM, Neale MC, Myers JM, Prescott CA, Kendler KS: **A population-based twin study of the relationship between neuroticism and internalizing disorders.** *Am J Psychiatry* 2006, **163:**857–864.
51. Hettema JM, Webb BT, Guo AY, Zhao Z, Maher BS, Chen X, An SS, Sun C, Aggen SH, Kendler KS, *et al*: **Prioritization and association analysis of murine-derived candidate genes in anxiety-spectrum disorders.** *Biol Psychiatry* 2011, **70:**888–896.
52. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM: **Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.** *Cell* 1999, **98:**115–124.
53. Lin J, Handschin C, Spiegelman BM: **Metabolic control through the PGC-1 family of transcription coactivators.** *Cell Metab* 2005, **1:**361–370.
54. Bouayed J, Rammal H, Soulimani R: **Oxidative stress and anxiety: relationship and cellular pathways.** *Oxid Med Cell Longev* 2009, **2:**63–67.
55. Hovatta I, Juhila J, Donner J: **Oxidative stress in anxiety and comorbid disorders.** *Neurosci Res* 2010, **68:**261–275.
56. Parker CC, Sokoloff G, Cheng R, Palmer AA: **Genome-wide association for fear conditioning in an advanced intercross mouse line.** *Behav Genet* 2012, **42:**437–448.
57. Nelson EC, Agrawal A, Pergadia ML, Lynskey MT, Todorov AA, Wang JC, Todd RD, Martin NG, Heath AC, Goate AM, *et al*: **Association of childhood trauma exposure and GABRA2 polymorphisms with risk of posttraumatic stress disorder in adults.** *Mol Psychiatry* 2009, **14:**234–235.
58. Liberzon I, Taylor SF, Phan KL, Britton JC, Fig LM, Bueller JA, Koeppe RA, Zubieta JK: **Altered central micro-opioid receptor binding after psychological trauma.** *Biol Psychiatry* 2007, **61:**1030–1038.
59. Fernandes C, Paya-Cano JL, Sluyter F, D'Souza U, Plomin R, Schalkwyk LC: **Hippocampal gene expression profiling across eight mouse inbred strains: towards understanding the molecular basis for behaviour.** *Eur J Neurosci* 2004, **19:**2576–2582.
60. Hovatta I, Tennant RS, Helton R, Marr RA, Singer O, Redwine JM, Schadt EE, Ellison JA, Verma IM, Lockhart DJ, Barlow C: **Glyoxalase 1 and glutathione reductase regulate anxiety in mice.** *Nature* 2005, **438:**662–666.
61. Letwin NE, Kafkafi N, Benjamini Y, Mayo C, Frank BC, Luu T, Lee NH, Elmer GI: **Combined application of behavior genetics and microarray analysis to identify regional expression themes and gene-behavior associations.** *J Neurosci* 2006, **26:**5277–5287.
62. Donner J, Pirkola S, Silander K, Kananen L, Terwilliger JD, Lonnqvist J, Peltonen L, Hovatta I: **An association analysis of murine anxiety genes in humans implicates novel candidate genes for anxiety disorders.** *Biol Psychiatry* 2008, **64:**672–680.
63. Dodman NH, Karlsson EK, Moon-Fanelli A, Galdzicka M, Perloski M, Shuster L, Lindblad-Toh K, Ginns EI: **A canine chromosome 7 locus confers compulsive disorder susceptibility.** *Mol Psychiatry* 2010, **15:**8–10.
64. Williams R, Lim JE, Harr B, Wing C, Walters R, Distler MG, Teschke M, Wu C, Wiltshire T, Su AI, *et al*: **A common and unstable copy number variant is associated with differences in Glo1 expression and anxiety-like behavior.** *PLoS One* 2009, **4:**e4649.
65. Distler MG, Plant LD, Sokoloff G, Hawk AJ, Aneas I, Wuenschell GE, Termini J, Meredith SC, Nobrega MA, Palmer AA: **Glyoxalase 1 increases anxiety by reducing GABAA receptor agonist methylglyoxal.** *J Clin Invest* 2012, **122:**2306–2315.
66. Thornalley PJ: **The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life.** *Biochem J* 1990, **269:**1–11.
67. Mannervik B: **Molecular enzymology of the glyoxalase system.** *Drug Metabol Drug Interact* 2008, **23:**13–27.
68. Kromer SA, Kessler MS, Milfay D, Birg IN, Bunck M, Czibere L, Panhuysen M, Putz B, Deussing JM, Holsboer F, *et al*: **Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety.** *J Neurosci* 2005, **25:**4375–4384.
69. Szego EM, Janaky T, Szabo Z, Csorba A, Kompagne H, Muller G, Levay G, Simor A, Juhasz G, Kekesi KA: **A mouse model of anxiety molecularly characterized by altered protein networks in the brain proteome.** *Eur Neuropsychopharmacol* 2010, **20:**96–111.
70. Distler MG, Palmer AA: **Role of Glyoxalase 1 (Glo1) and methylglyoxal (MG) in behavior: recent advances and mechanistic insights.** *Front Genet* 2012, **3:**250.
71. Fujimoto M, Uchida S, Watanuki T, Wakabayashi Y, Otsuki K, Matsubara T, Suetsugi M, Funato H, Watanabe Y: **Reduced expression of glyoxalase-1 mRNA in mood disorder patients.** *Neurosci Lett* 2008, **438:**196–199.
72. Eser D, Uhr M, Leicht G, Asmus M, Langer A, Schule C, Baghai TC, Mulert C, Rupprecht R: **Glyoxalase-I mRNA expression and CCK-4 induced panic attacks.** *J Psychiatr Res* 2011, **45:**60–63.
73. Arai M, Yuzawa H, Nohara I, Ohnishi T, Obata N, Iwayama Y, Haga S, Toyota T, Ujike H, Ichikawa T, *et al*: **Enhanced carbonyl stress in a subpopulation of schizophrenia.** *Arch Gen Psychiatry* 2010, **67:**589–597.
74. Politi P, Minoretti P, Falcone C, Martinelli V, Emanuele E: **Association analysis of the functional Ala111Glu polymorphism of the glyoxalase I gene in panic disorder.** *Neurosci Lett* 2006, **396:**163–166.
75. Ditzen C, Varadarajulu J, Czibere L, Gonik M, Targosz BS, Hambsch B, Bettecken T, Kessler MS, Frank E, Bunck M, *et al*: **Proteomic-based genotyping in a mouse model of trait anxiety exposes disease-relevant pathways.** *Mol Psychiatry* 2010, **15:**702–711.
76. Carboni L, Piubelli C, Pozzato C, Astner H, Arban R, Righetti PG, Hamdan M, Domenici E: **Proteomic analysis of rat hippocampus after repeated psychosocial stress.** *Neuroscience* 2006, **137:**1237–1246.

doi:10.1186/2045-5380-3-9
Cite this article as: Sokolowska and Hovatta: **Anxiety genetics – findings from cross-species genome-wide approaches.** *Biology of Mood & Anxiety Disorders* 2013 **3**:9.