

# Genome-Wide Linkage Analysis of Multiple Measures of Neuroticism of 2 Large Cohorts From Australia and the Netherlands

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**Context:** People meeting diagnostic criteria for anxiety or depressive disorders tend to score high on the personality scale of neuroticism. Studying this personality dimension can give insights into the etiology of these important psychiatric disorders.

**Objectives:** To undertake a comprehensive genome-wide linkage study of neuroticism using large study samples that have been measured multiple times and to compare the results between countries for replication and across time within countries for consistency.

**Design:** Genome-wide linkage scan.

**Setting:** Twin individuals and their family members from Australia and the Netherlands.

**Participants:** Nineteen thousand six hundred thirty-five sibling pairs completed self-report questionnaires for neuroticism up to 5 times over a period of up to 22 years. Five thousand sixty-nine sibling pairs were genotyped with microsatellite markers.

**Methods:** Nonparametric linkage analyses were conducted in MERLIN-REGRESS for the mean neuroticism scores averaged across time. Additional analyses were con-

ducted for the time-specific measures of neuroticism from each country to investigate consistency of linkage results.

**Results:** Three chromosomal regions exceeded empirically derived thresholds for suggestive linkage using mean neuroticism scores: 10p 5 Kosambi cM (cM) (Dutch study sample), 14q 103 cM (Dutch study sample), and 18q 117 cM (combined Australian and Dutch study sample), but only 14q retained significance after correction for multiple testing. These regions all showed evidence for linkage in individual time-specific measures of neuroticism and 1 (18q) showed some evidence for replication between countries. Linkage intervals for these regions all overlap with regions identified in other studies of neuroticism or related traits and/or in studies of anxiety in mice.

**Conclusions:** Our results demonstrate the value of the availability of multiple measures over time and add to the optimism reported in recent reviews for replication of linkage regions for neuroticism. These regions are likely to harbor causal variants for neuroticism and its related psychiatric disorders and can inform prioritization of results from genome-wide association studies.

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**T**HE PERSONALITY TRAIT OF neuroticism is defined as a tendency to experience psychological distress. Individuals with high neuroticism scores are characterized by emotional instability, low self-esteem, and feelings of anxiety, depression, and guilt.<sup>1</sup> Neuroticism scores are found to be high in those with psychiatric disorders such as major depression and anxiety disorders<sup>2</sup> and this association appears to be reciprocal. Prospective studies demonstrate that neuroticism or neuroticism-like traits predict future major depression<sup>3-7</sup> and not merely because of overlap with prodromal symp-

toms of major depression. Self-report questionnaires can be used to score neuroticism as a quantitative trait measurable on large population cohorts.<sup>8,9</sup> Therefore, study of neuroticism in large populations is relatively easy and can give insights into the etiology of important psychiatric disorders.

Neuroticism scores have been found to be robust measures, with test-retest correlations of 0.79<sup>10</sup> to more than 0.90<sup>9,11</sup> for scores measured up to 2 years apart and approximately 0.60 for scores measured up to 6 years<sup>12</sup> or 19 years<sup>11</sup> apart. It is well established that neuroticism is partially under genetic control,<sup>13,14</sup> with heritability

estimates of 30% to 54%.<sup>8,12,15,16</sup> Twin studies have consistently shown no evidence for a shared common environmental component.<sup>12,15,17</sup> Genetic correlations between neuroticism scores taken over a 6-year period were higher than 0.88 for all age groups.<sup>12</sup> On average, women score higher for neuroticism than men, but heritability estimates are mostly consistent across sex.<sup>14-16</sup> However, opposite-sex sibling correlations<sup>16,17</sup> and mother-son correlations<sup>15</sup> have been reported as lower, suggesting that different genes may be important in men and women. Estimates of the genetic correlation between neuroticism and depression or anxiety range from 0.4 to 0.8.<sup>17-20</sup>

Four previous linkage studies of neuroticism have been published<sup>10,16,21,22</sup>; 3 of these studies used a single measure of neuroticism and 1 study<sup>10</sup> used an average of 2 measures taken 6 months apart. For 2 of the studies, the linkage analyses for neuroticism were secondary to the analyses of the ascertainment criteria of their study cohorts, namely alcohol<sup>22</sup> or nicotine<sup>21</sup> dependence. Recent reviews<sup>14,23</sup> summarized the linkage analysis results from the 3 earliest published of these studies and from an additional 14 studies of psychiatric disorders considered to be genetically related to neuroticism and concluded that some consistency is starting to emerge across studies.

Examples of genetic linkage analysis of longitudinal data on any trait in adults are rare,<sup>24</sup> despite recognition that use of multiple measures can increase power by reducing between-sib residual nonshared variance.<sup>25</sup> Consistency in linkage regions across repeated measures cannot be considered a replication, as this requires identification of the same linkage region in independent data sets. Nonetheless, inconsistency in linkage regions identified from repeated measures might indicate type I error and biological implausibility of the putative region.

In this study, we report a linkage analysis of neuroticism from 2 large study samples of twin families from Australia and the Netherlands. Individuals in the Australian study have been measured up to 4 times over a 22-year period and on different scales. Individuals in the Dutch sample have been measured up to 5 times over an 11-year period using the same scale. These data sets are independent between countries and therefore provide an opportunity to investigate replication of linkage results. Within countries, there are partly overlapping samples of participants at each measurement occasion, providing an opportunity to investigate consistency of linkage results.

## METHODS

### PARTICIPANTS AND MEASURES OF NEUROTICISM

#### Australian Study Sample

All participants were adult twins and they and their families were recruited through the Australian Twin Registry and were of North European ancestry. All provided written informed consent under study protocols approved by the Queensland Institute of Medical Research Human Research Ethics Committee. Participants completed 1 or more personality questionnaires: the 23-item Eysenck Personality Questionnaire-Revised (EPQ-R),<sup>26</sup> the shortened 12-item subset (EPQ-RS), or the NEO Five-

Factor Inventory personality questionnaire,<sup>27</sup> which includes 12 items in the neuroticism domain and, compared with the EPQ-R, probes angry hostility, self-consciousness, impulsiveness, and vulnerability as well as anxiety and depression. Each individual could have up to 4 measures of neuroticism measured at 4 different times; these (or their transformations, discussed later) are referred to as AU<sub>80</sub> (EPQ-R), AU<sub>89</sub> (EPQ-RS), AU<sub>99</sub> (EPQ-R), and AU<sub>02</sub> (NEO), with these trait codes reflecting the approximate year in which the scores were collected. The participants contributing AU<sub>80</sub>, AU<sub>89</sub>, and AU<sub>99</sub> measures are described in detail elsewhere.<sup>8</sup> Briefly, participants contributing AU<sub>80</sub> or AU<sub>89</sub> scores were ascertained solely on the status of being a twin registered through the Australian Twin Registry or, in the case of AU<sub>89</sub>, being a family member of a registered twin. The participants contributing AU<sub>99</sub> measures were ascertained as siblings pairs selected for discordance or concordance with respect to extreme neuroticism or anxiety or depression scores: one sibling in the top or bottom decile and the other sibling in the top or bottom quintile, excluding monozygotic twin pairs and allowing for selection of multiple siblings per family in an extreme discordant and concordant (EDAC) design.<sup>8,28</sup> The EDAC design identifies the sibling pairs who are most informative for genetic studies.<sup>29</sup> The participants in the 1999 study had the opportunity to complete the EPQ-R by telephone interview and/or by mail; approximately 80% completed both within 6 months, with a test-retest correlation of 0.9.<sup>8,11</sup> The 2 scores were averaged for analysis in this study. The long-term stability of the AU<sub>80</sub>, AU<sub>89</sub>, and AU<sub>99</sub> measures are reported in Birley et al<sup>11</sup> (in which the 1980, 1989, and 1999 studies are named Canberra, alcohol cohorts [where "alcohol" does not refer to any ascertainment criteria], and anxiety studies). The participants contributing AU<sub>02</sub> measures were ascertained as being extended twin families with a high incidence of smoking as part of an ongoing nicotine addiction genetics study.<sup>30</sup> Where possible, blood (or buccal) samples were obtained from the study participants and their parents.

#### Dutch Study Sample

Families with adolescent and adult twins have been assessed roughly every 2 years since 1991 as part of an ongoing longitudinal survey study of the Netherlands Twin Register. Participants are of Dutch ancestry<sup>31</sup> and were recruited under informed consent. Each survey, with the exception of the 1995 wave, collected information on personality and psychopathology<sup>31,32</sup> and was conducted under protocols approved by the ethics committee of the Free University Hospital, Amsterdam, the Netherlands. Consequently, each individual could have up to 5 measures of neuroticism measured at 5 different times; these (or their transformations, discussed later) are referred to as NL<sub>91</sub>, NL<sub>93</sub>, NL<sub>97</sub>, NL<sub>99</sub>, and NL<sub>02</sub>, with subscript codes reflecting the approximate year in which the scores were collected (corresponding to waves 1, 2, and 4-6 of data collection<sup>32</sup>). Neuroticism was measured using the Amsterdamse Biografische Vragenlijst,<sup>33</sup> a self-report questionnaire similar in content to the EPQ-R.<sup>34</sup> The neuroticism scale comprises 30 questions with a 3-item response scale (no, don't know, yes). The neuroticism score is a weighted sum of the item responses.

### NEUROTICISM SCORES

Neuroticism scores are sum scores and such data typically deviate from normality by having heavy tails. The averaged angular transformation<sup>35</sup> was used to normalize the distribution, as in other studies.<sup>8,11,16,36</sup> The neuroticism scores used in the analysis were residuals from regression of the transformed neuroticism scores on age, sex, and age × sex (and age<sup>2</sup> and age<sup>2</sup> × sex

**Table 1. Description of Data Sets<sup>a</sup>**

	AU <sup>b</sup>					NL <sup>b</sup>					AU and NL	
	AU <sub>80</sub>	AU <sub>89</sub>	AU <sub>99</sub>	AU <sub>02</sub>	Total	NL <sub>91</sub>	NL <sub>93</sub>	NL <sub>97</sub>	NL <sub>99</sub>	NL <sub>02</sub>		Total
<b>Phenotype Data Set</b>												
No. of families	2017	4253	938	576	4999	1661	2014	1283	1332	2069	3808	8807
No. of sib pairs	2033	10513	1709	1589	12772	997	1148	3220	2691	2553	6863	19635
Age, mean, y	33.4	35.1	43.1	40.2		17.7	19.2	26.6	30.9	32.8		
Sib correlations	0.18	0.14	0.27 <sup>c</sup>	0.10	0.14	0.25	0.28	0.16	0.20	0.17	0.20	0.16
Female-female	0.25	0.16	0.31 <sup>c</sup>	0.13	0.14	0.25	0.34	0.20	0.19	0.25	0.23	0.17
Male-male	0.18	0.13	0.30 <sup>c</sup>	0.15	0.18	0.27	0.34	0.07	0.18	0.13	0.17	0.18
Female-male	0.12	0.12	0.24 <sup>c</sup>	0.07	0.11	0.23	0.21	0.18	0.21	0.12	0.19	0.14
<b>Linkage Analysis Data Set</b>												
No. of families	1035	1634	802	306	1945	133	224	410	367	359	564	2509
No. of individuals with GS	3209	5523	2862	1378	6522	558	834	1533	1405	1376	2030	8552
No. of full-sib pairs <sup>d</sup>	1046	2988	1350	912	3870	133	226	825	678	613	1199	5069
No. of female-female	427	11191	512	325	1501	47	86	319	268	261	461	3542
No. of male-male	168	431	202	154	586	30	48	161	124	106	219	805
Proportion of families with												
2 Sibs	1.00	0.75	0.75	0.52	0.73	1.00	1.00	0.65	0.69	0.72	0.62	
3 Sibs	0	0.17	0.19	0.24	0.17	0	0	0.23	0.22	0.21	0.23	
≥ 4 Sibs	0	0.08	0.06	0.24	0.10	0	0	0.12	0.09	0.08	0.12	
LOD suggestive <sup>e</sup>	1.6	1.7	1.7	1.7	1.7	1.5	1.5	1.7	1.7	1.7	1.6	1.7
LOD significant <sup>e</sup>	2.9	3.0	3.0	3.5	3.1	2.7	2.8	3.1	3.4	3.3	3.1	3.1
ELOD20 <sup>f</sup>	1.4	8.0	2.3	1.3	11.9	0.4	0.9	2.0	1.7	1.6	10.0	20.9 <sup>g</sup>

Abbreviations: AU, Australian study sample; GS, genome scan data; LOD, logarithm of odds; NL, Dutch study sample.

<sup>a</sup>For inclusion in the phenotype data set, families needed to have at least 2 individuals with neuroticism scores and known age and only 1 of a monozygotic pair included; for inclusion in the linkage analysis data set, family size was limited to 2 to 5 siblings with neuroticism scores, known age, and genotype data on more than 280 markers.

<sup>b</sup>The subscript trait codes for the study samples reflect the approximate year in which the neuroticism scores were collected.

<sup>c</sup>The high full-sib correlation reflects the ascertainment for inclusion in the Australian 1999 study.

<sup>d</sup>Monozygotic pairs excluded; a small number of families only contributed half-sib pairs.

<sup>e</sup>Empirical suggestive and significant LOD score thresholds from 1000 gene-drop simulation genome scans.

<sup>f</sup>Expected LOD score of a quantitative trait locus that accounts for 20% of the phenotypic variance.

<sup>g</sup>ELOD20 for AU and NL ≠ ELOD20 for AU + ELOD20 for NL because of different heritabilities used.

**Table 2. Overlap in Data Sets Used in Linkage Analysis<sup>a</sup>**

	AU <sub>80</sub>	AU <sub>89</sub>	AU <sub>99</sub>	AU <sub>02</sub>	NL <sub>91</sub>	NL <sub>93</sub>	NL <sub>97</sub>	NL <sub>99</sub>	NL <sub>02</sub>
<b>AU<sub>80</sub></b>		0.82	0.17	0.06	<b>NL<sub>91</sub></b>	0.81	0.62	0.53	0.50
<b>AU<sub>89</sub></b>	0.28		0.43	0.12	<b>NL<sub>93</sub></b>	0.48	0.65	0.56	0.52
<b>AU<sub>99</sub></b>	0.13	0.97		0.04	<b>NL<sub>97</sub></b>	0.09	0.15	0.59	0.51
<b>AU<sub>02</sub></b>	0.06	0.33	0.06		<b>NL<sub>99</sub></b>	0.09	0.16	0.74	0.64
<b>Total AU</b>	0.26	0.76	0.34	0.26	<b>NL<sub>02</sub></b>	0.10	0.17	0.71	0.71
					<b>Total NL</b>	0.10	0.17	0.71	0.57

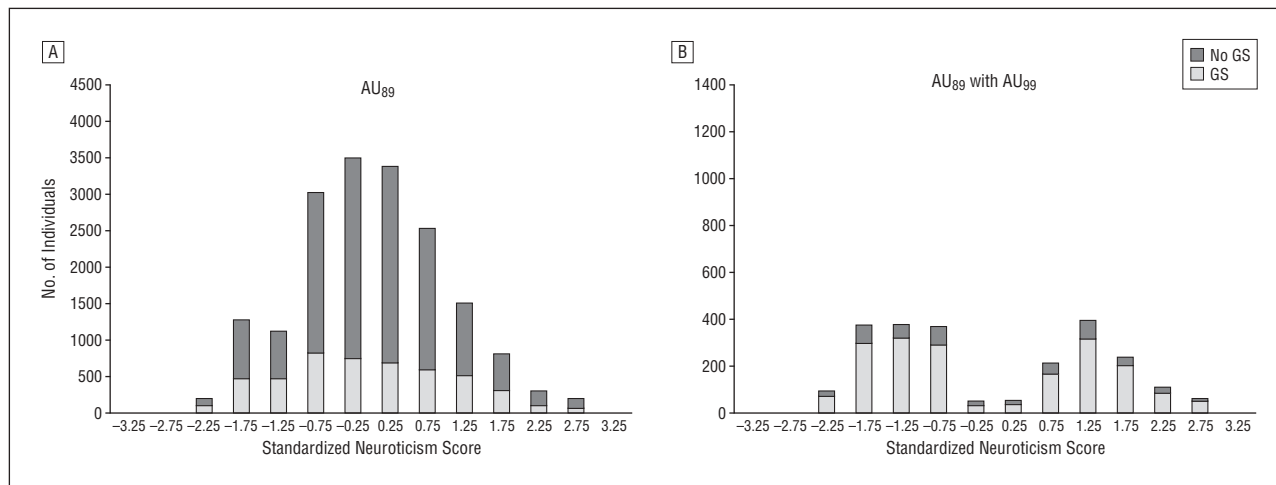
Abbreviations: AU, Australian study sample; NL, Dutch study sample.

<sup>a</sup>The proportion of full-sib pairs who have both the row measure of neuroticism and the column measure of neuroticism. The subscript trait codes for the study samples reflect the approximate year in which the neuroticism scores were collected.

for AU<sub>89</sub>), which were standardized separately for each sex. The mean AU<sub>89</sub> score of those selected for measurement in the AU<sub>99</sub> study sample was not significantly different from that of the full study group, but the variance was higher. Therefore, the AU<sub>99</sub> measures were standardized using the variance of the AU<sub>89</sub> cohort so that the higher variance of AU<sub>99</sub> measures was maintained. Finally, an average neuroticism score was calculated for each person within each country, denoted by AU and NL. The number of measures contributing to each average was recorded and used as a weight in the repeated-measures linkage analysis. Descriptions of the phenotype (all those measured) and genome scan (only those used in the linkage analysis) data sets are given in **Table 1** and **Table 2**.

## GENOTYPING

The genotypic data available for the Australian study sample resulted from submission of DNA samples to 1 or more of 6 genotyping centers: Gemini P/L, Sequana Therapeutics Inc, Leiden University Medical Centre, the Center for Mammalian Genetics Mammalian Genotyping Service of the Marshfield Clinic Research Foundation, the Australian Genome Research Facility, and the University of Helsinki Finnish Genome Center. Descriptions of the Gemini P/L, Leiden University Medical Centre, Mammalian Genotyping Service, and Sequana Therapeutics Inc genotyping and the subsequent merging and cleaning of the marker data sets are provided in detail elsewhere.<sup>37,38</sup> Since then, addi-



**Figure 1.** Distribution of standardized neuroticism scores in the Australian samples collected in 1989 (AU<sub>89</sub>): all participants (A) and those selected to have a measure collected in 1999 (B), for those in the genome scan (GS) and not in the GS.

tional Mammalian Genotyping Service, Australian Genome Research Facility, and Finnish Genome Center genotypes have been merged using the same protocol. Family members were submitted to the same genotyping facility. Participants with measure AU<sub>99</sub> were submitted preferentially for genotyping (Figure 1B), but this was not the sole criterion used to select families for genotyping, so the impact of the EDAC design was less marked for the AU<sub>89</sub> measure, which was available on the largest subset of samples (Figure 1A). Data cleaning based on mendelian errors, unlikely genotypes, and consistency of pedigree and marker relationships was undertaken as described by Cornes et al.<sup>37</sup>

Dutch samples were genotyped by the Mammalian Genotyping Service or Leiden University Medical Centre laboratories. The genotype data from these screens were combined. Allele calling and binning were equalized between markers that were present in multiple scans, using approximately 30 control samples. Data cleaning based on mendelian errors, unlikely genotypes, and consistency of pedigree and marker relationships was undertaken as described by Middeldorp et al.<sup>39</sup> The distributions of the neuroticism measures for those with and without genome scan data were similar.

Map positions of all genotyped markers were estimated in Kosambi cM (cM) by locally weighted linear regression (<http://www.qimr.edu.au/davidD>) from the NCBI build 35.1 physical map positions and published Decode and Marshfield genetic map positions.<sup>40</sup> Identical markers genotyped at different genotyping facilities were all included, separated by 0.001 cM on the genetic map. Using markers genotyped in common, the proportion of the total variance attributed to differences between the Australians and Dutch samples ( $F_{st}$ ) was estimated to be 0.30%, implying that these samples can be combined for joint genetic analysis.<sup>41</sup> Individuals were required to have genotypes on more than 280 markers resulting in an average distance of 8.2 cM (Australian sample) and 11.0 cM (Dutch sample) between genotyped markers of sib pairs. Thirty-eight percent (Australian sample) and 51% (Dutch sample) of parents were genotyped.

### PRELIMINARY ANALYSES

Phenotypic (test-retest) correlations between the EPQ measures AU<sub>80</sub>, AU<sub>89</sub>, and AU<sub>99</sub> ranged between 0.59 and 0.62.<sup>11,36</sup> Test-retest correlations of these measures with AU<sub>02</sub> were lower (range, 0.46-0.54),<sup>36</sup> reflecting the different emphasis of some of the items included in the NEO Personality Inventory neuroticism domain. The average phenotypic correlation between the Dutch measures was 0.65 (range, 0.56-0.77), with higher correlations be-

tween consecutive measures. The highest sib-pair correlations (estimated in Sib-Pair<sup>42</sup>) (Table 1) were for the youngest cohorts, NL<sub>91</sub> and NL<sub>93</sub>. The high sib correlation for AU<sub>99</sub> is a reflection of the EDAC selection. The lowest sib correlation was for AU<sub>02</sub>, scored on the NEO scale. Analyses of subsets of the Australian<sup>11,15</sup> and Dutch<sup>32</sup> data have consistently shown no evidence for the influence of common environmental effects. Genetic correlations were estimated in ASReml<sup>43</sup> and ranged between 0.91 and 0.95 between the EPQ measures (AU<sub>80</sub>, AU<sub>89</sub>, and AU<sub>99</sub>) and between 0.80 and 0.95 between these measures and the AU<sub>02</sub> NEO measure. Formal testing showed that the measures can be considered repeated measures of the same trait.<sup>36</sup> The genetic correlations between the 5 Dutch traits ranged from 0.84 to 0.95. Across all neuroticism measures, averaged estimates of heritability and phenotypic and genetic correlations were 0.32, 0.61, and 0.90, respectively. Preliminary linkage analyses conducted using a full multivariate model (not presented) suggested that there was little to be gained compared with the repeated-measures model with genetic correlations of this magnitude.

### LINKAGE ANALYSES

Genetic linkage analysis of the autosomes was conducted in MERLIN-REGRESS,<sup>44</sup> which regresses estimated identity by descent between relative pairs on the squared sums and squared differences of trait values of the pairs. Investigation of the properties of the method by simulation<sup>44</sup> showed it to be powerful and efficient even for selected samples (EDAC designs). It requires phenotypic measures to be standardized in the unselected population sample and uses the population parameters (mean, variance, heritability) derived from the full population sample rather than the selected or genotyped sample. The method is also appropriate for general pedigrees, including multiple sibs per family. However, simulation studies<sup>44</sup> showed that, although large sibships can increase power, the distribution of the test statistic can become distorted if the contributions from families become highly skewed. For this reason, sibships were limited to a maximum of 5, selecting sibs who maximized either the discordance or concordance of each family. Mean neuroticism scores were analyzed in MERLIN-REGRESS options *-mean 0 -var 1*, with heritabilities entered as twice the sib correlations (Table 1), and *-testretest*, with a correlation of 0.61. Analyses were repeated using mean measures from only males and only females because other studies have reported sex-specific linkage regions.<sup>16</sup> Analyses using scores of males or females only are denoted with subscripts in **Table 3**. Linkage analysis for the X chromosome was con-

**Table 3. Chromosomal Regions Where LOD Score Is Higher Than 1.5 for Dutch (NL), Australian (AU), and Combined Study Samples<sup>a</sup>**

Measure <sup>b</sup>	Chromosome	Position, cM <sup>c</sup>	LOD Score <sup>c</sup>	Linkage Interval, cM <sup>c</sup>	Linkage Interval, Cytogenetic Band	Single-Point Marker, LOD Score, Position <sup>d</sup>	LOD Score > 1.5 <sup>e</sup>	1.0 < LOD Score < 1.5 <sup>f</sup>	Region Identified by Fullerton <sup>14, g</sup>	Human Region-Homologous Mouse Linkage Region <sup>h</sup>	Primary Sources for Human Linkage Studies <sup>i</sup>
NL	6	75	1.5	59-111	6p21.2-6q21	D6S2410, 2.4, 75 cM	NL <sub>99</sub> <sup>j</sup> ; NL <sub>02</sub>	AU and NL		99-190 cM	
AU	8	134	1.6	125-145	8q24.12-8q24.21	D8S592, 1.7, 124 cM	AU <sub>89</sub> ; AU <sub>99</sub>	NL <sub>91</sub>		104-154 cM	
NL	10	5	2.0 <sup>j</sup>	0-29	10p15.3-10p14	D10S1412, 1.8, 25 cM	NL <sub>99</sub> <sup>j</sup> ; NL <sub>97</sub> <sup>j</sup> ; NL <sub>female</sub> <sup>j</sup> ; NL <sub>male</sub>				Neuroticism <sup>16, j</sup>
NL	14	103	2.6 <sup>j</sup>	94-118	14q32.12-14q32.31	D14S1434, 1.7, 104 cM	NL <sub>99</sub> <sup>j</sup> ; AU and NL <sup>j</sup>	NL <sub>02</sub>		76-134 cM	Anxiety, 105 cM <sup>39, j</sup> ; anxiety, 105 cM <sup>45</sup>
AU and NL	18	117	1.9 <sup>j</sup>	95-125	18q21.33-18qter	D18S61, 1.4, 99 cM	NL <sup>j</sup>	NL <sub>99</sub> ; NL <sub>02</sub> ; AU <sub>89</sub>	Reasonable support, 80 cM	85-109 cM	Neuroticism female, 91 cM <sup>22, k</sup> ; RE-MDD or anxiety, 73 cM <sup>46, j</sup> ; neuroticism female, 115 cM <sup>22</sup> ; harm avoidance, 109 cM <sup>47</sup> ; panic disorder, 104 cM <sup>48</sup>
<b>Sex Specific</b>											
AU <sub>male</sub> and NL <sub>male</sub>	2	112	1.6 <sup>j</sup>	94-118	2p13.2-2q11.2	D2S1790, 1.6, 111 cM	NL <sub>02</sub> <sup>j</sup>	AU <sub>male</sub> ; NL <sub>male</sub> ; AU <sub>89</sub>		102-151 cM	Suicide and RE-MDD, 99 cM <sup>50, k</sup>
NL <sub>male</sub>	5	191	2.2 <sup>j</sup>	185-199	5q35.1-5q35.2	D5S211, 1.4, 191 cM	NL <sub>99male</sub> <sup>j</sup> ; NL <sub>97male</sub>	NL <sub>02male</sub>			
AU <sub>male</sub>	8	45	1.6 <sup>j</sup>	34-53	8p22-8p21.1	D8S1771, 1.6, 49 cM		AU <sub>89male</sub>	Multiple studies, 50 cM		Harm avoidance, 60 cM <sup>49, k</sup> ; suicide and RE-MDD, 37 cM <sup>50</sup> ; anxiety, 21 cM <sup>45</sup> ; RE-MDD male pairs, 25 cM <sup>51</sup> ; neuroticism both sexes and male pairs <sup>14</sup>
NL <sub>male</sub>	10	175	1.7 <sup>j</sup>	164-175	10q26.3	D10S212, 1.1, 173 cM	NL <sub>99male</sub>			70-171 cM	
NL <sub>female</sub>	15	17	1.8 <sup>j</sup>	0-35	15p11.2-15q14	GTTTT001, 1.3, 24 cM	NL <sub>99female</sub> <sup>j</sup>	NL <sub>02female</sub>			

Abbreviations: cM, Kosambi centimorgan; LOD, logarithm of odds; RE-MDD, recurrent, early-onset major depression.

<sup>a</sup>Evidence for consistency of signal for individual measures within country and evidence for support from other studies. The subscript trait codes for the study samples reflect the approximate year in which the neuroticism scores were collected.

<sup>b</sup>Mean neuroticism measure with highest LOD score in region based on 5-cM grid search.

<sup>c</sup>Maximum LOD score from 5-cM grid search. Position and linkage interval (1 LOD less than the maximum LOD score) based on 1-cM grid search of identified regions; those marked with <sup>j</sup> are significant at the suggestive threshold for linkage.

<sup>d</sup>Marker within interval that shows largest single-point LOD score.

<sup>e</sup>Other measures within linkage interval boundaries with a LOD score higher than 1.5; those marked with <sup>j</sup> are significant at the suggestive threshold for linkage.

<sup>f</sup>Other individual measures with LOD scores higher than 1 but lower than 1.5 within linkage interval boundaries.

<sup>g</sup>Regions identified by Fullerton<sup>14</sup> as having support from multiple studies or having reasonable support for linkage from analyses of neuroticism, major depression, anxiety, and panic disorder as listed in the primary sources for results where linkage intervals overlap (or are likely to overlap if not presented).

<sup>h</sup>Regions identified by Smoller et al<sup>52</sup> as being homologous to linkage regions identified in studies of anxiety in mice.

<sup>i</sup>Linkage studies used in the review by Fullerton,<sup>14</sup> plus additional studies.<sup>22,39,45,48,50,54</sup>

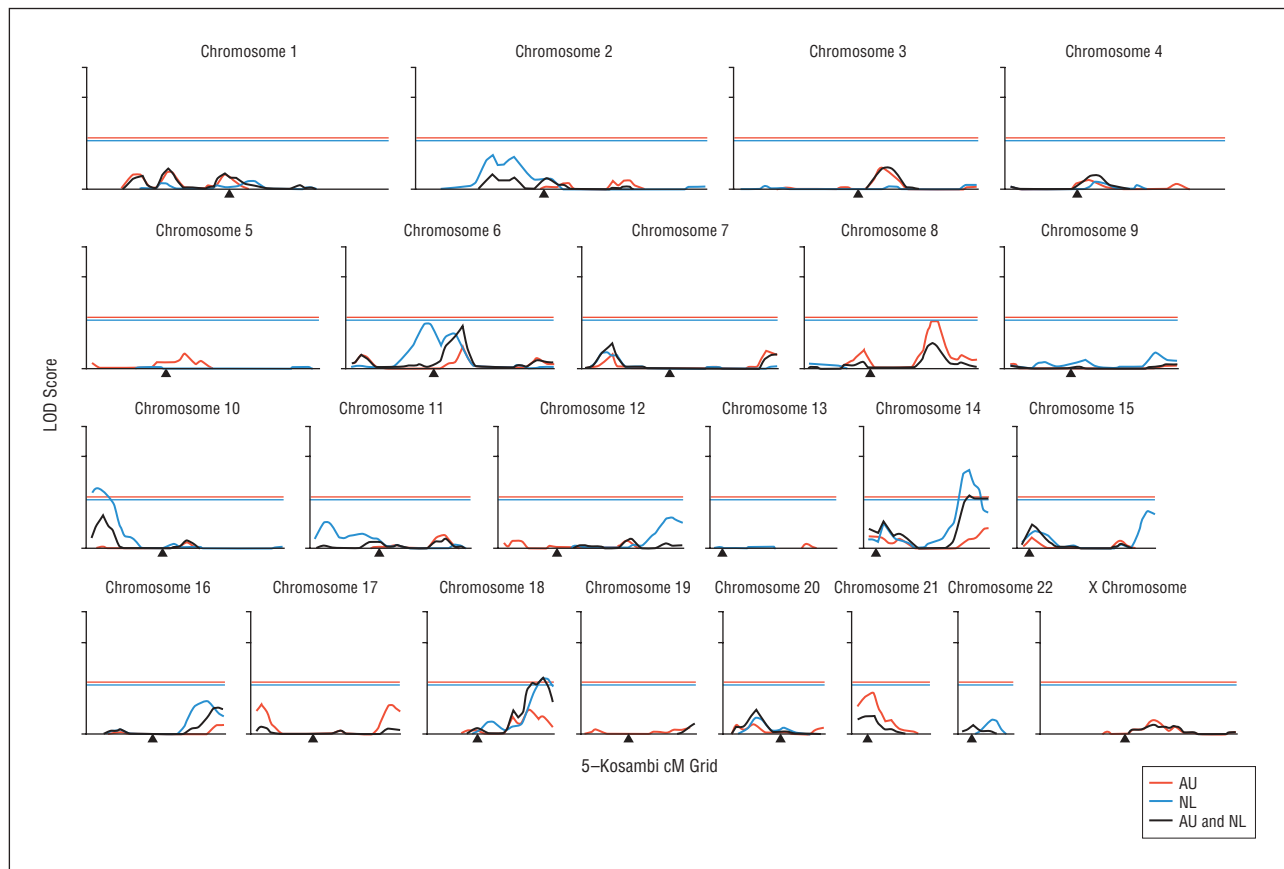
<sup>j</sup>Suggestive (these include estimated suggestive regions from the linkage study of Fullerton et al,<sup>16</sup> who discussed in detail only significant linkage results; from their Figure 2, we have estimated which additional peaks may have exceeded a suggestive threshold for linkage [ $\log P > 2.5$ ]).

<sup>k</sup>Significant.

ducted in MERLIN MINX. In all analyses, multipoint logarithm of odds (LOD) scores for the presence of a quantitative trait locus (QTL) were estimated every 5 cM (a 1-cM grid was used to determine linkage region confidence intervals, as the region bounded by 1 LOD score less than the maximum observed). Using the 5-cM grid allowed the linkage statistic to be collected over all families even when families were genotyped for different markers. Option *singlepoint* was used to identify the individual marker contributing most to regions showing evidence of linkage. Linkage analyses were repeated using individual measures of neuroti-

cism to allow examination of consistency in linkage signal between time-specific measures for each country.

Autosomal genome-wide empirical significance thresholds were derived from 1000 gene-drop simulations as implemented in MERLIN *simulate*, which uses the allele frequencies, marker positions, and missing genotype patterns of the real data set and simulates under a model that assumes random linkage between genotype and phenotypes. All phenotypes were analyzed using the same simulated data sets, which maintained the correlation structure between phenotypic measures. The maximum LOD



**Figure 2.** MERLIN-REGRESS linkage results of logarithm of odds (LOD) score (y-axis) for each chromosome (1-22 and X) based on a 5-Kosambi cM grid (x-axis) for mean neuroticism score of the Australian (AU), Dutch (NL), and combined AU and NL data sets. Empirical thresholds for suggestive linkage were 1.7 (red horizontal line) for AU and AU and NL combined and 1.9 (blue horizontal line) for NL.

scores from each chromosome of each simulation replicate were retained and were used to derive the empirical LOD score thresholds for Lander-Kruglyak<sup>53</sup> suggestive linkage (1 LOD score exceeding the threshold per genome scan) and significant linkage (1 LOD score exceeding the threshold per 20 genome scans) for each neuroticism measure analyzed and for the 9 mean measures of neuroticism simultaneously to derive thresholds that accounted for multiple testing.

Within MERLIN-REGRESS, option *-rankFamilies* gives an ELOD20 score for each phenotypic measure. The ELOD20 score is the LOD score expected given the data of a QTL that accounts for 20% of the phenotypic variance, assuming fully informative markers. Observed marker informativeness (I) was estimated as the average information content of the 5-cM estimates across the autosomes. The ELOD20 scores corrected for observed marker informativeness were calculated as  $ELOD20_{(I)} = ELOD20 \times I$ . Both  $ELOD20_{(I)}$  and  $ELOD10_{(I)}$  scores were used to calculate the power of our study samples given the phenotypic and genotypic information to detect a QTL that accounted for 20% and 10% of the total variance at the empirical significant<sup>53</sup> or suggestive<sup>53</sup> thresholds for linkage using the Probability Function Calculator of the Genetic Power Calculator,<sup>55</sup> where  $ELOD10_{(I)} = ELOD20_{(I)}/4$ .

## RESULTS

Empirically derived suggestive and significant LOD score thresholds for samples with each level of neuroticism are listed in Table 1. The lowest thresholds are for the samples composed predominantly of a single sib pair per family: AU<sub>80</sub>, NL<sub>91</sub>, and NL<sub>93</sub>. The empirical thresholds for sug-

gestive and significant linkage accounting for the multiple testing of the 9 mean measures of neuroticism were 2.5 and 4.1, respectively.

The means (SD) of the information content across the autosomes as calculated every 5 cM in MERLIN-REGRESS were 0.73 (0.08) (Australian samples) and 0.51 (0.10) (Dutch samples), the difference reflecting the average distance of genotyped markers between sib pairs. The ELOD20 scores are listed in Table 1. By accounting for the observed informativeness of the genotyped markers, we estimate that the Dutch, Australian, and combined study samples have 86%, 99%, and 100% power, respectively, to detect a QTL that accounts for 20% of the total variance at the significant threshold of linkage. These samples have powers of 9%, 27%, and 60% to detect a QTL that accounts for 10% of the total variance at the significant thresholds and 37%, 65%, and 89% at the suggestive thresholds. The power of sex-specific analyses is much lower, as expected from the number of same-sex sib pairs contributing to the analysis. The sex-specific Dutch, Australian, and combined study sample measures have for females 64%, 86%, and 99% and for males 24%, 40%, and 69% power to detect a QTL that accounts for 20% of the total variance at the suggestive threshold of linkage.

The genome-wide linkage plot for the Australian and Dutch samples and the joint analysis of the Australian and Dutch samples (**Figure 2**) show 3 regions that exceed the empirical threshold for suggestive linkage for their respec-

tive measures: 18q 117 cM for the combined Australian and Dutch sample, 14q 103 cM for the Dutch sample, and 10p 5 cM for the Dutch sample. An additional 2 regions just fail to reach the this threshold: 8q 134 cM for the Australian sample and 6cen 75 cM for the Dutch sample. The chromosomal position with the maximum LOD score based on a 1-cM grid scan, linkage intervals, corresponding cytogenetic band, and the marker with the maximum LOD score within the region based on a single-point analysis are listed in Table 3. To investigate these results, we looked for consistency in linkage signal in time-specific measures of neuroticism within the linkage intervals and found that for all 5 regions, at least 2 individual measures achieved a LOD score higher than 1 (Table 3). In contrast, an additional 10 chromosomal regions (NL<sub>97</sub>, 14q 22 cM; AU<sub>80</sub>, 16p 56 cM; AU<sub>02</sub>, 3q 14 cM; AU<sub>80</sub>, 6q 178 cM; NL<sub>02</sub>, 9q 149 cM; NL<sub>97</sub>, 16q 124 cM; AU<sub>99</sub>, 17p 14 cM; NL<sub>93</sub>, 18q 60 cM; AU<sub>80</sub>, 19q 104 cM; and AU<sub>89</sub>, 21p 21 cM) achieved LOD scores that exceeded the empirical suggestive threshold for significance for an individual measure of neuroticism, but only the first 2 listed achieved a LOD score higher than 1 for any other individual measure within the 1-LOD score drop confidence interval. Within country, some data sets were longitudinal (eg, >90% of those included in the AU<sub>80</sub> or AU<sub>99</sub> analyses were also included in the AU<sub>89</sub> analysis [Table 2]), while some data sets were largely independent (eg, <10% of participants with AU<sub>80</sub> or AU<sub>99</sub> scores were also scored for AU<sub>02</sub> [Table 2]). The most extreme example of inconsistency was for AU<sub>89</sub>, which achieved a LOD score of 2.7 for 21p 21 cM, yet no evidence for linkage was found with the AU<sub>99</sub> measure (maximum LOD score within the region of 0.1), a difference that persisted when the analysis was limited to include only phenotypes of individuals who were measured in both studies. Examination of the sib-pair phenotypic scores and the identity by descent of marker alleles in families who contributed most to these linkage signals showed nothing that could not reasonably be attributed to stochastic variation.

Five regions that exceeded empirical suggestive thresholds of linkage are reported for analyses of single-sex average neuroticism scores (Table 3). For the linkage interval of these regions, analyses were conducted for the relevant sex for the 9 time-specific data sets. Of the 5 regions, 3 (2p, 5q, and 15cen) were supported by more than 1 sex-specific individual measure, with a LOD score higher than 1.0 (Table 3). Of the 8 regions listed in Table 3 that exceeded the empirical suggestive threshold for significance, only the 14q region exceeded the threshold that accounts for multiple testing of the 9 mean measures.

#### COMMENT

We performed a linkage analysis for neuroticism using 2 large independent study samples of North European descent. In total, 5069 sibling pairs contributed to the linkage analysis, which used mean neuroticism scores from both Australia and the Netherlands to maximize sample size and power. Linkage analyses of the mean neuroticism score for each country separately allowed us to look for replication between independent data sets. The mean neuroticism measure of each participant could comprise between 1 and 5

individual measures and we used individual neuroticism scores to look for consistency of linkage results. Although individuals with more than 1 measure of neuroticism age over time, the high genetic correlations between measures would not lead us to expect different genetic variants to be identified in the linkage analysis of different measures. Using mean neuroticism score, we identified 5 regions where the LOD score was higher than 1.5; for 3 of these, the LOD score exceeded the empirical threshold for significance. All 5 regions showed some consistency in linkage scores for individual time-specific measures within country, and 2 regions (8q 134 cM and 18q 117 cM) showed some evidence for replication between countries. Other studies that have reported linkage to these regions are listed in Table 3; we include studies reviewed by Fullerton<sup>14</sup> plus a small number of additional, mostly subsequent, publications. Region 18q 117 cM overlaps the linkage intervals reported by 3 other studies: recurrent early-onset and major depression,<sup>46</sup> 73 cM; neuroticism in females,<sup>22</sup> 91 cM and 115 cM; and harm avoidance,<sup>47</sup> 109 cM. Region 14q 103 cM has previously been identified in a linkage analysis of the Dutch study samples for a broad anxiety phenotype<sup>39</sup> but also in an independent study of extended families with a high occurrence of anxiety disorders.<sup>45</sup> Region 10p 5 cM was estimated from the linkage graph for EPQ neuroticism presented by Fullerton et al<sup>16</sup> to have exceeded the level of suggestive linkage. Only the confidence interval of the 18q region overlapped with a region considered to have "reasonable support for linkage" by Fullerton<sup>14</sup> (10 regions were identified, representing about 9% of the genome). Also listed in Table 3 are human chromosomal regions homologous to linkage regions from studies of anxiety in mice as summarized by Smoller et al<sup>52</sup>; 11 homologous human chromosomal regions were identified, which totaled about 17% of the human genome. Linkage studies in mice are relevant because similar brain processes are likely to exist for anxiety in mice and neuroticism in humans<sup>13</sup> and the powerful design of studies that are possible in mice can lead to highly significant linkage regions bounded by tight confidence intervals. Of the 5 regions, we identified (Table 3) 4 that overlapped with regions identified by Smoller et al,<sup>52</sup> an overlap that exceeds chance expectations (binomial  $P = .003$ ).

Five sex-specific linkage regions exceeded thresholds of suggestive linkage (Table 3), of which 2cen 112 cM (males) showed evidence for replication between countries and 5q 191 cM (males) showed evidence for consistency between the Dutch time-specific measures. Of these, region 8p has previously been identified in other linkage studies, including 2 male-specific reports (Table 3), and linkage with suicide and recurrent early major depression has been reported for 2p.<sup>50</sup> Two of the 5 sex-specific regions overlapped with homologous regions identified by Smoller et al<sup>52</sup> from mouse linkage studies. Analyses of male and female mean scores separately had much reduced power compared with the joint-sex analyses, particularly the male-specific analyses, and so we place less emphasis on the sex-specific results.

For a study of its kind, our sample size is large (**Table 4**), yet the number of linkage regions that we identified for the Dutch, Australian, and combined study samples were 3, 0, and 2 respectively, not very different from the 1 per link-

**Table 4. Summary of Linkage Studies of Neuroticism Ordered by Number of Sib Pairs Measured for Neuroticism**

Country, Study	Source	Neuroticism Measure	No. of Measures If > 1	Base Population	No. of Sib Pairs Measured	Criterion for Inclusion in Linkage Analysis	No. of Sib Pairs in Linkage Analysis/ Intermarker Distance	Analysis	No. of Linkage Peaks Identified			
									SIG	SIGsex	SUG	SUGsex
England	Fullerton et al <sup>16</sup>	EPQ-R <sup>26</sup>		Community-based sample	34 580	Used neuroticism score to select 2.5% most discordant and 2.5% most concordant, 78% response rate <sup>a</sup>	629/10 cM <sup>b</sup>	Visscher-Hopper <sup>56</sup> regression	2	3	8 <sup>c</sup>	3 <sup>c</sup>
Australia and the Netherlands	Current study				19 635		5424	MERLIN-REGRESS <sup>44</sup>	0	0	2	1
Australia	Current study	EPQ-R; EPQ-RS; NEO <sup>27</sup>	Mean score of up to 4 measures over 22 y	Twin individuals and their families and large families with high incidence of smokers	Twin individuals: 11 665; large families: 1107 independent	Twin individuals: although based on an EDAC design in which the neuroticism score was used to select 1 sib in top/bottom 10% and the other sib in top/bottom 20% (91% response rate, <sup>8,43</sup> ), the selective genotyping overall was weak (Figure 1); large families: none	3364 and 702/8 cM <sup>d</sup>	MERLIN-REGRESS	0	0	0	1
The Netherlands, NETSAD	Current study	ABV <sup>33</sup>	Mean score of up to 5 measures over 11 y	Twin individuals and their families	6863	None; no difference in distribution of neuroticism scores for those with and without genotypes	1358/11 cM <sup>d</sup>	MERLIN-REGRESS	0	0	3	3
England, GENESIS	Nash et al <sup>10</sup>	EPQ-RS	Mean score of up to 2 (78% with 2 measures) 6 mo apart	Community-based sample	4824	Most informative 10%; 65% response rate <sup>a</sup>	297/9 cM	MERLIN-REGRESS	0	1	0 <sup>e</sup>	2
Ireland and Northern Ireland	Kuo et al <sup>22</sup>	EPQ-RS		Sibships concordant for alcohol dependence	714	None	714/4 cM	MERLIN-REGRESS	1	4	2 <sup>f</sup>	4 <sup>f</sup>
New Zealand	Neale et al <sup>21</sup>	EPQ-RS		Sibships concordant for nicotine dependence	201	None	201/10 cM <sup>b</sup>	MERLIN-REGRESS	0	NA	5	NA

Abbreviations: ABV, Amsterdamse Biografische Vragenlijst; EDAC, extreme discordant and concordant; EPQ-R, 23-item Eysenck Personality Questionnaire-Revised; EPQ-RS, shortened 12-item subset of EPQ-R; GENESIS, Genetic and Environmental Nature of Emotional States in Siblings; NA, not applicable; NEO, NEO Five-Factor Inventory personality questionnaire; NETSAD, Netherlands Twin Family Study of Anxious Depression; SIG, significant linkage; SIGsex, sex-specific linkage peak; SUG, suggestive linkage; SUGsex, sex-specific linkage peak.

<sup>a</sup> Response rates are for individuals and reflect the success of the selection criterion for entry into the linkage study; response rate for sib pairs are expected to be the square of this number.

<sup>b</sup> Estimated from number of markers.

<sup>c</sup> Estimated from Figure 2 in Fullerton et al.<sup>16</sup> assuming a threshold of  $-\log P = 2.5$ .

<sup>d</sup> Calculated as the average distance between markers genotyped in both members of sib pairs, which is likely to be higher than the average distance between markers reported for the other studies.

<sup>e</sup> One suggestive region identified but listed as significant sex-specific region.

<sup>f</sup> Estimated as those with a logarithm of the odds score greater than 1 (as empirical threshold for SIG was 1.29).

age scan expected by chance. Of the other linkage studies for neuroticism (Table 4), only the study of Fullerton et al<sup>16</sup> had more power to detect a QTL. Based on observed phenotypic and marker information, we had 100% (or 89%) power in the combined Australian and Dutch sample to detect a QTL that accounts for 20% (or 10%) of the total vari-

ance at the suggestive<sup>33</sup> threshold for linkage. For a trait with a heritability of 30%, these are perhaps optimistic power calculations; nonetheless, the next largest neuroticism linkage study<sup>10</sup> to date, assuming fully informative marker information, reports only 72% power for a QTL that accounts for 20% of the variance. Studies likely to have much



less power to detect a QTL have identified more suggestive and significant linkage regions (Table 4). Theoretically, sample sizes of more than 50 sib pairs should not result in a biased number of linkage statistics exceeding suggestive or significant linkage thresholds under the null hypothesis.<sup>53</sup> Although under the alternate hypothesis (when a QTL does exist), an inverse correlation between sample size and LOD score is expected.<sup>57</sup> One conclusion is that there simply are no variants that explain 10% or more of the genetic variance. When do our suggestive linkage peaks represent false positives and when does their low significance reflect variants of a smaller effect size? It is not possible to answer this question, but by considering multiple measures of neuroticism, we reduce the impact of the environmental noise surrounding chance extreme concordance or discordance of measures and therefore reduce one cause of the occurrence of false-positive linkage signals. The examination of linkage analyses from the individual measures of neuroticism provides some evidence for the robustness of our results using mean score.

Limitations of our study include different measures of neuroticism, both between countries and within the Australian sample. The Dutch participants came from a younger cohort than the Australian participants. A recent study has suggested that subtle differences in the EPQ-R and NEO neuroticism instruments may be important for genetic studies.<sup>58</sup> However, the high genetic correlations between different measures suggest the different measurement instruments are probing the same underlying trait, at least within country. As in other studies, we have undertaken some multiple testing (sex-dependent analyses, both between and within countries) that has not been accounted for in the empirical thresholds derived for each mean measure. The empirical threshold (LOD score, 2.5) derived to account for the multiple testing of the 9 mean neuroticism measures (including sex-specific means) is exceeded only for region 14q 103 cM. Nonetheless, the robustness of our results as measured by consistency in linkage score between time-specific measures, combined with the high rate of overlap with regions reported in other studies, adds to the optimism reported in recent reviews<sup>14,46</sup> for replication of linkage regions, even though the true effect sizes of underlying variants are unlikely to be large. A recent genome-wide association study of neuroticism using DNA pooling<sup>59</sup> failed to identify any loci that explained more than 1% of the variance. It is unlikely that the consensus in linkage signals across studies is driven by single variants of such a small magnitude but more likely implies allelic heterogeneity of causal variants within functionally important genes. Consistently identified regions from linkage analyses will be important in prioritizing results from genome-wide association studies. Time will tell if genome-wide association studies result in the identification of causal variants that account for most observed genetic variance. International collaborations compiling large family-based study samples for linkage analysis may well be necessary for identification of genes that contain multiple but rare causal variants.

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