A number of studies have suggested DNA sequence variability in the serotonin transporter gene (SLC6A4) between European-American (EA) and African-American (AA) populations, which could be clinically important, given the central role SLC6A4 has in serotonin transmission. However, these studies have had relatively small samples, used self-reported measures of race, and have only tested the promoter-linked polymorphism 5-HTTLPR. Here we genotype 5-HTTLPR and rs25531, a neighboring functional polymorphism, in 954 AA and 2622EA subjects from a National Institute of Mental Health repository sample. Genotyping was performed using fragment analysis by capillary electrophoresis. AA, as compared with EA, groups had lower frequencies of the S allele (0.25 vs 0.43) and SS genotype (0.06 vs 0.19) at 5-HTTLPR, and higher rates of the G allele at rs25531 (0.21 vs 0.079). A rare XL variant at 5-HTTLPR was also more common among AAs (0.017 vs 0.008). When the polymorphisms were redefined into a high- and low-transcription haplotypes, the AA group showed significantly fewer low-transcription variants ($\chi^2 = 4.8, P = 0.03$). No genotypes were associated with major depression, any anxiety disorder, or neuroticism in either EA or AA populations. This is the largest study to show SLC6A4 genotype differences between EA and AA populations, and the first to include rs25531. Lack of associations with clinical outcomes may reflect untested moderating environmental influences.

Keywords: African-American; 5-HTTLPR; major depression; rs25531; serotonin transporter
may moderate effects of genetic risks and are sometimes necessary for the development of the clinical phenotype of interest.11,12 Although the MGS sample has been widely used in genetics, including GWAS studies,13–15 this is the first study to our knowledge to directly genotype 5-HTTLPR in either EA or AA populations of the sample. All genotypes and results from this study will be made available for use by qualified investigators through the NIH Center for Genetic studies (https://www.nimhgenetics.org).

MATERIALS AND METHODS

Study population
All subjects were part of the MGS sample of the NIH Center for Collaborative Genomic Studies on Mental Disorders (https://www.nimhgenetics.org/). DNA was stored at, and provided by, the Rutgers University Cell and DNA repository (RUCDR) (www.rucdr.org; rucdr.rutgers.edu). The sample has been detailed previously.16

Assessment
As detailed elsewhere,9 all EA and 41% of AA subjects were recruited by Knowledge Networks, Inc. (Menlo Park, CA, USA) (KN) from a survey research panel that had been recruited using nationwide sampling (random digit dialing), whereas 59% of AA subjects were recruited by a subcontracting company (Survey Sampling International, Shelton, CT, USA) using banner ads on websites. Subjects were assessed using an on-line self-report version of the Composite International Diagnostic Interview-Short Form (CIDI-SF). Derived from the longer Composite International Diagnostic Interview (CIDI), the CIDI-SF is a structured set of scales assessing eight syndromes: major depressive episodes, panic attacks, agoraphobia, social phobia, specific phobia, generalized anxiety disorder, obsessive compulsive disorder, and alcohol and drug dependence.1 We examined major depression and any anxiety disorder (which included generalized anxiety disorder, obsessive compulsive disorder, panic attacks, social anxiety disorder and specific phobia). Questions were assessed on a lifetime basis, with subjects asked to refer to periods of peak symptoms. Initial gate questions were used to skip out subjects least likely to be cases; subjects who were not skipped out then completed remaining questions, and the total number of endorsed symptoms was then dichotomized into categorical diagnoses based on the CIDI-SF algorithms for cases.14 Total classification accuracy, that is, the number of respondents with concordant results on the CIDI and CIDI-SF was 93% for major depression, and above classification accuracy, that is, the number of respondents with concordant and the total number of endorsed symptoms was then dichotomized into.

Initial gate questions were used to skip out subjects least likely to be cases; 45.3 versus 50.2 years, $t = -9.9$, $P < 0.0001$ and included a greater proportion of females (61% vs 52%, $\chi^2 = 25.1$, $P < 0.0001$). AA subjects were also more likely to have some post-high school education (81% vs 64%, $\chi^2 = 103.1$, $P < 0.0001$) and to have never been married (28% vs 15%, $\chi^2 = 42.7$, $P < 0.0001$), differences that were likely because of the inclusion in the AA sample of individuals recruited through internet banner ads rather than using survey methods.

Classification of race
Ancestry of each AA subject was validated using the first principal component score (PC1) that was computed from genome-wide association study data and that reflected a geographical ancestry gradient from north (Europe) to south (Africa).13

Genotyping
DNA was extracted at RUCDR from Epstein-Barr virus-transformed lymphoblastoid cell lines or from fresh blood samples as described elsewhere.15 The 5-HTTLPR and rs25531 polymorphisms of the SLC6A4 gene were genotyped in the two sets of samples. The promoter region of the SLC6A4 gene was amplified in the EA sample using a 10 µl of PCR reagent mixture with a forward primer set of 0.25 µM VIC-labeled and 0.25 µM unlabeled, and 0.50 µM reverse primer and 250 µM dNTP on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR primers sequence and reaction conditions have been previously described.22 Twenty nanograms of DNA was used as a template. Resulting fragment was resolved in a 2.3% UltraPure Agarose (Invitrogen, Carlsbad, CA, USA) and visualized under the UV transilluminator. Here, 526 bp and 478 bp fragments were called as a L and a S allele at 5-HTTLPR, respectively. For the A/G SNP rs25531, the PCR amplicon was digested with restriction endonuclease MspI (New England Biolabs Inc., Boston, MA, USA). The resulting product was analyzed using an ABI3100 DNA Analyzer and the Peak Scan Software v1.0 (Applied Biosystems). Digested fragments with a size of 164–165 bp were determined as a G allele and indigested as an A allele. The AA sample was genotyped using a modified protocol. PCR amplification was performed with 0.50 µM VIC-labeled forward primer and 0.50 µM reverse primer and was digested with restriction endonuclease MspI (New England Biolabs Inc.). The resulting product was mixed with the same amount of the PCR amplicon and analyzed using an ABI3100 DNA Analyzer (Applied Biosystems) and Peak Scan Software v1.0.

Table 1. Genotype and allele frequencies of the 5-HTTLPR and rs25531 polymorphisms in EA and AA populations

| Populations | Genotype frequency | S-HTTLPR | rs25531 |
|-------------|--------------------|----------|---------|
|             |                    | ‘L/L’    | ‘L/S’   |
| EA          | 868 (33.1%)        | 1240 (47.39%) | 1 (0.04%) |
| AA          | 504 (52.8%)        | 358 (37.53%) | 27 (2.86%) |
|             |                    | ‘L/xL’   | ‘S/S’   |
| EA          | 510 (19.45%)       | 60 (6.29%) | 5 (0.52%) |
| AA          | 868 (33.1%)        | 371 (14.2%) | 12 (0.5%) |
|             |                    | ‘S/L/S’  | ‘xL/S’  |
| EA          | 35 (0.51%)         | 35 (0.51%) |
| AA          | 504 (52.8%)        | 329 (34.5%) | 35 (3.7%) |

Abbreviations: AA, African American; EA, European American; L, long allele; S, short allele.
RESULTS

Genotypes at 5-HTTLPR and rs25531

Genotype and allele frequencies in the full samples did not differ significantly from those predicted by Hardy–Weinberg equilibrium. The genotypic and allelic distributions for 5-HTTLPR and rs25531 in both EA and AA populations are shown in Table 1 and demonstrate significant differences across populations. Specifically, the frequency of the S allele at 5-HTTLPR was 25% in AA vs 43% in EA subjects, and frequency of the G allele at rs25531 was 7.5% in AA vs 21% in EA subjects. The less common XL allele frequency was 1.65% in AA vs 0.08% in EA. These frequency differences were all statistically significant. In AA subjects, 15-repeat, 18-repeat and 19-repeat alleles at 5-HTTLPR were identified in 1, 1 and 2 subjects, respectively, who were excluded from further analyses.

Haplotype analysis

Haplotype analyses for 5-HTTLPR and rs25531 were performed. EA and AA subjects were significantly different in their frequencies of L-G and S-A, but not L-A and S-G. These differences were all statistically significant. In AA subjects, 15-repeat, 18-repeat and 19-repeat alleles at 5-HTTLPR were identified in 1, 1 and 2 subjects, respectively, who were excluded from further analyses.

Table 2. Haplotype frequencies of the 5-HTTLPR and rs25531 polymorphisms

| S-HTTLPR/rs25531 haplotype | EA  | AA  | Test of difference | EA  | AA  | Test of difference |
|----------------------------|-----|-----|--------------------|-----|-----|--------------------|
| L-A                        | 0.50| 0.52| $\chi^2 = 115.32$, $df = 5$, $P = 0.0001$ | 0.50| 0.53| $\chi^2 = 4.7838$, $df = 1$, $P = 0.03$ |
| xL-G                       | 0.0006| 0.01|                               |     |     |                    |
| L-G                        | 0.07| 0.21|                               |     |     |                    |
| S-G                        | 0.43| 0.25|                               |     |     |                    |
| S-A                        | 0.005| 0.001|                        |     |     |                    |
| xL-A                       | 0.0004| 0.002|                        |     |     |                    |

Abbreviations: AA, African American; EA, European American; L, long allele; S, short allele.

*Not bold rows indicate a high-transcription group and bold rows display a low-transcription group.

Association of 5-HTTLPR and rs25531 with psychiatric outcomes

Finally, we examined whether 5-HTTLPR and rs25531 alleles and haplotypes were associated with presence/absence of major depression or of anxiety disorders, or with differences in clinical outcomes (specifically, major depression, anxiety disorders, and neuroticism scores), in EA or AA subjects separately, and, if so, whether the genetic differences predicted the rates of these disorders in the populations.

In the AA sample (Table 3a), 266 subjects met lifetime criteria for major depression and 485 for any anxiety disorder. Clinical outcome was not associated with any genotype or haplotype. PC1 scores of AA subjects were not associated with clinical outcomes or with S or G alleles. In EAs, 641 and 1069 met lifetime criteria for major depression and anxiety disorder, respectively (Table 3b). No significant association was found between clinical outcomes and either genotypes or haplotypes.

DISCUSSION

We report the largest published study of an AA sample to date examining functional 5-HTTLPR and rs25531 polymorphisms within the promoter region of the serotonin transporter gene. Our study shows that, compared with EA subjects, AA subjects have significantly lower rates of the S allele (0.25 vs 0.43) and its homozygous genotype SS (0.06 vs 0.19) at 5-HTTLPR. These rates are consistent with earlier studies. Lotrich et al.3 found the S allele frequency to be 0.25 in 54 AA subjects vs 0.47 in 16000 controls from 46 studies, showing a modest effect (odds ratio = 1.076, $P = 0.001$) consistent with a recessive model, but with many indications of possible methodological problems that might account for the results. Another hypothesis is that the S allele increases risk of depression only through an interaction with life stress.11 One recent meta-analysis failed to confirm this effect, whereas a
| Groups                   | 5-HTTLPR genotype frequency | rs25531 genotype frequency |
|--------------------------|-----------------------------|-----------------------------|
| AA                       | 'L/L'     | 'L/S'   | 'L/L'   | 'S/S'   | 'xL/S'   | SS vs other* (95% CI) | 'A/A'   | 'A/G'   | 'G/G'   | OR^ (95% CI) |
| Major Depression         |                        |                         |         |         |           | 0.987 (0.549–1.773)  |         |         |         |            |
| NO (668)                 | 351 (52.54%) | 253 (37.87%) | 19 (2.84%) | 43 (6.44%) | 2 (0.30%) | 0.97 | 408 (61.08%) | 235 (35.18%) | 25 (3.74%) | 0.87 (0.654–1.167) |
| YES (286)                | 153 (53.50%) | 105 (36.71%) | 8 (2.80%)  | 17 (5.94%)  | 3 (1.05%)  |         | 182 (63.64%) | 94 (32.87%)  | 10 (3.50%)  | P = 0.36 |
| Any anxiety disorder     |                        |                         |         |         |           | 0.87 (0.654–1.167)  |         |         |         |            |
| NO (485)                 | 250 (51.55%) | 187 (38.56%) | 12 (2.47%) | 33 (6.78%)  | 3 (0.62%)  | 0.883 (0.520–1.499) | 299 (61.65%) | 164 (33.81%) | 22 (4.54%)  | 1.033 (0.794–1.344) |
| YES (469)                | 254 (54.16%) | 171 (36.46%) | 15 (3.20%) | 27 (5.76%)  | 2 (0.43%)  |         | 291 (62.05%) | 165 (35.18%) | 13 (2.77%)  | P = 0.81 |
| Neuroticism              |                        |                         |         |         |           | 0.883 (0.520–1.499) |         |         |         |            |
| Mean                     | 45.4 ± 10.07 | 46.00 | 46.1 ± 11.38 | 45.95 ± 10.19 | 43.6 ± 7.21 | 0.60 (1.3) | P = 0.66 | 45.8 ± 10.33 | 45.5 ± 10.09 | 44.3 ± 10.50 | −1.57 (1.75) |
| EA                       |                        |                         |         |         |           |         |         |         | P = 0.39 |
| Major depression         |                        |                         |         |         |           | 0.845 (0.668–1.069) |         |         |         |            |
| NO (1981)                | 659 (33%)     | 922 (46.54%)  | 1 (0.05%)  | 396 (19.99%) | 3 (0.15%)  | 0.845 (0.668–1.069) | 1694 (85.56%) | 275 (13.89%) | 11 (0.56%)  | 1.057 (0.819–1.363) |
| YES (641)                | 209 (33%)     | 318 (49.61%)  | 0 (0.00%)  | 114 (17.78)  | 0 (0.00%)  |         | 542 (84.82%) | 96 (15.02%)  | 1 (0.16%)  | P = 0.67 |
| Any anxiety disorder     |                        |                         |         |         |           | 0.845 (0.668–1.069) |         |         |         |            |
| NO (1553)                | 530 (34%)     | 722 (46.49%)  | 0 (0.00%)  | 298 (19.19%) | 3 (0.19%)  | 1.028 (0.842–1.255) | 1323 (85.19%) | 222 (14.29%) | 8 (0.52%)  | 0.962 (0.769–1.205) |
| YES (1069)               | 338 (32%)     | 518 (48.46%)  | 1 (0.09%)  | 212 (19.83%) | 0 (0.00%)  |         | 913 (85.65%) | 149 (13.98%) | 4 (0.38%)  | P = 0.74 |
| Neuroticism              |                        |                         |         |         |           | 0.845 (0.668–1.069) |         |         |         |            |
| Mean                     | 44.3 ± 9.90   | 44.8 ± 9.84   | 60.6      | 45.1 ± 9.90 | 39.1 ± 3.83 | 0.39 (0.48) | P = 0.41 | 44.8 ± 9.90 | 44.1 ± 9.71 | 41.99 ± 7.74 | −2.2 (2.83) |

Abbreviations: AA, African American; EA, European American; L, long allele; S, short allele.
*Effect sizes compare SS, as compared with SL or LL genotypes, adjusted for subject age and gender. Data represent odds ratios and confidence intervals for diagnoses, and betas and s.e. for continuous neuroticism scores.
**Odds ratio for GG/AG, as compared with AA genotype, adjusted for subject age and gender. Data represent odds ratios and confidence intervals for diagnoses, and betas and s.e. for continuous neuroticism scores.
*Includes generalized anxiety disorder, panic attacks, social anxiety disorder, specific phobia, obsessive compulsive disorder.
econd one supported it,25 with the difference in outcome hinging on whether more restrictive or more inclusive criteria were used to define the phenotype (that is, the type of life stress and its measurement). No environmental measures were available for the present study. There are also several limitations of the present study that are related to the clinical sample. Because subjects were recruited from the community, cases may represent the milder end of the spectrum of depressive and anxiety disorders, for which genetic factors may have a less prominent role. Stronger genetic associations may potentially have been obtained had subjects been recruited from a treatment sample. The on-line diagnostic questionnaire did not provide information about several factors that are thought to predict a greater role for genetic factors, such as family history of depression and details of functional impairment.26,27 or conversely a less prominent role, such as the occurrence of depression only in the context of medical disorders, or substance use.

It is also unclear to what extent serotoninergic dysregulation leads to depressive phenotypes. Early experiments showed reduced SLC6A4 expression associated with the S variant of 5-HTTLPR when compared with the L variant, but a lack of information on distant regulatory elements could lead to misleading results.8,28–30 Lymphoblastoid cells have an altered gene expression associated with immortalization, and clonal expression of cells in these cultures could result in contradictory findings.31–34 The functional consequences of the genotype differences in multiple cell types remain unclear, and, indeed in postmortem brain, allelic ratios of serotonin transporter mRNA were not correlated with 5-HTTLPR genotypes.31,32 and PET studies have similarly failed to show serotonin transporter expression in the brain to correlate with allelic status of 5-HTTLPR.33,34 It is likely that the presence of the S allele alone is insufficient to cause a clinical phenotype. The SNP rs25531 is located within a repeat near the polymorphic segment of the 5-HTTLPR, and the G allele reportedly results in reduced transcription levels similar to those of the short allele.9 We therefore analyzed phenotypes in relation to low-transcription (S and L-G) and high-transcription (L-A) alleles, but found no significant associations. Martin et al.35 reported that there are additional SNPs that contribute to transcriptional variation of the serotonin transporter gene, but these are relatively rare so that larger samples would be required to detect any phenotypic effects. It is of interest that when the L allele containing the G allele of rs25531 was assumed to have low transcription, the total frequency of low-transcription (S and L-G) alleles was similar in AA (46%) and EA (50%) subjects, despite the substantial difference in S allele frequencies. All polymorphisms were in Hardy–Weinberg equilibrium. Assuming that these polymorphisms do have effects in expression in an at least some tissues, one speculative interpretation of this finding would be as follows. Both historical populations have experienced similar adaptive selective pressures that have shaped SLC6A4 expression levels. The result has been similar overall frequencies of high- and low-transcription common polymorphisms, but with the S allele (more common in Europeans) apparently partially replacing L-G (more common in Africans) over time. One might assume that alleles with large deleterious effects (such as a major increase in risk of severe depression) would have been subjected to negative selection, whereas the observed common polymorphisms may be relevant to the normal regulation of SLC6A4 expression.

CONFLICT OF INTEREST

In the past two years, Dr Weissman received funding from the NIMH, NIDA, NARSAD, the Sackler Foundation, the Templeton Foundation and the Interstitial Cystitis Association, and received royalties from the Oxford University Press, Perseus Press, the American Psychiatric Association Press and MultiHealth Systems. None of these present a conflict of interest with this manuscript. The remaining authors declare no conflict of interest.

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