Pfeifle, *Chromosoma* 98, 31 (1989)], digoxygenin-labeled probes were obtained by random-primer labeling of cDNA fragments comprising the *dmax* ORF, and nucleotides 1 to 2083 for *dmyc*, respectively.
2. J. Modolell, W. Bender, M. Meselson, *Proc. Natl. Acad. Sci. U.S.A.* 80, 1678 (1983).
3. D. L. Lindsley and G. G. Zimm, *The Genome of Drosophila melanogaster* (Academic Press, New York, 1992).
24. R. L. Marlor, S. M. Parkhurst, V. G. Corces, *Mol. Cell. Biol.* 6, 1129 (1986).
25. Genotypes of flies used in this study: wild-type refers to our isogenic Oregon R stock; RNA and genomic DNA were isolated from *Df(1)Pgd-kz/FM6*, *In(1)sc^{8} y^{sha1} dm^{1} B^{1}* and *Tp(3;1)N$^{264-6}$/y^{1} w^{1} dm^{1}* flies; the latter line was also used for RNA in situ hybridization to ovaries. Both *dm*^{1}-containing lines were obtained from the Bloomington stock center.
26. C. B. Moens, A. B. Auerbach, R. A. Conlon, A. L. Joyner, J. Rossant, *Genes Dev.* 6, 691 (1992).
27. K. Nakayama *et al.*, *Cell* 85, 707 (1996); H. Kiyokawa *et al.*, *ibid.* p. 721; M. L. Fero *et al.*, *ibid.* p. 733.
28. B. Mozer, R. Marlor, S. M. Parkhurst, V. Corces, *Mol. Cell. Biol.* 5, 885 (1985).
29. We thank N. H. Brown, R. Davis, J. C. J. Eeken, S. Elledge, J. Kiger, C. D. Laherty, A. P. Mahowald, M. Meyer, G. Poortinga, J. W. Tamkun, C. Thummel, P. P. Tolias, M. Watanabe, and the Bloomington Stock Center for reagents; D. Gottschling, S. Henikoff, T. Neufeld, and J. Roberts for critical readings of the manuscript; P. Goodwin for help with image analysis; J. Torgerson for assistance with the manuscript; and members of the Parkhurst, Eisenman, and Edgar laboratories for advice and support. Funded by National Institutes of Health–National Cancer Institute (NCI) grants RO1CA57138 (to R.N.E.), and RO1GM47852 (to S.M.P.), a Postdoctoral Fellowship from the Fonds National Suisse (to P.G.) and an NCI Japanese Foundation for Cancer Research Training Program in the U.S.–Japan Cooperative Cancer Committee (to Y.S.).

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Association of Anxiety-Related Traits with a Polymorphism in the Serotonin Transporter Gene Regulatory Region

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Transporter-facilitated uptake of serotonin (5-hydroxytryptamine or 5-HT) has been implicated in anxiety in humans and animal models and is the site of action of widely used uptake-inhibiting antidepressant and antianxiety drugs. Human 5-HT transporter (5-HTT) gene transcription is modulated by a common polymorphism in its upstream regulatory region. The short variant of the polymorphism reduces the transcriptional efficiency of the 5-HTT gene promoter, resulting in decreased 5-HTT expression and 5-HT uptake in lymphoblasts. Association studies in two independent samples totaling 505 individuals revealed that the 5-HTT polymorphism accounts for 3 to 4 percent of total variation and 7 to 9 percent of inherited variance in anxiety-related personality traits in individuals as well as sibships.

Anxiety-related traits are fundamental, enduring, and continuously distributed dimensions of normal human personality (*1–3*). Although twin studies have indicated that individual variation in measures of anxiety-related personality traits is 40 to 60% heritable (*4*), none of the relevant genes has yet been identified. Variance in personality traits, including those related to anxiety, is thought to be generated by a complex interaction of environmental and experiential factors with a number of gene products involving distinct brain systems such as the midbrain raphe serotonin (5-HT) system (*4*). Neurotransmission mediated by 5-HT contributes to many physiologic functions such as motor activity, food intake, sleep, and reproductive activity, as well as to cognition and emotional states including mood and anxiety (*5*). By regulating the magnitude and duration of serotonergic responses, the 5-HT transporter (5-HTT) is central to the fine-tuning of brain serotonergic neurotransmission and of the peripheral actions of 5-HT. In the brain, 5-HTT expression is particularly abundant in cortical and limbic areas involved in emotional aspects of behavior (*5*). The human 5-HTT is encoded by a single gene (*SLC6A4*) on chromosome 17q12 (*6–8*). Although 5-HTT has long been suspected to play a role in behavioral and psychiatric disorders, previous studies did not reveal any common, replicated 5-HTT gene sequence variation in either neuropsychiatric patients or healthy individuals (*9*).

Recently, we reported a polymorphism in the transcriptional control region upstream of the 5-HTT coding sequence (*10*). Initial experiments demonstrated that the long and short variants of this 5-HTT gene–linked polymorphic region (5-HTTLPR) had different transcriptional efficiencies when fused to a reporter gene and transfected into human placental choriocarcinoma (JAR) cells (*10*). The 5-HTTLPR is located ~1 kb upstream of the 5-HTT gene transcription initiation site and is composed of 16 repeat elements. The polymorphism consists of a 44–base pair (bp) insertion or deletion involving repeat elements 6 to 8 (Fig. 1A). In the present study, polymerase chain reaction (PCR)–based genotype analysis of 505 subjects revealed allele frequencies of 57% for the long (*l*) and 43% for the short (*s*) allele (*11*). The 5-HTTLPR genotypes were distributed according to Hardy-Weinberg equilibrium: 32% *l/l*, 49% *l/s*, and 19% *s/s*.

Because appropriate cell models for human serotonergic neurons do not exist and JAR cells are monozygotic for the 5-HTTLPR, we studied 5-HTT gene expression in human lymphoblastoid cell lines. Like 5-HT neurons and JAR cells, lymphoblasts constitutively express functional 5-HTT and exhibit adenosine 3′,5′-monophosphate (cAMP)–dependent and protein kinase C (PKC)–dependent 5-HTT gene regulation, but they do not express dopamine or norepinephrine transporters (*12*). Cell lines with the complete range of different 5-HTTLPR genotypes can readily be obtained (*13*).

Lymphoblast cell lines with different genotypes were first transfected with constructs in which a luciferase reporter gene was fused to ~1.4 kb of the 5′-flanking promoter sequence containing the *l* or *s* form of the 5-HTTLPR (*11*, *13*, *14*). The basal activity of the *l* variant was more than twice that of the *s* form of the 5-HTT gene promoter (Fig. 1B). Stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) or activation of adenylyl cyclase with forskolin-induced transcriptional activity was observed in both the *l* and *s* promoter variants, but the dose-dependent increases remained proportionally smaller in the *s* variant (Fig. 1B).

Although transfection experiments with reporter gene constructs are useful in assaying the transcriptional competence of a promoter sequence, they could conceivably give spurious results because of the absence of distant control elements or chromatin effects. Therefore, we next studied the expression of the native 5-HTT gene in lymphoblast cell lines cultured from subjects with different 5-HTTLPR genotypes (*15*). Cells homozygous for the *l* form of the

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5-HTTLPR produced steady-state concentrations of 5-HTT mRNA that were 1.4 to 1.7 times those in cells containing one or two copies of the *s* variant (Fig. 2C) (*15*).

The influence of the 5-HTTLPR on 5-HTT expression at the protein level was assayed by [^{125}I]RTI-55 binding and [^{3}H]5-HT uptake experiments (*16*). Membrane preparations from *l/l* lymphoblasts bound 30 to 40% more [^{125}I]RTI-55 than did membranes from *l/s* or *s/s* cells (Fig. 2B). Moreover, [^{3}H]5-HT uptake in cells homozygous for the *l* form of the 5-HTTLPR was 1.9 to 2.2 times that in cells carrying one or two endogenous copies of the *s* variant (Fig. 2A). The genotype-dependent differences in mRNA concentrations, [^{125}I]RTI-55 binding, and [^{3}H]5-HT uptake persisted proportionally when 5-HTT gene transcription was induced with forskolin or PMA (Fig. 2, A to C). In all of these studies, the data associated with the *s/s* and *l/s* genotype were similar, whereas both differed from the *l/l* genotype, suggesting that the polymorphism has more of a dominant-recessive than a codominant-additive effect.

We next evaluated the role of the 5-HTTLPR in personality traits by a combined population and family genetic study of two independently collected groups (505 total subjects) consisting predominantly of male siblings, other family members, and volunteers from two NIH protocols previously described (*17*, *18*). Personality traits were assessed with three different methods. The NEO personality inventory (NEO-PI-R), a self-report inventory based on the five-factor model of personality, was used as the primary psychometric instrument because it has high retest reliability, item validity, longitudinal stability, consistent correlations between self and observer ratings, and a robust factor structure that has been validated in a variety of populations and cultures (*19*, *20*). We predicted that the 5-HTTLPR genotype would be associated with the NEO-PI-R factor of Neuroticism, which is principally composed of anxiety- and depression-related subfactors, on the basis of several lines of evidence: 5-HT uptake inhibitors (also called serotonin reuptake inhibitors or SRIs) are an effective treatment for anxiety and depressive spectrum disorders; changes in 5-HT function are associated with these disorders; and manipulation of 5-HT alters anxiety-related behaviors in experimental animals (*21–23*). In addition, an anxiety-related personality trait, Harm Avoidance, was originally hypothesized to be mediated by serotonergic function (*1–3*).

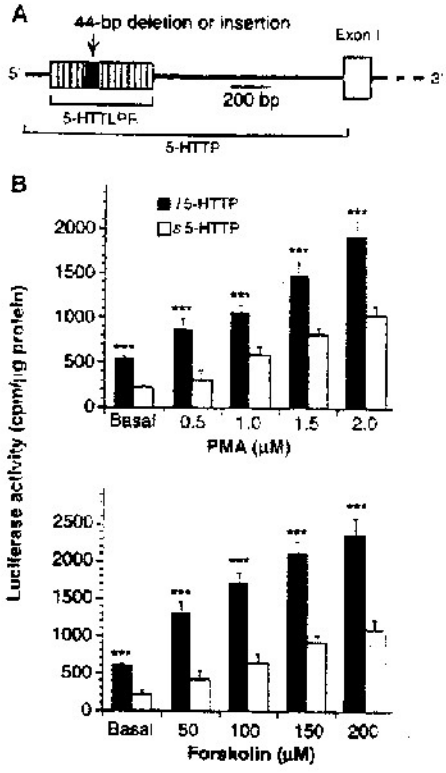

Fig. 1. (**A**) Map of the human 5-HT transporter gene promoter (5-HTTP) (EMBL–GenBank accession number X76753). The 5-HTTLPR comprises a repetitive sequence with an insertion-deletion variation indicated by a black box. (**B**) Basal and PKC- or cAMP-induced transcriptional activity of the long (*l*) and short (*s*) 5-HTTP variants in human lymphoblast cell lines with different 5-HTTLPR genotypes (*l* versus *s*: $^{***}P < 0.001$, one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test). Results are means ± SD for triplicate determinations and are representative of several cell lines with different 5-HTTLPR genotypes. The *l* variant (base pairs −1440 to +22 with respect to the transcription initiation site) and *s* variant (base pairs − 1396 to +22) of the 5-HTTP were ligated into a promoterless luciferase expression vector (*luc*+). Human lymphoblasts were transfected with 5 μg of the *l* and *s* 5-HTTP*luc*+ constructs and then treated with PMA or forskolin.

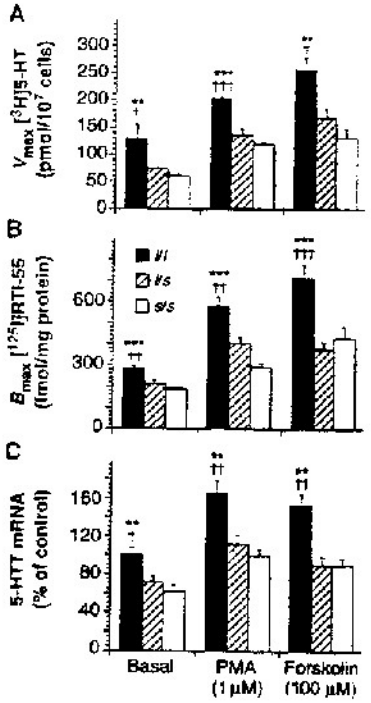

Fig. 2. [^{3}H]5-HT uptake (**A**), [^{125}I]RTI-55 binding (**B**), and 5-HTT mRNA concentrations (**C**) in human lymphoblast cell lines with the genotypes *l/l* (n = 4), *l/s* (n = 3), and *s/s* (n = 3) determined before and after treatment with PMA or forskolin (*l/l* versus *s/s*: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; *l/l* versus *l/s*: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$; one-way ANOVA followed by Fisher's P_LSD test). Results represent means ± SD for triplicate determinations. Kinetic analysis of [^{3}H]5-HT uptake in cell lines with different 5-HTTLPR genotypes yielded a Michaelis constant (K_m) range of 156 to 187 nM; the imipramine-insensitive uptake was 9.8 to 12.1%. The dissociation constants (K_d) for [^{125}I]RTI-55 binding to membranes of lymphoblasts were similar (0.27 to 0.34 nM). Nonspecific, paroxetine-insensitive binding was 8.7 to 10.1%.

In both groups of subjects (*18*), there was a significant association between 5-HTTLPR genotype and the Neuroticism factor (Table 1). Individuals with either one or two copies of the *s* form of the 5-HTTLPR (together referred to as group S) had higher Neuroticism scores than did individuals homozygous for the *l* variant of the 5-HTTLPR (group L). The scores for the *l/s* and *s/s* genotypes were not significantly different, which indicated, as in the biological measures of expression and function of 5-HTT described above, that this polymorphism has a dominant-recessive type of association with Neuroticism (Table 1). In the combined sample of 505 individuals, the distributions of Neuroticism scores in the S and L groups were overlapping but their means were separated by 3.4 *T*-score units, a difference of 0.29 SD units (Table 1 and Fig. 3).

The effect of the 5-HTTLPR genotype on personality was specific for Neuroticism. Scores on three of the other four major NEO personality factors (Extraversion, Openness, and Conscientiousness) were not significantly associated with the genetic

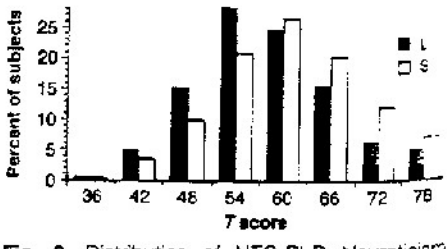

Fig. 3. Distribution of NEO-PI-R Neuroticism scores (separated into eight groups with the indicated median *T* scores) and percentages of subjects from the L (n = 163) and S (n = 342) groups in each of the eight *T*-score groups.

variant in either the separate or combined study groups. There was a negative association between the Agreeableness factor and 5-HTTLPR genotype in the combined sample, but this was not statistically significant in either of the separate groups (Table 1). The NEO-PI-R is based on a hierarchical model in which each of the five major personality factors comprises several related facets (*24*). An analysis of the six facets of the Neuroticism personality factor in the combined study population of 505 subjects (using the S versus L groups) revealed significant associations between 5-HTTLPR genotype and the facets of Anxiety (P = 0.027), Angry Hostility (P = 0.002), Depression (P = 0.007), and Impulsiveness (P = 0.008), but not Self-consciousness or Vulnerability (*24*).

Cattell's 16PF personality inventory was used as a secondary psychometric instrument. This self-report inventory is based on a factor analytic model in which personality is considered in terms of 16 core traits that constitute five second-order factors (*25*). We predicted that the 5-HTTLPR genotype would be associated with the second-order factor of Anxiety, which is the closest 16PF analog of Neuroticism and was strongly correlated with NEO-PI-R Neuroticism (correlation coefficient r = 0.77, P < 0.001). A significant and specific association between 5-HTTLPR genotype and the 16PF Anxiety factor was found (P = 0.023); this was primarily attributable to associations with the two anxiety-related 16PF primary factors of Tension (P = 0.001) and Suspiciousness (P = 0.002).

The third method of personality assessment was based on Cloninger's biosocial model, which conceptualizes temperament as consisting of the four genetically and biochemically distinct traits of Harm Avoidance, Reward Dependence, Novelty Seeking, and Persistence (*1*–*3*). Although our subjects did not complete the Tridimensional Personality Questionnaire (TPQ), weighted regression equations can be used to obtain estimated TPQ scores from the NEO-PI-R data (*17*, *20*, *26*). We predicted that the 5-HTTLPR genotype would be related to Harm Avoidance, which incorporates many aspects of anxiety and was correlated with both NEO-PI-R Neuroticism (r = 0.80, P < 0.001) and 16PF Anxiety (r = 0.63, P < 0.001). The 5-HTTLPR genotype was found to be associated with estimated scores for Harm Avoidance (P = 0.023) but not the other three TPQ traits. Analysis of the subscales for Harm Avoidance showed significant associations with the scales for Worry and Pessimism (P = 0.011), Fear of Uncertainty (P = 0.043), and Fatigability (P = 0.009), but not Shyness.

These results with three different personality assessment scales show that the 5-HTTLPR influences a constellation of traits related to anxiety. Across the three personality measures, the 5-HTT polymorphism contributes a modest but replicable 3 to 4% of the total variance and 7 to 9% of the genetic variance. These percentages are based on estimates from twin studies, using these and related measures, that have consistently demonstrated that genetic factors contribute 40 to 60% of the variance in Neuroticism, Harm Avoidance, and other anxiety-related personality traits in large population samples (*4*).

Population associations between a genetic marker and a phenotypic trait can arise either from population stratification or from genetic transmission. Because sibling pairs are by definition ethnically and racially homogeneous, any difference in trait scores between genetically discordant siblings must reflect true genetic transmission. Accordingly, our study was designed to allow family-based as well as population-based measurements of gene-trait associations. The combined study population included 459 siblings from 210 independent families, of which 78 sib-pairs from 61 in-

Table 1. Population association between the 5-HTTLPR and NEO five-factor personality traits (*17*, *20*, *26*). NEO-PI-R (form S) questionnaire results are given as *T* scores, which are standardized to have a mean (±SD) of 50 ± 10 in the normative population. *F* is the *F* value for one-way ANOVA comparing S and L genotype groups. Significance levels are reported as direct probabilities because there was a prior hypothesis of association to Neuroticism. S − L is the mean score for S genotypes (*s/s* and *l/s*) minus the mean score for L genotypes (*l/l*); ns, not significant (P > 0.05).

Genotype	n	NEO *T* score (mean ± SD)				
		Neuroticism	Extraversion	Openness	Agreeableness	Conscientiousness
			NIMH (n = 221)			
l/l (group L)	72	53.4 ± 12.0	52.5 ± 10.5	57.2 ± 12.9	45.4 ± 11.3	43.5 ± 11.6
l/s	106	57.8 ± 13.2	53.2 ± 12.2	56.1 ± 12.3	42.6 ± 11.8	40.5 ± 13.5
s/s	43	56.6 ± 11.2	52.3 ± 10.4	55.9 ± 14.1	40.9 ± 11.7	42.1 ± 12.4
l/s + *s/s* (group S)	149	57.4 ± 12.6	52.9 ± 11.7	56.1 ± 12.8	42.1 ± 11.7	41.0 ± 13.2
F		5.1	0.1	0.4	3.8	2.0
S − L		4.0	ns	ns	ns	ns
P		0.024	ns	ns	ns	ns
			NCI (n = 284)			
l/l (group L)	91	52.8 ± 11.1	55.6 ± 10.5	61.0 ± 10.1	50.5 ± 9.7	47.8 ± 10.0
l/s	141	55.5 ± 11.0	53.5 ± 10.4	59.8 ± 10.1	48.8 ± 10.2	46.6 ± 10.6
s/s	52	56.1 ± 11.3	52.1 ± 11.1	59.3 ± 8.9	49.8 ± 11.2	45.4 ± 10.8
l/s + *s/s* (group S)	193	55.7 ± 11.0	53.1 ± 10.6	59.7 ± 9.8	49.1 ± 10.5	47.7 ± 10.7
F		4.2	3.6	1.1	1.1	0
S − L		2.9	ns	ns	ns	ns
P		0.042	ns	ns	ns	ns
			Total (n = 505)			
l/l (group L)	163	53.1 ± 11.5	54.2 ± 10.6	59.4 ± 11.6	48.2 ± 10.7	45.9 ± 10.9
l/s	247	56.5 ± 12.0	53.3 ± 11.2	58.2 ± 11.2	46.2 ± 11.3	45.1 ± 12.5
s/s	95	56.3 ± 11.2	52.2 ± 10.7	57.8 ± 11.6	45.8 ± 12.2	43.9 ± 11.6
l/s + *s/s* (group S)	342	56.4 ± 11.8	53.0 ± 11.1	58.1 ± 11.3	46.1 ± 11.6	44.8 ± 12.3
F		9.3	1.4	1.3	4.0	1.0
S − L		3.4	ns	ns	−2.2	ns
P		0.002	ns	ns	0.045	ns

Table 2. Familial association between the 5-HTTLPR and anxiety-related traits (*27*). Results for the NEO factor of Neuroticism and the estimated TPQ factor of Harm Avoidance are in *T* score units (as in Table 1); those for the 16PF factor of Tension (Q4) are in Sten score units (which have a mean of 5.5 and SD of 1 in the normative population). For association across pedigrees, S − L is the maximum likelihood estimate of [(score for S individuals) − (score for L individuals)] across all families; $-2 \ln L = -2$[log(likelihood of data without 5-HTTLPR effect) − log(likelihood of data with 5-HTTLPR effect)]; and P was calculated by taking $-2 \ln L$ to be distributed as a χ^2 statistic at one degree of freedom. For association within pedigrees, S − L is the mean of [(score for S sib) − (score for L sib)] within each nuclear family; t^* is the *t* score, conservatively corrected for the nonindependence of sib-pairs from a single family; and P was calculated by a two-sided *t* test.

Statistic	Factor		
	Neuroticism	Tension	Harm Avoidance
Across-pedigrees test (n = 488 family members, 37 unrelated individuals)			
S − L	3.4	0.8	2.6
$-2 \ln L$	8.7	11.3	6.3
P	0.0031	0.0008	0.0119
Within-pedigrees test (n = 78 sib-pairs)			
S − L	4.6	0.8	5.1
t^*	2.2	2.4	3.0
P	0.028	0.022	0.004

dependent families had discordant (that is, *l/l* versus *l/s* or *s/s*) 5-HTTLPR genotypes. It was first necessary to analyze the association between 5-HTTLPR genotype and anxiety-related measures after correcting for the statistical nonindependence of family members resulting from factors unrelated to 5-HTT. Elston and colleagues have described a maximum likelihood method for estimating quantitative trait associations that takes into account polygenic inheritance (*27*). An across-pedigrees analysis of the major 5-HTTLPR–associated traits of Neuroticism (NEO-PI-R), Tension (16PF), and Harm Avoidance (TPQ) (Table 2) revealed that there was a significant association for each trait with 5-HTTLPR genotype, and that the effect sizes and significance levels were comparable to those obtained by population association analysis. In the 76 sib-pairs that were discordant for the 5-HTTLPR, the average difference in Neuroticism scores between the L and S siblings was 4.6 *T*-score units (Table 2), which was indistinguishable from the 3.4 *T*-score difference seen in all L and S individuals. Despite the reduction in sample size, the difference between the L and S siblings was statistically significant, even after conservatively correcting for the nonindependence of sib-pairs from the same family (*17*, *20*, *26*). Similar results were obtained for Tension and Harm Avoidance; the scores of group S probands were significantly higher than those of their group L siblings, and the effect sizes were similar to those obtained by population-based or across-pedigrees analyses (Table 2). These within-pedigrees results demonstrate that the observed associations between 5-HTTLPR genotype and personality are the result of genetic transmission rather than population stratification. Overall, however, the associations reported here represent only a small portion of the genetic contribution to anxiety-related traits observed in this nonrandom population sample.

Considerable evidence indicates that increased serotonergic neurotransmission (which would be an evident consequence of the reduced 5-HT uptake capacity found in individuals with the short allele of the 5-HTT polymorphism) is anxiogenic in animal models as well as in humans (*2*, *3*, *22*, *23*, *28*). At the clinical level, reduced 5-HT uptake or reduced inhibitor binding to 5-HTT has been one of the most consistent biological findings in individuals with depression and several anxiety disorders (*29*). Our findings that individuals with the short 5-HTTLPR allele and reduced 5-HTT function have greater anxiety-related personality characteristics would at first seem to conflict with the fact that SRIs such as fluoxetine, which competitively inhibit 5-HT uptake, are therapeutic agents in anxiety and depressive disorders (*21*–*23*). However, the therapeutic effects of the SRIs have primarily been demonstrated in neuropsychiatric patients, who may have some primary 5-HT or other neurotransmitter dysfunction that is ameliorated by the SRIs, whereas our findings are in a sample of the general population. The SRIs also have other pharmacological properties that may contribute to their therapeutic effects (*30*). The lifelong duration of the genetically driven differences in 5-HT uptake, including possible influences during early brain development (*31*), may also lead to different effects from those produced by SRI administration later in life.

The associations reported here represent only a small portion of the genetic contribution to anxiety-related personality traits. If other genes were hypothesized to contribute similar gene dosage effects to anxiety, approximately 10 to 15 genes might be predicted to be involved. Small, additive, or interactive contributions of this size have been found in studies of other quantitative traits in plants and vertebrates, including humans (*17*, *32*). As other anxiety-related genes are identified, including perhaps some with effects that are larger than or interact with this polymorphism, it might become possible to use this information to enhance individualized pharmacologic treatment of neuropsychiatric disorders, just as for other medical disorders (*17*, *32*). Whether this particular polymorphism contributes to the general tendency for individuals who score higher on neuroticism or anxiety factors in different personality tests to be at higher risk for anxiety or personality disorders as well as depression will require further study (*33*). It likewise remains to be seen whether therapeutic responses to serotonergic agents are influenced by this polymorphism.

REFERENCES AND NOTES

1. J. A. Gray, *The Neuropsychology of Anxiety. An Inquiry into the Functions of the Septo-Hippocampal System* (Oxford Science, New York, 1982); C. R. Cloninger, *Psychiatr. Dev.* **4**, 167 (1986); H. J. Eysenck, in *Handbook of Abnormal Psychology*, H. J. Eysenck, Ed. (Pitman, London, 1957), pp. 130–156.
2. C. R. Cloninger, *Arch. Gen. Psychiatry* **44**, 573 (1987).
3. ———, *Psychiatr. Dev.* **6**, 83 (1988).
4. J. C. Loehlin, *Am. Psychol.* **44**, 1285 (1989); A. C. Heath, C. R. Cloninger, N. G. Martin, *J. Pers. Soc. Psychol.* **66**, 762 (1994); R. Plomin, M. J. Owen, P. McGuffin, *Science* **264**, 1733 (1994); C. S. Bergeman *et al.*, *Psychol. Aging* **3**, 399 (1988); N. L. Pedersen *et al.*, *J. Pers. Soc. Psychol.* **55**, 950 (1988); E. S. Lander and N. J. Schork, *Science* **265**, 2037 (1994).
5. H. T. Chen, M. Clark, D. Goldman, *J. Pharmacol. Toxicol. Methods* **27**, 209 (1992); J. G. Hensler *et al.*, *Synapse* **17**, 1 (1994); P. Whitaker-Azmitia and S. Peroutka, *Ann. N.Y. Acad. Sci.* **600**, 4 (1990); H. G. Westenberg, D. L. Murphy, J. A. Den Boer, Eds., *Advances in the Neurobiology of Anxiety Disorders* (Wiley, New York, 1996).
6. K. P. Lesch *et al.*, *J. Neural Transm.* **91**, 67 (1993); K. P. Lesch *et al.*, *ibid.* **95**, 157 (1994).
7. J. Gelernter, A. J. Pakstis, K. K. Kidd, *Hum. Genet.* **95**, 677 (1995).
8. S. Ramamoorthy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2542 (1993).
9. K. P. Lesch *et al.*, *Biol. Psychiatry* **37**, 215 (1995); M. Altemus *et al.*, *Am. J. Med. Genet. Neuropsychiatr. Genet.* **67**, 409 (1996); D. Di Bella *et al.*, *Psychiatr. Genet.* **5**, S100 (1996); A. D. Ogilvie *et al.*, *Lancet* **347**, 731 (1996); D. Collier *et al.*, *Neuroreport* **7**, 1675 (1996).
10. A. Heils *et al.*, *J. Neural Transm.* **102**, 247 (1995); A. Heils *et al.*, *J. Neurochem.* **66**, 2621 (1996).
11. Blood for DNA isolation and analysis was obtained from healthy human volunteers. Oligonucleotide primers flanking the 5-HTTLPR and corresponding to the nucleotide positions −1416 to −1397 (stpr5, 5′-GGCGTTGCCGCTCTGAATGC) and −910 to −885 (stpr3, 5′-GAGGGACTGAGCTGGACAACCAC) of the 5-HTT gene 5′-flanking regulatory region were used to generate 484- or 528-bp fragments. PCR amplification was carried out in a final volume of 30 μl consisting of 50 ng of genomic DNA, 2.5 mM deoxyribonucleotides (dGTP/7-deaza-2′-dGTP = *l/l*), 0.1 μg of sense and antisense primers, 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase. Annealing was carried out at 61°C for 30 s, extension at 72°C for 1 min, and denaturation at 95°C for 30 s for 35 cycles.
12. K. Iken *et al.*, *Cell. Immunol.* **163**, 1 (1995); S. A. Faraj, Z. L. Olkowski, R. T. Jackson, *Int. J. Immunopharmacol.* **16**, 561 (1994).
13. Epstein-Barr virus–transformed lymphoblasts with the genotypes *l/l* (*n* = 4), *l/s* (*n* = 3), and *s/s* (*n* = 3) were grown in RPMI 1640 supplemented with 10% newborn calf serum at 37°C in a humidified atmosphere at 5% CO_2. For induction of 5-HTT expression, lymphoblasts were treated with 50 to 200 μM forskolin or 0.5 to 2 μM PMA and grown for an additional 24 hours.
14. The human 5-HTT gene 5′ regulatory sequence (promoter) is derived from the ~1.7-kb clone λHG5-HTT/P-HB (EMBL-GenBank accession number X76753) that was isolated from a human genomic library in λZAP express (Stratagene) as described (*6*). The long variant of the 5-HTT regulatory sequence (5-HTTP, base pairs −1440 to +22 with respect to the transcription initiation site) was ligated into the promoterless luciferase (*luc*+) expression vector pGL3 basic (Promega). The short variant of the 5-HTT gene promoter (s 5-HTTP, base pairs −1396 to +22) was generated by cleaving the short, deletion-containing 484-bp PCR product with Pst I and ligating it into the Pst I site of the 5-HTTP *luc*+ construct after the fragments flanked by the Pst I sites at nucleotide positions −1366 and −1192 had been removed. Inserts and insert-vector boundaries were verified by sequence analysis. Long and short human 5-HTT *luc*+ constructs and controls were transiently expressed in lymphoblasts with different genotypes (*13*), and *luc*− gene expression was studied relative to the pGL3 basic and pGL3 control vectors. Transfection efficiency was assessed by co-transfection with pSV-βGal (Promega). For transient expression, lymphoblasts (2 × 10^6 cells) were exposed for 24 hours to 5 μg of construct DNA complexed with 5 μl of Transfectam lipofectin reagent (Promega) in 5 ml of RPMI 1640. Cells were grown for an additional 24 hours before harvest in 1 ml of luciferase lysis buffer. Extracts were assayed for luciferase activity by addition of 10 μl of cell lysate samples at 15-s intervals to 100 ml of luciferin reagent. Chemiluminescence was counted for 15 s at a constant time (90 s) after reagent mixing in a multiple scintillation spectrometer.
15. Total RNA was isolated from lymphoblasts (*13*) by guanidine thiocyanate column purification (Qiagen). The 28S/18S bands of ribosomal RNA were analyzed by densitometry to control for variations in RNA concentration, and single-stranded cDNA (37°C, 60 min) was synthesized with random primer. 5-HTT mRNA was measured by semiquantitative compet-

tive reverse transcription PCR with a 5-HTT cDNA–derived template containing a 172-bp deletion (base pairs 1635 to 1806) as internal standard. The PCR amplification (30 s at 95°C, 30 s at 61°C, 1 min at 72°C for 35 cycles) of 355- or 527-bp fragments was carried out with the amplimers se3 (5′-ATGCAGAAGCGATAGCCAACATG, base pairs 1437 to 1459 with respect to the transcription initiation site) and 3re (5′-AGATGAGGTTCCTATGCAGTAAC, base pairs 2147 to 2167). 5-HTT mRNA concentrations of lymphoblast cell lines with the *l/l* genotype were first titrated against incremental concentrations of competitive template ranging from 0.01 to 1.0 ng. The concentration of the competitive template at target/template equilibrium was then used to compare mRNA concentrations semiquantitatively in lymphoblast cell lines with different genotypes (*13*) before and after induction of 5-HTT gene transcription. To control for differences in the efficiency of reverse transcription of mRNA, we performed cDNA synthesis and subsequent competitive PCR in quadruplicate. The reaction products were electrophoresed through 2% agarose, visualized by ultraviolet illumination in the presence of ethidium bromide, and quantified by densitometric analysis.

Inhibitor binding to the 5-HTT protein was assayed by incubating membranes from different lymphoblast cell lines (*13*) with [^{125}I]RTI-55 (0.05 to 1 nM) for 1 hour at 37°C as described [J. D. Ramamoorthy *et al.*, *J. Biol. Chem.* **270**, 17189 (1995)]. Nonspecific binding was determined in the presence of 5 μM paroxetine. RTI-55 [3β-(4-iodophenyl)tropan-2β-carboxylic acid methyl ester tartrate] is a cocaine analog that potently inhibits 5-HT uptake and binds to 5-HTT with high sensitivity (*8*) [J. W. Boja *et al.*, in *Dopamine Receptors and Transporters*, H. B. Niznik, Ed. (Dekker, New York, 1994), pp. 611–644]. We determined 5-HT uptake by incubating 1 × 10^7 suspended lymphoblasts with 0.1 to 1 μM [^{3}H]5-HT for 30 min at 25°C in the absence or presence of 0.1 mM imipramine.

J. Benjamin *et al.*, *Nature Genet.* 12, 81 (1996).

Two independent groups of predominantly male siblings, other family members, and unrelated individuals were studied: (i) The NIMH sample (*17*) was recruited from the NIH and local college campuses by advertising for pairs of brothers and pairs of sisters for a study of personality traits and chromosomes. The sample consisted of 221 subjects, of whom 93% were male and 7% were female. The average age was 23.3 ± 5.8 years (range 18 to 64 years), the average educational level was 15.6 ± 2.1 years (range 12 to 20 years), and the average Kinsey score was 0.2 ± 0.7 (range 0 to 5.6, where 0 is exclusively heterosexual and 6 is exclusively homosexual). The ethnic composition was 79.1% white non-Hispanic, 10.0% Asian/Pacific Islander, 4.1% Hispanic/Latino, 4.1% African American/Black, and 2.7% other. The family structure of the NIMH sample was 208 siblings from 104 families and 13 unrelated individuals. (ii) The NCI sample [D. H. Hamer *et al.*, *Science* 261, 321 (1993); S. Hu *et al.*, *Nature Genet.* 11, 248 (1995)] was collected from NIH clinics and local and national homophile organizations for a study of sexual orientation, HIV progression, and psychological traits. The sample consisted of 284 subjects of whom 92% were male and 8% were female. The average age was 37.6 ± 9.7 years (range 18 to 72 years), the average educational level was 17.3 ± 2.6 years (range 12 to 20 years), and the average Kinsey score was 4.8 ± 2.0 (range 0 to 6). The ethnic composition was 93.6% white non-Hispanic, 5.3% Hispanic/Latino, 0.7% African American/Black, 0.4% Native American/Alaskan, and 0.4% other. The family structure of the NCI sample was 251 siblings from 106 families, 9 parents, and 24 unrelated individuals.

R. R. McCrae and P. T. J. Costa, *Personality in Adulthood* (Guilford, New York, 1990).

P. T. J. Costa and R. R. McCrae, in *Handbook of Personality: Inventories*, J. Cheek and E. M. Donahue, Eds. (Plenum, New York, in press).

G. F. Heninger, *Psychopharmacology: The Fourth Generation of Progress*, F. E. Bloom and D. J. Kupfer, Eds. (Raven, New York, 1995), p. 471; D. L. Murphy *et al.*, in *Serotonin: From Cell Biology to Pharmacology and Therapeutics II* (Kluwer Academic, Dordrecht, Netherlands, 1993), pp. 223–230; O. Benkert, H. Wetzel, A. Szegedi, *Int. Clin. Psychopharmacol.* **8** (suppl. 1), 3 (1993).

22. G. Griebel, *Pharmacol. Ther.* **65**, 319 (1995).
23. S. L. Handley, *ibid.* **66**, 103 (1995).
24. A complete table of the S – L differences and F values for all of the NEO Neuroticism facets, 16PF second-order and primary factors, estimated TPQ factors, and TPQ Harm Avoidance subscales is available from the authors by request.
25. R. B. Cattell, *J. Abnorm. Soc. Psychol.* **38**, 476 (1943); R. B. Cattell, *The Description and Measurement of Personality* (World Book, Yonkers, NY, 1946); J. S. Wiggins and A. L. Pincus, *Annu. Rev. Psychol.* **43**, 473 (1992).
26. Population association was assessed by one-way analysis of variance (ANOVA) using SPSS Statistical Software.
27. V. T. George and R. C. Elston, *Genet. Epidemiol.* **4**, 193 (1987). Across-pedigrees association was assessed using the ASSOC program of the S.A.G.E. package [R. C. Elston, V. T. George, A. J. M. Sorant, in *S.A.G.E. Users Guide, Release 2.2* (Department of Biometry and Genetics, Louisiana State University Medical Center, New Orleans, 1994); T. G. Nick *et al.*, *Genet. Epidemiol.* **12**, 145 (1995)]. Within-pedigrees association was determined by a paired *t* test using genetically discordant sib-pairs and was conservatively corrected for the nonindependence of siblings from the same family (*17*).
28. J. A. Den Boer *et al.*, *Int. Clin. Psychopharmacol.* **9** (suppl. 4), 47 (1995).
29. M. J. Owens and C. B. Nemeroff, *Clin. Chem.* **40**, 288 (1994); L. J. Iny *et al.*, *Biol. Psychiatry* **36**, 281 (1994); G. Faludi, K. Tekes, L. Tothfalusi, *J. Psychiatry Neurosci.* **19**, 109 (1994).
30. K. Fuxe *et al.*, *Neuropharmacology* **22**, 389 (1983); C. M. Beasley, D. N. Masica, J. H. Polvin, *Psychopharmacology* **107**, 1 (1992); P. Blier and C. de Montigny, *Trends Pharmacol. Sci.* **15**, 220 (1994); E. H. Cook Jr. *et al.*, *Neuropharmacol. Neurotoxicol.* **5**, 1745 (1994); J. Maj and E. Moryl, *J. Neural Transm.* **88**, 143 (1992).
31. D. L. Shuey, T. W. Sadler, J. M. Lauder, *Teratology* **46**, 367 (1992).
32. A. H. Paterson *et al.*, *Genetics* **127**, 181 (1991); C. W. Stuber *et al.*, *ibid.* **132**, 823 (1992); J. Flint *et al.*, *Science* **269**, 1432 (1995); R. P. Ebstein *et al.*, *Nature Genet.* **12**, 78 (1996); T. Ohno, S. Kawazu, S. Tomono, *Metabolism* **45**, 218 (1996); F. Cambien *et al.*, *Nature* **359**, 641 (1992); E. Arbustini *et al.*, *Br. Heart J.* **74**, 584 (1995); F. Cambien, *Clin. Genet.* **46**, 94 (1994); A. Gardemann *et al.*, *Circulation* **92**, 2786 (1995).
33. R. T. Mulder, P. R. Joyce, C. R. Cloninger, *Compr. Psychiatry* **35**, 225 (1994); D. M. Svrakic *et al.*, *Arch. Gen. Psychiatry* **50**, 991 (1993); G. Andrews *et al.*, *J. Affective Disord.* **19**, 23 (1990); K. S. Kendler *et al.*, *Arch. Gen. Psychiatry* **50**, 863 (1993); A. MacKinnon and P. M. Mitchell, in *Handbook of Depression and Anxiety*, J. Den Boer and J. M. Ad Sitsen, Eds. (Dekker, New York, 1994), pp. 71–119; K. S. Kendler *et al.*, *Arch. Gen. Psychiatry* **44**, 451 (1987); D. Collier *et al.*, *Mol. Psychiatry*, in press.
34. We thank M. Schad, G. Ortega, and S. Jatzke for technical assistance, W. Davis and D. Drake for editorial assistance, and M. Altemus, J. Mizrahi, and A. Jaffe for logistical support. Supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung, the European Commission, and the intramural Research Programs of NIMH and NCI. K.P.L. is supported by the Hermann and Lilly Schilling Foundation.

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Discovering High-Affinity Ligands for Proteins: SAR by NMR

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A nuclear magnetic resonance (NMR)–based method is described in which small organic molecules that bind to proximal subsites of a protein are identified, optimized, and linked together to produce high-affinity ligands. The approach is called "SAR by NMR" because structure-activity relationships (SAR) are obtained from NMR. With this technique, compounds with nanomolar affinities for the FK506 binding protein were rapidly discovered by tethering two ligands with micromolar affinities. The method reduces the amount of chemical synthesis and time required for the discovery of high-affinity ligands and appears particularly useful in target-directed drug research.

Drugs are typically discovered by identifying active compounds from screening chemical libraries or natural products and optimizing their properties through the synthesis of structurally related analogs. This is a costly and time-consuming process. Suitable compounds with the requisite potency, compound availability, or desired chemical and physical properties cannot always be found. Furthermore, even when such compounds are found, optimization often requires the synthesis of many analogs.

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We now describe a method for identifying high-affinity ligands that can aid in the drug discovery process. The technique, which is called "SAR by NMR," is a linked-fragment approach wherein ligands are constructed from building blocks that have been optimized for binding to individual protein subsites (Fig. 1). In the first step of this process, a library of low molecular weight compounds (*1*) is screened to identify molecules that bind to the protein. Binding is determined by the observation of ^{15}N- or ^{1}H-amide chemical shift changes in two-dimensional ^{15}N-heteronuclear single-quantum correlation (^{15}N-HSQC) spectra (*2*) (Fig. 2) upon the addition of a ligand to