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ORIGINAL RESEARCH ARTICLE

Anorexia nervosa (restrictive subtype) is associated with a polymorphism in the novel norepinephrine transporter gene promoter polymorphic region

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Long-term weight-restored patients with anorexia nervosa (AN) have lower norepinephrine levels than controls.[1,2] Since this may reflect altered reuptake by the norepinephrine transporter (NET), we hypothesised that the NET gene was involved in the genetic component[3] of AN. PCR-amplification of an AAGG repeat island (AAGG1) in the NET gene promoter region revealed a novel 343-bp sequence with five additional AAGG repeat islands (AAGG2–AAGG6). We named the sequence from AAGG1 to AAGG6 inclusive, the NET gene promoter polymorphic region (NETpPR). A 4-bp deletion (S4) or insertion (L4) in AAGG4 resulted in the net loss or gain, respectively, of a putative Elk-1 transcription factor site. The transmission disequilibrium test[4] (TDT) with 87 Australian trios (patient plus parents) demonstrated significant preferential transmission of L4 (McNemar's χ^2 = 7.806, df = 1, P = 0.0052, odds ratio: 2.1) from parent to child with restricting AN (AN-R), suggesting that L4 or a DNA variant in linkage disequilibrium with it, doubles the risk for developing AN-R.
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The noradrenergic system is involved in regulating each of the neuroendocrine systems that become disrupted in AN.[5–7] AN patients at normal weight for 6–72 months have significantly lower blood and CSF levels of norepinephrine[1,2] and 3-methoxy-4-hydroxyphenylglycol[1] than normal controls, suggesting that reduced noradrenergic activity is associated with long-term weight restoration. It is not clear, however, whether alterations in metabolism, secretion, or NET reuptake of norepinephrine are responsible for this reduced activity. The NET gene has been cloned and sequenced[8] and polymorphisms have been identified in the coding and intronic regions[9–11] but not in the promoter. We hypothesised that DNA variation in the NET gene promoter was involved in the genetic susceptibility to develop AN.

We observed tandem AAGG repeats from positions –4297 to –4282 (AAGG1 in Figure 1a) in the only published sequence of the far 5′ end of the NET gene promoter reported earlier by Kim *et al*.[12] To investigate this repeat region, an expected 517-bp fragment was PCR-amplified from genomic DNA. However, gel sizing revealed a >800-bp band (data not shown) which was confirmed to be 860 bp after bidirectional DNA sequencing of the PCR product. Only the 860-bp fragment was observed in 50 anonymous (48 Caucasian: two East Asian) genomic DNA samples (data not shown). The 860 bp consisted of 517 bp from the Kim *et al*[12] sequence together with a novel 343-bp GA-rich sequence. Blast searches of GenBank with the novel sequence produced no matches with any known gene and only a single gene encoding the human NET is known to exist.[13] After we submitted our novel sequence to GenBank (accession number AF330628), The Human Genome Project working draft confirmed the existence of the novel sequence.

To study the 343-bp novel sequence, primers were designed to amplify a 525-bp region including the novel sequence (Figure 1a). Bidirectional sequencing of the PCR product revealed six discrete islands of consensus AAGG tetranucleotide repeats which we desig-

Figure 1 Organisation of the NETpPR, and NETpPR polymorphic variants. (a) 525-bp genomic DNA sequence from current study deposited in GenBank accession AF330628; numbering relates to the start site (+1) in GenBank sequence AF061198;[12] NETpPR located from –4297 to –3935; imperfect repeat sequences (RepeatA and RepeatB) are underlined; 343-bp novel sequence is in uppercase (–4277 to –3935); AAGG1–AAGG6 are boxed; identified polymorphisms are labelled L1, S1, L4, and S4; Elk-1 transcription factor sites lost in S1 and S4 are shown with core sequence in bold; primer sequences are shaded. (b), (c), and (d) Demonstrate detection of polymorphisms through heteroduplex (heterodup.) analysis: (b) and (c) electrophoresed using mini-PROTEAN II/III systems; (d) electrophoresed using Hoefer SE600.

a

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                                                                                  Elk-1
                                                                          aaggaaGGAAggaa
                                                               RepeatA         AAGG1    L1
-4357 gcagtgtaatatatgcctattgtccctgatcaagacagaaagacagaatgaaaaggaagaaggaaggaaggaagg
      Netp1Fins                                                      aaggaaggaagg....
                                                                                 S1
                                                                     -4297
-4281 gaggGAGGGAGGGAAAAAAGAGGAAGGAAAGAAATCAGGGAGAGAAAGAAGGAAAGGATGGAAGGAAGGTGGGAAA
      -4277                                                            AAGG2
                                              AAGG3
-4205 GGAGAGAGAGAAAGAAAGGGACTAAGGGAGAAAGAAAAAAGGAAGAAAGGAAAGAAAAGAGAAAGAAAAAGAAAGAAA
                                                                   RepeatA
                  Elk-1
              aaagaaGGAAggaagg
        RepeatB              L4
-4129 GAAAAAAGAAAAGAAAAGAAAGAAGGAAGGAAGGGAGGGAGGGAGGGAGGAAGAGGAAGGAAAGAAGTGAGGGAGG
                           AAGGAAGG....
                            AAGG4  S4
-4053 GAAAGAAGCAAAGGAAGGAAGGTAGGAAAGGAGAGAGAAAGAAAGGGAGGAAAGGAGAAAGAAAAAAAGGAAGAAA
               AAGG5                         -3935
                          AAGG6                             RepeatB
-3977 GAAAAGAGAAAGAGAAAGAAAAGAAAGAAGGAAGGAAGGAAGGaaagaaaagagggatggaggaaacaaatatgta
                                                                  Netp1Rins
-3901 ggcaaacctctcctcctttttttccttagtctcctcattggtgccatggaggtgtaggttctgatagcg -3833
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b

ladder L4/S4

Origin

1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp

L4/S4 heterodup.

525 + 521 bp

c

L4/S4 + L1/S1 L4/S4

S1/S4 heterodup.

L4/S4 heterodup. (very faint)

L1/S1 heterodup. (very faint)

521 bp

L4/S4 heterodup.

525 + 521 bp

d

L1/S1 L4/S4

L4/S4 heterodup.

L1/S1 heterodup.

525 + 521 bp

525 bp
521 bp

nated AAGG1, AAGG2, AAGG3, AAGG4, AAGG5 and AAGG6 (Figure 1a). Two imperfect repeats (RepeatA and RepeatB in Figure 1a) extended through the AAGG islands. RepeatA contains (in the 5′ to 3′ direction) AAGG1, AAGG2 and AAGG3. RepeatB contains AAGG4, AAGG5 and AAGG6. These repeat elements point to instability of the region probably resulting in the loss of the 343-bp novel sequence in the Kim *et al*[12] sequence during cloning. Amplification of the 50 anonymous DNA samples above revealed a common heteroduplex after polyacrylamide gel electrophoresis (PAGE) which suggested a heterozygous genotype (Figure 1b). Bidirectional sequencing of the PCR products confirmed that one of three AAGG repeats in AAGG4 was deleted which destroyed a putative Elk-1 transcription factor site (Figure 1a). Long AAGG4 (three AAGG repeats) and short AAGG4 (two AAGG repeats) were referred to as L4 and S4, respectively. The anonymous DNA samples revealed frequencies of 0.74 and 0.26 for L4 and S4, respectively. Genotype frequencies were in Hardy–Weinberg equilibrium (χ^2 = 0.008, df = 1, P = 0.9274). Genotyping the AN families revealed another variant present on only five of 404 unrelated parental chromosomes. Bidirectional sequencing of the PCR products confirmed this to be a deletion of one of four AAGG repeats in AAGG1 (Figure 1a), which destroyed another canonical Elk-1 transcription factor site. We called this variant S1. A L1/S1 heterozygote generated a characteristic heteroduplex on PAGE gels (Figure 1d). We never observed S1 and S4 on the same chromosome. S1 and S4 were in negative linkage disequilibrium (delta = –0.0018). L4/S4 in combination with L1/S1 was seen in one parent and produced three heteroduplexes probably due to multiple annealing possibilities and/or different conformations of the various ssDNA fragments[14] (Figure 1c). Due to its polymorphic nature, we named the DNA sequence from AAGG1 to AAGG6 inclusive, the NETpPR. Transcription factor binding sites were identified in the vicinity of the two polymorphic AAGG islands with MatInspector Public Domain[15] at http://genomatix.gsf.de/cgi-bin/matinspector/ matinspector.pl. This detected potential barbie boxes and putative MZF1, Elk-1, GKLF, Ik-2, NFAT, and Evi-1 sites (Table 1). These may play a role in NET gene expression.

We recruited 101 trios into our association study of the novel AAGG4 alleles: 87 patients with DSM-IV AN-R and 14 with DSM-IV binge/purging anorexia nervosa (AN-BP), plus both biological parents. AN-R and AN-BP were analysed separately due to the reported differences between these patients[16] that has led to them being sub-classified in DSM-IV. Genotype frequencies of patients and parents were in Hardy–Weinberg equilibrium (data not shown) (all P-values > 0.69). Haplotype relative risk (HRR)[17] and haplotype-based haplotype relative risk (HHRR)[18] analyses (Table 2) were performed for genotypes and alleles, respectively. For HRR analysis, L4/S4 heterozygotes + S4/S4 homozygotes were collapsed into one group and compared with L4/L4 homozygotes. For AN-R there were significantly more L4 homozygotes among the patients than in the non-transmitted parental genotypes (66% *vs* 46%, χ^2 = 5.964, df = 1, P = 0.0146, odds ratio: 2.23, 95% CI 1.21–4.11). L4 alleles were also significantly more common in AN-R patients than in their parents (82% *vs* 69%, χ^2 = 6.811, df = 1, P = 0.0091, odds ratio: 2.00, 95% CI 1.21–3.29) in the HHRR analysis. The TDT (Table 3) supported these results. L4/S4 heterozygous parents transmitted 42 L4 alleles and 20 S4 alleles to their AN-R children (McNemar's χ^2 = 7.806, df = 1, P = 0.0052, odds ratio: 2.10, 95% CI 1.21–3.78). In contrast, HRR and HHRR analysis demonstrated that both L4 homozygotes and L4 alleles, respectively, were less common in AN-BP patients than in their parents (43% *vs* 71%, and 68% *vs* 86%, respectively) although neither comparison reached statistical significance (Fisher's P = 0.2519, and P = 0.2047 respectively). The TDT (Table 3) showed preferential transmission of the S4 allele from L4/S4 heterozygous parents to AN-BP children but was also not significant (McNemar's χ^2 = 2.273, df = 1, P = 0.1317). We had 81% power to detect a significant association of AN-R with L4 (assuming L4 frequency: 0.74, genotypic relative risk: 2.2, 2 allele locus) but only 27% power to detect a significant association of AN-BP with S4 (assuming S4 frequency: 0.26, genotypic relative risk: 2.2, 2 allele locus) using published equations.[19] We then tested for a difference between AN-R and AN-BP by comparing the transmission of alleles from L4/S4 heterozygous parents of AN-R and AN-BP patients (Table 3). This was significant (Fisher's P = 0.0173).

Due to the rarity of S1 we could not perform an association study with the AAGG1 alleles. However, only one out of five L1/S1 parents transmitted S1 to their AN child.

We make three conclusions from our findings. Firstly, AN-R is associated with L4 due to this variant being involved in the disease process or being in linkage disequilibrium with a disease locus nearby. Secondly, when compared with each other there is a significant difference in allelic transmissions to AN-R and AN-BP patients due to preferential transmission of L4 to AN-R patients, and S4 to AN-BP patients, although the sample size of AN-BP decreases the power of this observation. Thirdly, preferential transmission of *different* alleles to AN-R and AN-BP patients suggests that our association of AN-R with L4 is not due to segregation distortion (a rare phenomenon[20] resulting in preferential transmission of an allele regardless of disease status). Segregation distortion would require preferential transmission of the same allele to both AN-R and AN-BP patients.

Premorbidly, AN patients often have anxiety disorders.[21] Accumulated evidence suggests that noradrenergic brain systems are involved in the pathophysiology of human anxiety.[22] In addition, expression of the NET is regulated by norepinephrine.[23] Due to the critical role played by the NET in noradrenergic transmission and therefore in anxiety it is reasonable to hypothesise that the NET contributes to the aetiology of AN.

Table 1 Putative transcription factor binding sites in the vicinity of the NETpPR polymorphisms

Locus[a]	*Strand*[b]	*Transcription factor*	*Sequence*[c]	*Locus*[a]	*Strand*[b]	*Transcription factor*	*Sequence*[c]
−4335	−	MZF1	TcaGGGGa	−4127	+	NFAT	aaaaaGAAAaga
−4309	+	Barbie	AtgaAAAGgaagaag	−4123	+	Evi-1	agaaAAGAaaa
−4308	+	Elk-1	TgaaaaGGAAgaag	−4118	+	Evi-1	agaaAAGAaag
−4301	+	Elk-1	gaagaaGGAAggaa	−4115	+	Barbie	aaagAAAGaaggaag
−4297	+	Elk-1[d]	aaggaaGGAAggaa	−4111	+	Elk-1[e]	aaagaaGGAAggaa
−4294	+	GKLF	gaaggaaggaAGGG	−4108	+	GKLF	gaaggaaggaAGGG
−4293	+	Elk-1	aaggaaGGAAggga	−4107	+	Elk-1	aaggaaGGAAggga
−4290	+	GKLF	gaaggaagggAGGG	−4104	+	GKLF	gaaggaagggAGGG
−4287	+	IK-2	ggaaGGGAggga	−4101	+	IK-2	ggaaGGGAggga

[a]Set for 100% core sequence similarity and greater than or equal to 85% matrix similarity; numbering relates to Kim *et al*[12] sequence; sense strand numbering used but all sequences are shown in the 5′–3′ direction.
[b]Sense strand +; anti-sense strand −.
[c]Core sequence in uppercase.
[d]Site lost in S1 allele.
[e]Site lost in S4 allele.

Table 2 Distribution of transmitted and non-transmitted NETpPR AAGG4 alleles and genotypes in AN family trios

	No. trios		*Allele*			*Genotype*			
			L4 (%)	*S4 (%)*	*n*	*L4/L4 (%)*	*L4/S4 (%)*	*S4/S4 (%)*	*n*
AN-R	87	Transmitted	142 (82)	32 (18)	174	57 (66)	28 (32)	2 (2)	87
		Non-transmitted	120 (69)	54 (31)	174	40 (46)	40 (46)	7 (8)	87
AN-BP	14	Transmitted	19 (68)	9 (32)	28	6 (43)	7 (50)	1 (7)	14
		Non-transmitted	24 (86)	4 (14)	28	10 (71)	4 (29)	0 (0)	14

Table 3 TDT of NETpPR AAGG4 alleles in AN family trios

	No. trios			*Not transmitted*	
				L4	*S4*
AN-R[a]	87	Transmitted	L4	100	42
			S4	20	12
AN-BP[b]	14	Transmitted	L4	16	3
			S4	8	1

[a]McNemar's $\chi^2 = 7.806$, df = 1, $P = 0.0052$, odds ratio: 2.10, 95% CI 1.21–3.78.
[b]McNemar's $\chi^2 = 2.273$, df = 1, $P = 0.1317$ (not significant).

A recent study suggests that atypical AN responds to venlafaxine,[24] a serotonin and norepinephrine reuptake inhibitor. Antidepressant treatment with serotonin and norepinephrine reuptake inhibitors results in upregulation of brain-derived neurotrophic factor gene expression[25] resulting in activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase cascade.[26] This results in Elk-1 phosphorylation, increasing its ability to directly bind to its transcription factor site in the promoters of target genes where it has an activating role in gene transcription.[27] Elk-1 is expressed throughout the human brain and is localised exclusively to neurons.[28] Insertion/deletion of potential Elk-1 sites as we have observed in the NETpPR may have important implications for NET gene expression and consequently AN.

In the current study we report the discovery of the NETpPR, a previously undescribed polymorphic region in the 5′ promoter region of the NET gene. Knowledge of the full structure of the promoter is essential for meaningful expression studies. Additionally, within the NETpPR we identified a polymorphism which produced a positive association with AN-R. Replication studies with other groups of AN-R and larger samples of AN-BP are required, as are functional studies to investigate the effect of the NETpPR on NET gene expression. Since the NET gene has pharmacological importance for major depressive disorder it is imperative that association studies be carried out with this condition and with other disorders thought to be influenced by the norepinephrine system.

Materials and methods

Subjects

There were 101 unrelated trios (DSM-IV AN patient + both biological parents) of mainly Caucasian ethnicity

(97 Caucasian: four East Asian) recruited from a total of six sites in Sydney and Melbourne, Australia. Ethics approval had been gained from the appropriate ethics committees and all participants gave written informed consent (parents signed for those under 14 years). Patients were consecutive consenting inpatients (n = 93) or outpatients (n = 8) admitted for treatment of AN. All patients currently fulfilled or had previously fulfilled, the criteria for DSM-IV AN except that the amenorrhoea criteria was waived for the two males and for the prepubertal females. Patients were excluded if one or both parents were not available for testing. Diagnostic information *was/had been* collected over a longitudinal period (ie assessment was ongoing) which allowed accurate classification of patients into the AN subtypes (AN-R and AN-BP). The diagnosis was made (blind to genotype) by clinicians who specialised in eating disorders. In addition, another researcher searched the case notes of each patient to ensure that each of the DSM-IV criteria was present at the time of admission or during treatment, and to check the accuracy of subtype classification. The diagnosis was confirmed by one of three psychiatrists: the principal clinical investigator (KN) or by one of two others (JR, PB) using all previously accumulated data, and by personally interviewing the patient to confirm the presence or absence of DSM-IV criteria for AN and to check that subtyping into AN-R and AN-BP was accurate. The consensus rate between the original and the confirmatory diagnosis was 95%. Where any diagnostic ambiguity existed, KN personally reviewed the case notes to confirm or disconfirm fulfilment of all DSM-IV AN and AN subtype criteria. Ages and BMI were recorded during treatment. BMI was determined from weight and height measurements made by hospital staff, the weight criterion for DSM-IV AN being based on the minimum BMI recorded. All AN patients aged 11–15 years weighed less than or equal to their age and sex-specific 5th percentile BMI cutoff as determined in an Australian population study.[29] Above age 15 years a BMI of 17.5 was used as the cutoff.[30] There were 87 (all female) DSM-IV AN-R, and 14 (12 females: two males) DSM-IV AN-BP. Of the 87 AN-R, 28 were under 15 years of age (mean age 13.63 ± 1.24 years) at their minimum BMI (mean minimum BMI 14.29 ± 1.66) reached at a mean of 0.94 ± 0.58 years after onset of AN, and 59 were aged 15 years and over (mean age 17.92 ± 3.25 years) at minimum BMI (mean minimum BMI 14.69 ± 1.69) reached at a mean of 2.14 ± 2.06 years after onset of AN. All AN-BP females were at least 15 years of age (mean age 18.15 ± 2.22 years) at minimum BMI (mean minimum BMI 14.16 ± 1.39) reached at a mean of 2.58 ± 1.62 years after onset of AN. The two male AN-BP were aged 19.1 and 22.4 years at minimum BMI of 12.5 and 15.4 respectively, reached 2.00 and 2.40 years after onset of AN, respectively.

Molecular genetic methods
A 10 ml EDTA venous blood sample was collected from patients and parents. Genomic DNA was extracted from the blood using standard methods.[31] Primers were designed to PCR-amplify a 525-bp DNA fragment containing the NETpPR: forward primer NETp1Fins 5′GCAGTGTAATATATGCCTATTGTCC (positions −4357 to −4333) and reverse primer NET-p1Rins 5′CGCTATCAGAACCTACACCTC (positions −3833 to −3853). Base numbering related to the start site of a published sequence.[12] PCR reactions were carried out in 10-μl volumes containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP, 0.25 U Ampli-Taq Gold (Applied Biosystems, Foster City, CA, USA), and 50 ng DNA template. The GeneAmp PCR System 9700 (Applied Biosystems) was used for thermal cycling: 95°C for 10 min; 30 cycles of 95°C for 30 s, 67–52.5°C for 30 s (annealing temperature decreased by 0.5°C/cycle over the 30 cycles from 67°C to 52.5°C), 72°C for 40 s; 10 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 40 s; and finally 72°C for 10 min. To allow heteroduplex formation this was followed by 95°C for 3 min, then 60 cycles of 94.5–65°C for 30 s (temperature decreased by 0.5°C/cycle over 60 cycles from 94.5°C to 65°C). PCR products (2-μl) were separated on 8% polyacrylamide (Acryl:Bis 29:1) gels. L4 and S4 alleles were identified by both size and visualisation of the characteristic L4/S4 heteroduplex by electrophoresing at 500 V for 2 h 15 min at room temperature with the Hoefer Model SE600 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with 0.75-mm spacers, followed by staining with ethidium bromide and visualisation of the DNA bands under UV light. Heteroduplexes could also be detected with the Mini-PROTEAN II & III electrophoresis systems (Biorad, Hercules, CA, USA). Detection of L4 and S4 homozygotes was confirmed by separately mixing with a known S4 homozygote and L4 homozygote followed by repeating the 'heteroduplex formation' part of the thermal cycling protocol. This strategy detected the L4/S4 heteroduplex in one of the mixed samples after subsequent electrophoresis, and differentiated the S4 and L4 homozygotes.

The ABI Prism Big Dye Terminator Ready Reaction Kit with Ampli-Taq DNA polymerase, FS (Applied Biosystems) and ABI Prism 310 Genetic Analyser (Applied Biosystems) were used for sequencing the PCR products.

Statistical analysis
The TDT, HRR, and HHRR tests were used to detect associations. The TDT uses only data derived from parents who are heterozygous and detects preferential transmission of one allele to affected children. The HRR test analyses the distribution of genotypes (composed of one allele from each parent) that are transmitted and not transmitted from parent to child. The HHRR test analyses the distribution of alleles that are transmitted and not transmitted from parent to child. In these three tests, the non-transmitted parental alleles (or genotype in the case of the HRR test) act as the control group. This prevents spurious associations resulting from ethnic differences between cases and controls.

As we could not predict which allele or genotype would be preferentially transmitted, two-sided *P*-values were used for all statistical tests. Since there were only two known alleles for AAGG4 the required significance level was set at 0.05. A χ^2 goodness-of-fit test was used for determining Hardy–Weinberg equilibrium and for the HRR and HHRR tests with Yate's correction being applied when appropriate. Fisher's Exact test was used when the expected cell size was less than 5, and when sample sizes were small. The TDT was performed using McNemar's χ^2. The linkage disequilibrium delta value[32] was used to determine whether the S1 and S4 alleles were in linkage disequilibrium.

GenBank/EMBL accession numbers

The DNA sequence of a 525-bp genomic DNA fragment including the 343-bp novel sequence has been deposited in GenBank, accession number AF330628.

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References

1 Kaye WH, Jimerson DC, Lake CR, Ebert MH. Altered norepinephrine metabolism following long-term weight recovery in patients with anorexia nervosa. *Psychiatry Res* 1985; **14**: 333–342.
2 Pirke KM, Kellner M, Philipp E, Laessle R, Krieg JC, Fichter MM. Plasma norepinephrine after a standardized test meal in acute and remitted patients with anorexia nervosa and in healthy controls. *Biol Psychiatry* 1992; **31**: 1074–1077.
3 Holland AJ, Sicotte N, Treasure J. Anorexia nervosa: evidence for a genetic basis. *J Psychosom Res* 1988; **32**: 561–571.
4 Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; **52**: 506–516.
5 Weiner RI, Ganong WF. Role of brain monoamines and histamine in regulation of anterior pituitary secretion. *Physiol Rev* 1978; **58**: 905–976.
6 Kimura T, Share L, Wang BC, Crofton JT. The role of central adrenoreceptors in the control of vasopressin release and blood pressure. *Endocrinology* 1981; **108**: 1829–1836.
7 Krulich L, Giachetti A, Marchlewska-Koj A, Hefco E, Jameson HE. On the role of the central noradrenergic and dopaminergic systems in the regulation of TSH secretion in the rat. *Endocrinology* 1977; **100**: 496–505.
8 Porzgen P, Bonisch H, Bruss M. Molecular cloning and organization of the coding region of the human norepinephrine transporter gene. *Biochem Biophys Res Commun* 1995; **215**: 1145–1150.
9 Shannon JR, Flattem NL, Jordan J, Jacob G, Black BK, Biaggioni I *et al*. Orthostatic intolerance and tachycardia associated with norepinephrine-transporter deficiency. *N Engl J Med* 2000; **342**: 541–549.
10 Stober G, Nöthen MM, Pörzgen P, Brüss M, Bönisch H, Knapp M *et al*. Systematic search for variation in the human norepinephrine transporter gene: identification of five naturally occurring missense mutations and study of association with major psychiatric disorders. *Am J Med Genet* 1996; **67**: 523–532.
11 Iwasa H, Kurabayashi M, Nagai R, Nakamura Y, Tanaka T. Genetic variations in five genes involved in the excitement of cardiomyocytes. *J Hum Genet* 2001; **46**: 549–552.
12 Kim CH, Kim HS, Cubells JF, Kim KS. A previously undescribed intron and extensive 5′ upstream sequence, but not Phox2a-mediated transactivation, are necessary for high level cell type-specific expression of the human norepinephrine transporter gene. *J Biol Chem* 1999; **274**: 6507–6518.
13 Brüss M, Kunz J, Lingen B, Bönisch H. Chromosomal mapping of the human gene for the tricyclic antidepressant-sensitive noradrenaline transporter. *Hum Genet* 1993; **91**: 278–280.
14 Lilley DMJ. Kinking of DNA and RNA by base bulges. *Proc Natl Acad Sci U S A* 1995; **92**: 7140–7142.
15 Quandt K, Frech K, Karas H, Wingender E, Werner T. Matlnd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995; **23**: 4878–4884.
16 Garfinkel PE, Kennedy SH, Kaplan AS. Views on classification and diagnosis of eating disorders. *Can J Psychiatry* 1995; **40**: 445–456.
17 Falk CT, Rubinstein P. Haplotype relative risks: an easy reliable way to construct a proper control sample for risk calculations. *Ann Hum Genet* 1987; **51**: 227–233.
18 Terwilliger JD, Ott J. A haplotype-based 'haplotype relative risk' approach to detecting allelic associations. *Hum Hered* 1992; **42**: 337–346.
19 Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996; **273**: 1516–1517.
20 Lyttle TW. Cheaters sometimes prosper: distortion of mendelian segregation by meiotic drive. *Trends Genet* 1993; **9**: 205–210.
21 Deep AL, Nagy LM, Weltzin TE, Rao R, Kaye WH. Premorbid onset of psychopathology in long-term recovered anorexia nervosa. *Int J Eat Disord* 1995; **17**: 291–297.
22 Charney DS, Bremner JD, Redmond DE Jr. Noradrenergic neural substrates for anxiety and fear. In: Bloom FE, Kupfer DJ (eds). *Psychopharmacology: the Fourth Generation of Progress*. Raven Press: New York, 1995, pp 387–395.
23 Lee CM, Javitch JA, Snyder SH. Recognition sites for norepinephrine uptake: regulation by neurotransmitter. *Science* 1983; **220**: 626–629.
24 Ricca V, Mannucci E, Paionni A, Di Bernardo M, Cellini M, Cabras PL *et al*. Venlafaxine versus fluoxetine in the treatment of atypical anorectic outpatients: a preliminary study. *Eat Weight Disord* 1999; **4**: 10–14.
25 Duman RS, Heninger GR, Nestler EJ. A molecular and cellular theory of depression. *Arch Gen Psychiatry* 1997; **54**: 597–606.
26 Nestler EJ. Antidepressant treatments in the 21st century. *Biol Psychiatry* 1998; **44**: 526–533.
27 Janknecht R, Ernst WH, Pingoud V, Nordheim A. Activation of ternary complex factor Elk-1 by MAP kinases. *EMBO J* 1993; **12**: 5097–5104.
28 Pastorcic M, Das HK. An upstream element containing an ETS binding site is crucial for transcription of the human presenilin-1 gene. *J Biol Chem* 1999; **274**: 24297–24307.
29 Harvey PWJ, Althaus M. The distribution of body mass index in Australian children aged 7–15 years. *Aust J Nutr Diet* 1993; **50**: 151–153.
30 WHO. *The ICD-10 Classification of Mental and Behavioral Disorders—Clinical Descriptions and Diagnostic Guidelines*. World Health Organisation: Geneva, 1992, pp 176–177.
31 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.
32 Mattiuz PL, Ihde D, Piazza A, Ceppellini R, Bodmer WF. New approaches to the population genetic and segregation analysis of the HLA-A system. In: Terasaki PI (ed). *Histocompatibility Testing*. Munksgaard: Copenhagen, 1970, pp 193–205.

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