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The human serotonin transporter gene polymorphism–basic research and clinical implications

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Summary. Mood, emotion, and cognition are modulated by the serotonergic midbrain raphe system, which seems to be involved in the pathogenesis of psychiatric disorders like those of the affective spectrum.

Since a dysregulation of serotonin transporter expression might be important in the course of those disorders, we isolated and cloned the 5′-regulatory region of the serotonin transporter gene, which is inducible by cAMP-and PKC-dependend signal transduction pathways.

Systematic screening for length variations and functional promoter analyses revealed a genetic polymorphism with allelic variation in transcriptional activity and protein expression. This 5-HTT gene polymorphism comprises a tandemly repeated sequence in the 5′-regulatory promoter region of the serotonin transporter gene.

Recent population association studies demonstrated the 5-HTT gene promoter polymorphism accounting for 4–5% of population variation of anxiety-related behavioral traits.

Keywords: Serotonin transporter, gene polymorphism, allelic association, genetics.

Abbreviations

5-HT serotonin, *5-HTT* serotonin transporter, *PKC* protein kinase C, *EBV* Epstein-Barr-Virus.

Introduction

One of the recent advances in neuroscience has been the elucidation of the mechanisms underlying neurotransmitter uptake. In particular, there has been progress in molecular cloning and characterization of multiple neurotransmitter transporters.

These neurotransporters are high affinity carrier proteins, localized in the plasma membrane or vesicle membrane of the presynaptic neuron termina-

tion. They mediate the removal of the neurotransmitter from the synaptic cleft, thereby terminating their action at the receptor. Intracellular vesicular transport systems reaccumulate the neurotransmitters in synaptic vesicles for additional cycles of release. The transmitter influx during the gating process is coupled directly to transmembrane ion gradients, thus providing the energy for the transport against the concentration gradient.

There is an increasing body of evidence, that different neurotransporters are involved in the pathogenesis of several neuropsychiatric disorders, such as neurodegenerative disorders or those of the affective spectrum.

For example, decreased inhibitor binding and 5-HT uptake in platelets and braintissue of patients with affective disorders or suicide victims have been consistently replicated (Ellis and Salmond, 1994). Dysregulation of the 5-HT transporter function or expression may therefore be involved in the pathogenesis of these disorders. Furthermore, the 5-HT transporter is the initial target site of antidepressant drugs but also of drugs of abuse such as cocaine and amphetamines and of neurotoxic drugs like MDMA, called "ecstasy".

Taken together the elucidation of mechanisms underlying the disease-related reduction of 5-HT transporter expression are of clinical interest, and the investigation of physiological and pathophysiological aspects of 5-HTT expression and its regulation or genetic control is warranted.

The 5-HT transporter-gene

Sequence analysis of a cDNA encoding the human 5-HTT revealed the identity of the human platelet-and brain serotonin transporter gene and it has been assigned to the chromosome 17 (Lesch et al., 1994).

With three overlapping DNA fragments we analysed the genomic exon-intron organization demonstrating the 5-HTT gene being composed of 14 exons spanning about 35.000 base pairs. Primer extension studies revealed, that the transcriptional start site is located 207 basepairs upstream of the initiator ATG codon, indicating that the first exon and the beginning of the second exon are normally not translated to a corresponding protein structure (Fig. 1).

Further investigations demonstrated a VNTR in the second intron of the 5-HTT gene. Within a caucasian population, this 17 base pair repetitive element is composed of 9, 10 or 12 repeat units (Lesch et al., 1994).

Furthermore we isolated the 5′-regulatory region as a 1,700 bp genomic insert from a 5-HTT-exon 1-enriched human genomic library. Sequence analysis of this putative promoter region identified a TATA-like motif upstream from the transcription start site. This single start site is located 208 basepairs upstream from the translation initiation codon located in exon 2 (Heils et al., 1995).

Systematic PCR-assisted screening revealed a genetic polymorphism with an additional short variant allele. The variant allele is deficient of the repeat unit 7 and to some extent of unit 6 and 8. In a normal control population of

about 300 caucasians, the frequency of this short allele is 39% with a heterozygosity of 52% (Heils et al., 1996).

In the present study we investigated the effect of the polymorphic promoter element on constitutive transcriptional activity and pharmacological activation by involving a reporter gene system. Allelic variation of serotonin transporter expression was assayed within EBV-transformed B-lymphoblastoid cells which were recently described as a 5-HTT-expressing cell model (Faraj et al., 1994).

Experimental procedures

To elucidate some of the mechanisms of 5-HTT gene regulation, we applied the procedure of transfection-and reporter gene studies with the possibility of pharmacological stimulation. Allelic variation of constitutive serotonin transporter expression was assayed within a B-lymphoblastoid cell model by measuring 5-HT uptake and inhibitor binding.

Reportergene constructs

The long (bp −1428 to +217 with respect to the transcription start site) and short allelic promoter variants were ligated into the promoterless luciferase expression vector pGL3

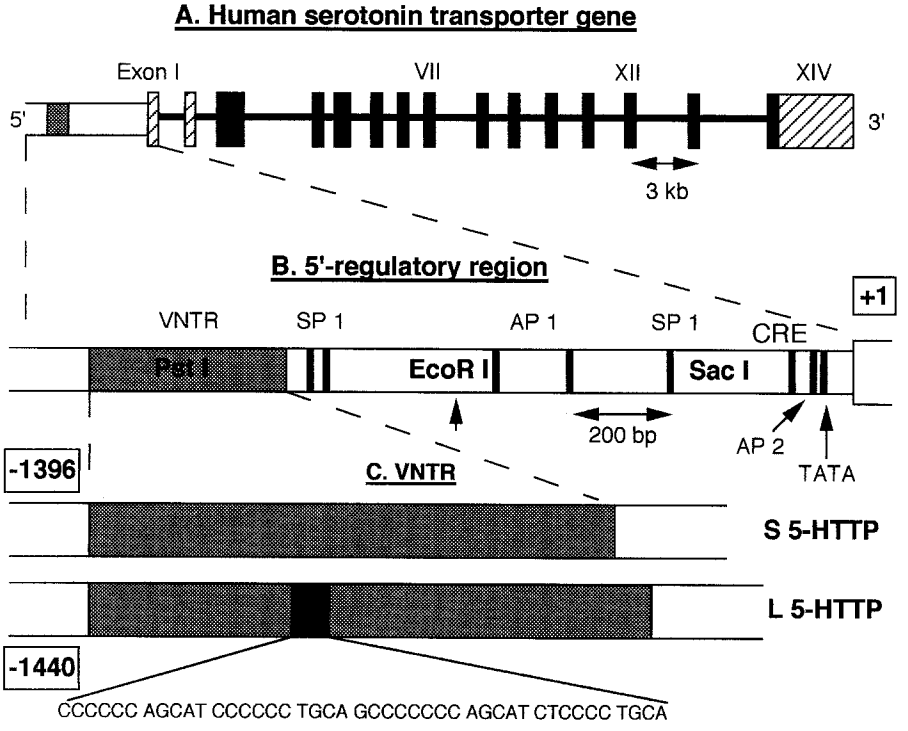

Fig. 1. Organization of the human 5-HTT gene and its 5′ flanking regulatory promoter region. **A** Solid and hatched boxes represent coding and noncoding regions. **B** The 5-HTT gene promoter is definied by a TATA-like motif and several potential transcription factor binding sites. **C** The polymorphic tandem repeat of the 5-HTT gene promoter is defined by a length variation of a repetitive sequence comprising a GC-rich, 20–23-bp-long repeat elements. The polymorphism is generated by a deletion with an overall length of 44 bp

basic (Promega). A series of deletional mutants was performed to determine functional enhancer/silencer elements: P-PB (bp −1186 to +217), P-EB (bp −734 to +217) and P-SB (bp −78 to +217) (Fig. 1).

Heterologous constructs were designed by ligating the long and short repetitive promoter element into the SmaI site of the pGL3 control vector (Promega) containing an SV 40 enhancer/promoter unit (Heils et al., 1995, 1996).

Transfection studies and reporter gene assays

HeLa cells, SK-N-SH cells and JAR human placental choriocarcinoma cells (ATCC HTB 144, which constitutively express the 5-HTT) were grown in RPM1 1640 medium with 10% FCS, gentamycin and L-glutamine at 37°C in a humified atmosphere at 5% CO_2. For transfection 2×10^5 JAR cells/35 mm-diameter plate were exposed for 24 h to 5 µg of construct DNA complexed with 5 µl Transfectam lipofectin reagent (Promega). After transfecting the reporter gene constructs, luciferase activity was measured in comparison to transfection efficiency, which was determined by cotransfection with the pSV-ßGal (Promega). Pharmacological stimulation was carried out 24 hours after transfection with a defined concentration range of PMA and Forskolin.

Another 24 hours after pharmacological stimulation cells were harvested in 1 ml of luciferase lysis buffer and assayed for luciferase activity by addition of 10 µl cell lysate to 100 µl of luciferin reagent. Chemiluminescence was measured as cpm/µg protein in a liquid scintillation counter (Heils et al., 1996).

EBV-transformation of human B-lymphoblastoid cells

Lymphocytes of healthy blood donors with different genotypes concerning the described promoter polymorphism were obtained by a Ficoll-gradient and subsequently infected with a EBV-(Epstein-Barr-virus) containing RPMI 1640 medium. B-cell propagation and T-cell depletion was carried out by administration of cyclosporin.

4 cell lines with a homozygous genotype for the long allele, 3 homozygous for the short variant allele and 3 heterozygote cell lines were established and grown in RPMI 1640 medium, containing 15% FCS. Genotype-dependend [^{125}I] RTI-55 binding- and [^{3}H] seortonin uptake measures were subsequently determined (Lesch et al., 1996).

[^{125}I]RTI-55 binding

RTI-55 [3β-(4-iodophenyl)tropan-2β-carboxyl acid methyl ester tartrate] is a concaine analog which potently inhibits serotonin uptake and binds to the serotonin transporter with high affinity. B-lymphoblastoid cells were washed twice with PBS and desintegrated by ultrasound in a membrane preparation buffer containing 10 mM MOPS, 1 mM sodium EDTA, 0,1 mM benzethoniumchloride, 1 mM benzamidine, 0,1% aprotinin (Trasylol®), and 8% saccharose at 4°C. The homogenate was centrifuged at 3.000 × g and the resulting supernatant again at 60.000 × g.

Equilibrium binding of [^{125}I]RTI-55 to cell membranes was assayed by incubating 50 mg of the protein preparation within a concentration range of the radioligand from 0,05 to 1 nM for 1 hour at room temperature in triplicate. Non-specific binding was determined in the presence of 5 µM paroxetine. Kd-values and Bmax, expressed as fmol/mg protein, were obtained by Scatchard plot analysis (Lesch et al., 1996).

[^{3}H]serotonin uptake

Serotonin uptake of 10^7 lymphoblastoid cells, washed twice with PBS, was measured by incubation the suspended cells with 0.1 to 1 µM [^{3}H]serotonin in triplicate for 10 min at 37°C in the presence or absence of 0,1 mM imipramine in 0,9% sodium-chloride. Incubation was stopped by 0,9% sodium-chloride/1,5% formaldehyde using a cell harvester and

Whatman GF/B filters. Bounded radioactivity was determined in a Beckman liquid scintillation counter. Kinetic analysis revealed Km-and Vmax-values as pmol/10^7 cells × 10 min (Lesch et al., 1996).

Results

The allelic variant long and short reporter gene constructs displayed constitutive promoter activity in 5-HTT expressing JAR human placental choriocarcinoma cells (Fig. 2A and B). Comparison of the polymorphic alleles revealed that the basal activity of the long variant is about threefold higher than that of the 5-HTT promoter with the deletion ($p < 0.001$ by Student's t test). Cyclic AMP-and protein kinase C-dependent mechanisms induced tran-

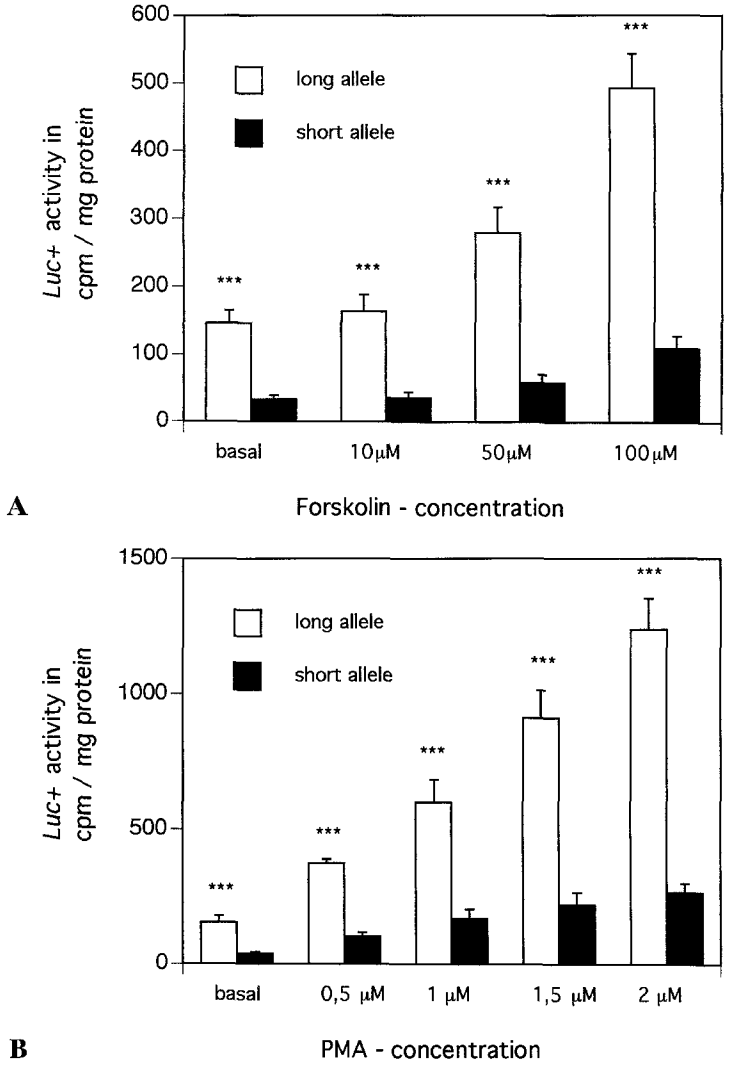

Fig. 2. Luciferase activity in cpm/mg protein after transfection of the long and short promoter allele constructs into JAR cells and induced by cyclic AMP (**A**) and protein kinase C (**B**). ***$p < 0{,}001$ by one-way ANOVA followed by t tests

scriptional activity in both variants of the 5-HTT promoter constructs, but the dose-dependent increase following induction with a concentration range of forskolin or phorbol ester remained proportionally smaller in the allele with the deletion ($p < 0.001$ by one-way ANOVA followed by t test).

We next examined the potential of the two allelic variants to confer differential silencing and/or activating action on the 5-HTT regulatory unit by fusing both the long and short form to a heterologous SV40 enhancer/promoter unit that is active in JAR cells (Fig. 3). The long variant repressed promoter activity to a basal activity of about 19%, but the deleted form was significantly less potent (about 54%; $p < 0.005$ by one-way ANOVA; Fig. 3).

The long promoter construct's transcriptional activity was exclusively expressed in JAR cells. Transfection into 5-HTT-deficient cell lines (HeLa-and SK-N-SH cells; Heils et al., 1996) didn't confer any reporter gene activity. A series of deletional mutants revealed the loss of cell specific gene expression within the EcoRI-and SacI-deletional constructs (Fig. 4). The short variant allele was not examined because of the polymorphic sequence being upstream from the deletional restriction sites.

The influence of the 5-HTT gene promoter polymorphism on 5-HTT expression was assayed by [^{125}I]RTI-55 binding and [^{3}H]5-HT uptake experiments in 5-HTT-expressing EBV-transformed human B-lymphoblastoid cell lines, available with different genotypes (L/L, n = 4; L/S, n = 3; S/S, n = 4) (Fig. 5A and B).

Membrane preparations from L/L lymphoblasts bound 30–40% more [^{125}I]RTI-55 than did membranes from L/S or S/S cells (Fig. 5A).

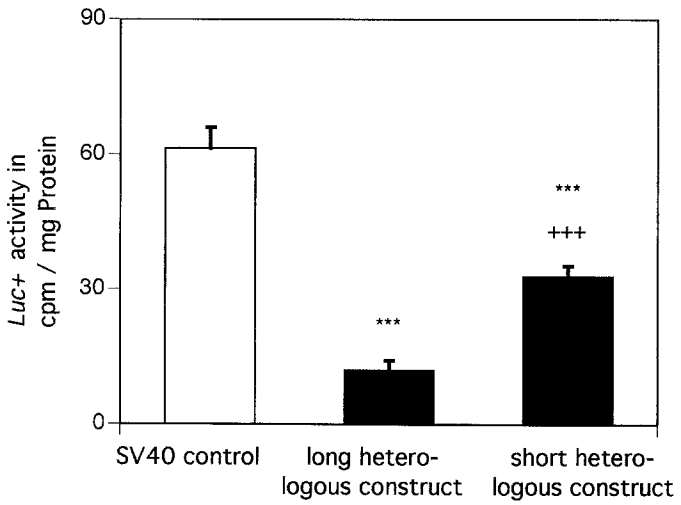

Fig. 3. Transcriptional activity of the long and short allelic variants within the heterologous system compared to the SV 40 promoter activity of the pGL3 control plasmid. The long variant of the polymorphic promoter repeat suppressed the SV 40 promoter more than the short variant did. Long or short variant vs. SV 40 control, ***$p < 0{,}001$ by one-way ANOVA followed by t tests. Long vs. short variant, $^{+++}p < 0{,}005$ by Student's t test

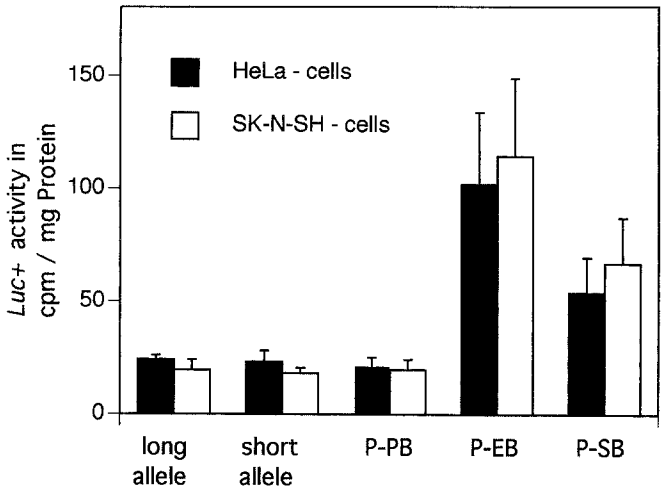

Fig. 4. The information contained within 1,4 kb of the 5′-flanking sequence was sufficient to confer its expression in human JAR cells (data not shown, Heils et al., 1995) but it was inactive in serotonin transporter deficient cell lines like HeLa cells and SK-N-SH cells. A deletion of about 660 bp of the 5′-termination (P-EB deletional mutant) lead to a loss of cell-specific transcriptional activity indicating the localization of cell-specific enhancer/silencer element within the deleted sequence

Moreover, [^{3}H]5-HT uptake in cells homozygous for the long variant promoter allele was 1.9 to 2.2 times that in cells carrying one or two endogenous copies of the short form.

Uptake-and binding parameters persisted proportionally when 5-HTT gene transcription was induced with forskolin or PMA (Fig. 5A and B).

Discussion

We have shown that the 5-HTT gene promoter is defined by a TATA-like motif and several potential binding sites for transcription factors including AP1, AP2, SP1, and a CRE-like motif. The role of these transcription factor motifs in the regulation of 5-HTT gene expression has been established by transfection studies using reporter gene fusion constructs of 5-HTT 5′-flanking sequences. Functional promoter mapping revealed both constitutive and forskolin/PMA-promoter activity and a role for negative attenuating elements and positive elements in the transcriptional regulation of the 5-HTT gene.

The information contained within 1.4 kb of the 5′-flanking sequence was sufficient to confer its cell-specific expression in human JAR cells, but inactive in 5-HTT-deficient human SK-N-SH neuroblastoma and HeLa cells.

Decreased platelet 5-HT transport and reduced [^{3}H]imipramine or [^{3}H]paroxetine binding to brain and platelet 5-HT uptake sites in patients with affective disorders or suicide victims are one of the few relatively consistent findings in psychobilogical research. Since no structural defects in the coding region has been detected (Di Bella et al., 1995), the disease-associated decrease in 5-HT uptake and inhibitor binding is likely to reflect dysregulated

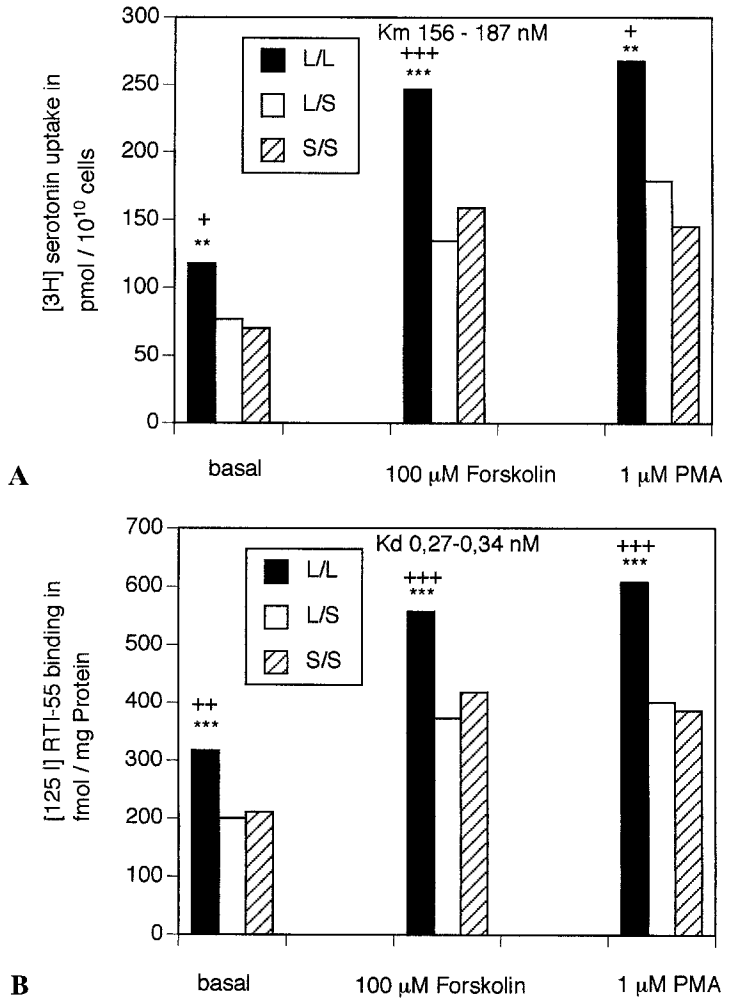

Fig. 5. [^{3}H] serotonin uptake (**A**) and [^{125}I] RTI-55 binding (**B**) in human B-lymphoblastoid cell lines with the genotype long/long (n = 4), long/short (n = 3), and short/short (n = 3). Data were determined basal and after treatment with 100 µM forskolin and 1 µM PMA. (long/long vs. short/short: *$p < 0{,}05$, **$p < 0{,}001$, ***$p < 0{,}001$; $^{+}p < 0{,}05$, $^{++}p < 0{,}01$, $^{+++}p < 0{,}001$; one-way ANOVA followed by Fisher's PLSD test)

expression of the 5-HT transporter gene. In the view of these findings the promoter polymorphism of the 5-HTT gene with different transcriptional activities will provide a basis for a more conclusive answer to emerge as to whether a genetic defect involving the 5-HTT is present in affective disorders.

Because of not reflecting a closed regulatory loop reporter gene assays might give spurious results. We examined native 5-HTT expression in human EBV-transformed B-lymphoblastoid cell lines, and we found that the genotype-dependent differences in 5-HTT expression were not of the same amount but significantly of the same direction as we detected in reporter gene assays.

It was anticipated that differential transcriptional activities of 5-HTT promoter variants contribute to quantitative traits. Therefore, identification of

allelic variation in 5-HTT expression provided the first step toward better understanding of the molecular basis for the previously reported genetic control of 5-HTT function and its potential relevance for complex traits and disease.

To clarify the role of the described classical candidate locus, association studies of allelic variations in 5-HTT gene expression and quantitative traits were performed.

As previously reported, Lesch et al. evaluated the role of the 5-HTT gene promoter polymorphism in personality traits by a combined population and family genetic study of two independently collected groups with 505 subjects in total (Lesch et al., 1996).

The results demonstrated that the polymorphism influences a constellation of traits related to anxiety. Across three personality measures the polymorphism contributes a modest but replicable 3 to 4% of the total variance and 7 to 9% of the genetic variance.

The associations Lesch et al. reported about represent only a small portion of the genetic contribution to anxiety-related personality traits.

If other genes were hypothesized to contribute similar gene dose effects to anxiety, approximately 10 to 15 genes might be predicted to be involved.

In conclusion our findings indicate allelic variation in functional 5-HTT gene promoter activities, which seems to involved in contributing to a small portion of the genetic control of anxiety-related personality traits.

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