Vol. 210, No. 2, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 16, 1995 Pages 530-536

TWO NATURALLY OCCURRING AMINO ACID SUBSTITUTIONS IN THE HUMAN 5-HT_{1A} RECEPTOR: GLYCINE 22 TO SERINE 22 AND ISOLEUCINE 28 TO VALINE 28

Bita Nakhai*, David A.Nielsen, Markku Linnoila and David Goldman

Section of Molecular Genetics, Laboratory of Neurogenetics, NIAAA,
National Institutes of Health, Rockville, MD 20852

Received April 4, 1995

SUMMARY: The human 5-HT_{1A} receptor was screened for naturally occurring mutations. The PCR product of the 5-HT_{1A} receptor gene was digested with several restriction enzymes and evaluated by single-strand conformational polymorphism (SSCP) analysis. Comparison of the SSCP electrophoretic pattern with a restriction map of the 5-HT_{1A} receptor allowed localization of the polymorphic sites facilitating their identification by sequence analysis. Two polymorphisms were identified in the human 5-HT_{1A} receptor gene that altered amino acid composition. The polymorphisms encode amino acid substitutions in the 5-HT_{1A} receptor of a glycine to serine at amino acid 22 and an isoleucine to valine at amino acid 28, respectively. Both polymorphisms alter the extracellular amino terminal domain of the 5- HT_{1A} receptor. The polymorphic 5-HT_{1A} alleles have been found in American and Finnish Caucasians and in native American Indians. This is the first report of a polymorphism in the human 5-HT_{1A} receptor gene that alters the structure of the 5-HT_{1A} receptor protein composition.

The serotonin 1A (5-hydroxytryptamine) receptor gene is located at chromosome 5, q11.2-q13 and encodes the 5-HT_{1A} receptor (1). It belongs to the family of G-protein coupled receptor genes (1) which includes the β adrenergic receptor and the majority of serotonin receptors. The 5-HT_{1A} receptor is expressed presynaptically in cell bodies and dendrites of serotonergic neurons of the dorsal raphe nucleus and postsynaptically in neurons of the hippocampus. Expression of the 5-HT_{1A} gene in the brain is highest in raphe nucleus, septum, hippocampus, entorhinal cortex and interpeduncular nucleus (2). 5-HT_{1A} receptor function is mediated through several interrelated G-protein coupled mechanisms. Upon binding of serotonin, cystosolic Ca^{2+} levels become elevated (3), neuronal firing decreases (4), sodium-dependent phosphate transport is activated via activation of protein kinase C (5), phospholipase C is stimulated to increase inositol phosphates (6), potassium channels open (7) and adenylyl cyclase is inhibited thereby reducing intracellular cAMP levels (8). Furthermore, binding of serotonin to the 5-HT_{1A} receptor inhibits serotonin release (9).

*Corresponding author. FAX: (301) 443-8579.

0006-291X/95 $5.00

Serotonin has been demonstrated to play a major role in many physiological and behavioral functions. These include behaviors characterized by an intolerance to delay, such as impulsivity, self destructive behaviors and appetite control (10). Physiological functions in which serotonin is involved include control of body temperature and sleep (11). These behaviors and physiological functions are modulated, in part, by binding of serotonin to the 5-HT_{1A} receptor. There is evidence in rodents that the 5-HT_{1A} receptor may be involved in alcohol consumption (12). Density of 5-HT_{1A} receptors in frontal cerebral cortex and hippocampus of alcohol-preferring rats was significantly higher than in alcohol non-preferring rats. This was accompanied by a concomitant decrease in the K_D of the 5-HT_{1A} receptor for serotonin. Furthermore the 5-HT_{1A} receptor appears to be involved in aggressive behavior. Specific 5-HT_{1A} agonists can block resident-intruder and maternal aggression (13).

Although the 5-HT_{1A} receptor has been implicated in many behavioral and physiological functions the genetic factors determining individual differences are unknown. Genetic variants of the 5-HT_{1A} receptor could be involved in the predisposition to certain behaviors such as alcoholism and aggression as well as in differences in pharmacokinetic responses to drugs which act via 5-HT_{1A} receptor binding.

To understand the role of the 5-HT_{1A} receptor in behavioral and physiological responses we have examined the 5-HT_{1A} receptor gene in several human populations. Two DNA transitions were identified which altered the protein sequence of the human 5-HT_{1A} receptor.

MATERIALS AND METHODS

PCR Amplification: Genomic human DNA was analyzed for polymorphisms in the 5-HT_{1A} gene by single-strand conformational polymorphism analysis (SSCP) followed by direct sequencing of amplified DNA. PCR amplification was performed with 100 ng DNA, 100 nM of primer A 5'-TTCGCGCTGCTTTTTCTTCC-3' and primer B 5'-CTACTCCTCCGTCATCACTG-3', 250 μM each of dCTP, dGTP, dTTP and dATP, 15 mM $(NH_4)SO_4$, 200 μM spermidine, 2 mM $MgCl_2$, 60 mM Tris, pH 10.0, 2.5 μCi [α-^{32}P] dCTP and 1.25 units Taq polymerase (Boehringer Mannheim) in 12.5 μl. Samples were amplified for 30 cycles, each cycle consisting of 1 min at 95°C, 2 min. at 60°C and 3 min. at 72°C, followed by 7 min. at 72°C.

Restriction Enzyme Analysis of PCR Product by SSCP Analysis: The PCR product was digested with *Alu*I, *Hae*III and *Hinf*I (by single or double digestion) according to manufacturers' recommendations. The samples were then denatured with 9.5 mM NaOH and 90% formamide at 100°C for 2 minutes. Samples were electrophoresed on a 6% nondenaturing polyacrylamide gel (14) overnight at 200V and dried. DNA bands were detected by autoradiography or by using a Molecular Dynamics PhosphorImager 400.

DNA Sequencing: The polymorphic region was sequenced using the afore mentioned primers or primer C 5'-TGCACCATTAGCAAGGATCAT-3'. Amplified DNA was purified (Wizard™ PCR prep DNA purification system, Promega) and sequenced with ^{32}P end-labeled primers using the dideoxy cycle sequencing kit (Life Technology). Samples were electrophoresed in a 6% polyacrylamide gel, dried and visualized using a Molecular Dynamics PhosphorImager 400 or by autoradiography on x-ray film.

RESULTS

Identification and characterization of 5-HT_{1A} polymorphisms by restriction digestion.

SSCP analysis of the full length PCR product identified two polymorphisms in the coding region of the human 5-HT_{1A} receptor gene (data not shown). Since the initial PCR product was

1330 bp in length we employed a novel restriction mapping technique to localize the polymorphic site. The polymorphic alleles and several control DNAs were PCR amplified, digested with restriction enzymes and analyzed by SSCP analysis. The restriction enzymes *Alu*I, *Hae*III and *Hinf*I were used for single and double digestions. The resulting SSCP gel of the digested PCR product was compared with the restriction map of the 5-HT_{1A} gene (Fig. 1C). Digestion of the PCR product with *Alu*I produced five fragments out of which only one of the bands showed altered mobilities compared to the control DNA (Fig 1A lane 1&2, 1B lane 3). Digestion of the PCR product with *Hae*III plus *Alu*I (Fig1A lane 2, 1B, lane 2), *Hinf*I plus *Alu*I (Fig1A lane 2, 1B, lane 2), or *Hinf*I, *Hae*III plus *Alu*I (Fig1A lane 3, 1B, lane 3), did not alter the mobility of the fragment containing the polymorphism as compared to digestion with *Alu*I alone. These results indicated that the mutations reside within one of the two *Alu*I - *Alu*I fragments or the 3' or 5' terminal *Alu*I fragments of the 5-HT_{1A} PCR product (Fig. 1C).

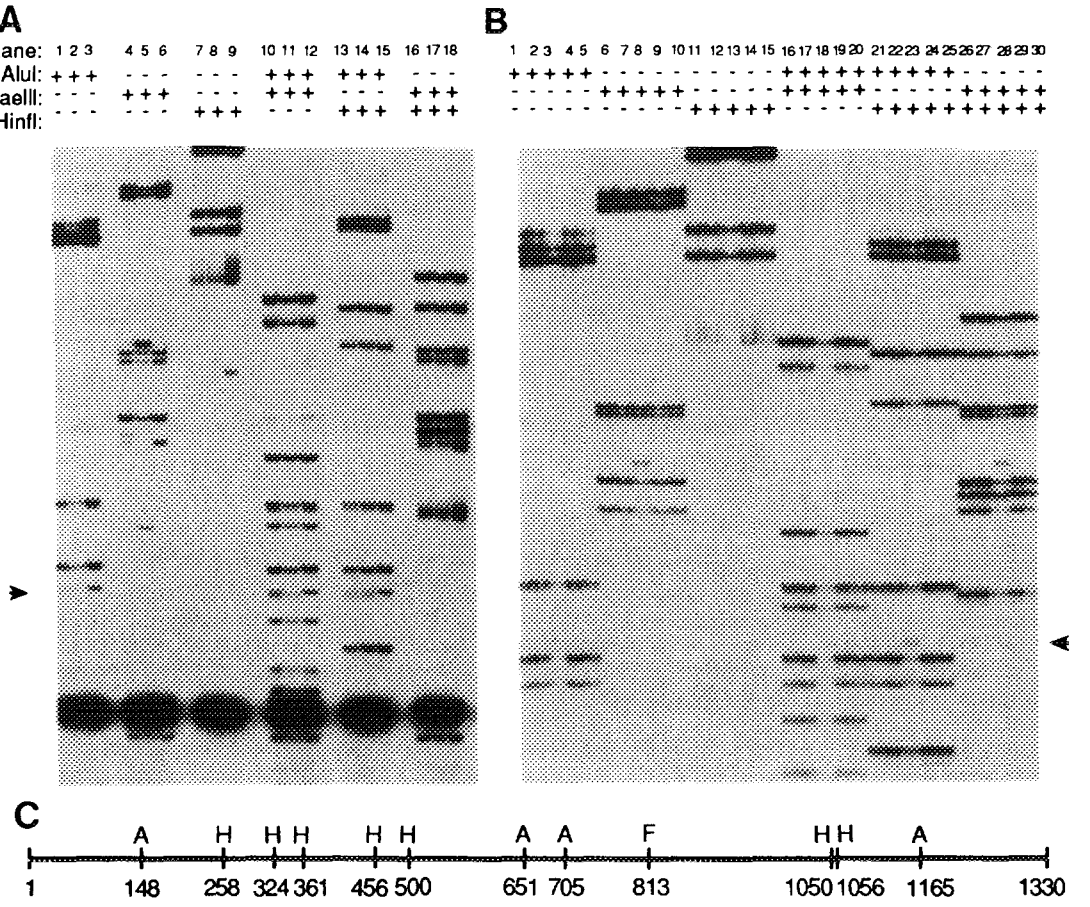

Figure 1. SSCP/restriction enzyme analysis of the 5-HT_{1A} alleles. (**A**) Characterization of the 5-HT_{1A}-746A variant. Amplified DNA was digested with *Alu*I, *Hae*III and *Hinf*I in single and double digestions as indicated. Lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, 14 ,16 and 17 are heterozygous for the 5-HT_{1A}-746A allele and lanes 3, 6, 9, 12, 15 and 18 are homozygous for the wild type allele. The arrow indicates the 5' *Alu*I fragment with altered SSCP mobility. (**B**) Characterization of the 5-HT_{1A}-764 mutation. Lanes 3, 8, 13, 18, 23 and 28 are heterozygous for the 5-HT_{1A}-764 allele and lanes 1, 2, 4-7, 9-12, 14-17, 19-22, 24-27, 29 and 30 are homozygous for the wild type allele. The arrow indicates the 5' *Alu*I fragment with altered SSCP mobility.

Sequence analysis and identification of the 5-HT$_{1A}$ allele polymorphisms.

The two *Alu*I - *Alu*I fragments and 5' *Alu*I fragment of the polymorphic alleles and control DNAs were sequenced by cycle sequencing. Two polymorphisms were identified by sequencing in the amino terminal coding region of the human 5-HT$_{1A}$ receptor gene (Fig. 2). The first polymorphism was a G to A transition at nucleotide 746 (5-HT$_{1A}$-746A). This nucleotide transition codes for an amino acid substitution of serine for glycine at amino acid 22. The second polymorphism was an A to G transition at nucleotide 764 (5-HT$_{1A}$-764G). This polymorphism produces a valine for isoleucine substitution at amino acid 28. In addition, the nucleotide 746 G to A transition creates a restriction fragment length polymorphism (RFLP) in the 5-HT$_{1A}$ gene with the *Nsp*B II restriction enzyme. Both amino acid substitutions were found near the amino-terminus, in the extracellular domain of the receptor.

Population frequencies of the 5-HT$_{1A}$-746A and the 5-HT$_{1A}$-764G alleles.

Table I lists the abundances of the polymorphic 5-HT$_{1A}$ alleles in three populations. Although the polymorphisms identified are relatively rare, both polymorphic alleles were found in more than one population. The 5-HT$_{1A}$-746A allele was found in one Cheyenne Indian and in one American Caucasian. The 5-HT$_{1A}$-764G allele was found in one Finnish subject and has been observed in other populations (15).

DISCUSSION

Two nucleotide substitutions have been identified in the 5-HT$_{1A}$ receptor gene. Both mutations alter the amino acid coding sequence of the 5-HT$_{1A}$ receptor gene. The first polymorphism identified encodes an amino acid substitutions of a glycine to serine at amino acid 22. The second mutation encodes an isoleucine to valine substitution at amino acid 28. When expressed these polymorphisms will alter the extracellular amino terminal domain of the 5-HT$_{1A}$ receptor. Both these amino acid residues, 22 and 28, are conserved between human and rat 5-HT$_{1A}$ receptors (16), perhaps indicating a conservation of function. We believe this report is the

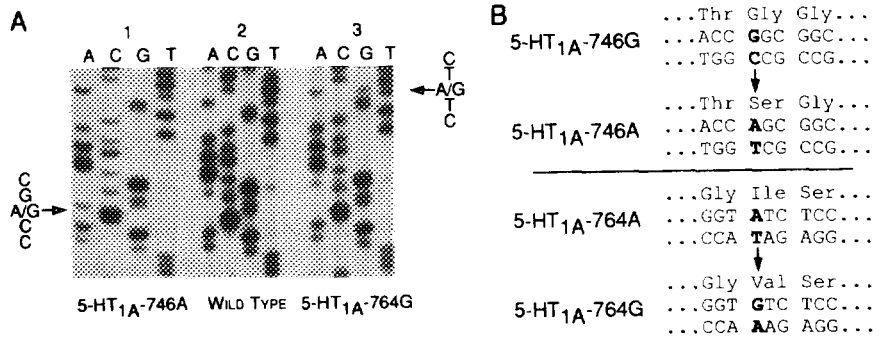

Figure 2. Sequence analysis of the 5-HT$_{1A}$-746A, 5-HT$_{1A}$ wild type and 5-HT$_{1A}$-764G alleles. (**A**) Sequencing autoradiogram of part of the coding region of the wild type gene and variants. The arrows indicate nucleotide substitutions in the coding region of the 5-HT$_{1A}$ receptor gene. Panel 1 displays the 5-HT$_{1A}$-746A transition, panel 2 displays the wild type sequence and panel 3 displays the 5-HT$_{1A}$-764G variant. Sequencing was performed as described in text. (**B**) Partial nucleotide and amino acid sequences of the coding regions of the wild type and the two variants with their amino acid sequences.

Table I

Population distribution of the 5-HT_{1A}-746A and the 5-HT_{1A}-764G alleles *

Population	Wild type	5-HT_{1A}-746A	5-HT_{1A}-764G
American Caucasian	82	1	0
Cheyenne Indians	65	1	0
Finns	189	0	1

* Numbers in the table are individuals.

first to identify naturally occurring mutations in the 5-HT_{1A} receptor gene that alter 5-HT_{1A} receptor amino acid composition.

It is somewhat surprising that the 5-HT_{1A}-746A allele was found in two distant populations; Cheyenne Indians and American Caucasians. 5-HT_{1A}-764G was also found in multiple populations (15). This could either indicate that these variants are ancient or that the mutations arose independently. However, if the origins of the variants are independent in the different populations it is very curious that only these two rare amino acid substitutions are observed with the same nucleotide substitutions involved.

In the closely related human $ß_2$-adrenergic receptor, naturally-occurring substitutions have also been identified in the extracellular domain of the protein near the amino terminus (17). The $ß_2$-adrenergic genes containing these substitutions, at residues 16 and 27, have been expressed in cultured cells. The mutated $ß_2$-adrenergic receptors expressed in the transfected cells displayed normal agonist binding and functional coupling to G_S resulting in stimulation of adenylyl cyclase. Both mutations, however, altered agonist-promoted receptor downregulation and receptor degradation. These results indicate that this region in the $ß_2$-adrenergic receptor is not involved in agonist binding or signal transduction but functions in modulating receptor density. Since the 5-HT_{1A} amino acid substitutions identified here are also located in the extracellular amino terminus of the receptor, we can speculate that they may alter receptor function in a fashion similar to the $ß_2$-adrenergic receptor variants.

The 5-HT_{1A} receptor has been implicated in alcohol-preference (12) and in aggressive behavior (13) in rodent models. Therefore, it is possible that the glycine 22 to serine 22 and isolucine 28 to valine 28 substitutions in the 5-HT_{1A} receptor could be determinants of interindividual variation in these behaviors in the human.

The method used in this study of combining SSCP analysis with restriction enzyme mapping localized the polymorphic site to a subregion of the 5-HT_{1A} receptor gene. This eliminated the need for the complete sequencing of the 1330 bp of the 5-HT_{1A} receptor cDNA(18).

Experiments are in progress to characterize the mutant 5-HT_{1A} alleles. The human 5-HT_{1A} receptor cDNA was cloned into a mammalian expression vector. Site-directed mutagenesis (19) was used to introduce the glycine 22 to serine 22 and the isoleucine 28 to valine 28 amino acid substitutions. Cultured cells were transfected with these constructs (20) and cell lines

expressing these receptors have been established. These cell lines are being screened for 5-HT_{1A} expression. Experiments are in progress to determine receptor binding affinities and to characterize receptor downregulation and degradation. In preliminary experiments, the mutant 5-HT_{1A} receptors displayed normal agonist binding which was similar to the binding to the normal 5-HT_{1A} receptor. This was expected for the polymorphic 5-HT_{1A} receptors since in similar experiments with the human β_2-adrenergic receptor agonist binding was unaffected by mutations in this region .

ACKNOWLEDGMENTS

We would like to thank Longina Akhtar and Gary L. Jenkins for their excellent technical assistance.

REFERENCES

1. Kobilka, B., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T., Francke, U., Lefkowitz, R.J. and Caron, M.G. (1987). Nature 329, 75-79.
2. El Mestikawy, S., Fargin, A., Raymond, J.R., Gozlan, H. and Hnatowich, M. (1991) Neurochemical Res. 16, 1-10.
3. Aune, T.M., McGarth, K.M., Sarr, T., Bombara, M.P. and Kelly, K.A. (1993) J. Immunology 151,1175-1183.
4. VanderMaelen, C.P., Matheson, G.K., Wilderman, R.C. and Patterson, L.A. (1986) Eur. J. Pharmcol. 129, 123-130.
5. Middleton, J.P., Raymond, J.R., Whorton, A.R. and Dennis, V.W. (1990) J. Clin. Invest. 86, 1799-1805.
6. Fargin, A., Raymond, J.R., Regan, R.W., Cotecchia, S., Lefkowitz, R.J. and Caron, M.G. (1989) J. Biol. Chem. 264, 14848-14852.
7. Andrade, R., Malenka, R.C. and Nicoll, R.A. (1986) Science 234, 1261-1265.
8. Albert, P.R., Zhou, Q-Y., Van Tol, H.H.M., Bunzow, J.R. and Civelli, O. (1990) J. Biol. Chem. 265, 5825-5832.
9. Sharp, T., Bramwell, S.R. and Grahame-Smith, D.G. (1989) Br. J. Pharmacol. 96, 283-290.
10. Soubrie, P. (1986) Behav. Brain, Sci. 9, 319-364.
11. Linnoila, M., Virkkunen, M., George, T., Eckardt, M., Higley, J.D., Nielsen, D. and Goldman, D. (1994) Toward a Molecular Basis of Alcohol Use and Abuse (Janson B., Jornvall H., Rydberg U., Terenius L. and Vallee B.L. Ed), pp 154-163, Birkhauser Verlag Basel, Switzerland.
12. Wong, D.T., Threlkeld, P. G., Lawrence L. and Li, T-K. (1990) Life Sciences 46, 231-235.
13. Mos, J., Olivier, B and Tulp, M.Th.M. (1992) Drug Development Research 26, 343-360.
14. Nielsen, D.A., Dean, M. and Goldman, D. (1992) Amer. J. Human. Genet. 51, 1366-1371.

15. Bergen, A.W., Wang, C.-Y., Nakhai, B. and Goldman, D.A. (1995) Human Mutation (In press).
16. Fujiwara, Y., Nelson, D.L., Kashihara, K., Varga, E., Roeske, W.R. and Yamamura, H.I. (1990) Life Science 47, PL-127-PL-132.
17. Green, S.A., Turk, J., Innis, M. and Liggett, S.B. (1994) Biochemistry 33, 9414-9419.
18. Nakhai, B., Goldman, D. and Nielsen, D.A. (1995) (manuscript submitted).
19. Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488-92.
20. Nielsen, D.A., Chou, J., MacKrell, A.J., Casadaban, M. and Steiner, D.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5198-5202.