Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 107-114

POLYMORPHISMS OF THE D4 DOPAMINE RECEPTOR ALLELES IN CHRONIC ALCOHOLISM

Susan R. George, Regina Cheng, Tuan Nguyen, Yedy Israel and Brian F. O'Dowd

Primary Mechanisms Department, Addiction Research Foundation, Toronto,and the Departments of Pharmacology and Medicine, University of Toronto, Toronto M5S 1A8, CANADA

Received August 17, 1993

We have screened genomic DNA for the identification of D4 dopamine receptor polymorphisms. We show that the D4 dopamine receptor genotype in 72 severely affected chronic alcoholics is heterogenous, with individuals homozygous and heterozygous for the various D4 receptor alleles. Alcoholics demonstrated a greater prevalence of the D4(3) ($p<0.005$) and D4(6) ($p<0.005$) alleles than has been reported in normals. There was a high prevalence of nicotine abuse among all D4 genotypes. The frequency of other drug abuse was higher in the D4(3,3) and the D4(4,7) groups, and the family history was strongly positive in the D4(2,4) group. The distribution of the D2 alleles showed equivalence in all D4 genotypes, except in D4(4,6) and D4(4,7) in whom the prevalence of the D2 A1A2 allele was 2-fold higher. The polymorphic variations of the D4 receptor genes should be among the factors considered in the assessment of individual differences in susceptibility to disorders such as alcohol abuse or drug addiction that may be mediated through central dopaminergic systems.

It is generally agreed that alcohol abuse and alcoholism are most likely due to the interaction of both genetic and environmental factors (1). However, even though there is growing evidence supporting an important genetic contribution to the etiology of alcohol abuse, the molecular basis for the genetic predisposition remains largely unknown. The reinforcing properties of many substances of abuse such as alcohol, may be mediated through common neurochemical substrates in brain. In this regard, the dopamine neuronal system has been implicated in reward and reinforcement (2,3), and a role for dopamine in mediating the rewarding effects of alcohol has been advanced (4).

The functions of dopamine in brain are mediated by receptors that transduce the signal across the cell membrane to alter neuronal function. Differences of receptor expression, structure, or allelic composition may possibly affect transmitter function and may potentially be implicated in the pathophysiology of diseases in which the transmitter system plays a role. The identification by molecular cloning of the genes encoding the receptors for dopamine in recent years has revealed the remarkable heterogeneity of the proteins that mediate its function (5) and suggest the potential for genetic differences in the receptor proteins to be linked to differences in the perception or mediation of reward, and has enabled the molecular genetic analysis of genotype differences in disorders such as alcohol abuse.

The human dopamine D2 receptor has been reported to show an allelic association with alcoholism in some studies (6,7), but not in others (8), and although still controversial, the overall

0006-291X/93 $4.00

survey of these studies does not suggest causality, but rather a modifying influence on the clinical expression of the disorder (9). The cloning of the human D4 dopamine receptor (10) has revealed the structural similarity of this receptor gene with the D2 receptor gene and the very similar pharmacological profile of these two receptors. A most interesting aspect of the structure of the D4 dopamine receptor is the heterogeneity of the third cytoplasmic loop, between transmembrane regions 5 and 6, imparted by the number of variable 16 amino acid repeat sequences present (11), as shown in Figure 1A. The significance of the polymorphism of the D4 receptor is not yet known. In the present study, we present (i) a method to identify and characterize dopamine D4 receptor polymorphisms, (ii) data on the association between polymorphism at D4 alleles and alcoholism in a population of chronic alcoholics, and (iii) data relating the D4 receptor polymorphisms with the different allelic variants of D2 dopamine receptors in alcoholics.

METHODS

Study Population

The sample population consisted of 72 chronic alcoholics of Caucasian origin, of average age 40.5 ± 1.41 years, with males representing 86% and females 14%, admitted to the Emergency departments of 3 University teaching hospitals in Toronto for medical complications of alcoholism. The subjects were all habitual consumers of alcohol, ranging from 120-700 gm/day (average 315 ± 145 gm/day) for 6-7 days per week. The average duration of abstinence was 0.4 days, and 49% were acutely intoxicated at the time of presentation. After detoxification, informed consent was obtained from the patients, allowing the use of a blood sample for a study of markers of alcoholism. Details of the demographics and other population characteristics are summarized in Table I.

For this initial study, the alcoholics were compared to large databases for control populations, available from the literature. A general problem in this field has been the identification of an appropriate control group for comparison. Ideally, the control group should involve non-affected relatives in the families of the proband alcoholics.

Southern Blot Analysis

Human DNA was isolated from blood samples, digested with Pst1 and HincII (12), and electrophoresed on 2% agarose gels, and transferred onto nylon membrane. The blots were probed with a radiolabelled cDNA fragment (0.5kb) encoding the third cytoplasmic loop of D4 and washed with 2XSSC, 0.1% SDS at 50°C for 20 min.and with 0.1XSSC, 0.1% SDS and exposed to X-ray film overnight at -70°C with an intensifying screen.

Genotyping by PCR

Human genomic DNA was subjected to amplification by PCR. The methods were as follows: the DNA sample (in a final volume of 10% DMSO) was submitted to 30 cycles in the PCR with oligonucleotides A1 (5'-CTG CGG GTC TGC GGT GGA GTC TGG-3') and A2 (5'-GCT CAT GCT GCT GCT CTA CTG GGC-3') . These oligonucleotides were devised from the D4 sequence flanking the repeat sequence in the third cytoplasmic loop (figure 1). The timing of PCR was 1.5 min. at 93°C, 2 min. at 55°C, and 3 min. at 72°C as described (13). The DNA was

Table I: Characteristics of the chronic alcoholic subjects

Age (yrs)	Quantity (gm/day)	Frequency (days/wk)	BAC (mmol/L)	AST (IU/L)	ALT (IU/L)	GGT (U/L)
40.51 ±1.41	**315.72 ±17.29**	**6.76 ±0.09**	**20.01 ±3.20**	**123.28 ±43.49**	**73.29 ±13.32**	**293.85 ±2.39**

Quantity = alcohol consumed per day
Frequency = alcohol drinking days per week
BAC = blood alcohol concentration
AST = aspartate aminotransferase (≤37 IU/L)
ALT = alanine aminotransferase (≤40 IU/L)
GGT = gamma glutamyl transferase (≤50 U/L)

electrophoresed in a 1.5% agarose gel and visualized by UV light after staining with ethidium bromide.

Statistical Analysis

Comparisons between populations or groups were performed using the Chi square distribution test, and designated as significant if $p<0.05$.

RESULTS

Genetic typing of the D4 dopamine receptor in the 72 alcoholic subjects was carried out by two different methods: Southern blot and PCR analysis. The results of the Southern blotting technique were confirmed by PCR analysis. A typical Southern blot analysis of the human genomic DNA isolated from 7 different unrelated individuals, after digestion of the DNA with the restriction endonucleases, and probed with a D4 cDNA clone is shown in Fig. 1B. The D4 polymorphisms resulted in hybridizing bands of varying sizes and the different allelic fragments were sized at 520 bp, 570 bp, 620 bp, 670 bp, 720 bp and 760 bp by DNA size markers on the gel electrophoresis, compatible with a pattern of the 48 bp repeats of 2, 3, 4, 5, 6, and 7 respectively. This pattern of polymorphisms is in keeping with a previous report of the D4 receptor variants in the human population (11). PCR analysis of the alcoholic subjects confirmed the genotyping results obtained from the Southern blot analysis; examples are shown in Fig. 1C.

The distribution of the D4 receptor allelic variants in our population of severe chronic alcoholics is shown in Fig. 2. The results from the 144 chromosomes from the alcoholic subjects is contrasted to the reported distribution of the D4 alleles in 462 chromosomes from normal individuals, combined from 2 separate studies (14, 15) The most frequently occuring allele in both the normal and alcoholic populations was the 4-repeat of the 48 bp segment D4(4), present in 73%

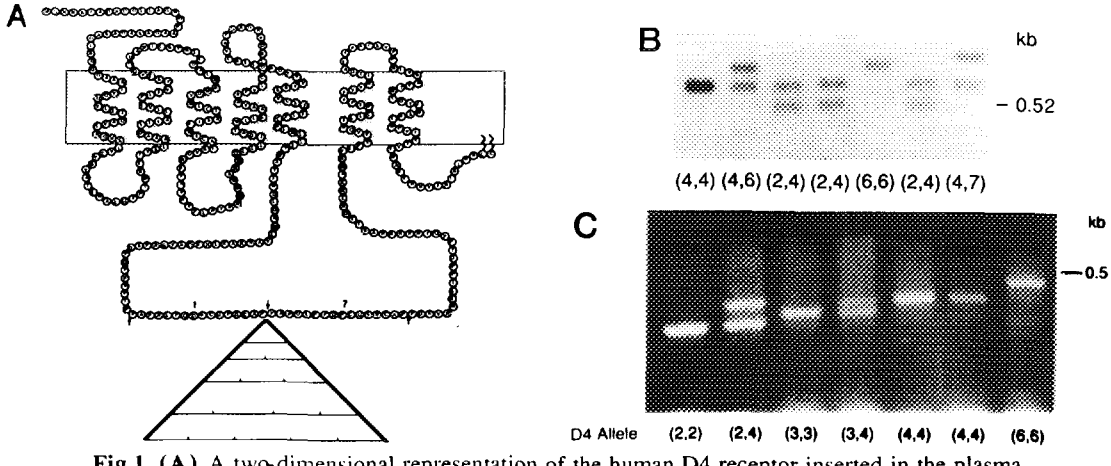

Fig.1. **(A)** A two-dimensional representation of the human D4 receptor inserted in the plasma membrane. The position of the 5 variable (48 bp) repeat sequences in the third cytoplasmic loop of the D4 receptor are indicated by the insert, while the repeats 1 and 7 are indicated by the arrows. **(B)** Southern blot analysis. Human genomic DNA (5μg) was digested with PstI/HincII and electrophoresed on a 2% agarose gel, transferred onto nylon membrane and hybridized with a D4 cDNA probe. The numbers below each lane indicates the D4 allelic composition of the sample. **(C)** PCR analysis of the human genomic DNA samples. A segment of the D4 genes in each sample was amplified as described in Methods, electrophoresed on a 2% agarose gel and visualized by ethidium bromide. The numbers below each lane indicate the D4 allelic composition.

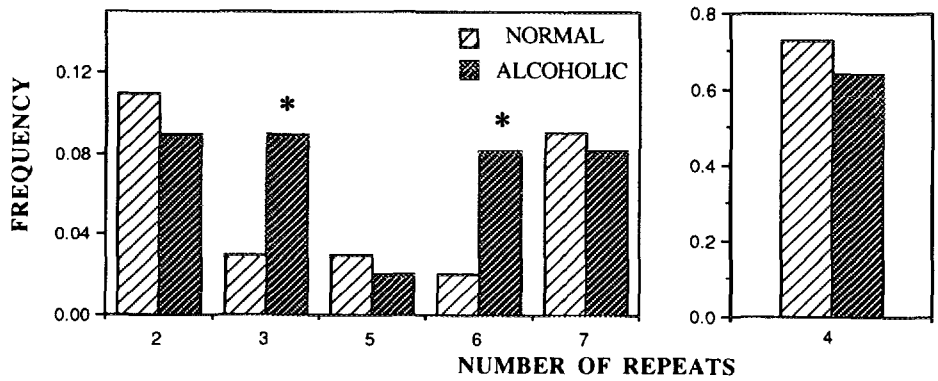

Fig. 2. Comparison of the distribution of the D4 repeats in the normal population and the alcoholic subjects. * denotes $p<0.005$ using the Chi square test.

of normal chromosomes and 64% of alcoholics. The frequency of the 3-repeat sequence allele, D4(3) was 3 fold higher ($p<0.005$), and the 6-repeat D4(6) was 4 fold higher ($p<0.005$) in the alcoholics compared to the normals. The D4(2), D4(4) D4(5) and D4(7) alleles were present in proportions equivalent to that documented in the normal population. Certain combinations of alleles were not seen at all in the alcoholic population.

The frequency of the A1 and A2 alleles of the D2 receptor gene in our population were 22% and 78% respectively, with the D2 genotypes represented as follows: A1A1 (4%), A1A2 (38%) and A2A2 (58%) as shown in Fig. 3A and B. The distribution of the D2 alleles relative to the D4

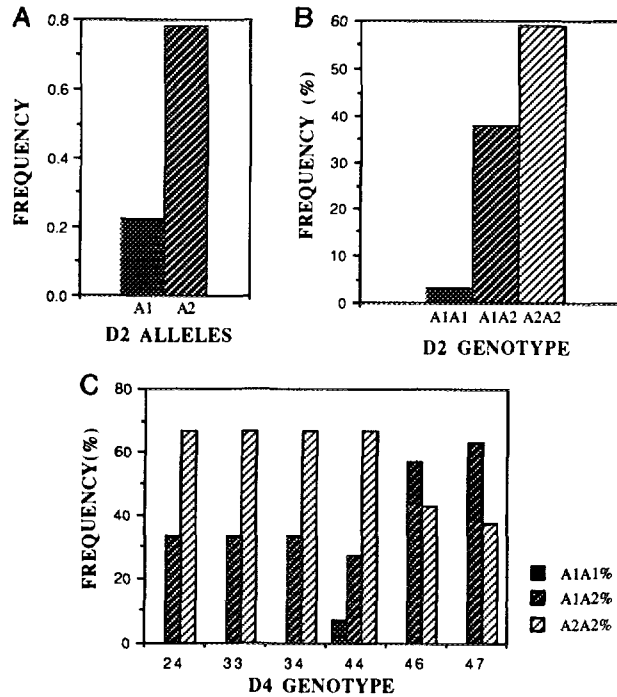

Fig. 3. The frequency of (A) the D2 alleles, (B) the D2 genotype and (C) the co-association of the D2 and D4 genotypes in our population of chronic alcoholics.

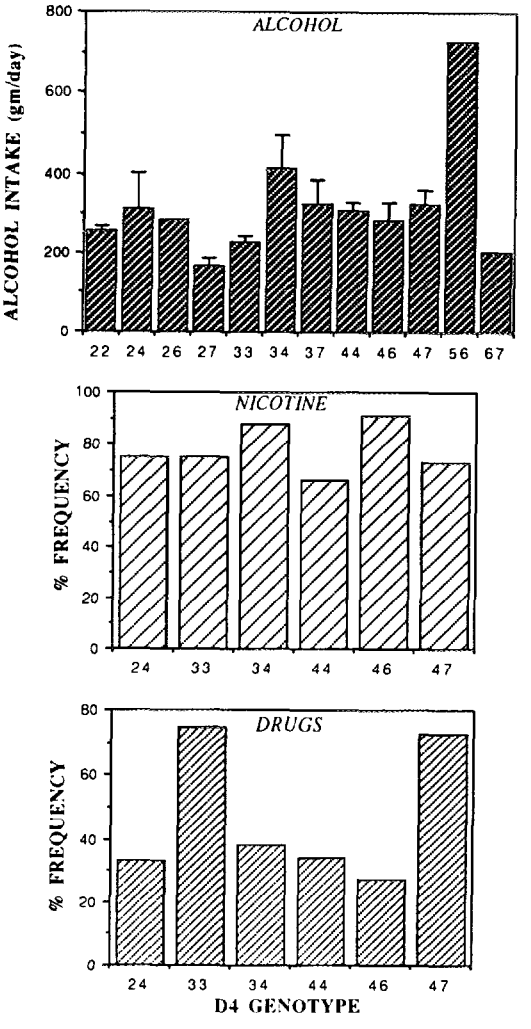

Fig. 4. Comparison of the alcohol intake and the frequency of cigarette smoking (>25/day) and other drug abuse in the alcoholics, grouped according to D4 genotype.

genotype revealed that in the individuals with the D4(2,4), D4(3,3), D4(3,4) and D4(4,4) genotypes, the proportion of A1A2:A2A2 was ~30:70 as shown in Fig. 3C. However, for the genotypes D4(4,6) and D4(4,7), the proportions were drastically altered with the A1A2 genotype accounting for ~60% of the individuals. The individuals with the A1A1 genotype all had the D4(4,4) alleles.

The mean daily intake of alcohol in this group of severe alcoholics is depicted in Fig. 4. As shown, the average intake of alcohol exceeded 300 gm/day, but for all D4 genotypes were in the range of 200-400 gm/day. The highest alcohol consumption was in an individual with the D4(5,6) allelic pattern; however there was only one subject with this genotype.

The pattern of other substance abuse in this population of chronic alcoholics was determined, and as shown in Fig. 4, there was an extremely high incidence of cigarette smoking, reported in 70-90% of the individuals in all of the represented D4 allelic combinations. Similarly, the abuse of other drugs was documented, and 30-40% of subjects with the D4(2,4), (3,4), (4,4)

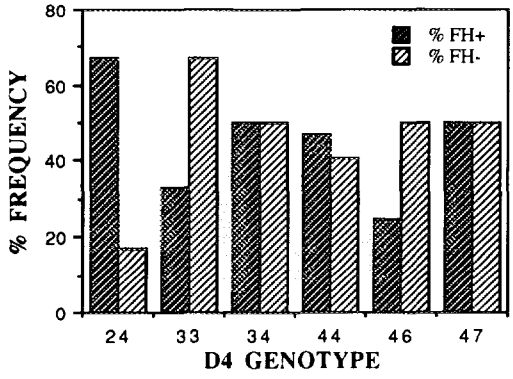

Fig. 5. The incidence of a positive (FH+) and negative (FH-) family history of alcohol abuse in first degree relatives of the alcoholic subjects.

and (4,6) alleles reported a history of drug abuse. The incidence of drug abuse in D4(3,3) and D4(4,7) was higher, in the range of 70-80%. The presence of a positive family history appeared more likely in the D4(2,4) group, at ~70%, whereas the incidence of a positive family history in the other groups ranged 30-50%, as shown in Fig. 5.

DISCUSSION

We have used an efficient method for the characterization of the polymorphisms of the D4 dopamine receptor, for which we have validated the reliability and accuracy by PCR. This technique should facilitate the rapid screening of populations to examine D4 receptor polymorphisms.

The alcoholics in our study met the criteria for alcohol dependence. Ancillary parameters determined e.g. cost of alcohol per week, liver function tests and blood biochemistry were used to confirm the severity of the alcohol abuse problem. In the alcoholic subjects, the distribution of the D4 receptor gene alleles differed significantly from normals, with D4(3) and D4(6) present at 3- and 4-fold higher prevalence respectively. The overall incidence of the D2 alleles, A1 and A2 in our population were 22% and 78%. This differs from the incidence of 9% and 91% reported in white nonalcoholic subjects , and is more in keeping with what has been reported in alcoholics (6,7), although another report found no differences (8). The D2 genotype prevalence of 4% A1A1, 38% A1A2 and 58% A2A2 in our population is comparable with what has been reported for alcoholics, rather than for nonalcoholic subjects (7). Interestingly, the co-association of the D4 and D2 receptor gene alleles revealed a fairly uniform distribution of the A1A2 and A2A2 genotypes in a 30:70% ratio across most of the D4 genotypes. However, in the D4(4,6) and D4(4,7) groups, there was a striking preponderance of the A1A2 genotype in ≥60% of subjects, suggesting that the D4 genotype, in association with the D2 genotype may represent an added risk factor.

Among this group of heavy drinkers, there did not appear to be a correlation between D4 genotype and amount of alcohol consumed, as the average daily intake was very high. A positive family history of alcoholism has been reported to be one of the most powerful predictors of risk

(16), and our study shows a positive family history in 30-50% of all the D4 genotypes, except D4(2,4) which had a much higher incidence of ~70%. The role of the D4 alleles in the overall propensity for substance abuse was addressed by examining the incidence of cigarette smoking and other drug use. The prevalence of cigarette smoking or nicotine abuse was uniformly high, ranging between 70-90% among all the D4 allelic combinations represented. Other drug abuse was less prevalent, present in 30-40% of most D4 genotypes, but had a 2 fold higher prevalence in the D4(3,3) and (4,7) groups. There were no other factors identified that distinguished this group from the other D4 genotypes, such as amount of alcohol consumed, family history or D2 genotype.

The attempts to link specific genotypes with alcoholism or with an increased risk for alcoholism has been furthered by the finding of the higher frequency of the D2 dopamine receptor allele A1 in alcoholic populations. In the present study, we have documented the polymorphisms of the closely related D2-like receptor, the D4 dopamine receptor in a population of alcoholics, to demonstrate that the allelic frequency of the polymorphisms represented vary from that reported in the normal population, and that certain allelic combinations may have a higher propensity for other drug abuse or for a positive family history of alcohol abuse. The close association of a significantly higher prevalence of the D2 A1 allele in a subpopulation of D4 genotypes is intriguing and merits further investigation.

In summary, the present study demonstrates that the D4 receptor genotype in 72 severely affected chronic alcoholics is a heterogenous mixture, containing individuals that are homozygous and heterozygous for the various D4 alleles. The D4 receptor gene allelic composition in chronic alcoholic subjects demonstrates a significantly greater prevalence of the D4(3) anf D4(6) alleles than has been reported in normals. Among the group of alcoholics, there was no correlation of D4 genotype with amount of alcohol consumed per day, and there was an equally high prevalence of nicotine abuse among all D4 genotypes. The frequency of other drug abuse was higher in the D4(3,3) and the D4(4,7) groups, and the family history was strongly positive in the D4(2,4) group. The distribution of the D2 alleles showed equivalence in all D4 genotypes, except in D4(4,6) and D4(4,7) in whom the prevalence of the D2 A1A2 allele was 2 fold higher. Further studies are needed to determine the role of the D4 receptor polymorphism in alcoholism, the influence on the D2 alleles and in co-existing morbidities such as other substance abuse.

ACKNOWLEDGMENTS

This study was supported by grants from the Addiction Research Foundation and the Medical Research Council of Canada.

REFERENCES

1. Cloninger, C. (1987) Science 236, 410-416.
2. Koob, G.G. and Bloom, F.E. (1988) Science 242, 715-723.
3. Wise, R.A. and Rompre, P.P. (1989) Annu. Rev. Psychol. 40, 191-225.
4. Imperato, A. and DiChiara, G. (1986) J. Pharm. Exp. Ther. 239, 219-228.
5. O'Dowd, B.F. (1993) J. Neurochem. 660, 804-816.
6. Blum, K., Noble, E.P., Sheridan, P.J., Montgomery, A., Ritchie, T., Jagadeeswaran, P., Nogami, H., Briggs, A.H.and Cohn, J.B. (1990) J. Am. Med. Assoc. 263, 2055-2060.

7. Blum, K., Noble, E.P., Sheridan, P.J., Finley, O., Montgomery, A., Ritchie, T., Ozkaragoz, T., Fitch, R.J., Sadlack, L., Sheffield, D., Dahlmann, T., Halbardier, S. and Nogami, H. (1991) Alcohol 8, 406-416.
8. Bolos, A.M., Dean, M., Lucas-Derse, G., Ramsburg, M., Brown, G.L.and Goldman D. (1990) J. Am. Med. Assoc. 264, 3156-3160.
9. Cloninger, C.R. (1991) J. Am. Med. Assoc. 266, 1833-1834.
10. Van Tol, H.H.M., Bunzow, J.R., Guan, H-C.,Sunahara, R.K., Seeman, P., Niznik, H. and Civelli, O. Nature (1991) 350, 610-614.
11. Van Tol, H.H.M., Wu, C.M., Guan, H-C., Ohara, K., Bunzow, J.R., Civelli, O., Kennedy, J., Seeman, P., Niznik, H.B. and Jovanovich, V. (1992) Nature 358, 149-152.
12. Miller, S.A. (1988) Nucleic Acid Res. 16, 1215.
13. Nguyen, T., Sunahara, R., Marchese, A., Van Tol, H.H.M., Seeman, P. and O'Dowd, B.F. (1991) Biochem. Biophys. Res. Commun. 181, 16-21.
14. Petronis, A., Van Tol, H.H.M., Livak, K.J., Sidenberg, D.G., Macciardi, F.M. and Kennedy, J.L. (1992) Am. J. Hum. Genetics, Abstr. 779.16.
15. Nanko, S., Hattori, M., Ikeda, K., Sasaki, T., Kazamatsuri, H. and Kuwata, S. (1993) Lancet 341, 689-690.
16. Cotton, N.S. (1979) J. Stud. Alcohol 40, 89-116.