

A missense mutation in the vasopressin – neurophysin precursor gene cosegregates with human autosomal dominant neurohypophyseal diabetes insipidus

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Familial neurohypophyseal diabetes insipidus in humans is a rare disease transmitted as an autosomal dominant trait. Affected individuals have very low or undetectable levels of circulating vasopressin and suffer from polydipsia and polyuria. An obvious candidate gene for the disease is the vasopressin–neurophysin (AVP-NP) precursor gene on human chromosome 20. The 2 kb gene with three exons encodes a composite precursor protein consisting of the neuropeptide vasopressin and two associated proteins, neurophysin and a glycopeptide. Cloning and nucleotide sequence analysis of both alleles of the AVP-NP gene present in a Dutch ADNDI family reveals a point mutation in one allele of the affected family members. Comparison of the nucleotide sequences shows a G → T transversion within the neurophysin-encoding exon B. This missense mutation converts a highly conserved glycine (Gly17 of neurophysin) to a valine residue. RFLP analysis of six related family members indicates cosegregation of the mutant allele with the DI phenotype. The mutation is not present in 96 chromosomes of an unrelated control group. These data suggest that a single amino acid exchange within a highly conserved domain of the human vasopressin-associated neurophysin is the primary cause of one form of ADNDI.
Key words: Brattleboro rat/chromosome 20/inherited disease/neurophysin/vasopressin

Introduction

The nonapeptide arginine vasopressin (AVP) is produced in hypothalamic neurones and exerts an important endocrine function as antidiuretic hormone that controls effective water reabsorption in the kidney. Partial or complete absence of circulating AVP results in the syndrome of diabetes insipidus that is characterized by the clinical symptoms of excessive thirst and polyuria (Moses, 1985). Among other possible causes for AVP deficiencies, autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) represents an inherited form of the disease in humans (Pender and Fraser, 1953; Levinger and Escamilla, 1955). Affected individuals have low or undetectable levels of endogenous AVP but respond to substitution therapy with exogenous AVP or its

analogues. Diabetes insipidus shows clinical variability both within and between families in respect of onset and severity of the symptoms (Martin, 1959). The few histological post-mortem studies on hypothalamic tissue of diabetes insipidus patients report conflicting results ranging from striking decrease in cell number in supraoptic and paraventricular nuclei (Braverman *et al.*, 1965) to histologically intact nerve cells in that region (Nagai *et al.*, 1984).

AVP is synthesized as part of the larger vasopressin–neurophysin (AVP-NP) hormone precursor that includes the cysteine-rich carrier protein neurophysin and a C-terminal glycoprotein (Richter, 1988). The organization of the precursor genes and the amino acid sequences especially at the central part of the neurophysins are highly conserved among mammals (Acher and Chauvet, 1988). Following synthesis on membrane-bound ribosomes, the precursor is targeted to the Golgi where it is packaged for axonal transport into vesicles of the regulated secretory pathway. These neurosecretory granules contain all processing enzymes necessary for proteolytic release of the biologically active peptides.

Mutations in the AVP-NP precursor gene can lead to vasopressin deficiency without altering directly the hormone encoding sequence as has been shown for the Brattleboro rat, the best studied model of inherited diabetes insipidus. Brattleboro rats exhibit an autosomal recessive genetic defect characterized by a single base deletion in the neurophysin-encoding exon B of the AVP-NP precursor (Schmale and Richter, 1984). The resulting frame shift mutation completely alters the C terminus of the AVP-NP precursor. As a consequence, the mutant protein is retained in the endoplasmic reticulum because its conformation prevents normal intracellular transport (Schmale *et al.*, 1989).

To determine whether the human AVP-NP precursor is defective in patients with ADNDI we searched for mutations in the coding sequences after molecular cloning of both alleles of the respective gene from affected members of a Dutch ADNDI family. Here we demonstrate that a single point mutation in one allele of affected individuals results in an amino acid exchange in the neurophysin carrier protein.

Results

Pedigree and clinical assessment of the Dutch ADNDI family

The ADNDI phenotype in the family under study exhibits a clearly dominant mode of inheritance that can be traced back for five generations (Meinders and Bijlsma, 1970) (Figure 1). It was crucial to have a recent accurate assessment of the DI status in the six members III-1, IV-3, IV-Y, V-7 to -9 of the family, who agreed to participate in the study. To this end, urine production and osmolality after overnight fluid deprivation were measured. All members of the family diagnosed with DI earlier (in 1978) showed values typical for DI patients and the individuals IV-Y and V-7 were

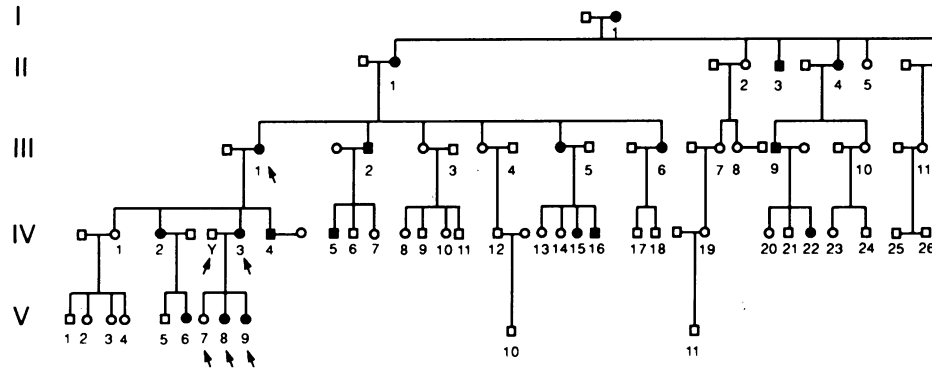


Fig. 1. Pedigree of the Dutch ADNDI family comprising five generations. Black symbols denote affected individuals, females are indicated by circles and males by squares. Samples were available from individuals marked by the arrows.

unaffected (Table I). Interestingly, patient V-9 who was apparently unaffected at the age of 2.5 years at the time of the initial clinical assessment developed the DI phenotype later (Schmale *et al.*, 1991). The late onset of DI observed before in the family under study has also been reported for other cases of familial DI (Martin, 1959).

Sequence analysis of both AVP-NP gene alleles of an ADNDI carrier

Leukocyte DNA prepared from blood samples of the six individuals was studied in order to detect major changes in the AVP-NP gene. Southern blot analysis using various restriction enzymes and hybridization with human AVP-NP cDNA probe (Mohr *et al.*, 1985) revealed no indications for major deletions, insertions or rearrangements within the vasopressin locus of the affected individuals. To investigate whether a point mutation is present in the AVP-NP gene we decided to isolate and compare both alleles of the affected person IV-3 because in autosomal dominant traits by definition affected individuals are heterozygous for the mutant locus. A small subgenomic library was constructed from a genomic DNA preparation enriched for 3.5 kb *Bam*HI–*Kpn*I fragments containing the entire AVP-NP gene (Nicholls *et al.*, 1985). Screening of 8000 recombinant bacterial clones with the human AVP-NP cDNA probe yielded four positives, three of which were subjected to exon and intron sequence analysis. Two clones turned out to be completely identical, whereas the third one revealed two sequence differences indicating that it represented the second allele. Besides the insertion of a G residue in intron 2, we detected a G → T transversion in exon B that predicts a substitution of the Gly17 residue to Val in the corresponding neurophysin amino acid sequence (Figure 2). Furthermore, the mutation abolishes a recognition site for the restriction endonuclease *Bgl*I (GC₄G₅C → GC₄G₄TC) thereby allowing a distinction between both alleles by RFLP analysis. The map of *Bgl*I sites in the AVP-NP gene locus (Figure 3) predicts the generation of a 1.56 kb fragment in the mutant case instead of a 1.44 kb fragment present in the normal allele.

The segregation of the two alleles could be followed in three generations of the ADNDI family. As shown in Figure 4, all four affected individuals (III-1, IV-3, V-8 and V-9) were heterozygous for the mutation as indicated by the presence of both the 1.44 and 1.56 kb *Bgl*I fragments whereas the two non-affected persons (IV-Y and V-7) exhibited only the normal 1.44 kb species. These data are

Table I. Urinary output and osmolality after dehydration in six members of the Dutch ADNDI family

	Sex	Age	Urine production l/24 h	ADNDI	DDAVP therapy	Urine osmolality mOsm/l
III-1	F	73	16	+	+	123
IV-3	F	46	16–20	+	+	167
IV-Y	M	47	1–1.5	–	–	837
V-7	F	20	1–1.5	–	–	707
V-8	F	19	>10	+	+	95
V-9	F	16	>10	+	+	210

The urine samples were taken 24 h after the last DDAVP treatment and a water intake deprivation of 8–10 h. F = female; M = male.

entirely consistent with cosegregation of the Gly → Val mutation with the DI phenotype. To exclude the possibility that the G → T transversion represents a frequent polymorphism in the general population we have studied 96 chromosomes of an unrelated normal human control group by Southern blot analysis following *Bgl*I digest of genomic DNA. Among these, all samples exhibited the normal pattern of *Bgl*I fragments.

Genetic heterogeneity in ADNDI families

In order to determine whether in an unrelated ADNDI family affected persons exhibited the same mutation detectable by *Bgl*I restriction we examined four members (three affected) of a French kindred. Southern blot analysis after *Bgl*I digest and hybridization with an AVP-NP specific probe yielded in all persons only the 1.44 kb fragment indicative of the normal sequence in that part of the AVP-NP gene (not shown).

The finding that different mutations in the AVP-NP gene of unrelated ADNDI families might be responsible for the phenotype is supported by two recent reports linking familial DI to the vasopressin gene locus in families with different genetic backgrounds. Two American ADNDI families exhibited RFLPs detectable with an oxytocin (OT) gene probe that cosegregated with the DI phenotype (Repaske *et al.*, 1990). Since the OT-NP gene is located adjacent to the AVP-NP gene only separated by 8 kb (Sausville *et al.*, 1985) on human chromosome 20 (Riddell *et al.*, 1985) the observed RFLPs can serve as markers for the chromosomal region comprising both genes and suggest that a gene in that genetic locus is responsible for the disease phenotype. As

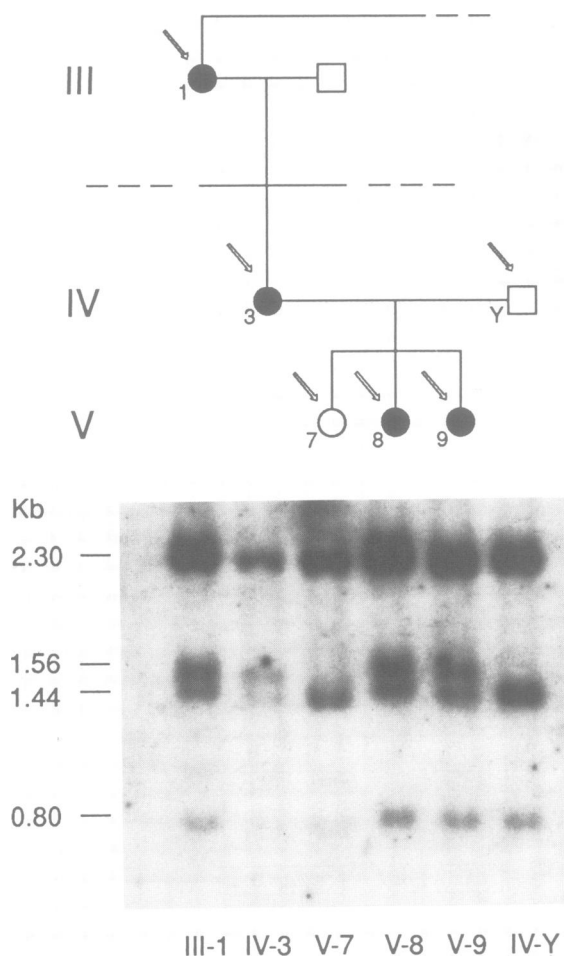


Fig. 4. Pedigree of part of the Dutch ADNDI family and corresponding autoradiograph of a Southern blot of *Bgl*I-digested genomic DNAs hybridized to a human genomic AVP-NP gene probe. Black symbols denote affected individuals, females are indicated by circles and males by squares. Samples were available from individuals marked by the arrows. Heterozygous carriers of the mutant allele exhibit two bands (1.44 and 1.56 kb) whereas in homozygous persons only the 1.44 kb fragment is present.

the adjacent Cys21-Pro24 peptide backbone depends on correct β -turn folding. Hormone binding has allosteric effects facilitating dimerization not only of the processed hormone–neurophysin complex but also of the polyprotein precursor (Breslow and Burman, 1990). As revealed by the crystal structure, the basic aggregates of the hormone–neurophysin complex are tetramers that can self-associate to higher oligomers, a process that might have important implications for precursor packaging and precipitation of the complex in neurosecretory granules (Chen *et al.*, 1991). Binding of AVP, dimerization of neurophysin, or correct proteolytic cleavage of the precursor (Kanmera and Chaiken, 1985) might be interdependent processes adversely influenced by the presence of aberrant protein products.

Alterations of protein conformation also have an impact on the intracellular transport of the secretory AVP-NP precursor as has been shown previously with inherited DI in a rat model (Schmale *et al.*, 1989). In contrast to the Brattleboro rat, where only homozygous animals suffer from DI, in human ADNDI the protein product of the mutant allele is sufficient to prevent production of AVP even in the

presence of the normal allele. A possible explanation might be that the presence of the aberrant protein interferes with the self-aggregation process of the AVP-NP precursor in the trans-Golgi suggested to be required for targeting to secretory dense core granules (Pfeffer and Rothman 1987). This hypothesis would imply that the subtle alteration of the mutant human precursor would allow its transport beyond the ER. In contrast, the Brattleboro precursor due to its more dramatic conformational change is retained in the ER and thus has no access to cellular compartments where interaction between normal and mutant precursor molecules occurs. This mechanism would allow unaffected expression of the normal allele in heterozygous Brattleboro rats.

Understanding of the mechanisms by which point mutations in the human AVP-NP gene produce the pathology characteristic of ADNDI may provide further insights into the function of neurophysins and into mechanisms of protein targeting and secretion in neurosecretory cells.

Materials and methods

Southern blot analysis

Genomic DNA was prepared from blood samples by standard methods (Herrmann and Frischauf, 1987). 10 μ g of restricted genomic DNA were separated electrophoretically through agarose gels and transferred to nylon membranes (Hybond N) according to standard methods by neutral capillary blotting in $20 \times$ SSC. DNA was immobilized by UV radiation (120 000 μ J/cm²) using a crosslinking device (Stratalinker 1800, Stratagene). Blots were hybridized with an α -³²P-labelled human AVP cDNA probe (Mohr *et al.*, 1985) or a human genomic AVP probe (1×10^6 c.p.m./ml 50% formamide, $6 \times$ SSC, $5 \times$ Denhardt's, 0.5% SDS, 100 μ g/ml denatured herring sperm DNA). DNA probes were labelled with [α -³²P]dCTP by the random primer method (Hodgson and Fisk, 1987) to a specific activity of 1×10^9 c.p.m./ μ g.

Cloning of AVP-NP precursor gene alleles

For cloning of the human VP gene the fragment enrichment strategy (Nicholls *et al.*, 1985) was adopted with slight modifications: 180 μ g genomic DNA was subjected to endonuclease digestion with *Bam*HI and *Kpn*I. The digested DNA was separated on a 0.7% agarose gel in $0.5 \times$ TAE and the 3.5 kb fragment harbouring the entire AVP-NP gene was cut off the gel in a slice containing DNA fragments from 4.5 to 2.5 kb. The DNA was recovered by electroelution (Biotrap, Schleicher and Schuell) in $0.25 \times$ TAE at 100 V. The eluted material was digested with *Dra*I, *Eco*RI, *Eco*RV, *Hpa*I, *Sst*I and *Xba*I and further enriched for the 3.5 kb AVP-NP fragment by agarose gel electrophoresis and electroelution as described above. The genomic DNA fragments were ligated to pBluescript DNA (Stratagene), previously linearized with *Bam*HI and *Kpn*I. High competence *Escherichia coli* PLK-F' (Stratagene) were transformed using 2.7 ng ligated vector per 100 μ l cells. The resulting subgenomic library was plated on LB (Amp/Tet) agar. Double replica filters were screened with α -³²P-labelled human cDNA probe (1×10^6 c.p.m./ml 50% formamide, $6 \times$ SSC, $5 \times$ Denhardt's, 0.5% SDS, 100 μ g/ml denatured herring sperm DNA). For sequencing, template DNA was isolated by R408 helper phage assisted single strand rescue from positive pBluescript clones according to the manufacturer's instructions (Stratagene) or by subcloning positive inserts into M13mp18 and mp19 vectors. Dideoxy DNA sequencing was performed using a Sequenase 2.0 DNA sequencing kit (USB). Sequencing primers covering the entire 3.5 kb fragment were synthesized on a DNA synthesizer 480B (Applied Biosystems) and were derived in part from the published sequence of the human VP gene (Sausville *et al.*, 1985). Both strands were sequenced.

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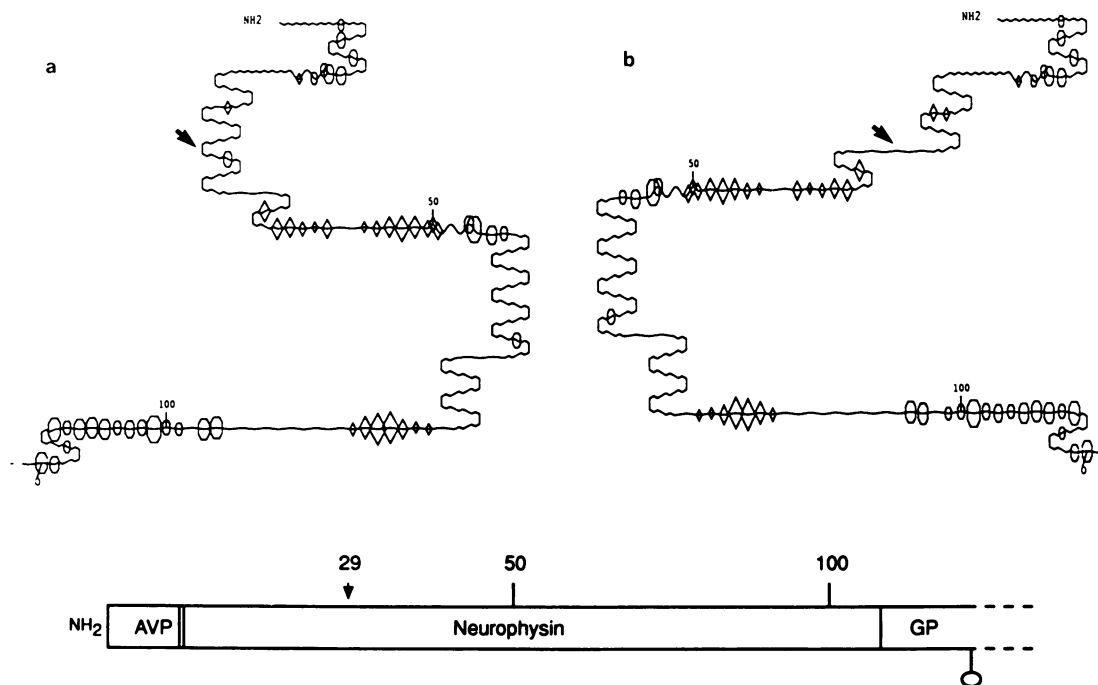


Fig. 5. Secondary structure prediction according to Chou and Fasman of the normal (a) and mutant (b) human AVP-NP precursor. The arrow marks the β -turn around amino acid 29 in the normal protein. This β -turn is not present in the mutant precursor. Numbering of amino acids refers to the AVP-NP precursor, where amino acid 29 corresponds to amino acid 17 of the neurophysin moiety. Only part of the protein sequence is shown as indicated by the schematic representation of the precursor. \circ , K_D hydrophilicity ≥ 1.3 ; \diamond , K_D hydrophobicity ≥ 1.3 ; \odot , glycosylation; GP, glycopeptide.

secondary structure analysis. This work is part of a PhD thesis (U.B.) and was supported by Deutsche Forschungsgemeinschaft (Ri 192/17-6,17-8).

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Note added in proof

The nucleotide sequence reported in this paper has been deposited in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X62890 and X62891, respectively.