Clinical Endocrinology (1996) **44**, 45–51

A novel mutation in the coding region for neurophysin-II is associated with autosomal dominant neurohypophyseal diabetes insipidus*

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(Received 10 May 1995; returned for revision 22 June 1995; finally revised 19 July 1995; accepted 11 August 1995)

Summary

OBJECTIVE Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) is a rare cause of diabetes insipidus, in which AVP serum levels are insufficient. AVP is synthesized along with neurophysin-II (NPII) as an AVP-NPII precursor polypeptide in the hypothalamus. After proteolytic cleavage during axonal transport, AVP and NPII are reassembled and stored loosely bound to each other in the posterior pituitary until both are released into the circulation. In this study, we investigated the genetic basis of ADNDI in a German kindred with 10 affected members spanning three generations.

DESIGN Genomic DNA was isolated from peripheral blood leucocytes. The entire coding region of the AVP-NPII gene of one of the affected persons was amplified by polymerase chain reaction (PCR) and subjected to nucleotide sequence analysis. Sequencing results were confirmed by restriction enzyme analysis of PCR products.

PATIENTS Six affected and two unaffected members of a family with ADNDI and 54 unrelated healthy control subjects were studied.

RESULTS The index patient was found by direct sequencing to be heterozygous for a G to T transversion at nucleotide position 1884 (exon 2) of the AVP-NPII gene. This mutation introduced a new recognition site for the restriction enzyme Ava II, which was used to test for the presence of the mutation in other family members and in control subjects. The mutation was detected in all family members with ADNDI, but was not found in unaffected family members or in control subjects. The mutation encodes a valine in place of the normal glycine at amino acid 65 of NPII, which is known to be highly conserved during evolution.

CONCLUSIONS In this family, the autosomal dominant neurohypophyseal diabetes insipidus phenotype cosegregates with a point mutation in a region of the AVP-neurophysin-II gene which codes for the carboxy-terminal domain of neurophysin-II. Although the altered amino acid is not directly involved in AVP binding, the mutation might lead to conformational changes that impair the dimerization of neurophysin-II molecules. This could in turn affect the AVP binding affinity of neurophysin-II or might interfere with the transport of the AVP-neurophysin-II precursor in the AVP-producing cells of the hypothalamus.

Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) is a hereditary form of diabetes insipidus (DI) which results from an insufficient serum concentration of AVP (Pedersen *et al.*, 1985). Affected individuals have polyuria and polydipsia, usually recognized in the first few years of life (McLeod *et al.*, 1993). The renal response to AVP is normal in ADNDI; consequently, affected subjects respond to exogenous AVP or its analogues with a reduction of free water loss.

AVP is a nonapeptide encoded by the 2·5 kilobase AVP-neurophysin-II (AVP-NPII) gene (Sausville *et al.*, 1985) located on the distal short arm of chromosome 20 (Summar *et al.*, 1990). The AVP-NPII gene product is synthesized as a precursor polypeptide, pre-pro-AVP-NPII, which includes the AVP peptide, its 93-amino acid carrier protein neurophysin-II (NPII), and copeptin (Richter, 1988). The latter is a 39-amino acid glycopeptide of unknown function. The AVP-NPII precursor polypeptide is synthesized in the magnocellular neurones of the supraoptic and paraventricular nuclei of the hypothalamus. After post-translational processing in the cell bodies, AVP, NPII and copeptin are proteolytically cleaved from one another during axonal transport to the posterior pituitary (Richter, 1988). In the neurohypophysis, AVP and NPII interact to form non-covalent complexes within neurosecretory granules (Richter, 1988), which are released into the circulation in response to serum hyperosmolality, hypotension or hypovolaemia.

The molecular basis of ADNDI appears heterogeneous, as six mutations within exon 2 of the AVP-NPII gene and

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* Presented in part at the 34th Annual Meeting of the European Society for Pediatric Endocrinology, Edinburgh, June 1995.

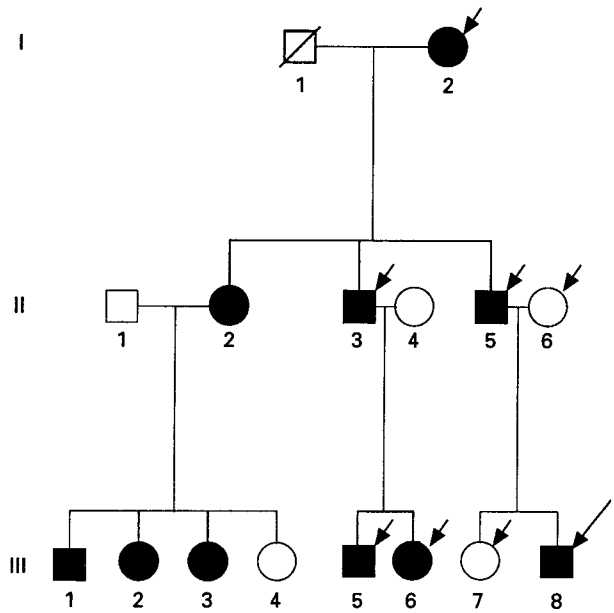

Fig. 1 Pedigree with ADNDI. Arrows indicate family members who participated in the molecular studies. ●, Affected; ○, unaffected; ⍁ deceased.

one mutation in exon 1 have now been identified in different kindreds with ADNDI (Ito *et al.*, 1991;1993; Bahnsen *et al.*, 1992; Yuasa *et al.*, 1993; Krishnamani *et al.*, 1993; McLeod *et al.*, 1993; Repaske & Browning, 1994; Nagasaki *et al.*, 1995).

This report describes studies of a novel exon 2 mutation in a German family with ADNDI.

Materials and methods

Patients

The index patient (Fig. 1, subject III-8) was a 10-year-old boy with a history of polyuria (4–6 l/day), nocturia and polydipsia first recognized at ≈18 months of age. A water deprivation test demonstrated a defect in urine concentrating ability, and intranasal desmopressin therapy led to resolution of symptoms. His father (subject II-5) had a history of polyuria and polydipsia (5–10 l/day) since infancy, but had not sought medical attention for this disorder until after the diagnosis of DI had been established in his son. The index patient's mother (subject II-6) and 12-year-old sister (III-7) had never had symptoms of DI. Subsequently, the other living members of the pedigree underwent the diagnostic work-up for diabetes insipidus, including a water deprivation test. This revealed a history typical of DI and a defect in urine concentrating ability in the index patient's grandmother (subject I-2), his aunt (II-2) and his uncle (II-3), as well as in five of his six cousins (Fig. 1). With the exception of subject I-2, who refused the medication, symptoms were controlled with desmopressin in all affected members of the kindred.

Eight years after the initial diagnosis, blood samples were obtained from eight members of the kindred (indicated by an arrow in Fig. 1), including six individuals affected with ADNDI. One part of the family with four affected subjects had moved away and DNA was not available for analysis. Informed written consent was obtained from all participants of the study.

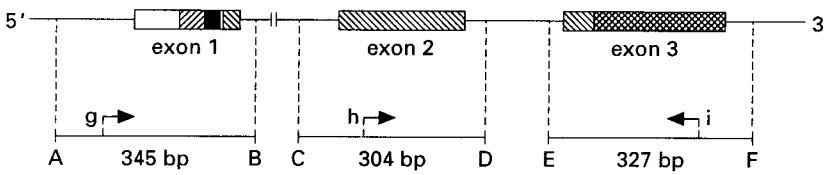

Fig. 2 Sequencing strategy of the AVP-NPII gene. The primers used for PCR and sequencing are indicated below. ▨, Signal peptide; ■, vasopressin; ▧, neurophysin-II; ▩, glycoprotein.
A: 5′-TGCCTGAATCACTGCTGACC GCTGGGGACC;
B: 5′-GCTATGGCTGCCCTGAGAT GGCCCACAGTG;
C: 5′-TCGCTGCGTTCCCCTCCAA CCCCTCGACTC;
D: 5′-CGCCCCCCCCCAGGCCCG CCCCCGCCGCGC;
E: 5′-CCCAGGCGCCCGTGCTC ACACGTCCTCCCG;
F: 5′-CCTCTCTCCCCTTCCCTCTT CCCGCCAGAG;
g: 5′-TGGCGGCCGCGTCTCGCCTCC ACGGGAACA;
h: 5′-TGCTTCGGGCCCAGCATCT GCTGCG;
i: 5′-TAGGCGTCGGGCTGGGC GGGCTCGA.

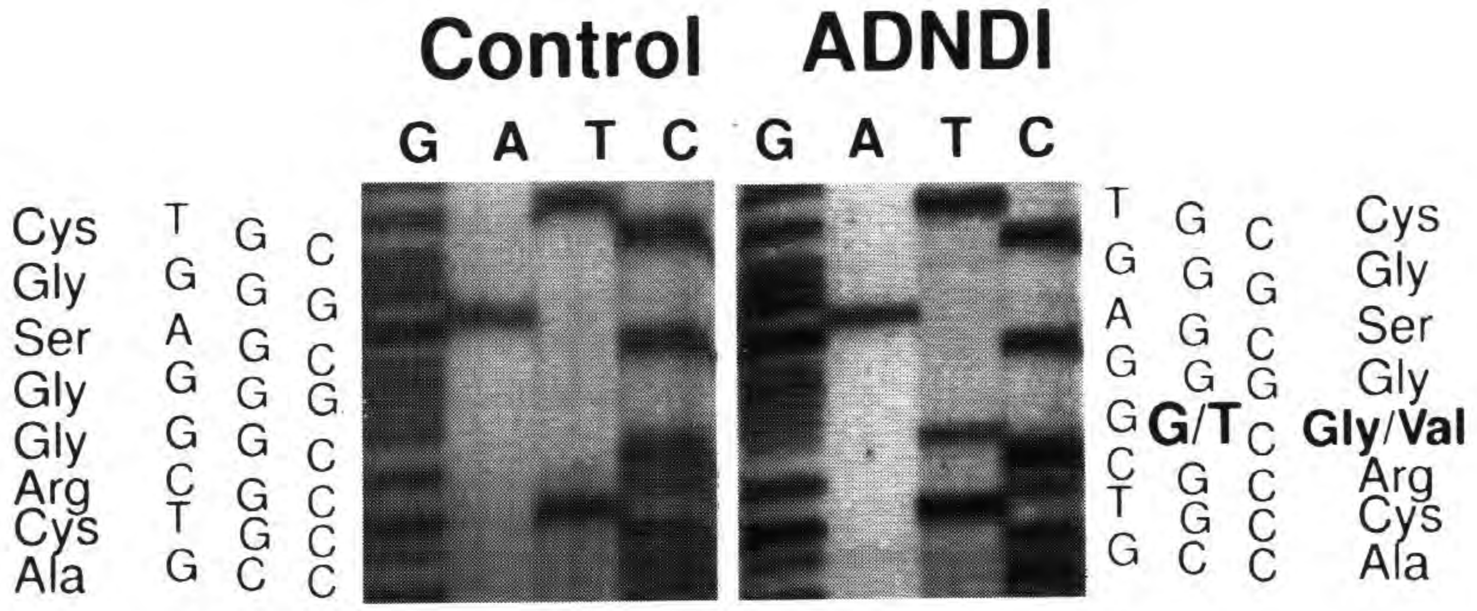

Fig. 3 Sequence determination at the mutation site in exon 2 of the AVP-NPII gene in the index patient and in a healthy unrelated control. The mutation is printed in bold.

PCR assay

Genomic DNA was isolated from peripheral blood leucocytes with a commercial kit (Nucleon II extraction kit, Scotlab, Strathclyde, Scotland). As shown in Fig. 2, each exon of the AVP-NPII gene was amplified separately by polymerase chain reaction (PCR) using primers A and B for exon 1, C and D for exon 2 and E and F for exon 3 (sequences as given by Ito *et al.* (1991), see legend to Fig. 2). Primers B, D and E were biotinylated. The PCR reaction was performed in a 50-μl reaction volume containing 400 ng purified genomic DNA, 0·4 mM of each deoxynucleotide triphosphate, 5 μl 10 × PCR-buffer (Boehringer Mannheim, Germany and 2·5 U of Taq DNA polymerase (Boehringer Mannheim, Germany). In contrast to Ito *et al.* (1991), addition of dimethoxysulphoxide was not found to be necessary in the PCR assay. The reaction was carried out using a Biometra TRIO thermocycler with 33 temperature cycles of 1 min at 95°C (exon 1) or 20 s at 98°C (exons 2 and 3), 1 min at 69°C (exon 1), 68°C (exon 2) or 58°C (exon 3), respectively, and 1·5 min at 72°C, followed by a final extension step at 72°C for 7 min.

Sequencing of single stranded DNA

The biotinylated PCR products were conjugated to streptavidin magnetic beads (Dynal Corp., Oslo, Norway) and subjected to alkali denaturation to produce single-stranded DNA (Hultman *et al.*, 1989). For sequencing, non-biotinylated PCR primers and internal primers g (exon 1), h (exon 2), and i (exon 3) were used (Fig. 2). Sequencing reactions were done by the dideoxy-nucleotide chain termination method (Sanger *et al.*, 1977) using T7 polymerase, 7-deaza dGTP mixes (Pharmacia, Uppsala, Sweden) and α-^{33}P-dATP (DuPont NEN Research Products, Boston, MA, USA). The samples were analysed by electrophoresis on a 8% polyacrylamide/7 M urea gel. Amino acid and nucleotide numbering are according to Sausville *et al.* (1985).

Digestion of amplified DNA by restriction enzyme

PCR-amplified fragments were directly digested with Ava II (Boehringer Mannheim, Germany) according to the manufacturer's instructions and analysed on a 2% agarose gel (SERVA, Heidelberg, Germany). The gel was stained with ethidium bromide before visualization under ultraviolet light.

Results

Detection of the mutation in AVP-NPII gene exon 2 in subjects with ADNDI

PCR-amplification products of all three exons of affected

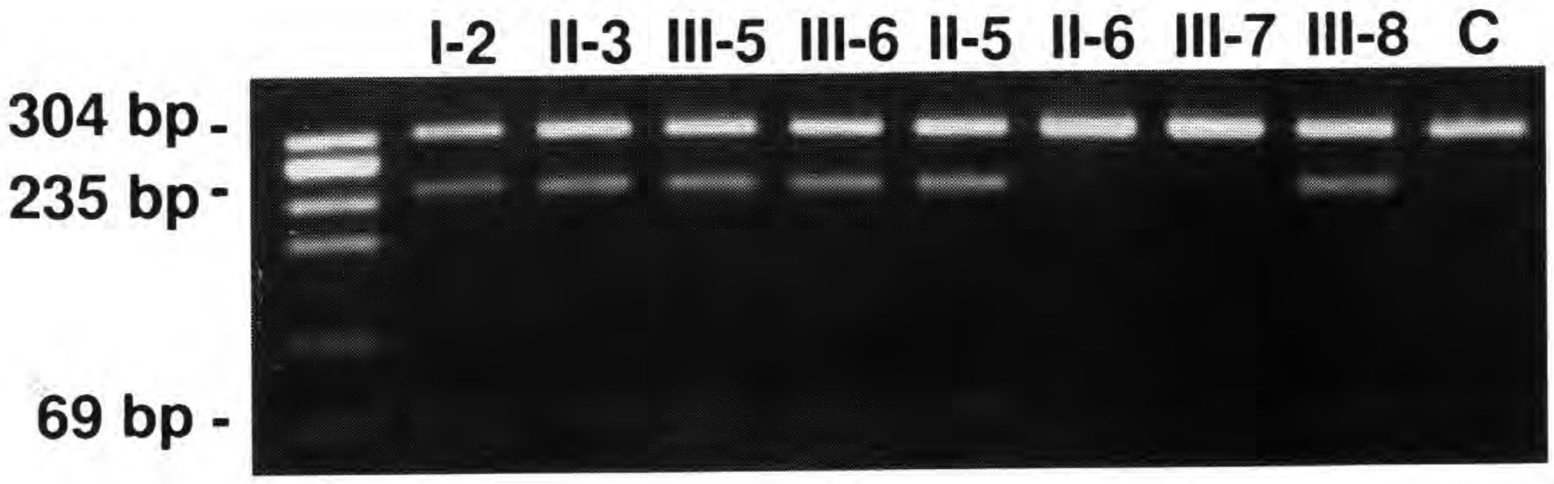

Fig. 4 Results of Ava II restriction enzyme digest of PCR-amplified exon 2 of the AVP-NPII gene in a family with ADNDI and in a healthy unrelated control subject (C). Subjects are numbered as indicated in Fig. 1.

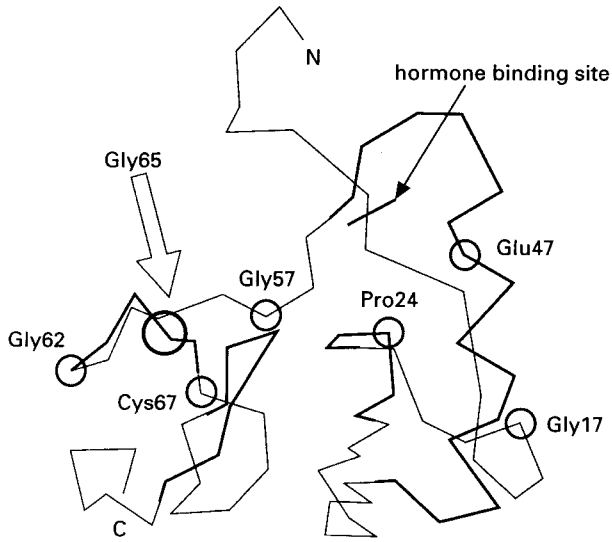

Fig. 5 The ADNDI associated mutations in NPII. The C^{α} backbone of bovine NPII complexed with dipeptide I-Phe-Tyr-NH_2 (Chen *et al.*, 1991) is used to demonstrate the positions of mutated amino acids in man (circles): $Gly^{17} \rightarrow$ Val, $Pro^{24} \rightarrow$ Leu, $Glu^{47} \rightarrow$ del, $Gly^{57} \rightarrow$ Ser, and $Gly^{65} \rightarrow$ Val. The carboxy-terminal domain starts left of Gly^{57}. The short bold line above Pro^{24} represents the bound dipeptide analogue of the hormone.

and unaffected subjects had the expected size, thus excluding major insertions and deletions (not shown). Each of the three exons of the AVP-NPII gene from the index patient was completely sequenced. In exons 1 and 3, no discrepancy from the published sequences was found (Sausville *et al.*, 1985; Ito *et al.*, 1991). In exon 2, a mutation was detected at nucleotide position 1884, where the sequence determination indicated the presence of a T in addition to the expected G (Fig. 3). Sequencing of exon 2 revealed the same mutation in the proband's affected father (subject II-5), while sequencing of his unaffected sister's (III-7) and mother's DNA (II-6) and the DNA of a healthy unrelated control person (Fig. 3) revealed only the normal nucleotide sequence. The mutant nucleotide sequence encodes a valine (GTC) in place of the normal glycine (GGC) at amino acid 65 of NPII.

Confirmation of the mutation by restriction enzyme analysis

To confirm the presence of the single nucleotide mutation in affected family members, restriction enzyme analysis of exon 2 of the AVP-NPII gene was performed. The G → T mutation at nucleotide position 1884 introduces a recognition site for the restriction enzyme Ava II at positions 1882–1886 (GGGCC → GGTCC), whereas the unmutated sequence contains no Ava II site. Therefore, digestion of the 304-bp exon 2 PCR product from heterozygotes should produce three DNA fragments (304, 235 and 69 bp), while DNA from normal subjects should produce a single undigested fragment of 304 bp. As shown in Fig. 4, Ava II digestion of exon 2 PCR products from the six affected family members tested produced three fragments of the predicted length, indicating the presence of the mutation-associated Ava II site. In the two unaffected family members and in an unrelated healthy control subject, digestion with Ava II did not alter the length of the PCR products of exon 2. The Ava II recognition site thus cosegregated with the ADNDI phenotype.

To exclude the possibility that the G → T mutation at nucleotide position 1884 represents a frequent polymorphism in the general population, the same restriction enzyme analysis was performed on 54 unrelated healthy subjects of German descent. In none of these subjects was the exon 2 PCR product cut by Ava II (data not shown).

Figure 5 shows the known changes of the NPII molecule associated to ADNDI. The mutation found here alters an amino acid in the carboxy-terminal domain of the molecule (situated on the left side in Fig. 5). Figure 6 demonstrates the influence of the $Gly^{65} \rightarrow$ Val mutation on the molecular structure of NPII in a three-dimensional way. To generate this drawing, atomic coordinates were used, as deposited in the Protein Data Bank (Bernstein *et al.*, 1977; Chen *et al.*, 1991).

Discussion

In this report we describe a German family with ADNDI. Interestingly, the youngest member of the kindred was the first to be thoroughly tested for the disorder. The other affected family members had not sought medical attention, although most of them had noticed polydipsia. This is in accordance with reports that the ADNDI phenotype may be variable within the same family (McLeod *et al.*, 1993).

A missense mutation in the AVP-NPII gene was present in this pedigree, which cosegregated with the ADNDI phenotype among six affected and two unaffected family members. As expected from the autosomal dominant mode of inheritance observed in this family, affected subjects were heterozygous for the mutation. The mutation was a G → T transversion at nucleotide 1884 of the AVP-NPII gene, predicting a Gly → Val substitution at amino acid position 65 in the NPII moiety of the AVP precursor. We demonstrated that the mutation is not a polymorphism occurring frequently in the German population by screening 108 chromosomes of unrelated healthy subjects. These

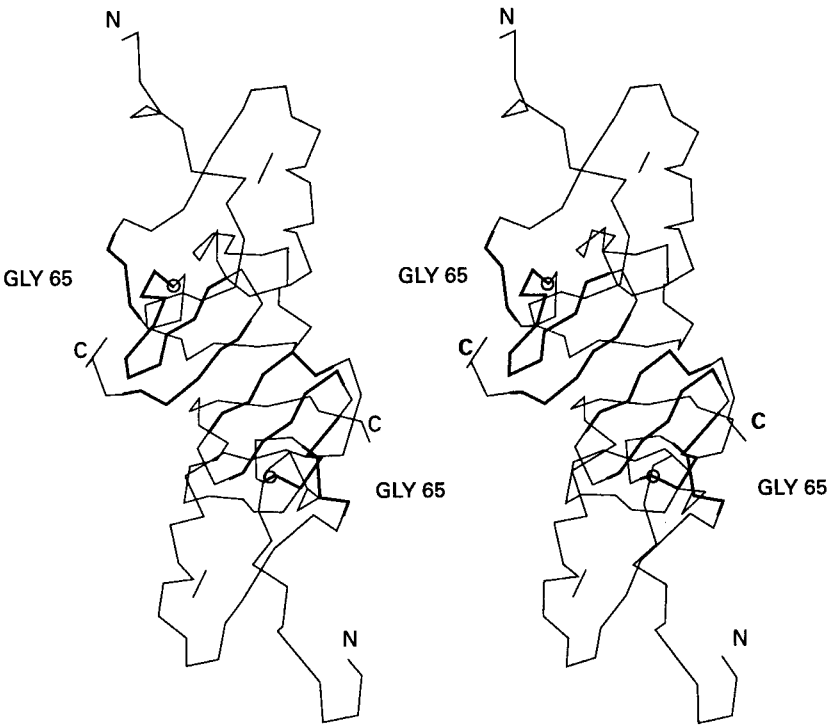

Fig. 6 Stereo drawing of the C^{α} backbone of one NPII dimer showing the site of mutation of Gly^{65} (marked by small circles). Antiparallel β-sheets of the carboxy domain are drawn by bold lines.

findings strongly suggest that the mutation indeed plays a decisive role in the aetiology of ADNDI in this family.

The Gly^{65}→Val mutation does not affect the coding sequence of the AVP hormone itself. Instead, as in six out of the seven published mutations in the AVP-NPII gene associated to ADNDI, it falls within the coding sequence of NPII, the AVP carrier protein (Ito *et al.*, 1991; Bahnsen *et al.*, 1992; Yuasa *et al.*, 1993; Repaske & Browning, 1994; Nagasaki *et al.*, 1995). Gly^{65} is the initial residue of the second β-strand of the carboxy-terminal domain (Fig. 6). The impact of its substitution by Val on the structure and function of NPII is difficult to assess precisely. Due to the large distance to the principal hormone binding site, which is formed by the amino-terminal domain, a direct interaction between Gly^{65} and AVP appears unlikely. Furthermore, the general folding of the NPII molecule is fixed by disulphide bridges (Chen *et al.*, 1991). According to calculations of mobility within the three-dimensional molecular structure, these disulphide bridges could form without tension even in the presence of Val instead of Gly (data not shown). However, the substitution of Val for Gly brings about a significant increase in side-chain volume, which leads to a conformational change in the amino acid loop from position 61 to 65. This could interfere with the dimerization of NPII molecules via a shift of the β-sheets (see Fig. 6), which in turn would influence the binding strength for AVP (Breslow *et al.*, 1991; Chen *et al.*, 1991). Whatever the exact molecular mechanisms, Gly^{65} appears to be of significant structural importance, since it has been estimated that during 600 million years of evolution no exchange has occurred at this position of the NPII molecule (van Kesteren *et al.*, 1992).

The autosomal dominant nature of ADNDI is unusual for a hereditary hormone deficiency. As the patients are heterozygous for the mutation, the normal NPII molecule is also expected to be synthesized in their hypothalamus. Indeed, in the animal model for inherited neurohypophyseal DI, the Brattleboro rat, DI is inherited in an autosomal recessive fashion (Richter, 1988). In these animals, a single base deletion in exon 2 of the AVP-NPII gene results in an AVP precursor with an altered C-terminal region starting at amino acid residue 64, which causes the precursor protein to be retained in the endoplasmatic reticulum (Schmale & Richter, 1984; Schmale *et al.*, 1989). In man, however, the pathophysiological mechanisms by which the mutant protein interferes with the function of the normal molecule are not fully understood.

A supposed function of NPII is to protect AVP from degradation by proteolytic enzymes (Breslow & Burman, 1990). If the mutant NPII were able to bind AVP but unable to protect it from proteolysis, the mutant NPII would act like a catalyst for the proteolytic degradation of all

AVP stored in a neurosecretory granule (Repaske & Browning, 1994).

Also, impairment of dimerization of NPII molecules alone could lead to autosomal dominant disease: if 50% of NPII monomers are of the wild type, theoretically only 6·25% of NPII tetramers will contain no mutated molecule. As even higher oligomer complexes might be required for storage of AVP in the neurosecretory granules of the posterior pituitary, the percentage of hormone stored normally could be much lower than that (Chen *et al.*, 1991). Alternatively, the mutation might interfere with the post-translational modification or transport of the AVP-NPII precursor in the AVP producing cells of the hypothalamus. The abnormal precursor molecules could accumulate within these cells, finally leading to their destruction. This hypothesis is compatible with the clinical observation that the AVP deficiency often develops progessively during childhood (McLeod *et al.*, 1993) and explains the pathological finding that the number of AVP-producing cells is reduced in patients with ADNDI (Braverman *et al.*, 1965; Nagai *et al.*, 1984; Bergeron *et al.*, 1991).

Acknowledgements

We are grateful to Dr S. Scharenberg for bringing the index patient to our attention, to Ms J. Seedat for technical assistance and to Dr N. Robinson for reviewing the manuscript.

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