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In this issue: Experience to date suggests that prenatal treatment of affected female fetuses is generally efficient in minimizing virilization of external genitalia. However, there is still a need to collect more data concerning possible rare unfavorable effects of this therapy on mother and child. (See page 3872.)

Also in this issue: These results indicate that 11 β -hydroxysteroid dehydrogenase (11 β -HSD) colocalizes with mineralocorticoid receptors in human airway epithelia and suggest that 11 β -HSD plays an important role in pulmonary mineralocorticoid activity such as sodium and fluid transport. (See page 4022.)

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The Vasopressin Precursor Is Not Processed in the Hypothalamus of Wolfram Syndrome Patients with Diabetes Insipidus: Evidence for the Involvement of PC2 and 7B2

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ABSTRACT

Wolfram syndrome (WS) is characterized by optic atrophy, insulin-dependent diabetes mellitus, vasopressin (VP)-sensitive diabetes insipidus, and neurosensory hearing loss. Here we report a disturbance in VP precursor processing in the supraoptic and paraventricular nuclei of WS patients. In these patients with diabetes insipidus we could hardly detect any cellular immunoreactivity for processed VP in the supraoptic and paraventricular nuclei. On the other hand, in the paraventricular nucleus a considerable number of cells immuno-

reactive for the VP precursor were present. In addition, the proprotein convertase PC2 and the molecular chaperone 7B2 were absent. As expression of PC2 and 7B2 was detected in the nearby nucleus basalis of Meynert of one WS patient and in the anterior lobe of the other WS patient, the absence of the two proteins in the paraventricular nucleus was not due to mutations in their genes. These results indicate that in WS patients with diabetes insipidus, not only does VP neuron loss occur in the supraoptic nucleus, but there is also a defect in VP precursor processing. (*J Clin Endocrinol Metab* 83: 4026–4033, 1998)

WOLFRAM syndrome (WS; MIM 222300) was first described in 1938 in four siblings and was characterized by insulin-dependent diabetes mellitus (IDDM) and bilateral progressive primary optic atrophy (OA) (1), although the association between OA and diabetes mellitus (DM) had been established a long time previously (2, 3). The acronym DIDMOAD was later applied to such patients to denote the common co-occurrence of diabetes insipidus (DI), diabetes mellitus, OA, and sensori-neural deafness (4). A recent cross-sectional study of WS found that all four features occurred in 54% of the patients (5). Urinary tract abnormalities and neurological complications such as cerebellar ataxia and myoclonus may also be present (5). The most common causes of morbidity and mortality are the neurological manifestations of this syndrome and the complications of urinary tract atony (6).

WS is an autosomal, recessively inherited disorder. Genetic studies have established linkage to chromosome 4p16.1, albeit with some evidence for heterogeneity (7, 8). In addition, Barrientos (9, 10) suggested that the chromosomal

defect on 4p16 might predispose to mitochondrial DNA deletions.

Endocrinological studies have shown the DI of WS to arise at the hypothalamo-pituitary level, to be either partial or complete, to have no additional nephrogenic component, and to be present in all patients in whom it is actively sought (11, 12). The cause of the DI at the molecular and cellular levels, however, is currently unknown. The possibility of a defect in the preprovasopressin gene itself lying on human chromosome 20 (13, 14), akin to that described for autosomal dominant DI (14) would, for instance, appear to be excluded by the genetic data implicating chromosome 4 instead. Therefore, we looked for a disruption in the biosynthetic pathway of vasopressin (VP).

As we found no processed VP staining in the paraventricular nucleus (PVN) of a WS patient, whereas the precursor appeared to be present, we investigated whether there is a VP processing disturbance in the WS, *e.g.* at the level of the responsible processing enzymes of the regulated secretory pathway. At present, the prohormone convertase family of processing enzymes consists of furin, PC1/PC3, PC2, PACE4, PC4, PC5/PC6, and PC7 (15). All of these endoproteases cleave proproteins at pairs of basic amino acids and at selected single basic residues in specific compartments of the secretory pathway (16). The convertases PC1/PC3 and PC2 are selectively present in (neuro)endocrine cells (*e.g.* hypo-

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thalamus), and there is now ample evidence that these endoproteases are the prohormone-converting enzymes in the regulated secretory pathway (17).

In the present study we investigated the processing of the VP precursor by examining the expression of the VP prohormone and its various processing products, *viz.* VP, neuropeptide Y (NP) and the C-terminal glycopeptide (GP) in the supraoptic nucleus (SON) and PVN. The expression of oxytocin (OT) was also investigated because it is one of the other major peptides of the SON and PVN (18). In addition, we investigated the expression of the neuroendocrine prohormone convertases PC1/PC3 and PC2, and the neuroendocrine polypeptide 7B2, which can act as a molecular chaperone, preventing premature activation of the PC2 precursor in the regulated secretory pathway (19). To evaluate the regional specificity of the processing disturbance, we also investigated the pituitary and the nucleus basalis of Meynert (NBM), which is one of the major sources of cholinergic innervation of the cerebral cortex (20) but is also a nucleus in which peptides are synthesized (21).

Materials and Methods

For the present study, 10 hypothalami and 1 pituitary from control subjects without any primary neurological, psychiatric, or (neuro)endocrine disease and ranging in age from 19–88 yr were obtained at autopsy. Two hypothalami from control subjects without any primary neurological or psychiatric disease were matched for extreme postmortem delay and fixation time with 3 hypothalami and 1 pituitary from clinically diagnosed WS patients (see below). After weighing the brain, the hypothalamus was dissected out and fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) on a rocking table at room temperature. Subsequently, tissue was routinely dehydrated and embedded in paraffin. Serial 6- μ m frontal sections were cut and mounted on chrome-alum-coated glass slides. Sections for microwave treatment were mounted on Superfrost/Plus glass slides (Menzel Gläser, Braunschweig, Germany). For anatomical orientation, every 50th section was stained with 0.1% thionine in acetate buffer (pH 4.0).

Case 1: 96-46 (Department of Pathology and Department of Ophthalmology, University of Nijmegen, Nijmegen, The Netherlands)

A 16-yr-old male was diagnosed as having WS upon development of IDDM, hypothalamic DI, and OA. No hearing loss was reported. There was sclerosis of the ostium urethra causing hydronephrosis, for which he underwent an endoresection. He died postoperatively from septic shock caused by a urinary tract infection. There was a positive family history for WS, with one sibling with OA, DM, DI, and deafness. At autopsy, the pancreas was reported to contain fewer than normal islets of Langerhans. Neuropathology of this patient was described by Mtanda *et al.* (22).

Case 2: 95-68 (Institute of Psychiatry, Department of Neuropathology, London, UK)

A 30-yr-old woman was diagnosed as having WS upon development of DM and OA at age 6 yr. She subsequently developed autonomic neuropathy, cerebellar uncoordination, and Parkinsonian signs at age 29 yr. Neuroradiological examination at about the same time demonstrated olivopontocerebellar degeneration. One year before death, sensorineural deafness was noted, and DI was diagnosed. She had undergone several hospital admissions for hypoglycemic coma and respiratory arrest, and she died of the latter. There was a positive family history of WS; a brother had developed OA and IDDM at age 6 yr, and DI 5 years before death at age 40 yr. At autopsy, the pancreas was fatty but showed no obvious abnormality.

Case 3: 94-133 (Institute of Psychiatry, Department of Neuropathology, London, UK)

A 49-yr-old man, who was described when he was 19 yr old by Rose *et al.* (23), developed DM at 15 yr, OA at 19 yr, sensorineural deafness at 26 yr, and DI at 39 yr. He also had features of autonomic neuropathy. He underwent emergency hospital admission for diabetic ketoacidosis, renal impairment, sepsis, and gastrointestinal bleeding. Despite treatment, he deteriorated and died. A brother had also been diagnosed with WS, having developed OA at 10 yr and DM at 15 yr. At autopsy, death was ascribed to bronchopneumonia and chronic pyelonephritis. Lymphocyte-derived mitochondrial DNA was negative for major rearrangements and deletions.

Immunocytochemistry

For immunocytochemistry, deparaffinized sections were incubated with the following antibodies as previously described (24).

VP antibodies. Monoclonal mouse anti-VP was used, which recognizes Phe in position 3, the most important determinant in the VP ring (VP III-D-7, diluted 1:200, provided by Dr. A. Hou-Yu, Columbia University, New York, NY) (25). In the nonprocessing cell line HEK-293 transfected with the human VP gene, there was no reaction with this antibody. However, in the Neuro2A cell line (26), which shows regulated pathway processing, transfected with the human VP gene, an intense reaction was obtained, indicating that only processed VP was recognized.

Polyclonal rabbit anti-VP preabsorbed with OT, which preferentially recognizes VP in its processed form (Truus, 29-01-1986) was diluted 1:1000 (27).

NP antibodies. Monoclonal mouse anti-NP, with the most likely epitope located between amino acids 75 and 76 of the NP moiety (28) (PS41, diluted 1:200, provided by Dr. H. Gainer, NIH, Bethesda, MD), was used (29). In immunoprecipitation assays the antibody brought down NP as well as its precursor molecule synthesized *in vivo*, suggesting that this antibody recognizes both the VP precursor and processed NP (29).

Polyclonal rabbit anti-NP against a synthetic human N-terminal NP fragment representing the residues 1–12+Tyr [N-Term NP, diluted 1:500, prepared by Dr. A. G. Robinson, University of California (Los Angeles, CA) and supplied by the Pituitary Hormones and Antisera Center, Director A. F. Parlow] was used. In tricine SDS-PAGE (30) and subsequent Western blotting, the antibody recognizes predominantly processed NP.

Polyclonal rabbit anti-NP against a synthetic human C-terminal NP fragment of the residues 80–91+Tyr (C-Term NP, diluted 1:500, prepared by Dr. A. G. Robinson, University of California-Los Angeles, and supplied by the Pituitary Hormones and Antisera Center) was used (31). In tricine SDS-PAGE and subsequent Western blotting, the antibody recognizes predominantly processed NP.

GP antibodies. Polyclonal rabbit anti-GP against a synthetic human GP-(22–39) (Boris, diluted 1:1000, provided by W. G. North, Dartmouth Medical School, Lebanon, NH) was used (32). This antibody reacts in both HEK-293 and Neuro2A cell lines (26), so it at least recognizes the VP precursor. In tricine SDS-PAGE and subsequent Western blotting, the antibody recognizes predominantly the VP precursor.

Polyclonal rabbit anti-GP directed against the guinea pig glycoprotein moiety of the VP precursor (K 1.7, diluted 1:500, provided by I. C. A. F. Robinson, National Institute for Medical Research, London, UK) was used (33). This antibody reacts in both HEK-293 and Neuro2A cell lines (26), which indicates that it at least recognizes the VP precursor. In tricine SDS-PAGE and subsequent Western blotting, the antibody recognizes predominantly the VP precursor.

OT antibody. Monoclonal mouse anti-OT with three different antigenic determinants on the OT molecule, the Ile in position 3, Pro in position 7, and Leu in position 8 (A-I-28, diluted 1:200, provided by Dr. A. Hou-Yu, Columbia University, New York, NY) was used (34).

7B2 antibodies. Polyclonal rabbit anti-7B2 against synthetic human 7B2-(23–39) (RB-7, diluted 1:500) was used (35, 36).

Monoclonal mouse anti-human 7B2, recognizing 7B2 sequence 64–94, [MON-144 (supernatant), diluted 1:10, provided by H. L. P. van Duynhoven, Helmond, The Netherlands] was used (37).

Monoclonal mouse anti-human 7B2, recognizing 7B2 sequence 128–143 [MON-102 (ascites), diluted 1:1000, kindly by H. L. P. van Duynhoven) was used (37).

Polyclonal rabbit anti-mouse 7B2 against residues 156–186, recognizing 156–171 of the C-terminus of 7B2 (CT 7B2, Rb-4, 14-2-1991, diluted 1:250) was used (38).

PC1 antibodies. Polyclonal rabbit anti-PC1 against a human PC1 fragment 43–628 was used (PC1, diluted 1:500). In Western blot, it was positive for AtT20 cell lysate and negative for bacterial fusion proteins with parts of PC2, PC4, PACE4, PC6A, C-terminus of PC6B, and prodomain of furin. It was positive in immunoprecipitation [transfected PK (15) cells, no reaction in untransfected cells]. It was immunofluorescence positive in AtT20 cells as endogenous activity and in transfected cells, but negative in COS-1 cells.

Polyclonal rabbit anti-mouse PC1 against residues 84–100 has 88% homology with human PC1 (2B6 N-terminal, diluted 1:500, provided by I. Lindberg, Louisiana State University, New Orleans, LA) (39).

Polyclonal rabbit anti-mouse PC1 against residues 629–726 (PC1 92-12-08 C-terminal, diluted 1:500) was used (40).

PC2 antibody. Polyclonal rabbit anti-human PC2, against residues 122–637 (PC2, diluted 1:500) was used. In Western blot, it was negative for AtT20 cell lysate and bacterial fusion proteins with parts of PC1, PC4, PACE4, PC6A, C-terminus of PC6B, and prodomain of furin. It was positive in immunoprecipitation [transfected PK (15) cells, no reaction in untransfected cells]. In immunofluorescence, it was positive in transfected PK (15) cells, but negative in untransfected PK (15) cells and AtT20 cells.

Glial fibrillary acidic protein (GFAP) antibody. Polyclonal rabbit anticow GFAP was used (diluted 1:500; provided by Dako Corp., for details see Dako Corp. specification sheet, code no. Z 0334, lot 119).

Those tissue sections that stained with some antibodies very faintly or not at all were pretreated with their controls in the microwave according to a modified procedure (41), as described by Lucassen *et al.* (42). After deparaffinizing, the sections were briefly treated according to the following procedure: 1) a 15-min wash in distilled water; 2) incubation in a microwave oven twice for 5 min in a citric acid solution (0.1 mol/L citric acid monohydrate and 0.1 mol/L trisodium citrate dihydrate), pH 6.0; 3) a 2-fold 5-min wash in TBS; and 4) incubation in 5% nonfat dried milk for 30 min (Elk, Campina, Eindhoven, The Netherlands). The intensity of the immunoreactivity was estimated semiquantitatively.

Controls

For specificity of the VP, NP, GP, OT, and GFAP antibodies, see the above-described procedures and mentioned references. The antibodies raised against different parts of 7B2 were adsorbed with recombinant 7B2 protein (prepared by Drs. D. W. Eib and M. Van Horssen; for details, see Ref. 37). PC1 and PC2 were absorbed with their respective recombinant PC1 and PC2 antigens. 2B6 N-terminal PC1 and 92-12-08 C-terminal PC1 were absorbed with their respective antigen residues (2B6 N-terminal PC1 antigen provided by I. Lindberg, Louisiana State University; PC1). After adsorption, no reaction was seen. When the first antibodies were omitted from the procedure, the reaction was absent as well.

Results

The SON and PVN cells of both the 10 control patients (43) and the 2 additional controls who were matched for extreme postmortem delay and fixation time with 2 WS patients, respectively (94-133 and 95-68), stained intensely with all antibodies against VP, NP, and GP regardless of the deliberately extended range of postmortem intervals and fixation times. The two WS patients (94-133 and 96-46) with DI had virtually no processed VP or NP immunoreactivity in the cells of either the SON or the PVN (Table 1 and Fig. 1, A and B). However, in these patients a normal number of cells displayed a clear GP (VP precursor) immunoreactivity in the

TABLE 1. Immunoreactivities with and without microwave for parts of the vasopressin precursor and oxytocin in magnocellular neurons of the PVN and SON of three Wolfgram patients and in the pituitary of one control patient

	Brain weight (g)	Age (yr)/sex	PMD	Fixation	VP proc. form III-D-7	MVP proc. form III-D-7	VP proc. form Truus	MVP proc. form Truus	NP PS41 75-76	MNP PS41 75-76	NP N-Term 1-12	MNP N-Term 1-12	NP C-Term 80-91	MNP C-Term 80-91	GP guinea pig K1.7	MGP guinea pig K1.7	GP human 22-39 Boris	MGP human 22-39 Boris	OT A1-28
Wolfgram patients																			
96-46	1500	16 m	12 h	Few weeks	-	-	-	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
95-68	1174	31 f	3 days	14-29 months	-	-	-	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
94-133	1369	49 m	5.85 days	2 months	-	-	-	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Pituitaries																			
Wolfgram 96-46	1500	16 m	12 h	Few weeks	-	-	-	-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Neural lobe									+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Anterior lobe									+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Control 92.001	1300	83 m	6.5 h	41 days	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Neural lobe									+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Anterior lobe									+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

f, Female; m, male; g, grams; VP, vasopressin; MVP, microwave vasopressin; proc., processed; NP, neurophysin; MNP, microwave neurophysin; GP, glycopeptide; MGP, microwave glycopeptide OT, oxytocin; PMD, postmortem delay. Stainability: -, no staining; +, faint; ++, moderate; +++, intense; +++++, very intense; A, high background; //, differential staining in SON and PVN is indicated by SON//PVN. The number of cells is per section: *, 1 cell; **, 2 cells. Twelve control patients stained intensely with all of the antibodies against VP, NP, GP, and OT.

FIG. 1. In paraffin sections through the PVN of WS patient 94-133, there was no immunoreactivity with the antibody III-D-7 recognizing processed VP (A) and also no immunoreactivity with the antibody PS41 predominantly recognizing the processed form of NP (B), but there were many positive cells with the antibody Boris recognizing predominantly the VP precursor (C). Bar = 25 μ m.

FIG. 2. In paraffin sections of WS patient 95-68, there was no immunoreactivity with the antibody Truus in the SON preferentially recognizing the processed form of VP (A), but after microwave pretreatment some staining was seen with Truus (B), showing that this patient still has VP expression. Bar = 25 μ m.

PVN (Table 1 and Fig. 1C), but the cells were clearly too small. There were no distinct subdivisions within the PVN (44), so there was a uniform distribution of GP immunoreactivity throughout the PVN. In the SON, only 1 or 2 GP-immunoreactive cells were observed. The lateral part of the SON of patients 94-133 and 96-46 did not show neurons but, rather, gliosis. There was, moreover, an intense staining with a GFAP antibody in the VP part of the SON. Microwave pretreatment did not retrieve VP or NP staining in these WS patients (Table 1). WS patient 95-68, who only showed DI in the last year of life, exhibited a different profile of immunoreactivity. In this patient there was no VP staining without microwave pretreatment (Fig. 2A), but after microwave pretreatment, intense VP staining was shown with the VP antibody Truus in the SON and PVN (Fig. 2B). Moreover, there was NP and GP staining with and without microwave pretreatment in this patient. Furthermore, no gliosis was observed in the SON of the same WS patient. OT immunoreactivity was present in all control and WS patients as very intense staining in a large number of cells of the SON and PVN (Table 1).

The 10 control patients and the 2 controls with extreme postmortem delay and fixation time stained with all PC1, PC2, and 7B2 antibodies in the SON, PVN, and NBM. Compared to the SON and PVN, the staining intensity in the NBM was generally lower for PC1. With the PC1 antibodies there was always staining in the SON and PVN of WS patients after microwave pretreatment (Table 2). However, the WS patients (94-133 and 96-46) had lower PC1 immunoreactivity than WS patient 95-68. With 4 different antibodies directed against different parts of 7B2, no clear staining in WS patients 94-133 and 96-46 was found, even not after microwave pretreatment in the SON or PVN (Table 2, only the 7B2 antibody MON-102 is shown, and Fig. 3A). Also, in these patients the PC2 antibody did not show any clear staining in the SON and PVN after microwave pretreatment (Table 2 and Fig. 3C). However, WS patient 95-68 showed staining with all 4 7B2 antibodies and the PC2 antibody in the SON and PVN after microwave pretreatment (Table 2, only the 7B2 antibody MON-102 is shown). As opposed to the SON and PVN, the NBM of WS patient 94-133 showed faint to moderate staining with different 7B2 antibodies (Table 2, only the 7B2 antibody MON-102 is shown, and Fig. 3B) and the anterior lobe of the pituitary of WS patient 96-46 displayed an intense staining with the 7B2 antibody MON-102 (Table 2 and Fig. 4C). The NBM (Table 2 and Fig. 3D) of WS patient 94-133 and the anterior lobe of WS patient 96-46 (Table 2 and Fig. 4D) also displayed reaction with the PC2 antibody.

The pituitary of a control patient (92.001) showed a very intense immunoreactivity for all VP, NP, and GP antibodies in the neural lobe (Table 1). The neural lobe of WS patient 96-46 showed very faint processed VP and NP staining, but moderate GP staining. OT immunoreactivity was very intense in the neural lobe of both pituitaries (Table 1). In the control pituitary, PC1 immunoreactivity was very intense in the neural lobe and in cells of the anterior lobe (Table 2). PC1 immunoreactivity with the three different antibodies was absent to moderate in the neural lobe and absent to very intense in the anterior lobe of the WS pituitary. In the anterior and neural lobes of the control pituitary (92.001), very intense 7B2 and PC2 im-

TABLE 2. Immunoreactivities with and without microwave (M) for PC₁, PC₂, and 7B2 in magnocellular neurons of the PVN, SON, and NBM of three Wolfram patients and in the pituitary of one Wolfram and one control patient

	PC1 43-628	MPC1 43-628	PC1 84-100 mouse 2B6 N-Term	MPC1 84- 100 mouse 2B6 N-Term	PC1 629-726 92-12-08 C-Term	MPC1 629-726 92-12-08 C-Term	7B2 128-143 MON102	M7B2 128-143 MON102	PC2 122-637	MPC2 122-637
Wolfram patients										
96-46	+	++++	-	++	-	+++	-//*	-//***	-	-
	NBM: +	NBM: +++++	NBM: -	NBM: +	NBM: -	NBM: +	NBM: ++	NBM: ++	NBM: -	NBM: -
95-68	++	++++	- ^a	+++	- ^b	++++	-	+++	-	++//+
	NBM: -	NBM: ++	NBM: -	NBM: -	NBM: -	NBM: +	NBM: +	NBM: +++	NBM: -	NBM: ++
94-133	-	+//++	-	++	-	+++//++	-	-//***	-	-
	NBM: -	NBM: ++	NBM: -	NBM: +	NBM: -	NBM: ++	NBM: ++	NBM: ++	NBM: -	NBM: +
Pituitaries										
96-46										
Neural lobe	+	++	-	+	-	++	-	-	-	-
Anterior lobe	+++	++++	-	++	-	++++	++++	++++	+++	++++
92.001										
Neural lobe	++++	++++	++	++++	++++	+++	++++	++++	++++	++++
Anterior lobe	++++	++++	+++ ^c	++++	+++ ^c	++	++++	++++	+++ ^c	++++

Stainability: -, no staining; +, faint; ++, moderate; +++, intense; +++++, very intense; //, differential staining in SON and PVN is indicated by SON//PVN. The number of cells is per section: *, 1 cell; ***, some cells.

^a SON few well stained cells.

^b One very faint cell in SON, some very faint cells in PVN.

^c Some cells stained. Twelve controls stained intensely with all PC₁, PC₂, and 7B2 antibodies.

FIG. 3. In paraffin sections, there was no immunoreactivity with MON-102 recognizing 7B2 in the PVN (A), but there was moderate staining with MON-102 in the NBM of WS patient 94-133 after microwave pretreatment (B). There was no immunoreactivity with antibody PC₂ in the PVN (C), but there was faint staining with this antibody in the NBM of WS patient 94-133 (D). In conclusion, the 7B2 and PC₂ genes are still expressed in this patient. Bar = 25 μ m.

munoreactivity was present (Table 2 and Fig. 4, A and B). 7B2 and PC₂ immunoreactivities were absent in the neural lobe of the pituitary of WS patient 96-46, but very intense in the anterior lobe (Table 2 and Fig. 4, C and D).

Discussion

The present study was directed toward elucidating the cellular and biochemical basis of hypothalamic DI occurring in at least 72% of WS cases (5, 12). A defect in the VP gene, located on chromosome 20 (13, 14), is not expected, because WS is associated with a defect on chromosome 4 (7). For the same reason, a defect in processing due to a point mutation within the cleavage sites of the VP precursor, as described for the cleavage site of proinsulin (45) and blood coagulation factor IX (46), was not considered.

We investigated the hypothalami of three WS patients. In

the two WS patients with DI (94-133 and 96-46), the SON showed no VP, NP, or GP, except for one or two positive cells per section. The lateral part of the SON exhibited gliosis, as shown by an extensive GFAP staining, which might be a reaction to VP cell death. However, the PVN of both patients showed the absence of VP and NP and the presence of the VP precursor, as apparent from GP immunoreactivity throughout the hypothalamus. Quantification showed that in the PVN the total number of VP neurons (as determined by GP immunoreactivity) of WS patient 94-133 was in the normal range (Swaab, D. F., personal communication), which points to the absence of VP neuron loss in this nucleus.

Two other conclusions may also be drawn from finding expression of the GP in the PVN. First, transcription is intact, as already anticipated from the linkage data. Secondly, the VP precursor has been translated entirely, because GP is at

FIG. 4. In paraffin sections from the pituitary of the control patient 92.001, there was intense immunoreactivity for MON-102 recognizing 7B2 (A) and for PC2 (B) in cells of the anterior lobe and fibers of the neural lobe. In WS patient 96-46, there was no fiber staining with MON-102 recognizing 7B2 (C) or PC2 (D) in fibers of the neural lobe, but there was very intense immunoreactivity for both antigens in cells of the anterior lobe of the pituitary. In conclusion, the 7B2 and PC2 gene are still expressed in this patient. AL, Anterior lobe; NL, neural lobe. Bar = 25 μ m.

the C-terminal end of the precursor. A splicing defect of the heteronuclear VP transcript, so that exons 1 and 2 are expelled, could theoretically explain the results. However, this seems highly unlikely in view of the existence of some residual VP and NP staining in patients 96-46 and 94-133 and the microwave-disclosable staining in patient 95-68. Instead, the data point toward disturbance of the enzyme-mediated processing of the VP precursor.

In agreement with this hypothesis is that in both WS patients who stained for the VP precursor but not for VP (96-46 and 94-133) also lacked stainable (neuro)endocrine proprotein convertase PC2 and its molecular chaperone 7B2 in the

SON and PVN, and the fact that PC2 is predominantly located in VP cells and almost not at all in OT cells (43), which suggests that PC2 is a good candidate enzyme for cleavage of the VP precursor at the dibasic site connecting VP-NP. The presence of PC1 in WS might indicate that PC1 is unable to cleave the VP-NP site.

Although an absence of PC2 and 7B2 would provide a biochemically plausible interpretation for the aberrant VP staining pattern and, in turn, the neuroendocrine abnormality, the more fundamental explanation is currently obscure. For instance, a straightforward defect in either the PC2 or 7B2 gene is not tenable for two reasons. First, the

human chromosomal location of both, 20 and 15 respectively (47, 48), is inconsistent with the WS linkage to chromosome 4 (7). Secondly, the NBM of WS patient 94-133 and the anterior lobe of WS patient 96-46 expresses apparently normal amounts of PC2 and 7B2. Ongoing studies are directed toward establishing whether targeted disruption of the PC2 and 7B2 axis reproduces the immunocytochemical profile of WS and what factors influence the respective aberrant enzyme activities.

To date, we have glossed over the fact that cases 96-46 and 94-133 have a consistent staining pattern, whereas 95-68 is somewhat different in exhibiting residual VP, PC2, and 7B2 with no gliosis in the SON. There are several possible explanations. First, there has been a clinical misdiagnosis. Second, there is etiologically heterogeneity even within an apparently homogeneous set of patients. Third, the pathology may evolve over the course of the disease. The present series, however, developed the syndrome defining OA and IDDM before the age of 20 yr, has no significant features to suggest a differential diagnosis and has a familial pattern of occurrence consistent with that of autosomal recessive transmission. The second possibility is raised by the existence of family K (8), who were excluded from linkage to chromosome 4. Even though the clinical features were atypical, it is not possible to establish genetic homogeneity until the underlying defect is isolated. The third possibility, evolution of the hypothalamic pathology, is consistent with the short duration of DI in WS patient 95-68, in contrast to the longer duration of DI symptoms in one other WS patient (94-133). Moreover, the occurrence of reactive gliosis only in the cases with the longest standing DI may be viewed in the same light. In accordance with this progressive deterioration hypothesis, one may suggest the following pathogenesis. First, there is a down-regulation of the amount of processed VP likely to result in partial DI (WS patient 95-68). Secondly there is no processed VP whatsoever; thus, there is complete DI, although there are still many VP precursor-containing cells (GP positive) present (WS patients 94-133 and 96-46). Thirdly, the VP cells disappear, as seen in the SONs of WS patients 94-133 and 96-46.

The results of this study not only hold the prospect of elucidating the cause of an intriguing facet of WS, but also have wider implications for understanding the mechanisms of (neuro)endocrine disease.

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