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In this issue: MK-677 reverses diet-induced nitrogen wasting, suggesting that, if these short-term anabolic effects are maintained in patients who are catabolic because of certain acute or chronic disease states, it may be useful in treating catabolic conditions. (See page 320.)

Also in this issue: A heterozygous C to T base change was identified at nucleotide position 1126, exchanging codon 373 from Ala to Val in the first transmembrane domain. The mutated receptor displayed an up to 7.5-fold increase of basal cyclic adenosine monophosphate production compared with the wild-type receptor in transiently transfected COS-7 cells. (See page 476.)

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Attenuation of the Polypeptide 7B2, Prohormone Convertase PC2, and Vasopressin in the Hypothalamus of Some Prader-Willi Patients: Indications for a Processing Defect

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ABSTRACT

7B2 is a neuroendocrine chaperone interacting with the prohormone convertase PC2 in the regulated secretory pathway. Its gene is located near the Prader-Willi syndrome (PWS) region on chromosome 15. In a previous study we were able to show 7B2 immunoreactivity in the supraoptic nucleus (SON) or the paraventricular nucleus (PVN) in only three of five PWS patients. Here we report that in contrast with five other PWS patients, the neurons in the hypothalamic SON and PVN of the two 7B2-immunonegative PWS patients also failed to

show any reaction using two antibodies directed against processed vasopressin (VP). On the other hand, even these two cases reacted normally with five antibodies that recognize different parts of the VP precursor. This finding pointed to a processing defect. Indeed, the same patients had no PC2 immunoreactivity in the SON or PVN, whereas PC1 immunoreactivity was only slightly diminished. In conclusion, in the VP neurons of two PWS patients, greatly reduced amounts of 7B2 and PC2 are present, resulting in diminished VP precursor processing. (*J Clin Endocrinol Metab* 83: 591–599, 1998)

PRADER-WILLI syndrome (PWS) was first described in 1956 on the basis of gross obesity (due to insatiable hunger), hypogonadism and hypogenitalism, short stature, mental retardation, and a tendency to develop diabetes mellitus in adolescence (1, 2). Major symptoms of this syndrome are the result of hypothalamic disturbances (3). For example, the number of oxytocin (OT) cells of the paraventricular nucleus (PVN) of the hypothalamus, the putative satiety neurons, is strongly diminished in PWS (4). Approximately 70% of the PWS cases are due to a 15q11–13 deletion in the paternally contributed chromosome, whereas about 28% of the PWS cases are due to maternal uniparental disomy. Fewer than 2% of the cases have an abnormality in the imprinting process, which causes nonexpression of the paternal genes in the PWS critical region (5). Close to the PWS region is the 7B2 gene localized on 15q13–14 (6). The molecular chaperone 7B2, a precursor consisting of 185 amino acids (7), is selectively expressed in (neuro)endocrine cells (8). Expression of 7B2 has previously been studied in the supraoptic nucleus (SON) and PVN of the hypothalamus of five subjects clinically diagnosed as PWS patients, of whom two PWS patients displayed a complete absence of 7B2 protein (9). The

hypothalami of these two PWS patients hardly stained with an antibody recognizing processed vasopressin (VP), but all five PWS patients stained well with an antibody against the VP precursor (4, 9). Braks *et al.* (10) showed that 7B2 transiently interacts with the prohormone convertase PC2, which acts as a molecular chaperone, preventing premature activation of pro-PC2 in the regulated secretory pathway. PC2 belongs to the Kex2-related family of eukaryotic endoproteases. At present this family consists of furin, PC1 (PC3), PC2, PACE4, PC4, PC5/PC6, and PC7 (11). These endoproteases cleave proproteins at pairs of basic amino acids and at particular single basic residues in specific compartments of the secretory pathway (12). The convertases PC1 and PC2 are selectively present in (neuro)endocrine cells, and there is ample evidence that these endoproteases are the prohormone-converting enzymes acting in the regulated secretory pathway (13).

The current study was initiated to investigate the expression of the major neuroendocrine peptides VP and OT in the SON and PVN (14), in particular in those two PWS patients that had a disturbed expression of 7B2. Therefore, we tested immunocytochemically the expression of different parts of the VP precursor and OT in the SON and PVN of seven PWS hypothalami. Because of the discrepancy found in the two 7B2-negative PWS patients, *viz.*; no staining of processed VP, whereas antibodies against other parts of the VP precursor revealed positive staining, we investigated the possibility of a processing defect by determining the expression of the

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TABLE 1. Immunoreactivities for parts of the vasopressin precursor and oxytocin in magnocellular neurons of the PVN and SON of 7 PWS patients and 11 controls

Subject no. ^a	Sex	Age (yr)	Brain wt (g)	Postmortem delay (h)	Fixation (days)	Clinical diagnosis	VP processed form III-D-7	VP processed form Truus	NP PS41 75-76	NP N-Term 1-12	NP C-Term 80-91	GP human 22-39 Boris	GP guinea pig K1.7	OT A-1-28
Patients														
96-34	f	25	1300	35	26	PWS	++++	++++	++++	++++	+++	++++	++++	++++
92-005/44282	m	28	1415	30	7-14	PWS	-	-	++++ ^b	ND	ND	++++	++++	++++
83-011/43830	f	30	1310	4.5	ND	PWS	++++	++++	++++	++++	++++	++++	++++	++++
91-058/91252	f	33	1223	5	33	PWS	+/+	+	++++	+	+	++++	++++	++++
93-056	m	38	1540	45	385	PWS	-	-	++++	++++	+/+	++++	++++	++++
95-104/	m	51	1570	16	32	PWS	++ ^c	+/++++ ^d	+/++ ^e	++	+/	++++	++++	++++
95-9090														
90-111/31790	f	64	1150	20	14	PWS	++++	++++	++++	++++	++++	++++	++++	++++
Controls														
87-63	m	19	1310	88	44	Multitraumata	++++	++++	++++	++++	++++	++++	++++	++++
95-83	m	25	1549	24	607	Congenital heart disease, ruptured aneurysma	++++	+++	++++	++++	+/++++	++++	++++	++++
84-15	m	29	1400	13	41	Congenital heart disease, heart failure	++++	++++	++++	++++	++++	++++	++++	++++
85-27	f	29	1150	24	60	Alcoholic-induced hepatitis	+/++++	+++	+++	+++	+++	+++	+++	+++
81-20	f	30	1460	24	39	Acute heart arrest	++++	++++	++++	++++	++++	++++	++++	++++
87-69	m	41	1440	120	44	Contusion cerebri, lung embolism	++++	++++	++++	++++	++++	++++	++++	++++
82-16	f	52	1370	5	33	Bronchopneumonia, medullary breast cancer, multiple cerebral and meningeal metastases	++++	++++	++++	++++	++++	++++	++++	++++
81-03	f	64	1090	8	44	Hemorrhagic peptic ulcer, hypovolemic shock, renal insufficiency	++++	++++	++++	++++	++++	++++	++++	++++
80-09	f	72	1200	8	59	Lung infarction, bronchopneumonia, highly differentiated squamous carcinoma	++++	++++	++++	++++	++++	++++	++++	++++
81-10	m	83	1280	22	42	Divericulitis	++++	++++	++++	++++	++++	++++	++++	++++
81-15	m	88	1370	47	60	Heart failures, cardiac asthma, myocard infarction	++++	++++	++++	++++	++++	++++	++++	++++

f, female; m, male; ND, not determined because of lack of material; VP, vasopressin; NP, neurophysin; GP, glycopeptide; OT, oxytocin; SON, supraoptic nucleus; PVN, paraventricular nucleus; PWS, Prader-Willi syndrome. Stainability: -, no staining; +/-, very faint; +, faint; ++, moderate; ++++, intense; +++++, very intense; //, differential staining in SON and PVN is indicated by SON/PVN.

^a Netherlands Brain Bank number/obduction number.

^b Few cells in PVN, one cell in SON.

^c Some cells in SON, one cell in PVN.

^d Large number of cells.

^e Some cells in SON/PVN.

^f Some cells in SON, no cells in PVN.

FIG. 1. Paraffin sections of the SON from PWS patient 93-056 shows no immunoreactivity with antibody III-D-7 recognizing processed VP (A), but very intense immunoreactivity with antibody PS41 recognizing NP predominantly in its processed form (B) and very intense immunoreactivity with antibody Boris recognizing predominantly the VP precursor (C). Bar = 25 μ m.

TABLE 2. Immunoreactivities for parts of the 7B2 precursor with and without microwave in the SON and PVN of seven PWS patients

Subject no.	Postm. delay (h)	Fix. (days)	RB-7 (23-39)	Microwave RB-7 (23-39)	MON144 (64-94)	Microwave MON144 (64-94)	MON102 (128-143)	Microwave MON102 (128-143)	CT 7B2 (156-186)	Microwave CT 7B2 (156-186)
96-34	35	26	-	++	-	+//+/-	+	+	++	++
92-005/44282	30	7-14	-	ND	-	ND	-	++	-	ND
83-011/43830	4.5	ND	++	+++	+++	+//-	++++	+//-	++	++
91-058/91252	5	33	-	++	+	+	- ^a	+	++	++
93-056	45	385	-	++	-	+	-	++	-	+//+/-
95-104/95-9090	16	32	-	++	+	+	-	++	+//++	++
90-111/31790	20	14	-	+++//+	+	++	++	+	+	+++//+

ND, Not determined because of lack of material; SON, supraoptic nucleus; PVN, paraventricular nucleus; PWS, Prader-Willi syndrome. Stainability: -, no staining; +/-, very faint; +, faint; ++, moderate; +++, intense; +++++, very intense; // differential staining in SON and PVN is indicated by SON//PVN.

^a One large solitary cell.

neuroendocrine convertases PC1 and PC2 in these hypothalamic cells.

Materials and Methods

For the present study, brains from 11 control subjects, ranging in age from 19-88 yr, without a primary neurological, psychiatric, or endocrinological disease (for clinicopathological details, see Table 1) were obtained at autopsy. In addition, the hypothalami of 7 clinically diagnosed PWS patients were studied (for details of 5 of the 7 PWS patients, see Refs. 4 and 9), the clinical data of the other two new patients are given below). The control patients were in the same range for age, postmortem delay, and fixation time. The required separate permission for brain autopsy was obtained either from the patients themselves or their partners or relatives. Neuropathological investigation of the control subjects was performed by Drs. W. Kamphorst (Free University, Amsterdam, The Netherlands), D. Troost (Academic Medical Center, Amsterdam, The Netherlands), or Prof. F. C. Stam (Netherlands Brain Bank, Amsterdam, The Netherlands). The hypothalamus was dissected out and fixed in 4% formaldehyde at room temperature. Subsequently, tissue

was routinely dehydrated and embedded in paraffin. Serial 6- μ m coronal 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).

Case 1: 96-34 (The John F. Kennedy Institute, Glostrup, Denmark)

The patient was a 25-yr-old woman. Pregnancy was uncomplicated. Nothing was reported about the quantity or quality of fetal movements by the mother. The patient was a floppy infant with a weak cry and was not able to suck during the first days of life. The patient became obese at the age of 2 yr and from that time had been kept on an energy-reduced diet (1200 Cal/day). She took food from everywhere, and the kitchen had to be locked. Her face was "typical for PWS," and her hands and feet were small. Pubarche occurred at 13 yr of age. She showed some mammary development but no menarche at 16 yr (last hospital records). At the age of 15 yr, the patient was able to read, but not to calculate. No task problems could be solved without guidance and support. There was no diabetes mellitus, and the glucose tolerance test was normal. Hypothy-

roidism was found and was treated with T_4 (0.05–0.1 mg/day). Other hormone levels were: FSH, 19 IE/L (normal levels, 2–30 IU); and LH, above 2 IE/L (normal levels, 3–120 IE/L depending on the stage of the menstrual cycle). There was a normal GH response on glucagon stimulation, a normal computed tomography scan and x-ray of the cranium, and no abnormalities on the electroencephalogram. At autopsy she had a length of 155 cm, and a weight of 52 kg. The uterus and ovaries were of normal size and shape. For one marker (D15S97), the patient showed a deletion in the PWS region. She had 9 points according to the diagnostic criteria of Holm (15), with at least 8 points being necessary for a positive PWS diagnosis.

Case 6: 95-104 (Mental Retardation Institute, Zwammerdam, The Netherlands)

The patient was a 51-yr-old man. There was no history of hypotonia or poor suckling reflex during the neonatal period. At the age of 5 yr he developed obesity. The patient had a "characteristic face" for PWS. In adulthood, a small penis and a left testis with a volume of 9 mL were reported. The right testis was removed curatively because of a seminoma at the age of 29 yr. There was a normal sexual hair pattern. His length was originally 1.65 m, but was reduced later on by scoliosis; his weight was 92 kg. Small hands and feet were observed. The patient was mentally retarded, with an IQ of 49, and exhibited a number of behavioral problems, such as temper fits, obstinate behavior, stealing food, and nicotine addiction. There was no apnea syndrome or rapid eye movement sleep disturbances, but there was an abnormal hypnogram, showing very little deep sleep, too many arousals, and excessive sleepiness during the day. He also developed hypertension and hypercholesterolemia without diabetes mellitus. Endocrinological investigations showed no thyroid or adrenal abnormalities or disturbances of the X-sella turcica. He probably died of cardiac arrhythmia from a 3-week-old infarct of the left chamber of the heart complicated by bronchopneumonia of the right lung. According to the diagnostic criteria of Holm *et al.* (15), the patient had 8.5 points of the required 8 for PWS diagnosis. Molecular genetic analysis revealed no deletion, but maternal disomy was shown with probe PW71, which is indicative of PWS.

Immunocytochemistry

For immunocytochemistry, deparaffinized sections were incubated with the following antibodies (for details of the procedure, see Ref. 9).

VP antibodies. Monoclonal mouse anti-VP (VP III-D-7) was used, which recognizes Phe in position 3 as the most important determinant in the VP ring, diluted 1:200 (provided by Dr. A. Hou-Yu, Columbia University, New York, NY) (16). 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 (17), 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 was used; it preferentially recognizes VP in its processed form (Truus, 29-01-1986; diluted 1:1000) (18).

Neurophysin (NP) antibodies. Monoclonal mouse anti-NP, with the most likely epitope located between amino acids 75 and 76 of the NP moiety, was used (19) (PS41, diluted 1:200, provided by Dr. H. Gainer, NIH, Bethesda, MD) (20). 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 (20).

Polyclonal rabbit anti-NP directed against a synthetic human N-terminal NP fragment representing the residues 1–12 plus Tyr (N-Term NP, diluted 1:500, prepared by Dr. A. G. Robinson, University of California-Los Angeles and supplied by Pituitary Hormones and Antisera Center, Director A.F. Parlow). In tricine SDS-PAGE according to Schägger (21) and subsequently Western blotting, the antibody recognizes predominantly processed NP.

Polyclonal rabbit anti-NP directed against a synthetic human C-terminal NP fragment of the residues 80–91 plus Tyr was used (C-Term NP, diluted 1:500, prepared by Dr. A. G. Robinson, University of California-Los Angeles and supplied by Pituitary Hormones and Antisera Center, Bethesda, MD) (22). In tricine SDS-PAGE and subsequently Western blotting, the antibody recognizes predominantly processed NP.

FIG. 2. After microwave pretreatment in the SON of PWS patient 93-056, there was moderate immunoreactivity with the antibody MON-102 recognizing 7B2 (A) and moderate immunoreactivity with antibody III-D-7 recognizing processed VP (B). Note that there is still 7B2 and processed VP immunoreactivity in this patient as well as in patient 92-005. Bar = 25 μ m.

FIG. 3. Paraffin section of PWS patient 93-056 with very intense OT immunoreactivity after incubation with antibody A-I-28 in cells and fibers of the SON. Bar = 25 μ m.

FIG. 4. Paraffin sections of a control patient staining with antibody III-D-7 recognizing processed VP (A) and in the consecutive section staining with antibody MON-102 recognizing 7B2 (B) are shown. Immunoreactivity in a control patient for antibody A-I-28 recognizing OT (C) and in the consecutive paraffin section immunoreactivity for the antibody MON-102 (D) is shown. Note that 7B2 is expressed in both VP and OT cells. For orientation, the same cells are indicated by arrowheads. Bar = 25 μ m.

Glycopeptide (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)] (23). This antibody reacts in both the HEK-293 and Neuro2A (17) cell lines, so it at least recognizes the VP precursor. In tricine SDS-PAGE and subsequently Western blotting, the antibody recognizes predominantly the VP precursor.

Polyclonal rabbit anti-GP directed against the guinea pig glycoprotein moiety of the VP precursor was used [K 1.7, diluted 1:500, provided by L.C.A.F. Robinson, National Institute for Medical Research (London, UK)] (24). This antibody reacts in both the HEK-293 and Neuro2A (17) cell lines, which indicates that it at least recognizes the VP precursor. In tricine SDS-PAGE and subsequently Western blotting, the antibody recognizes predominantly the VP precursor.

OT antibody. Monoclonal mouse anti-OT, with three different antigenic determinants on the OT molecule was used: the Ile in position 3, Pro in 7, and Leu in 8 [A-I-28, diluted 1:200, provided by Dr. A. Hou-Yu, Columbia University (New York, NY)] (25).

7B2 antibodies. Polyclonal rabbit anti-7B2 directed against synthetic human 7B2 23–39 was used (RB-7, diluted 1:500) (26).

Monoclonal mouse anti-human 7B2, recognizing 7B2 sequence 64–94, was used [MON-144 (supernatant), diluted 1:10, provided by H. L. P. van Duynhoven (Helmond, The Netherlands)] (27).

Monoclonal mouse anti-human 7B2, recognizing 7B2 sequence 128–143, was used [MON-102 (ascites), diluted 1:1000, provided by H. L. P. van Duynhoven] (27).

Polyclonal rabbit anti-mouse 7B2 directed against residues 156–186 and recognizing 156–171 of the C-terminus of 7B2 was used (CT 7B2, Rb-4, 14-2-1991, diluted 1:250) (28).

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

FIG. 5. Immunoreactivity in a control patient for antibody III-D-7 recognizing processed VP (A) and in the consecutive paraffin section immunoreactivity for the antibody PC1 (B) is shown. Staining in a control patient by antibody A-I-28 recognizing OT (C) and in the consecutive paraffin section staining by PC1 (D) is shown. Note that PC1 is predominantly expressed in VP cells. For orientation, the same cells are indicated by arrowheads. Bar = 25 μ m.

immunofluorescence positive in AtT20 cells as endogenous activity and in transfected cells, and negative in COS-1 cells.

Polyclonal rabbit antimouse PC1 directed against residues 84–100 was used [88% homology with human PC1; 2B6 N-terminal, diluted 1:500, provided by I. Lindberg, Louisiana State University, New Orleans, LA] (29).

Polyclonal rabbit antimouse PC1 directed against residues 629–726 was used (PC1 92-12-08 C-terminal, diluted 1:500) (30).

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

The intensity of the immunoreactivity was estimated semiquantitatively. The tissue, which stained very faintly or not at all with some antibodies, was applied to microwave pretreatment according to a modified procedure, based on that reported by Shi *et al.* (31) as described by Lucassen *et al.* (32), to increase the sensitivity. After deparaffinization, the sections were treated according to the following procedure: 1) a 15-min wash in distilled water; 2) incubation in a microwave oven twice for 5 min each time 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 Tris-buffered saline; and 4) incubation in 5% nonfat dried milk for 30 min (Elk, Campina, Eindhoven, The Netherlands).

To determine whether PC1, PC2, and 7B2 are coexpressed in VP or OT cells, we stained sections of a control subject with VP antibody III-D-7 or OT antibody A-I-28 and the consecutive section with, respectively, 7B2 antibody MON-102, PC1 antibody PC1, and PC2 antibody PC2.

Specificity controls

For specificity of the VP, NP, GP, and OT antibodies, see the procedures and references described above. The antibodies raised against

different parts of 7B2 were adsorbed with the recombinant glutathione-S-transferase-7B2 hybrid protein (prepared by D. W. Eib) (26). PC1 and PC2 were absorbed with their respective recombinant antigens PC1 and PC2. 2B6 N-terminal PC1 and PC1 92-12-08 C-terminal were absorbed with their respective antigen residues (2B6 N-terminal PC1 antigen, provided by I. Lindberg, Louisiana State University). After adsorption, no reaction was seen, nor was there any reaction when the first antibodies were omitted from the procedure.

Results

All control patients showed very intense immunoreactivity for processed VP, as demonstrated by the staining with VP antibodies (III-D-7 and Truus) in the SON and PVN (Table 1). No clear difference in staining was observed with regard to age, sex, postmortem delay, or fixation time. The SON and PVN of the hypothalami of two PWS patients (92-005 and 93-056) previously shown to be deficient for 7B2 did not display processed VP immunoreactivity with either III-D-7 (Fig. 1A) or Truus (see Table 1). However, these patients showed a very intense staining for the processed NP and GP parts of the VP precursor and the precursor itself, as demonstrated by the staining with NP antibodies (PS41, N-Term NP, and C-Term NP) and GP antibodies (Boris and K 1.7) in the SON and PVN (Table 1 and Fig. 1, B and C). The absence of processed VP staining in PWS patient 93-056 was not due to the long fixation time, because a control patient (95-83) with a much longer fixation time (607 days) had very intense processed VP immunoreactivity. Two PWS patients (91-058 and 95-104) showed moderate processed VP immunoreactivity and no MON-102 immunoreactivity, but there was staining with the other 7B2 antibodies, indicating some degree of VP processing (Tables 1 and 2). To improve the detection level of the immunocytochemical method, we used the microwave method and found moderate staining with antibody MON-102 in PWS patient (92-005) and only faint to moderate 7B2 staining for all four 7B2 antibodies in PWS patient (93-056; Table 2 and Fig. 2A). Unfortunately, there was no material available to perform the same procedure on a PWS patient (92-005) for the other 7B2 antibodies. With the same procedure, a very intense processed VP staining appeared in the PWS patient (92-005) and a moderate processed VP staining was found in PWS patient 93-056 (Fig. 2B). A normal staining intensity for OT was found in PWS patients compared with that in control patients (Table 1 and Fig. 3).

To investigate whether 7B2 (MON-102), PC1 (PC1), and PC2 (PC2) are expressed in both VP (III-D-7) and OT (A-I-28) cells, we performed colocalization studies in control patients with the respective antibodies. 7B2 was present in both VP and OT cells, but PC1 and PC2 were predominantly expressed in VP cells (Figs. 4, A-D; 5, A-D; and 6, A-D).

To elucidate a possible processing defect in the VP precursor, we checked patients and controls for the presence of the processing enzymes PC1 and PC2 in both SON and PVN (Table 3). PC1 immunoreactivity was present in all PWS patients and controls, but the intensity was lower for PC1 and absent for PC1 2B6 N-terminal in the 7B2-nonreacting PWS patients (92-005 and 93-056). PC2 immunoreactivity was not detectable in the 7B2-nonreacting PWS patients (Fig. 7A), but all other PWS and control patients showed clear PC2 staining (Fig. 7B). After microwave pretreatment, the PC2-nonreact-

FIG. 6. In a control patient, staining by antibody III-D-7 recognizing VP (A) and in the consecutive section staining by the antibody PC2 (B) are shown. Immunoreactivity in a control patient for antibody A-I-28 recognizing OT (C) and in the consecutive section immunoreactivity for PC2 (D) are shown. Note that PC2 is predominantly expressed in VP cells. For orientation, the same cells are indicated by arrowheads. Bar = 25 μ m.

ing PWS patients (92-005 and 93-056) showed only very faint to faint staining for PC2 (Table 3 and Fig. 7C).

Discussion

It has recently been shown that 20% of PWS patients have a complete absence of the posterior pituitary bright spot after magnetic resonance imaging (MRI), indicating that the hypothalamo-neurohypophyseal system is affected (33). The source of this MRI signal is the neurosecretory granules (34), and in patients with hypothalamic diabetes insipidus, this MRI high intensity signal in the posterior pituitary is seldom observed (35). The absence of the pituitary bright spot in some PWS patients may indicate a diminished amount of VP in the pituitary either by reduced transport or by accelerated release from the posterior pituitary. Our present results show a diminished amount of bioactive processed VP in the SON and PVN of some PWS patients, indicating a diminished transport of processed VP to the pituitary (36). This presumably leads to osmotic activation and, subsequently, increased release of mainly unprocessed VP from the posterior pituitary. The exact degree of disturbance of VP production in PWS patients with an absent MRI bright spot should be studied further.

The main finding of the present study is that the absence of the neuroendocrine polypeptide 7B2 in two PWS patients (92-005 and 93-056) coincides with the absence of processed VP and PC2 in the SON and PVN. In addition, our results showed that PC1 and PC2 are predominantly located in VP cells. In these two PWS patients, however, the VP precursor

TABLE 3. Immunoreactivities with and without microwave for 7B2, PC1, and PC2 in magnocellular neurons of the PVN and SON of 7 PWS patients and 11 controls

Subject no.	Postmortem delay (h)	Fixation (days)	7B2 MON102 128-143	Microwave 7B2 MON102 128-143	PC1 43-628	Microwave PC1 43-628	PC1 84-100 mouse 2B6 N-terminal	Microwave PC1 84-100 mouse 2B6 N-terminal	PC1 629-726 92.12.08 C-terminal	Microwave PC2 629-726 92.12.08 C-terminal	PC2 122-637	Microwave PC2 122-637
PWS												
96-34	35	26	+	+	+++	++++	++//+	+++	++//+	++++	++//+	++
92-005/44282	30	7-14	-	++	++	ND	-	ND	++//+	ND	-	+
83-011/43830	4.5	ND	++++	++/-	++++	++++	++++	++++	++++	++++	+++	++
91-058/91252	5	33	- ^a	+	+++	++++	+ ^b	+++	++	++++- ^f	- ^c	++//+
93-056	45	385	-	++	++	++++	-	++++	++//+	++++	-	+/-
95-104/95-9090	16	32	-	++	+++	++++	+	+++	+ ^d	++++	+/-	++
90-111/31790	20	14	++	+	++++	++++	++/+++	++++	++++	++++	+ ^e	++
Controls												
87-63	88	44	+++		+++		++ ^f		++ ^g		++//+	
95-83	24	607	+/-	++	+++	++++	-	+++	+	+++	+	++
84-15	13	41	++++		++++		++++		++++		++	
85-27	24	60	+++		+++		+++		+++		+	
81-20	24	39	++++		++++		++++ ^g		++++		++// ^g	
87-69	120	44	+++		++		++ ^g		++		++// ^h	
82-16	5	33	+++		++++		++++		++++		++//+	
81-03	8	44	++++		++++		++++		++++		++//++	
80-09	8	59	++++		++		++ ^g		++++		++ ^g	
81-10	22	42	++++		+++		++/+++		++++		++	
81-15	47	60	++		++++		++/+++		++++		++/+++	

ND, Not determined because of lack of material; SON, supraoptic nucleus; PVN, paraventricular nucleus; PWS, Prader-Willi syndrome. Stainability: -, no staining; +/-, very faint; +, faint; ++, moderate; +++, intense; +++++, very intense. No microwave on control patients, with the exception of no. 95-83, because all of these patients had already stained; //, differential staining in SON and PVN is indicated by SON/PVN.

^a One large solitary cell in SON.

^b One large solitary cell in SON within a cluster of faintly stained cells.

^c One cell in SON, few very light cells in SON.

^d Some cells in SON/PVN.

^e Few very light cells in SON/PVN.

^f One cell in SON.

^g SON, no PVN in section.

^h Few cells in PVN.

appeared to be present normally, as shown by antibodies against NP and GP. These observations strongly suggest a processing defect. The absence of 7B2 probably leads to a disturbed pro-PC2 transport from the endoplasmic reticulum to the Golgi complex and subsequently to a lack of activation of PC2. The described results fully agree with the *in vitro* finding of Braks *et al.* (10), that 7B2 is a molecular chaperone of pro-PC2. Our results indicate, moreover, that PC2 might be a candidate enzyme for cleaving the dibasic amino acid site between VP and NP in the VP precursor because the absence of PC2 leads to no processed VP.

The very intense (92-005) to moderate (93-056) staining of processed VP, a moderate reaction with 7B2, and faint to very faint PC2 immunoreaction after microwave pretreatment in both PWS patients indicate that although these proteins are expressed to an abnormally low degree, they are not totally absent. We can be confident that the microwave procedure itself does not induce staining, because in another group of patients deficient in processed VP (Wolfram syndrome) using the same procedure, no immunoreactivity for processed VP, 7B2, or PC2 (Gabreëls, B. A. Th. F., submitted for publication) was seen. One can at present only speculate about the mechanism behind the reduced expression of these proteins. It could be that PWS patients 92-005 and 93-056 have such a large deletion on the paternal allele of chromosome 15

that not only the PWS region (15q11-13) but also the near 7B2 gene (15q13-14) or its promotor region are disturbed. This region of chromosome 15 is known to be particularly unstable (37). In agreement with the appearance of some 7B2 immunoreactivity after improving the sensitivity of the staining by microwave treatment is the finding that the 7B2 gene is probably not part of the imprinting region on chromosome 15 (38); thus, the 7B2 allele of the mother in PWS patients (92-005 and 93-056) can still be expressed. Unfortunately, it is impossible at this moment to obtain a molecular genetic diagnosis of paraffin-embedded material to establish the deletion in these PWS patients. The DNA extracted from the cerebellum of one patient (93-056) appeared to be highly fragmented (Smeets, H. J. M., personal communication). In this regard, it is of interest that in patient 95-104, the pre-mortem molecular genetic diagnosis showed maternal disomy, and this patient had no deletion and stained well for 7B2.

Syndromes have been described with inherited defects in processing in an autosomal dominant pattern arising from mutations within the cleavage sites of certain proproteins, such as proinsulin (39) or blood coagulation factor IX (40). In addition, patients have been mentioned in the literature with a normal precursor, *viz.* POMC (41, 42) or proinsulin (42), but without detectable processing products. Recently, in the pa-

Fig. 7. In PWS patient 93-056, there was no staining by antibody PC2 in the SON (A), but there was moderate staining by the same antibody in the SON of PWS patient 43830 (B). However, after microwave pretreatment, very faint immunoreactivity for PC2 was seen in the SON of PWS patient 93-056 (C). Bar = 25 μ m.

tient described by O'Rahilly, a mutation in the PC1 gene was reported (43). However, the present report is the first involving patients showing a disturbance in the regulation of a processing enzyme itself.

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