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ORIGINAL ARTICLE

Glucocorticoid treatment is associated with decreased expression of processed AVP but not of proAVP, neurophysin or oxytocin in the human hypothalamus: *Are PC1 and PC2 involved?*

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Abstract

OBJECTIVES: We reported earlier that vasopressin (AVP) peptide expression is significantly decreased in the postmortem hypothalamus of glucocorticoid (GC) treated patients, while such a decrease was not observed in AVP prohormone (proAVP) expression. This indicated a GC-induced suppression of AVP synthesis at the posttranslational level. Here, we investigated in detail whether this decreased levels of AVP expression in GC treated patients might be due to the down regulation of the prohormone convertases PC-1 and PC-2, and the molecular chaperone 7B2, as was reported previously in some AVP-related disorders. **MATERIALS & METHODS**: An immunocytochemical study was performed on post-mortem hypothalami of GC exposed patients and controls, in which quantification of proAVP, AVP, neurophysin (NP) and oxytocin (OXT) expression were done along with the quantification of PC1, PC2 and 7B2 expression in the paraventricular nucleus, by using a computerized image analysis system. **RESULTS**: Expression of processed AVP in GC exposed patients was significantly decreased ($p=0.021$), while the amount of proAVP expression was unchanged. Despite the strong correlation between AVP and NP (the other cleavage product of proAVP) expression in the GC group ($r=0.917$, $p=0.004$), the mean NP immunoreactivity did not show a significant decrease in this group. Also the OXT expression was similar in both groups. Although in most of the GC treated patients, the expression intensities of PC1 and PC2 were decreased parallel to the decrease in AVP, the mean expression levels of neither of PC1 and PC2, nor of 7B2 were statistically different between the groups ($p=0.20–0.80$). **CONCLUSION**: We conclude that the suppression of AVP expression by GCs is not mediated solely by the down regulation of PC1, PC2 or 7B2. Other mechanisms, which may contribute to the GC-induced posttranslational suppression of AVP, are discussed.

Abbreviations and Units

AVP	Vasopressin (arginine vasopressin)
CRH	Corticotropin Relesaing Hormone
GC	Glucocorticoid
NP	Neurophysin
NPY	Neuropeptide Y
OXT	Oxytocin
PC	Prohormone Convertase
PVN	Paraventricular nucleus of the hypothalamus
TRH	Thyrotropin Releasing Hormone

Introduction

The classical mechanism of steroid action takes place at the genomic level, regulating gene transcription [1, 2]. However, some recent studies show that glucocorticoids (GCs) may also have non-genomic effects, regulating posttranscriptional and posttranslational processes [3–7].

The posttranscriptional steps of hormone synthesis are regulated by processing enzymes and numerous regulatory factors. In the last ten years the prohormone convertase (PC) family of processing enzymes has been discovered and studied extensively in mammals. Today, this family consists of furin (SPC1, PACE), PACE4, PC1 (PC3, SPC3), PC2 (SPC2), PC4 (SPC4), PC5 (PC6, SPC6) and PC7 (SPC7, LPC, PC8) [8–11]. In neuroendocrine cells of the hypothalamus, the convertases PC1 and PC2 colocalize with and cleave the precursors of corticotrophin releasing hormone (CRH), vasopressin (AVP), neurophysin (NP), oxytocin (OXT), thyrothropin releasing hormone (TRH), neuropeptide Y (NPY) and neurotensin [12–17]. In addition, it has been shown that the neuroendocrine polypeptide 7B2 can act as a molecular chaperone for PC2, preventing premature activation of the proenzyme in the secretory pathway and assisting it during the processing of the prohormones [18–21].

Our group has previously shown that PC1 and PC2 are predominantly expressed in the AVP cells of the paraventricular (PVN) and supraoptic nucleus (SON) of the human hypothalamus. We also reported a PC2- and 7B2-associated processing defect of AVP in the hypothalamus of Wolfram syndrome and some Prader-Willi patients [15, 22, 23]. These studies showed that, along with the significant decrease in processed AVP, both PC2 and 7B2 were also diminished in the hypothalamus of these patients, whereas the amount of AVP precursor (proAVP) was not different from control levels. These results indicated that reduced expression of PC2 and 7B2 could be responsible for reduced processing of proAVP in Wolfram and a group of Prader-Willi Syndrome patients.

In a separate study, we have shown that GC treatment was associated with a significant decrease in the expression levels of processed AVP in the human hypothalamus, which could be at the posttranscriptional level [24]. In the light of previous studies indicating an association of a defective PC1 and PC2 production with AVP processing disturbance [15, 16, 23], and the recent evidence showing that production of prohormone convertases can be differentially regulated by GCs [13], we reasoned that the suppression of AVP synthesis by GCs can be mediated through the suppression of PC1, PC2 or 7B2 production. In the present study, therefore, we have investigated the expression levels of PC1, PC2 and the molecular chaperone 7B2, in relation to proAVP, the processed AVP, NP and OXT in hypothalamic PVN neurons of GC-treated patients and controls.

Materials and Methods

Post-mortem hypothalami of 17 subjects were studied immunocytochemically (ICC). Eight patients without reported exposure to steroids during the last 3 months formed the control group and 7 patients with GC exposure until death formed the GC group. Mean age, postmortem delay (pmd) and fixation period (fxp) were statistically not different between the two groups. In addition, pmd and fxp of the subjects were found not to influence the results of ICC staining [24]. Two subjects, who were exposed to GCs until 2 weeks and 2 months before death, were evaluated separately. The formalin fixed (4%) and paraffin embedded hypothalami were cut in 6 μm thick serial sections. The ICC staining protocol was applied to serial sections at 600 μm intervals through the hypothalamus in order to detect the immunoreactivity for vasopressin precursor (proAVP), processed vasopressin (AVP), neurophysin (NP), oxytocin (OXT), prohormone convertase (PC)-1, PC-2, and its chaperone 7B2. It must be noted that III-D-7 is an highly specific antibody against processed AVP, unlike Truus, which was used in our previous experiments recognizing both processed AVP and proAVP. The clinicopathological data of patients and the information on the antibodies are summarized in table 1 and table 2, respectively.

Following deparaffinization in xylene and rehydration through graded ethanol, the previously described ICC protocol was applied and the staining was revealed by 3, 3'-diaminobenzidine (DAB) [15, 24]. DAB-nickel was not used as it might influence the immunoreactivity to amount of peptide ratio, which would destruct the optical analysis by the computerized image analysis system (IBAS). In each ICC session, hypothalamic sections from all subjects were immunocytochemically stained for one peptide or protein. Sections from one separate subject was included in each ICC session to serve as a positive control. Following the ICC staining, sections were analyzed by IBAS. On each section, the paraventricular nucleus

Table 1. Clinicopathological data of the subjects

Patient	sex	age	ctd	pmd	fxp	brw	Diagnosis, clinicopathological information, summary
CONTROLS							
1 (90–901)	m	30	18:00	4h50	46	1325	Fallot's tetralogy, bacterial endocarditis
2 (87–260)	m	37	09:25	36h	46	1510	Alcohol and benzodiazepine intoxication, cerebral edema
3 (86–403)	f	53	14:00	24h	17	1410	Chronic myeloid leukemia with dura mater metastasis
4 (92–046)	f	54	nd	13h	nd	1080	Traffic accident
5 (92–047)	m	54	nd	14h	31	1410	Bronchogenic carcinoma
6 (90–060)	m	68	15:30	7h	47	1365	Coronary by-pass, myocardial infarction
7 (94–191)	m	78	12:15	8h25	24	1442	Metastatic prostate carcinoma, renal insufficiency, death due to cardiac arrhythmia
8 (93–019)	m	78	12:10	52h50	70	1340	Bronchopneumonia, cardiopulmonary insufficiency
MEAN		56.5		20h10	40.1	1360.3	
± *SEM*		*6.2*		*5h55*	*6.7*	*45.2*	
CORTICOSTEROID GROUP							
9 (83–173)	f	46	06:10	5h50	33	1360	Metastatic adrenal carcinoma causing high levels of adrenal steroids (Urine 17-Ketosteroid= 4164 µmol/24h (normal=21–52); 17-OH-corticosteroid= 381 µmol/24h (normal=10–52); Plasma cortisol= 0.69 µmol/L at 10:00 am and 0.77 µmol/L at 3:00 pm (normal=0.14–0.55)). Perioperative corticosteroid supplement
10 (95–026)	m	62	10:15	6h35	35	1350	Metastatic adenocarcinoma. Prednisone 30 mg/day for last 18 days
11 (93–133)	m	64	06:00	8h10	30	1450	Chronic myeloid leukemia. Prednisone 60–80 mg/day for last 5 months
12 (95–120)	m	65	01:15	4h45	28	1500	Basal cell carcinoma, asthma bronchial. Chronic low-dose beclomethason inhalation, and 200 µg/day for last 7 days
13 (93–094)	f	67	nd	<17h	79	1340	Lung carcinoma with metastasis and thrombocytopenia. Prednisone 60 mg/day for last 7 days
14 (93–095)	m	75	12:00	53h	618	1280	Metastatic prostate carcinoma with pneumonia, lung edema and heart failure. Prednisone 30 mg/day last 2 days
15 (92–156)	f	76	09:25	<8h	269	1225	Ovarium adenocarcinoma with metastasis. Prednisone minimal 60 mg/day for last 8 days
MEAN		65.0		14h45	156.0	1357.9	
± *SEM*		*3.8*		*6h40*	*221.4*	*35.5*	
p=		***0.488***		***0.298***	***0.406***	***0.685***	
SUBJECTS LATELY EXPOSED TO CORTICOSTEROIDS							
16 (86–354)	f	33	nd	18–41h	20	1035	Metastatic lung carcinoma. Dexamethasone up to 9 mg/day, mostly 0.5–1.5mg/day for >1 month, gradually stopped 14 days before death
17 (95–132)	f	72	13:50	9h10	34	1075	Cardiac failure with respirator insufficiency , cachexia, dehydration. Chronic prednisone use of 5 mg/day, doses of 5–30 mg/day last 4 months, stopped 2 months before death

The variables age, pmd, fxt and brw were tested between the control and corticosteroid exposed group, and the p values are given according to the Mann-Whitney U test.
Abbreviations; brw=brain weight in grams; ctd=clock-time of death; f=female; fxp=fixation period in days; m=male; nd=not determined; pmd=postmortem delay of abduction in hours; SEM=standard error of mean.

Table 2. Information on the antibodies.

Ab code	species	Detects	Reference
VP-III-D-7	monocl. mouse	**processed AVP**, binding Phe in position 3	48
Boris	polycl. rabbit	predominantly **AVP precursor**, against human glycoprotein 22–39	45
C-term VP-NP	polycl. rabbit	predominantly **processed NP**, against synthetic human NP 80–91	49
A-I-28	monocl. mouse	**OXT**, against positions 3 (Ile), 7 (Pro) and 8 (Leu)	50
PC1	polycl. rabbit	**PC1**, against human PC1 43–628	15
PC2	polycl. rabbit	**PC2**, against human PC2 122–637	15
MON-102	monocl. mouse	**7B2**, against human 7B2 128–143	51

Ab=antibody; AVP=vasopressin, NP=neurophysin, OXT=oxytocin, PC=prohormone convertase

(PVN) was encircled manually as the area of interest, after which the image analysis system measured 1) the number of cell profiles expressing immunoreactivity 2) the total area of immunoreactivity and 3) the mean intensity of immunoreactivity (the average signal in pixels, after subtracting the background signal). For the statistical analysis, the mean intensity of immunoreactivity in whole PVN was used in this study, which is a reliable measure of peptide expression for quantitative comparison [24]. The mean intensity of immunoreactivity for each peptide, which was expressed as arbitrary units, was compared between the groups by Mann-Whitney's non-parametric U test (2-tailed). Correlations between the data were tested by Pearson's test. A value of $p<0.05$ was considered to be significant.

Results

A good immunocytochemical staining was obtained for all studied peptides and proteins in the PVN and SON of all subjects. Immunoreactivity for OXT was limited to its typical neuronal distribution pattern in central and lateral parts of the PVN, dorsal part of the SON and the accessory neurons [25], whereas the other peptides and proteins studied were distributed throughout the PVN and SON, without obvious compartmentalization. Representative micrographs of the expression of these compounds in adjacent hypothalamic sections are shown in figures 1 and 2.

Immunoreactivity for AVP, confirming our earlier observations, showed a significant 40% decrease in the PVN of GC exposed patients (mean ± SEM= 0.204 ± 0.013 vs 0.126 ± 0.024; $p=0.021$) while none of the other compounds showed a significant difference between the groups ($p>0.2$). The mean intensities of the ICC staining for the peptides and proteins studied are summarized in figure 3 and the correlations between the staining intensities of the peptides are summarized in figure 4. Except for the tendency for a correlation between AVP and NP ($r=0.677$, $p=0.065$), there were further no significant correlations between the staining intensities of the other compounds were observed in the control group (figure 4). However, a number of significant correlations are observed between the intensities of peptide/protein expressions in the GC exposed patients. In this group, AVP showed a strong correlation with NP ($r=0.92$, $p=0.004$), but not with the processing enzymes PC1 and PC2 ($p>0.2$). ProAVP, on the other hand, showed strong correlations with PC1 and PC2 ($r=0.84$, $p=0.017$ and $r=0.81$, $p=0.027$, respectively). There were also correlations between PC1 and OXT ($r=0.90$, $p=0.006$), and between PC1 and PC2 ($r=0.85$, $p=0.016$) in the GC group. No correlation was observed between PC2 and 7B2 in both groups ($p>0.7$).

The subject who stopped GC treatment two weeks prior to death (patient #16) showed a very strong immunoreactivity for processed AVP and proAVP, due to either a rebound effect after stopping the GC treatment, or a unique effect of dexamethasone. This patient also showed the strongest immunoreactivity for PC1, PC2 and 7B2 when compared to the rest of the subjects studied (figure 1). For patient # 17, who stopped GC treatment 2 months before death, expression intensities of the compounds studied were within the limits of control subjects.

Discussion

The possible suppressive effects of GCs on the expression levels of prohormone convertases PC1 and PC2, and the chaperone 7B2 were investigated in relation to the expression of the neurohypophysial hormones AVP, NP and OXT in the human hypothalamus. The present results, extending our earlier observations [24], showed a strong suppressive effect of GCs on the expression level of processed AVP, whereas the expression of the precursor of AVP (proAVP) was not decreased. The expression intensity of OXT was unaffected by GCs in these patients. Intensity of NP immunoreactivity showed a strong correlation with that of AVP, but the mean intensity of NP expression did not show a significant decrease by GCs as observed for AVP.

Animal studies point to a decrease in circulating AVP upon GC administration, whereas GC deficient states are associated with elevated plasma levels of AVP [26–28]. Also in humans, increase in AVP levels in GC deficient patients, such as patients with Addison's disease, and the suppression of AVP upon administration of GC is known for a long time [29–33].

Direct introduction of GC into the supraoptic nucleus was associated with diminished urinary AVP excretion in monkeys, indicating that GCs have a suppressing effect on AVP release at the hypothalamic level [34]. Besides, AVP immunoreactivity in the PVN and AVP levels in the portal circulation of different rat strains are increased after adrenalectomy, which is reversed by GC replacement [35–38].

It is important to note that while the vasopressinergic activity of parvocellular cells of PVN are regulated by GCs, the magnocellular neurons do not respond to GCs in rats [39]. Also there is no report on downregulation of magnocellular AVP cells of SON in experimental animals. In humans, however, there is no compartmentalization of parvo- and magnocellular neurons in the hypothalamic PVN. It is, therefore, impossible to differentially study the effects of GCs on these two types of vasopressinergic neurons of PVN in humans. However, as it is clear from figure 1, there is no selective preservation of

the vasopressinergic activity of magnocellular cells, and both parvocellular and magnocellular cells show similar expression intensities for AVP in GC treated patients. Also, as reported in our previous study [24], the neurons of the supraoptic nucleus (SON), which are dominantly magnocellular, were also suppressed by GCs. This indicates that there is a species difference in response of magnocellular AVP cells to GCs. Nevertheless, it is reported not only for humans, but also for experimental animals, that the peripheral AVP levels are diminished by GCs, supporting our observation that the magnocellular cells of PVN and the SON are also subject to suppression by GCs.

Despite the clear demonstrations of a suppressed AVP peptide production in the hypothalamus by GCs, there is a lack of enough evidence to show that this suppression of AVP occurs solely through direct actions of GCs on AVP gene transcription. Most of the studies are restricted to demonstration of that GCs can suppress the elevated AVP mRNA and hnRNA levels only back to the basal levels [37–43]. However, none of these studies provide evidence for a down regulation of basal AVP transcription by GCs. We could not study the effects of GCs on the mRNA levels of AVP, because a quantitative analysis of AVP mRNA was not possible on the patient group of the current study, as the variance in fixation period and storage times were too large for an reliable in-situ hybridization study [44]. The effects of GCs on AVP transcription are, therefore, subject to future studies.

In the current study, the mean expression level of proAVP was the same in control and GC groups, whereas there was some 40% decrease in the processed AVP expression after GC treatment ($p=0.021$). The antibody used against processed AVP in this study is highly specific, and gives a better estimation for the levels of processed AVP than was reported previously by using a less specific antibody (Truus). NP, the other neuropeptide cleaved from proAVP, also showed a 15% decrease, although not significantly different from control values. The most logical explanation for this discrepancy between expression levels of AVP and NP in the hypothalamic neurons after GC treatment is the fact that antibody for NP (C-terminal NP), although recognizing predominantly the processed NP, binds also to the NP-precursor. There is possibly a differential processing of proAVP to produce AVP and NP, as shown previously in some Prader-Willi patients [22]. The high correlation between intensities of AVP and NP immunoreactivities, on the other hand, ($r=0.917$, $p=0.004$ in GC group and $r=0.677$, $p=0.065$ in the control group; figure 4), strongly indicates that the major source of NP in the PVN is proAVP.

Posttranslational processing of prohormones is a long and complicated chain of modifications, including glycoslation, phosphorylation, sulphation, endoproteolytic cleavage, exoproteolytic cleavage, acetylation and amidation, which are essential for functional maturation. In different systems and tissues, the production of many biological products are suppressed by steroids during posttranslational processing [4, 7, 46]. It is not known, however, which of the posttranslational modification steps are affected by GCs to cause this suppression. Suppression of production or functioning of prohormone convertases is a possible mechanism for posttranslational suppression of neuropeptides and hormones by GCs. Our group has shown that some human diseases that are accompanied by defective AVP production, such as Wolfram's syndrome and Prader-Willi syndrome, are associated with a defective expression of the processing enzyme PC2 in the hypothalamus. An association between changes in the processing enzyme production and the suppression of AVP production by GCs can be expected on the basis of the results of Dong *et al* [13], who reported a suppression of PC1 mRNA by GC. However, in the present study no evidence was obtained for a significant GC induced suppression of PC1, PC2 or 7B2 levels, which leads us to alternative explanations for the observed posttranslational suppression of AVP production;

1. In order to be functional, the processing enzymes themselves have also to be processed into active forms from their precursors. At present, we do not know whether the antibodies to PC1, PC2 and 7B2 recognize only the active forms or the precursors of these peptides or both forms. It is possible that GCs suppress the maturation of the processing enzymes into the active forms, and therefore the expression levels detected in our study may not reflect their functionality.
2. Coates and Birch [16] showed that different isoforms of prohormone convertase PC1 are responsible for processing of proAVP into active hormones, and different production rates of AVP can be achieved by altering the activity of PC1 isoforms within the same tissue. Should some of these isoforms be GC sensitive, this would result in a fine tuning of hormone synthesis without a change in the transcription. However, antibodies to detect specifically the different isoforms of PCs on paraffin embedded postmortem human tissue are not available, and therefore we can not conclude on the basis of the present ICC study whether one or more isoforms of the processing enzymes are differentially affected by GC.
3. The complex processing of proAVP into its end-products involves a balanced co-action of several proenzyme convertases and other factors. Other processing enzymes can be taking place in the pro-

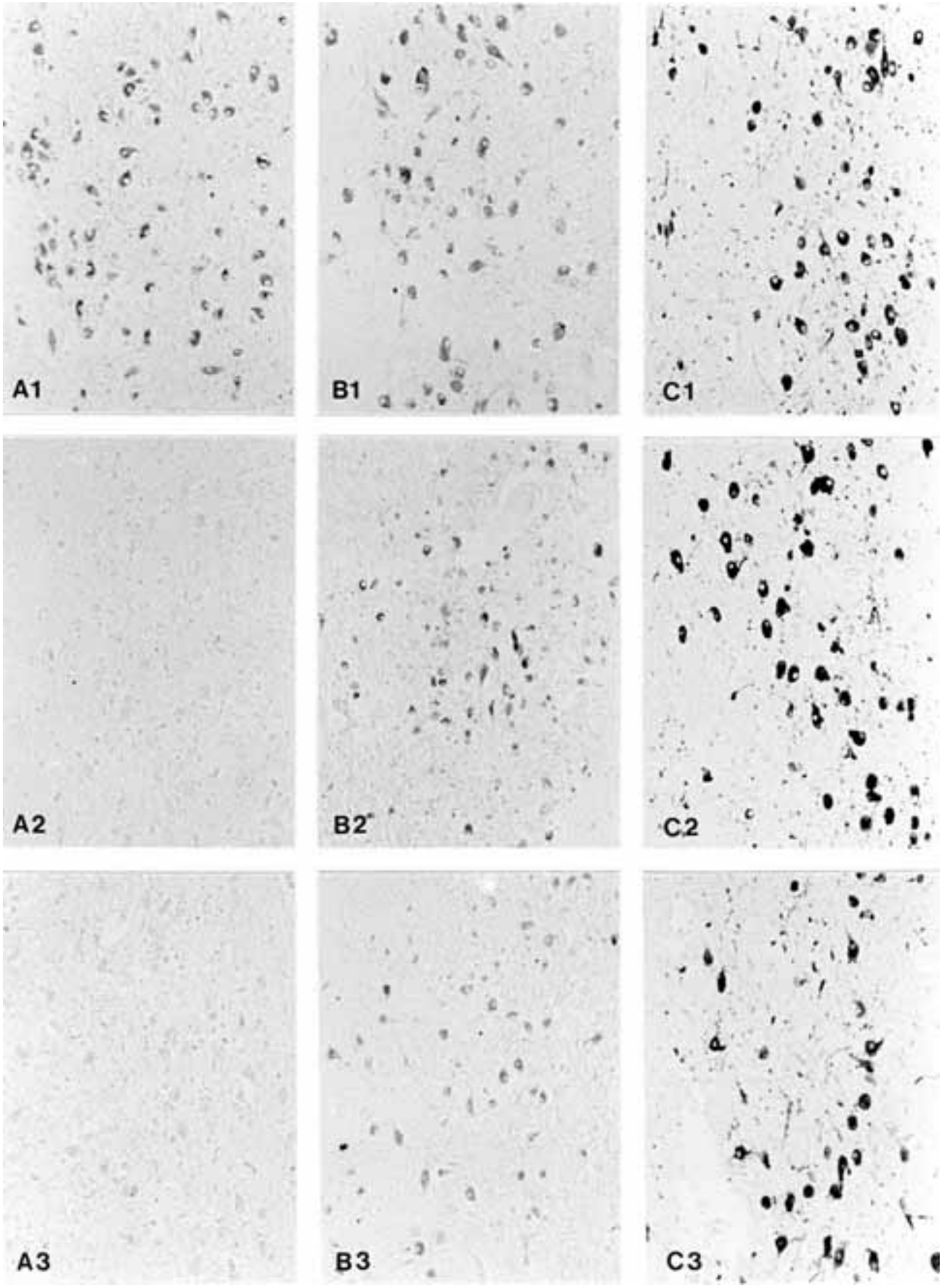
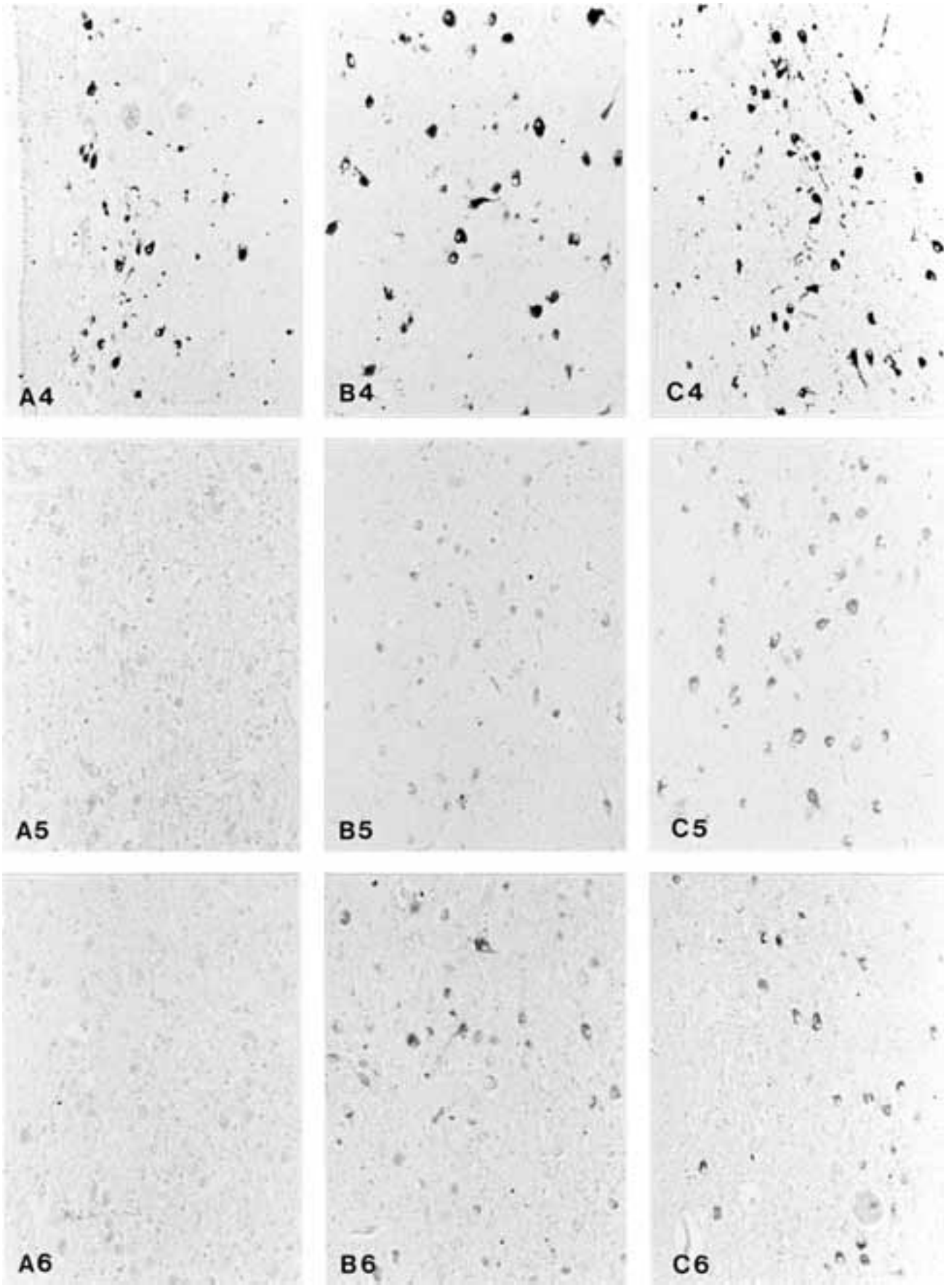


Figure 1. Serial sections from the PVN of 2 representative subjects, # 93–94 (colomn A) from GC treated group and # 92–47 (colomn B) from control group. The third colomn (C) shows sections from subject # 86–354, who shows the strongest immunoreactivity for all of the componds studied. Horizontally, immunoreactivity for proAVP (row 1), AVP (row 2), NP (row 3), OXT (row 4), PC1 (row 4) and PC2 (row 5) are presented. Note that NP, as well as PC1 and PC2 show faint immunocytochemical staining parallel to the decreased AVP



immunoreactivity in GC treated patient, although the mean expression levels are not significantly different from those in the control group. Also note that strong immunocytochemical expression of proAVP and processed AVP associated with strong expression for PC1 and PC2, and of 7B2 (not in the figure) in patient C. OXT immunoreactivity on the other hand, does not show a difference between the groups. 100 x magnification.

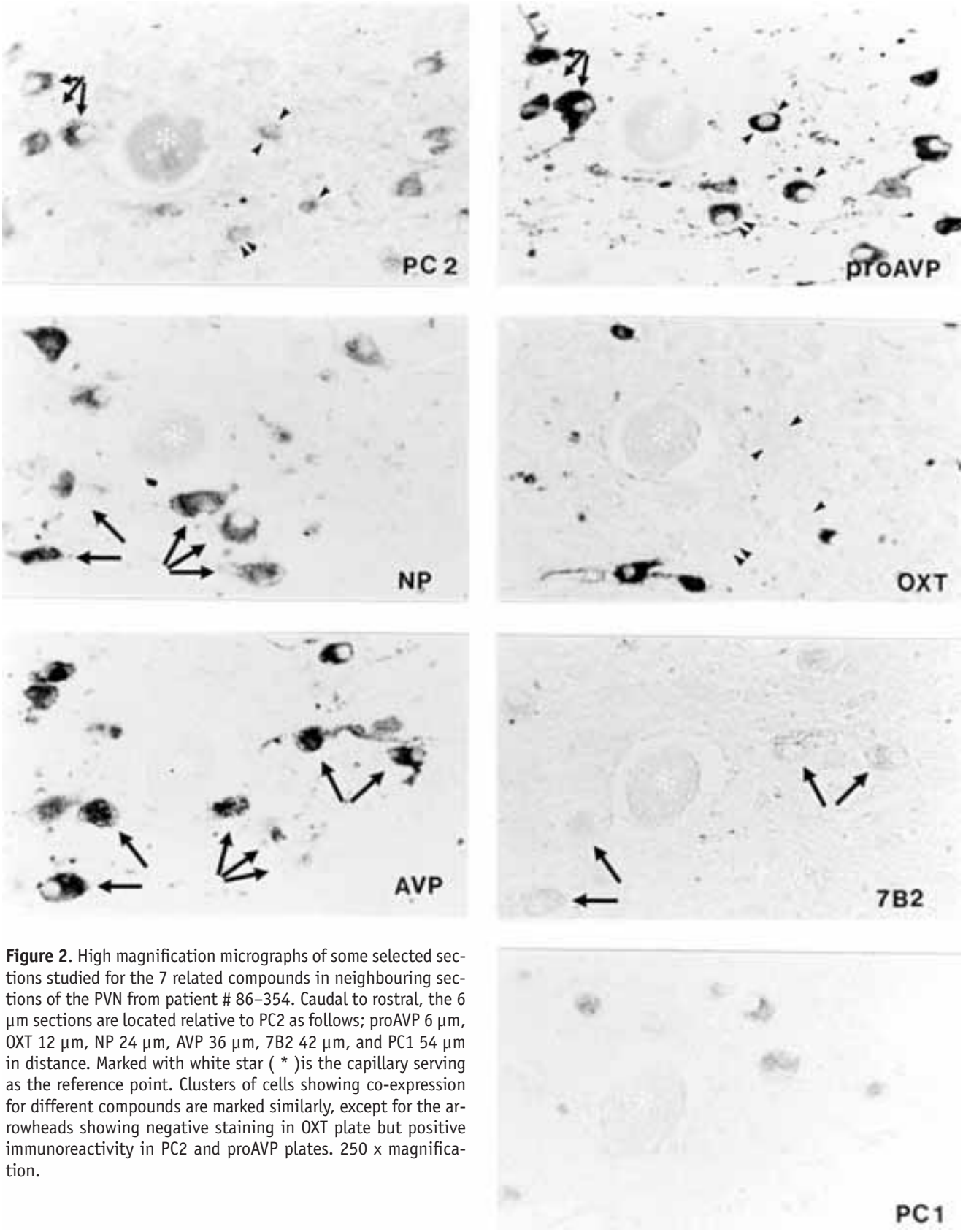


Figure 2. High magnification micrographs of some selected sections studied for the 7 related compounds in neighbouring sections of the PVN from patient # 86–354. Caudal to rostral, the 6 µm sections are located relative to PC2 as follows; proAVP 6 µm, OXT 12 µm, NP 24 µm, AVP 36 µm, 7B2 42 µm, and PC1 54 µm in distance. Marked with white star (*)is the capillary serving as the reference point. Clusters of cells showing co-expression for different compounds are marked similarly, except for the arrowheads showing negative staining in OXT plate but positive immunoreactivity in PC2 and proAVP plates. 250 x magnification.

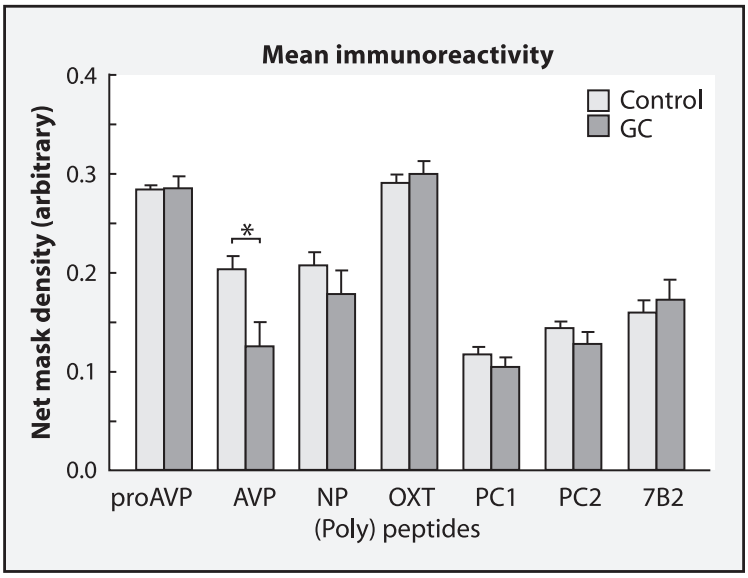


Figure 3. Mean immunocytochemical expression intensities in the PVN for the compounds studied in control and glucocorticoid treated (GC) groups. Only AVP immunostaining showed a significant difference between the groups according to the Mann-Whitney test (p=0.021).

duction process of AVP from proAVP. Furin and PC5, for example, were detected in the neurons of PVN and SON of the rat, indicating their role in processing of the neuropeptides produced in these regions. Unfortunately, there are no antibodies available that can be used for a comparative quantitative study on paraffin embedded human brain tissue. There are also other enzymes involved in the posttranslational modification of polypeptides, which can indeed be affected by GCs. Peptidyl-glycine α-amidating monooxygenase (PAM) for example is a posttranslational processing enzyme which catalyzes the formation of biologically active alpha-amidated peptides, including AVP. This enzyme was also shown to be affected by adrenalectomy along with the changes in AVP, returning to basal levels upon GC replacement [47].

Although the expression intensities of PC1 and PC2 showed a similar decrease as observed for processed AVP in GC-treated patients in comparison to that of controls (figure 1), this reduction was not statistically significant. Our groups were not large enough to perform a multiple regression analysis to see whether such small suppressions in PC1 and PC2 levels can altogether be responsible for the 40% reduction in expression of processed AVP. However, there was no significant correlation between AVP and PC1 or PC2 in either group in the current study as analyzed by IBAS, pleading in favor of additional factors other than PC1 and PC2 to play role in the processing of proAVP to AVP. Also, the strong correlation between PC1 and PC2, as well as between proAVP and both PCs in the GC group indicates the presence of other factors regulating proAVP processing of proAVP, and balancing the levels of these peptides and proteins in equilibrium in the synthesis pathways.

It is not clear whether an increase in proAVP transcription leads to an increase in PCs involved in its processing, or the stimulation to produce more AVP affects the production of all components of AVP transcription and posttranslational processing simultaneously. We observed a remarkably strong immunoreactivity not only for AVP and proAVP, but also for NP, PC1, PC2, 7B2 and OXT in patient # 16, who had stopped GC treatment 2 weeks prior to death (figures 1 and 2). These high levels of protein expression may be either the effect of dexamethasone or a rebound effect of GC withdrawal. Whether the high PC1 and PC2 levels in this patient are the consequence of increased proAVP levels, altogether leading to the increased AVP production, or an increase in PC1 and PC2 results in an excessive production of AVP, remains to be investigated.

Even though we do not fully understand the mechanism(s) by which GCs affect the post-translational steps of AVP production, it is clear from our present study that the suppression of AVP expression by GC follows a more complex mechanism than the processing disturbance of AVP observed in Prader-Willi and Wolfram syndrome patients [15, 23]. Further studies on the factors and steps affected by GCs in the processing pathway of proAVP and related hormones are needed, to solve the mystery of non-genomic effects of GCs on AVP processing.

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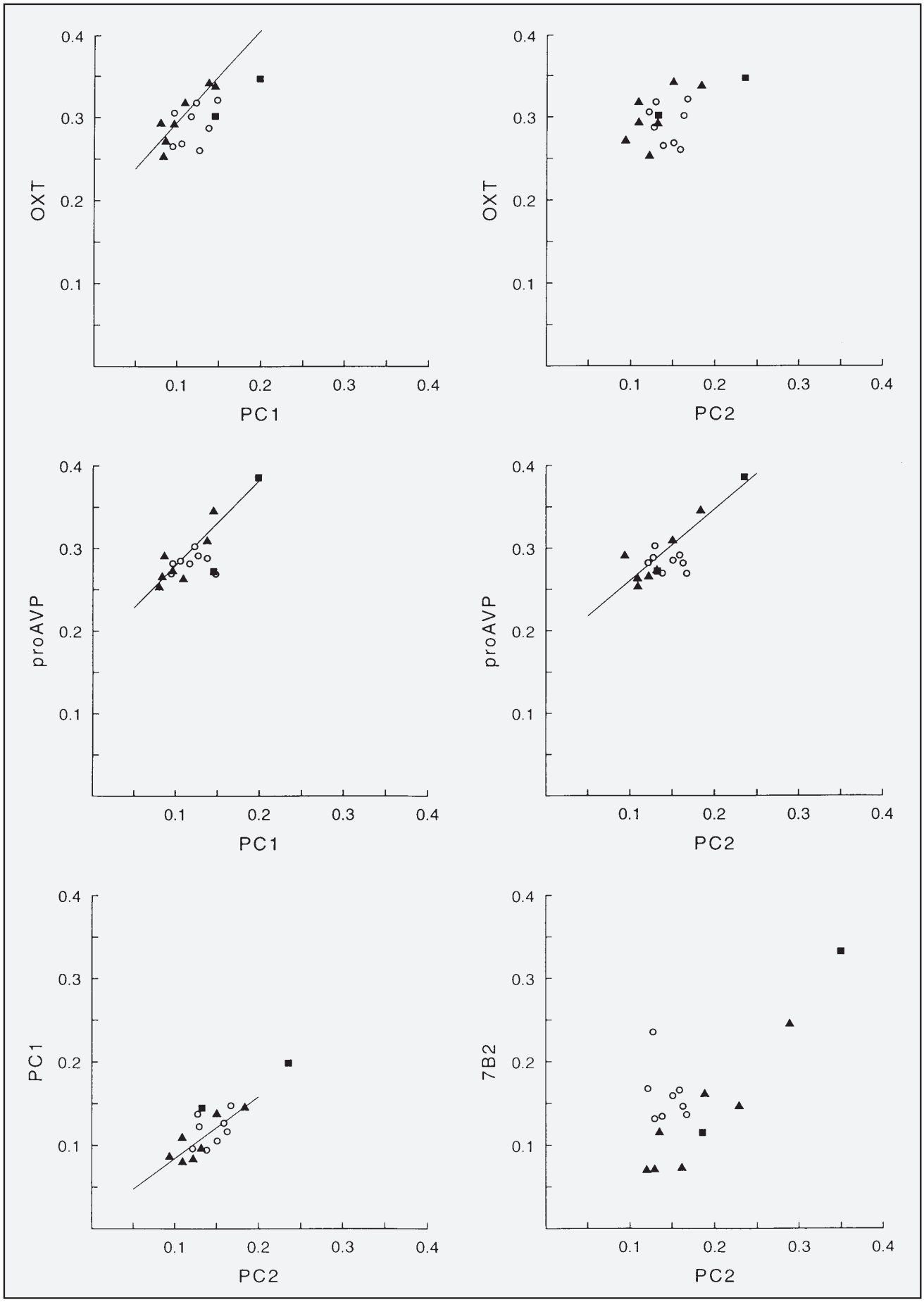
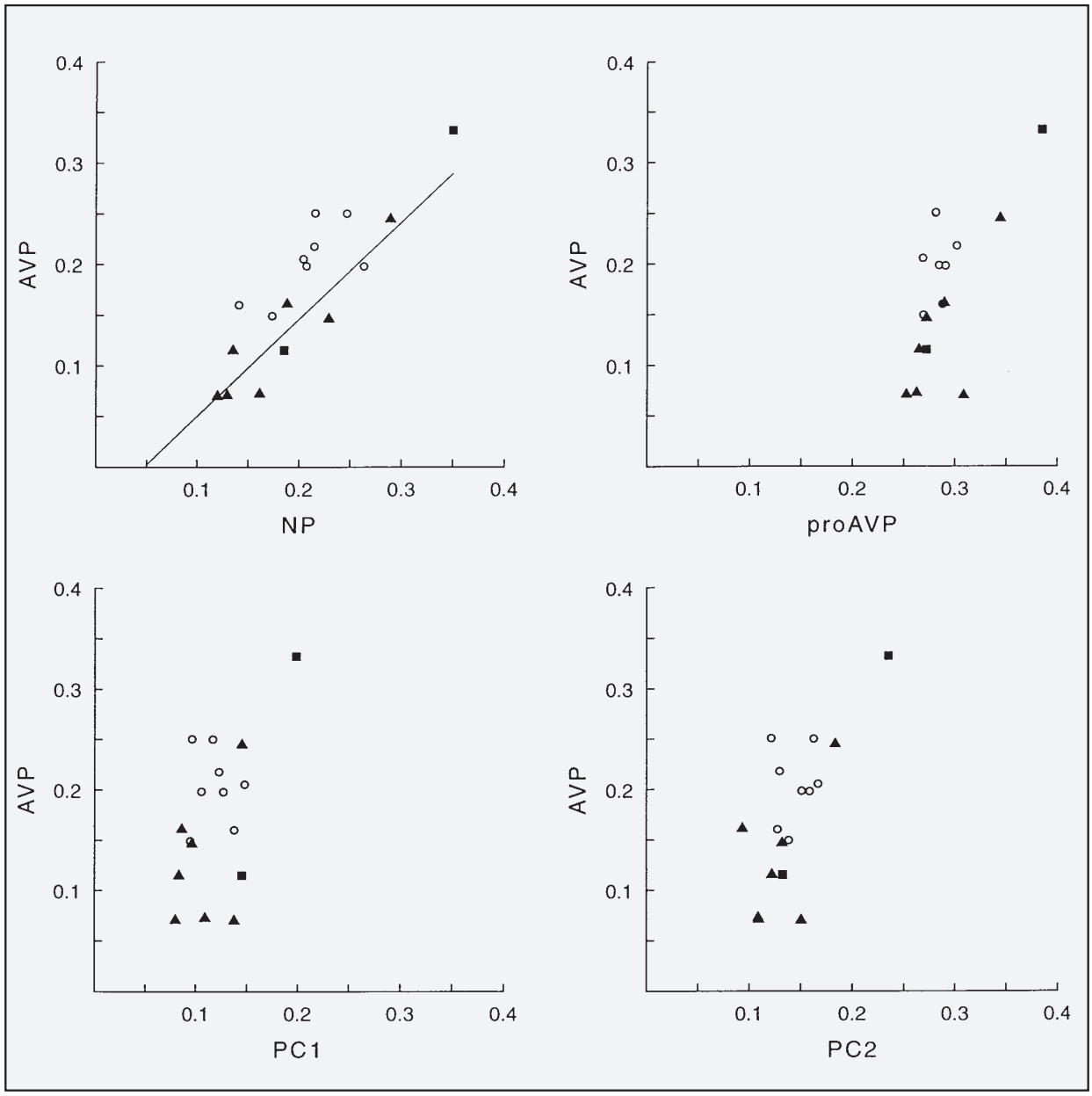


Figure 4. Spotplots showing correaltions between the immunocytochemical stainings of the compounds studied. (○) for controls, (▲) for glucocorticoid treated patients (GC),(■) for patients # 16 and 17. The lines represent significant linear correlations for the GC group.



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