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Increased vasopressin immunoreactivity in the rat brain after a postmortem interval of 6 hours

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Enhanced immunocytochemical staining of vasopressin-containing neurons was observed after incubation of rat brain slices in Ringer medium for 6 h at room temperature, as compared to brain tissue fixed immediately after death. Hypothalamic vasopressin neurons in the supraoptic nucleus, the paraventricular nucleus and the suprachiasmatic nucleus stained more intensely after a postmortem interval of 6 h. Extrahypothalamic vasopressin neurons (VP) in the bed nucleus of the stria terminalis, the medial amygdala and the locus coeruleus proved to be stained as well. Extrahypothalamic VP neurons in the locus coeruleus could, until now, only be visualized after *in vivo* pretreatment with colchicine. In addition, staining was observed at two new sites, the dorsal raphe nucleus and the lateral septum. Staining of VP was corroborated by application of different antibodies directed against the intact vasopressin molecule as well as by antibodies directed against the other parts of the vasopressin precursor molecule, i.e. neurophysin and glycopeptide. The specificity of the VP-staining was validated by using pre-immune serum and using Brattleboro rat brain tissue, resulting in a negative staining in both cases. Furthermore, homogenated punches of the suprachiasmatic nucleus were submitted to iso-electric focussing on polyacrylamide gel, followed by press blotting and subsequent immunocytochemical staining for vasopressin. Iso-electric focussing enabled us to characterize and quantify peptides in the suprachiasmatic nucleus. The vasopressin content increased 6 h postmortem, while c-terminal glycopeptide and neurophysin levels remained stable. Similar results were observed in the suprachiasmatic nucleus from decapitated rats whose brains were left intact in the skull for 6 h at room temperature.

INTRODUCTION

In various mammals, including man, vasopressin (VP) neurons can easily be stained using immunocytochemical techniques, in the supraoptic nucleus (SON), the paraventricular nucleus (PVN) and the suprachiasmatic nucleus (SCN)^{4,8,16,19}. However, in some extrahypothalamic regions, such as the dorsal hypothalamic nucleus, the locus coeruleus (LC) and the subcoeruleus^{2,3,21}, VP-containing neurons can so far only be visualized after pretreatment *in vivo* with colchicine. This compound blocks axonal transport, resulting in enhanced neuropeptide staining in neuronal cell bodies.

In preliminary experiments, aimed at trying to visualize VP binding sites in rat brain slices incubated *in vitro* at room temperature for 6 h in Ringer medium, an enhanced immunocytochemical staining of hypothalamic and extrahypothalamic VP neurons was observed as compared to brains fixed immediately after decapitation.

Because of our interest in immunocytochemical detection of VP in postmortem human brain, these observations initiated experiments addressing the issue of

whether the enhancement of immunocytochemical staining after a postmortem delay of 6 h is specific for VP.

The results obtained with anti-VP were corroborated using antibodies directed against other parts of the VP-precursor molecule, i.e. neurophysin (NF) and c-terminal glycopeptide (CPP). The specificity of VP-staining was validated using pre-immune serum, Brattleboro rat brain material, antibodies against related and non-related peptides (i.e. oxytocin, α -melanocyte-stimulating hormone (α -MSH)) and by applying iso-electric focussing (IEF) on homogenated punches of the SCN followed by press-blotting and subsequent immunocytochemical staining for peptides²⁰. The IEF procedure enabled us, besides investigating the specificity, to quantify the staining intensity, which correlated linearly with the amount of peptides present in the tissue²⁰.

MATERIALS AND METHODS

Tissue preparation

Male Wistar rats ($n = 39$), 3 months old, were used. Within a period of 15 min and starting at 11.00 h, all rats were sacrificed by decapitation.

Thirty-four rats were decapitated, the brains immediately removed and cut in a template by razor blade into coronal slices, 3 mm thick. The slices were either immediately fixed to obtain a reference value, or incubated for 6 h at room temperature in Ringer medium enriched with 0.1% bovine serum albumin, 2 mM MgCl₂ and buffered with Tris 0.05 M (pH 7.6). This medium was chosen as preliminary observations aimed at staining VP binding sites were made on tissue which was incubated in this medium. This medium has originally been used for autoradiographic binding studies¹³. Subsequently, slices to be used for immunocytochemical staining, were fixed by immersion fixation for 1 week in glutaraldehyde/paraformaldehyde (2.5%/1%) in phosphate-buffered saline (pH 7.4) (*n* = 12). Slices to be used in IEF were frozen in liquid nitrogen and stored at -20 °C (*n* = 22).

Five rats were decapitated, the brains being left intact in the skull for 6 h at room temperature. This time lapse was used to simulate the postmortem interval for human brain tissue obtained by rapid autopsies by the Netherlands Brain Bank.

Subsequently, the brains were removed, cut and frozen as described before, for use in IEF experiments.

Prior to immunocytochemical staining, the brain slices were extensively rinsed in Tris-buffered saline (TBS) and sliced on a vibratome (50 µm). Free-floating sections were again thoroughly rinsed with TBS and subsequently immunocytochemically stained.

Immunocytochemical staining

The peroxidase-antiperoxidase (PAP) procedure¹⁴ was applied to vibratome sections using various antibodies.

An additional step of methanol treatment was introduced before the incubation with the first antibody in order to prevent pseudoperoxidase staining of erythrocytes⁶. Stained sections were mounted on gelatin-chromalum-coated glasses, dehydrated in a series of alcohols, and embedded in Entellan.

Iso-electric focussing

Cryostat sections of frozen brains were sliced (25 µm) from rostral to caudal at -10 to -20 °C. Just before the rostral part of the SCN, a circular imprint was made around the nucleus by means of a punching needle with an inner diameter of 1.5 mm. Further slicing produced small discs on the knife, which were picked up and every fourth section was put on gelatin-chromalum-coated glass and dried on a warm plate for 10 min. These sections were stained with thionin for microscopic orientation. Once the SCN became visible in a thionin-stained section, the next 30 discs were collected in a plastic tube and homogenized after adding 40 µl medium consisting of 10% v/v dimethylformamide (BDH, Pöde, U.K.; for gas chromatography), 10% v/v glycerol (BDH), 2.5% v/v Nonidet P-40 (Sigma).

Two microliters of the homogenate, a concentration-range of synthetic VP (Sigma, V-9879) and cytochrome C (Sigma, C-2506) were subsequently applied onto the gel for focussing, followed by press-blotting of the gel onto a gelatin-coated nitrocellulose sheet using glutaraldehyde to cross-link the peptides²⁰. Cytochrome C served as a marker during the actual focussing and the synthetic VP range was used as a reference during the quantification procedure. The fixed peptides were visualized by immunocytochemical staining of the press-blot using the PAP-procedure. This procedure was slightly modified, the used incubation buffer in the immunostaining procedure was enriched with 0.25% w/v gelatin, and the peroxidase reaction was performed in normal Tris/HCl buffer enriched with 0.2% w/v nickel ammonium sulphate. The blots were made transparent with immersion oil (Zeiss, Oberkochen, F.R.G.) and were covered with a transparent plastic sheet. Optical densities of the individual bands were quantified on a Kontron/IBAS 2000 image processing system (Zeiss, München, F.R.G.) and the results expressed as integrated optical density²⁰.

Antibodies

The following first antibodies were used: anti-arginine-VP, Truus (10/4/86), 1:1000; anti-lysine-VP 121[4] 1:500; anti-oxytocin (OXT), O-1-V, (4/4/75) 1:1000; anti- α -MSH, 4394, (23/4/75) 1:1000; anti-

CPP (kindly provided by Dr. D. Smyth, London, U.K.), 1:1000; anti-CPP, C2 final and C3 final (kindly provided by Dr. M. Seger and Dr. P.H. Burbach, RMI, Utrecht, The Netherlands), 1:500, 1:2000 respectively; anti-rat neurophysin I + II, R4, (19/7/73) (kindly provided by Dr. A.G. Robinson, Pittsburgh, PA, U.S.A.; NIH Grant AM 16166) 1:2000; preimmune serum (Truus), (27/2/85) 1:1000.

Antibodies directed against glutaraldehyde-thyroglobulin components in the VP antiserum were removed by adsorption with thyroglobulin-Sepharose beads, treated with glutaraldehyde¹⁰. The second antibody was goat-anti-rabbit IgG (Betsy, 1:100) and as third antibody peroxidase-anti-peroxidase (PAP, 1:1000) was used¹⁴.

Statistics

The results of the iso-electric focussing experiments using two experimental groups were statistically evaluated with the Student's *t*-tests (two-tailed) with *P* < 0.05 as level of significance. Experiments involving 3 groups were evaluated using one-way analysis of variance. If a significant difference was present, the Student-Newman-Keuls test was used to identify the groups with a marked disparity.

RESULTS

Immunocytochemistry

Incubation of brain slices from Wistar rats for 6 h in the incubation medium (T6m) or leaving the brains intact in the skull for the same period (T6s), resulted in enhanced VP immunoreactivity of hypothalamic neurons in the SON, the PVN and the SCN (Fig. 1), as compared to the brains fixed immediately after decapitation (T0).

In extrahypothalamic brain regions such as the bed nucleus of the stria terminalis (BST), the medial amygdala (MA) and the LC, VP immunoreactivity was present after 6 h of incubation, whereas no or only minimal staining of these extrahypothalamic neurons were observed in the control group. In the T6m group, VP

TABLE I

Immunoreactivity of cell bodies in brain regions of Wistar rats (n = 12) after staining with vasopressin (VP), c-terminal glycopeptide (CPP) and neurophysin (NF) antibodies

R0 is compared to 6 h incubation in Ringer medium, R6m. anti-VP, Truus, 121[4]; anti-NF, Rnp-4; anti-CPP, Smyth, C2-final, C3-final. + + +, intense staining; + +, marked staining; +, moderate staining; $\pm$, weak staining; -, no staining.

Brain region	Antibodies directed against:		
	VP	NF	CPP
	R0/R6m	R0/R6m	R0/R6m
PVN	+/++	++/+++	+/++
SON	+/++	++/+++	+/++
SCN	+/++	++/+++	+/++
BST	-/+	-/+	-/+
MA	-/+	$\pm$ /+	-/+
LC	-/+	-/-	-/+
Septum	-/+	-/+	-/+
DR	-/+	-/ $\pm$	-/+

Fig. 1. Immunocytochemical staining of the suprachiasmatic nucleus (SCN) in Wistar rat brain, which was immediately fixed (A), incubated in Ringer medium for 6 h (B) and left intact in the skull for 6 h (C) after decapitation. Sections ($50\text{ }\mu\text{m}$, vibratome) were stained with an antibody directed against arginine-vasopressin (AVP) (Truus 1:1000). Note the enhancement of staining in B and C compared to A. Bar represents $100\text{ }\mu\text{m}$.

immunoreactive neuronal cell bodies were present in two brain regions that were, so far, not reported to contain VP neurons, i.e. the lateral septum and the dorsal raphe nucleus (DR).

Table I summarizes the results of these immunocytochemical stainings using antibodies directed against the intact VP molecule as well as other parts of the precursor molecule, i.e. NF and CPP.

Pre-immune serum and antibodies directed against OXT and α -MSH did not show any immunoreactivity in vasopressin-containing brain regions. However, the use of anti-OXT resulted in enhanced immunoreactivity of OXT-expressing neurons in the PVN and SON. Unfortunately,

these observations are based on two rats, one T0 and one T6m rat and can therefore only be regarded as very preliminary evidence for an enhanced postmortem immunoreactivity.

The brain of a Brattleboro rat did not show any vasopressin immunoreactivity following 6 h incubation in Ringer medium.

Iso-electric focussing

SCN-tissue punches of T0 and T6m were applied in iso-electric focussing experiments in order to establish the specificity of the increased VP staining and to quantify the difference in staining intensity of VP. The results of

Fig. 2. Mean integrated optical density of arginine-vasopressin (AVP), c-terminal glycopeptide (CPP) and neurophysin (NF) in the suprachiasmatic nucleus of the Wistar rat, in immediately fixed brain and in brain 6 h incubated in Ringer medium. Bars represent the mean value of 6 animals. Of each animal the 3 antigens, AVP, CPP and NF were analyzed on the same gel and each animal was analyzed on a different gel. Vertical lines represent S.E.M.

Fig. 3. Mean integrated optical density of arginine-vasopressin (AVP), c-terminal glycopeptide (CPP) and neurophysin (NF) in the suprachiasmatic nucleus of the Wistar rat, in immediately fixed brain, in brain 6 h incubated in Ringer medium and in brain left intact in the skull for 6 h. Bars represent mean values of 5 animals, for each antigen the values of the 5 animals were analyzed on the same gel. Vertical lines represent S.E.M.

this experiment are presented in Fig. 2. The VP content of the SCN in the rat brain, increased significantly ($P < 0.05$) after a postmortem interval of 6 h, CPP and NF levels did not change.

In the following experiments a third group was included, SCN tissue of brains left intact in the skull for 6 h (T6s). These results are presented in Fig. 3. The mean integrated optical density (IOD) of the T6m and the T6s groups were significantly different ($P < 0.05$ for both groups) from the immediately fixed group (T0). No significant difference was observed between T6m and T6s.

DISCUSSION

Postmortem changes have direct implications on the application of immunocytochemical staining procedures in human brain. Some brain compounds, e.g. P-creatine and ATP show considerable changes within seconds after death¹⁵, whereas others remain stable for days, e.g. RNA⁷. The various classes of neurotransmitter show different alterations during the postmortem interval. Acetylcholine and the catecholamines degrade rapidly after death¹, while most amino acids remain stable or even increase in concentration⁹. Neuropeptides are generally found to be remarkably stable after death^{12,17}.

Our results provide evidence for a postmortem stability of CPP and NF and an increase in VP, during an interval of 6 h. Rat brain slices incubated in a modified Ringer medium for 6 h as well as brain tissue from decapitated rat heads left at room temperature for a period of 6 h showed stable concentrations of CPP and NF and even a rise in VP content in the SCN. Since the two methods provided the same results, we concluded that the increase in VP level and the stability of the CPP and NF levels was due to the postmortem delay rather than influenced by the incubation in medium.

In the literature, postmortem stability in immunocytochemically detected neurohypophysial hormones has been reported. A stable VP content was found in the lateral septum of male rats, whose heads were kept at room temperature for 24 or 48 h following decapitation¹⁷. Furthermore, Fliers and co-workers reported no effect of a postmortem interval up to 30 h on AVP and OXT cell density in the PVN, SON, and SCN in human brain^{5,18}.

In the present experiments, the VP content in the SCN even increased after a postmortem interval of 6 h. Similar changes have been reported in the literature. For example, the concentration of thyrotropin-releasing hormone and pro-opiomelanocortin-related peptides in the mouse brain, showed an increase in concentration up to 72 h of postmortem interval¹¹. Furthermore, an increase in immunoreactivity of adrenocorticotropin (ACTH) containing cells in the anterior lobe of the rat pituitary was

observed after a postmortem interval of 48 h at 4 °C¹⁷.

These results indicate that in the case of neuropeptides there seems to be no need for a short postmortem interval in studying the brain. The postmortem elevation of neuropeptide content indicates that peptides are formed after death. A plausible explanation for this would be that during the postmortem period, the existing precursor molecules are processed into their peptide fractions. If this occurred in our material, one would presume that the levels of the other precursor fragments, viz. CPP and NF, would also increase. However, no evidence was found to support this assumption. Since the variation in these peptide contents was relatively high and the number of rats limited ($n = 6$), a rise in CPP and NF levels might have been masked.

A source of variation may be the place of focussing in the gel, i.e. the iso-electric point. The iso-electric point of VP (pH 10) is much higher than that of CPP (pH 6.3) and NF (pH 4.5). Consequently, CPP and NF focus in an area in which the bulk of tissue proteins are focussed. Thus, the high inter-assay variation of CPP and NF might be explained by interference of tissue proteins. Alternatively, the turnover rate of CPP and NF could be higher than that of VP which might, despite the high processing rate of the precursor molecule, result in a lack of rise of these compounds in the postmortem period.

In the present study, neurons in several extrahypothalamic regions, i.e. the bed nucleus of the BST, the MA and the LC, appeared to stain for VP. Such staining in the LC has so far only been reported following *in vivo* colchicine pretreatment of the rat^{2,3,21}. The rise in peptide levels during the postmortem interval may thus be used as an alternative to improve the sensitivity of immunocytochemistry. This may be a useful procedure since colchicine treatment causes, in our experience, a high mortality rate in aged animals so that many animals do not survive the colchicine treatment long enough to induce an enhanced staining in the cell bodies. Moreover, colchicine is a toxic compound inducing a considerable burden on the general condition of the rats.

Recently we applied the 6-h postmortem interval to investigate differences in the number of VP immunoreactive cells in extrahypothalamic regions in brains from aged Brown-Norway rats. This study indeed showed a useful staining in extrahypothalamic VP neurons and revealed a significant age-related decrease in VP neurons in several extrahypothalamic regions.

Surprisingly, in brain regions in which no VP had previously been detected, neurons were also positively stained, i.e. LS, DR and a few cells in the organum vasculosum of the lamina terminalis. As VP in these regions had never been detected, not even following colchicine treatment, the possibility was investigated

whether VP-related compounds were stained. A positive staining with antibodies directed against parts of the precursor molecule of VP, i.e. CPP and NF, provided further evidence that VP production sites had indeed been stained and excluded possible formation of aspecific epitopes during the postmortem interval. However, IEF of homogenized tissue punches from these brain regions showed no positive immunoreactivity so far. The IEF procedure was probably not sensitive enough to detect these levels of peptide even after 6 h postmortem.

The results of the IEF experiments using the SCN showed an increase in the VP content after 6 h of postmortem interval, providing further evidence that the immunocytochemical results were due to staining of the VP-molecule rather than an aspecific epitope.

A few points still remain to be resolved. Although the LC and DR did show positive staining for VP and CPP after 6 h postmortem interval there was a complete lack or at best a slight immunoreactivity of NF. It is not yet clear how this difference can be explained. Staining with different antibodies raised against NF will be performed. The postmortem processing rate of the VP-precursor

molecule might be different in various brain regions and therefore the NF content might decrease below the detection level of immunocytochemistry. Alternatively, it is possible that the degradation rates of NF in DR and LC exceed precursor processing, thus resulting in a negative staining.

In conclusion, the results of this study are in agreement with other reports in the literature, which indicate postmortem stability or even enhancement of neuropeptide content. Our study shows that a postmortem interval of several hours is not necessarily detrimental for immunocytochemical studies on brain material. Furthermore, in rat a postmortem interval may provide an alternative to the colchicine treatment in experimental designs involving aged animals.

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REFERENCES

- Bird, E.D. and Iversen, L.L., Human brain postmortem neurochemistry. In A. Lajtha (Ed.), *Handbook of Neurochemistry*, Vol. 2, Plenum, New York, 1982, pp. 225–251.
- Caffe, A.R. and Van Leeuwen, F.W., Vasopressin-immunoreactive cells in the dorsomedial hypothalamic region, medial amygdaloid nucleus and the locus coeruleus of the rat, *Cell Tissue Res.*, 233 (1983) 23–33.
- Caffe, A.R., Van Leeuwen, F.W., Buijs, R.M., De Vries, G.J. and Geffard, M., Coexistence of vasopressin, neurophysin and noradrenaline immunoreactivity in medium-sized cells of the locus coeruleus and subcoeruleus in the rat, *Brain Research*, 338 (1985) 160–164.
- Dierickx, K. and Vandesande, F., Immunocytochemical localization of the vasopressinergic and the oxytocinergic neurons in the human hypothalamus, *Cell Tissue Res.*, 184 (1977) 15–27.
- Fliers, E., Swaab, D.F., Pool, Chr.W. and Verwer, R.W.H., The vasopressin neurons in the human supraoptic and paraventricular nucleus; changes with aging and in senile dementia, *Brain Research*, 342 (1985) 45–53.
- Fliers, E., Guldenaar, S.E.F., v.d. Wal, N. and Swaab, D.F., Extrahypothalamic vasopressin and oxytocin in the human brain; presence of vasopressin cells in the bed nucleus of the stria terminalis, *Brain Research*, 375 (1986) 363–367.
- Johnson, S.A., Morgan, D.G. and Finch, C.E., Extensive postmortem stability of RNA from rat and human brain, *J. Neurosci. Res.*, 16 (1986) 267–280.
- Livett, B.G., Immunocytochemical localization of nervous system specific proteins and peptides, *Int. Rev. Cytol.*, Suppl. 7 (1978) 53–237.
- Perry, T.L., Hansen, S. and Gandham, S.S., Postmortem changes of amino compounds in human and rat brain, *J. Neurochem.*, 36 (1981) 406–412.
- Pool, C.W., Madlener, S., Diegenbach, P.C., Sluiter, A.A. and Van der Sluis, P., Quantification of antiserum reactivity in immunocytochemistry, *J. Histochem. Cytochem.*, 32 (1984) 921–928.
- Ratter, S.J., Ackland, J., Corder, R., Emson, P., Rossor, M., Tomlin, S. and Rees, L.H., Stability of pro-opiocortin-related peptides in postmortem mouse brain tissue, *Neuroendocrinology*, 35 (1982) 336–341.
- Rossor, M., Emson, P., Dawbarn, D., Dockray, G., Mountjoy, C. and Roth, M., Postmortem studies of peptides in Alzheimer's disease and Huntington's disease. In J.B. Martin and J.D. Barchas (Eds.), *Neuropeptides in Neurologic and Psychiatric Disease*, Raven, New York, 1986, pp. 259–277.
- Snijderwint, F.G.M., Van Leeuwen, F.W. and Boer, G.J., Ontogeny of vasopressin and oxytocin binding sites in the brain of Wistar and Brattleboro rats as demonstrated by light microscopical autoradiography, *J. Chem. Neuroanat.*, 2 (1989) 3–17.
- Sternberger, L.A., The unlabelled antibody peroxidase-antiperoxidase (PAP) method. In *Immunocytochemistry*, 2nd edn., Wiley, New York, 1979, pp. 104–170.
- Swaab, D.F. and Boer, K., The presence of biologically labile compounds during ischemia and their relationship to the EEG in rat cerebral cortex and hypothalamus, *J. Neurochem.*, 19 (1972) 2843–2853.
- Swaab, D.F., Pool, Chr.W. and Nijfeldt, F., Immunofluorescence of vasopressin and oxytocin in the rat hypothalamo-neurohypophyseal system, *J. Neural Transm.*, 36 (1975) 195–215.
- Swaab, D.F., Comments on the validity of immunocytochemical methods. In V. Chan-Palay and S.L. Palay (Eds.), *Cytochemical Methods in Neuroanatomy*, Liss, New York, 1982, pp. 423–440.
- Swaab, D.F., Fliers, E. and Partiman, T.S., The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia, *Brain Research*, 342 (1985) 37–44.
- Vandesande, F., Dierickx, K. and De Mey, J., Identification of the vasopressin-neurophysin II and the oxytocin-neurophysin I producing neurons in the bovine hypothalamus, *Cell Tissue Res.*, 156 (1975) 189–200.
- Van der Sluis, P.J., Pool, Chr.W. and Sluiter, A.A., Immunocytochemical detection of peptides and proteins on press-blot after direct tissue gel iso-electric focussing, *Electrophoresis*, 9 (1988) 654–661.
- Van Leeuwen, F.W. and Caffe, A.R., Vasopressin-immunoreactive cell bodies in the bed nucleus of the stria terminalis of the rat, *Cell Tissue Res.*, 228 (1983) 525–534.