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IMMUNOCYTOCHEMISTRY OF THE C-TERMINAL PEPTIDE OF PROPRESSOPHYSIN (CPP): RELATIONSHIP TO VASOPRESSIN, OXYTOCIN AND NEUROPHYSIN

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ABSTRACT

Arginine-vasopressin (AVP) and its associated neurophysin (AVP-NP) are synthesized via a precursor, propressophysin, which also contains a 39 amino acid glycopeptide at its C-terminus (C-terminus of propressophysin, or CPP). In the present study, immunocytochemical techniques were used to determine the cellular co-localization of CPP with AVP, oxytocin (OXY), AVP-NP and OXY-NP in the rat hypothalamus using colchicine pre-treatment and serial 5 μm section analysis. Extensive cross-competition studies of antisera raised against each peptide with the various antigens yielded no significant crossreactivity of the CPP, AVP, OXY and NP antisera. The NP antiserum, although directed against both AVP-NP and OXY-NP, demonstrated a preference for OXY-NP at a dilution of 1:20,000. CPP and AVP were always co-localized within the same magnocellular neurons of the supraoptic, paraventricular and circularis nuclei, and further showed very similar patterning in the suprachiasmatic nucleus as well. In conrast, no cellular overlap could be detected between CPP and OXY, in any of the above nuclei (the suprachiasmatic nucleus is devoid of OXY). Likewise, no examples of co-localization of CPP and OXY-NP were found in the magnocellular nuclei. These results are in strong agreement with a biosynthetic relationship between CPP, AVP and AVP-NP, and their separateness from the OXY and OXY-NP precursor.

INTRODUCTION

A 39 amino acid glycopeptide extracted from pituitary has been sequenced by three separate laboratories over the last few years (1-3). Based on its size, source and distribution, Seidah et al. (1) suggested it to be part of the vasopressin precursor. This peptide is highly conserved in four species: human, pig, sheep and ox. Recently the cDNA structure of the mRNA for the precursor to arginine-vasopressin (AVP) and its associated neurophysin (AVP-NP) have been sequenced (4). The general structure of that mRNA involves

coding for a signal sequence, followed by AVP, then NP, and finally as expected (1) the 39 amino acid glycopeptide described above, namely the C-terminal peptide of propressophysin (CPP). Using antisera directed against this C-terminal 39 amino acid glycopeptide, we had previously shown that the peptide is uniquely localized in the posterior lobe of pituitary, the internal layer of the median eminance, and in the magnocellular neurosecretory neurons of rat hypothalamus, and is absent in several magnocellular nuclei of the homozygous Brattleboro rat (a strain known not to contain AVP or AVP-NP) (5,6). Specifically, a subset of neurons within the supraoptic, paraventricular and circularis nuclei have been stained by the specific CPP antiserum, with fibers leading from these neurons into the internal layer of the median eminance. Further, upon analysis of the parvocellular component of the suprachiasmatic nucleus, which does not contain oxytocin (OXY) cell bodies, many small perikarya were detected with the C-terminal peptide antiserum. Using specific AVP antisera, we were able to demonstrate the co-localization of CPP and AVP in the supraoptic and paraventricular nuclei (5). Thus, several lines of evidence suggested an intimate relationship between CPP and AVP.

In this paper we report on an immunocytochemical study of the cellular localization of CPP-like immunoreactivity as compared to AVP, OXY and OXY-associated NP (OXY-NP), in several hypothalamic magnocellular and parvocellular nuclei. The results of this study strongly support the biosynthetic relationship between CPP, AVP, and AVP-NP, but do not support a relationship between CPP and OXY.

MATERIALS AND METHODS

CPP was extracted as reported elsewhere (1). The peptide was conjugated via carbodiimide to ovalbumin, and injected in multiple subcutaneous sites on the back of rabbits. The serum harvested was used for immunocytochemical study at a titer of approximately 1:1000 in the immunocytochemical paradigm reported elsewhere (5). Under the immunocytochemical conditions studied, the binding of CPP to the CPP antiserum was not blocked by up to 50 μM concentrations of AVP, OXY, a combination of OXY-NP and AVP-NP, β-endorphin, α-MSH, [Met]- and [Leu]enkephalin, or dynorphin A. It could be blocked by as little as 10 nM concentration of CPP itself.

AVP and OXY antisera (from EZ and GN; and TvWG) were produced by linking their respective peptides via carbodiimide to thyroglobulin for rabbit immunization. The resulting antisera were used in immunocytochemical titer of 1:1000. The binding of AVP to AVP antisera could not be blocked by OXY, CPP, OXY-NP and AVP-NP, [Met]- and [Leu]enkephalin, dynorphin A, β-endorphin, ACTH or α-MSH in concentrations up to 50 μ M. However, AVP could block the immunocytochemical demonstration by AVP antiserum in concentrations as low as 100 nM. Similar characterization for the OXY antibodies was obtained. AVP, CPP, OXY-NP, AVP-NP, [Met]- and Leu]enkephalin, dynorphin, β-endorphin, ACTH, or α-MSH were unable to block the binding of OXY to OXY antisera at concentrations up to 50 μM. However, OXY antibodies could be blocked by OXY itself at approximately 100 nM concentration.

The NP antiserum used in this study (Robinson #4) is directed to both rat neurophysins, and has been previously characterized (7). It was used at

immunocytochemical titer of 1:20,000. None of the peptides listed above could block the binding of NP to its antiserum at concentrations of up to 50μM, with the exception of a mixture of rat neurophysins (a generous gift of J. Russell and H. Gainer, NIH) at 1 nM. Although directed against both neurophysins, at a dilution of 1:20,000 the antiserum generally stained OXY cells and was therefore assumed to be largely directed against OXY-NP.

Five animals were prepared from each of the following groups: normal and colchicine pretreated Sprague-Dawley male rats. Colchicine pretreatment was carried out 48 hours prior to sacrifice by the ICV administration of 50μg of colchicine in 50μl of normal saline under ether anesthesia. All animals were perfused via the ascending aorta with cold neutral-buffered 4% formaldehyde and prepared for immunocytochemistry as described elsewhere (8). Brains from all animals were sectioned in the frontal plane through the supraoptic, paraventricular, circularius, and suprachiasmatic nuclei. The tissue was prepared for immunohistochemistry using the concentrations of primary antiserum described above. The PAP technique as described by Sternberger (9) was modified for tissue staining (10). All the blocking studies were carried out in the presence of up to 50μM peptide.

The specific staining paradigm involved the use of serial 5 μm frozen sections in groups of twelve. Sections 1 and 12 were used for the peptide blocking control for the two peptides to be studied (e.g., AVP and CPP). The remaining sections (2-11) were stained alternately for each of the peptides. For example, sections 2,4,6,8, and 10 were stained for CPP, whereas 3,5,7,9 and 11 were stained for AVP. The goal of this paradigm was the attempted co-localization of the two peptides within the same cell. For a particular cell to be seen as positively stained for both peptides, it must occur in at least three consecutive sections and be clearly stained by both antisera. Because of the size of individual magnocellular neurons, any one cell often occurs in two, three or more serial 5μm sections. Comparisons were carried out for sera against the following peptides: AVP versus CPP, OXY versus CPP, NP versus CPP, and NP versus AVP. Sections through the supraoptic nucleus, paraventricular nucleus, nucleus circularis, and suprachiasmatic nucleus were taken for the study of these several staining comparisons.

RESULTS

CPP and AVP were seen to stain similar regions of the supraoptic, paraventricular and circularis nuclei. Upon careful analysis of serial sections prepared as described above, it became clear that CPP and AVP immunoreactivities are localized to the same cells within the supraoptic nucleus (Fig. 1), paraventricular nucleus (Fig. 2), and nucleus circularis (Fig. 3). Because of the small size of the cells in the suprachismatic nucleus, it was not possible to co-localize the two peptides. However, it should be noted that there are no OXY staining cells in that nucleus (see Figure 4) and that the pattern of staining using CPP and AVP antisera is very similar.

Within the limits of the resolution of this type of serial section analysis, no examples could be detected of CPP or AVP staining independent of

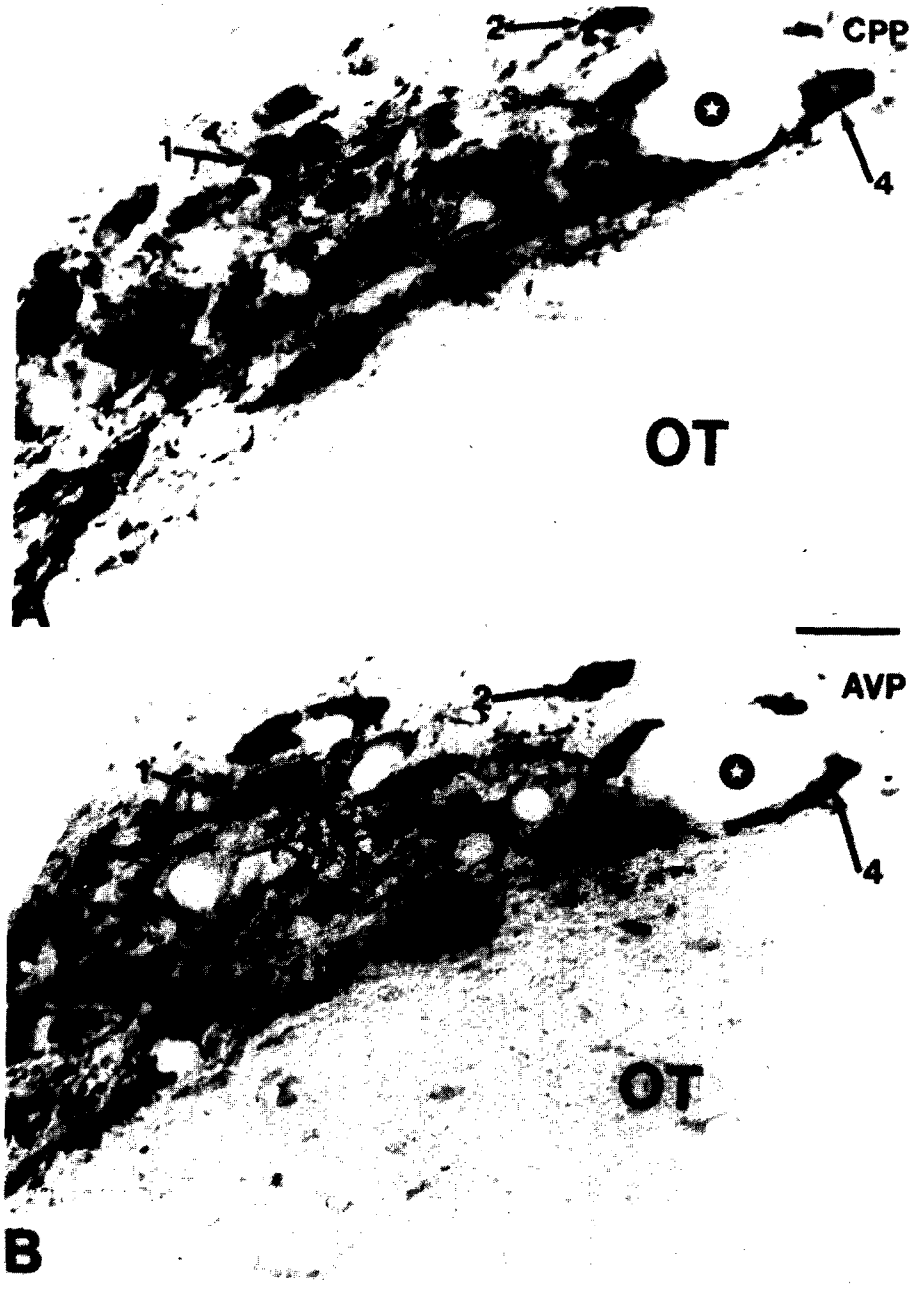

Figure 1. Supraoptic nucleus: This nucleus has cells which contain both AVP and CPP immunoreactivity. Serial 5 μm sections were taken from a colchicine pretreated rat (50 μg ICV, 48 hours prior to sacrifice). Sections were stained with antiserum against CPP (panel A) or AVP (panel B). Many cells, stained with both sera, can be identified in both sections (see numbered cells). Star is in a common vessel. OT: optic tract. Calibration bar equals 20 μm.

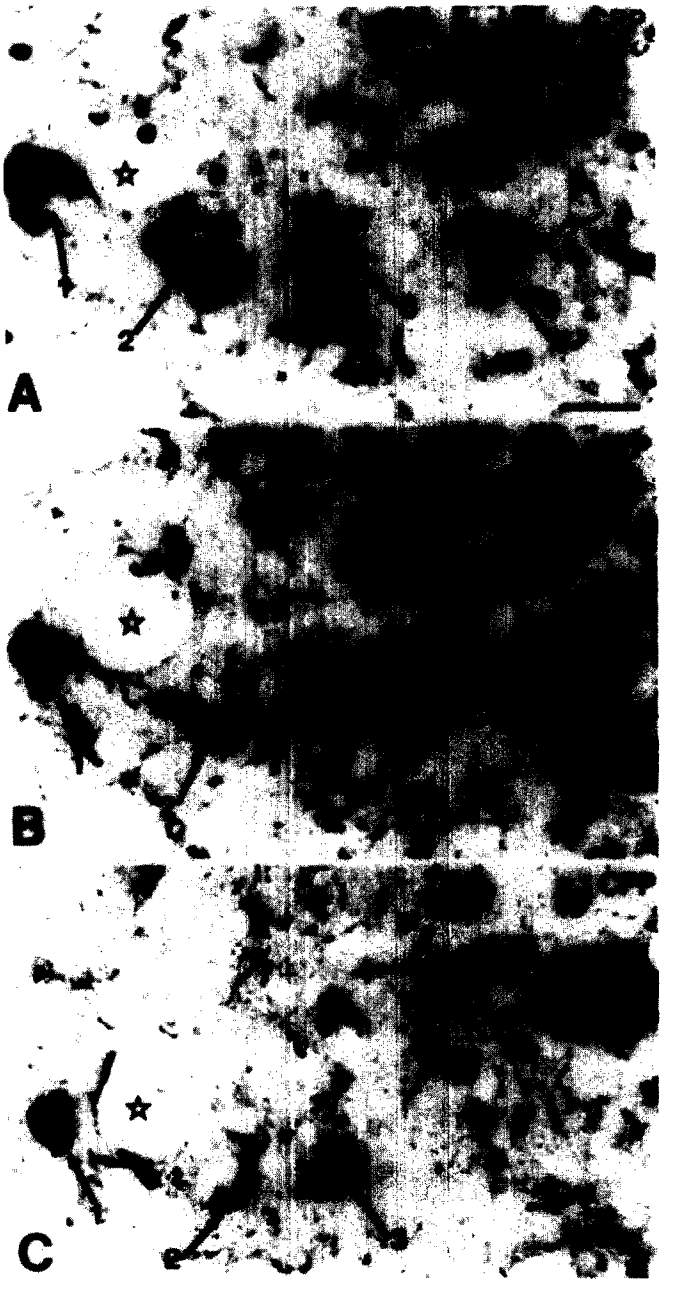

Figure 2. Paraventricular nucleus: This nucleus has cells which contain both AVP and CPP immunoreactivity. The same animal and methods were used as in Figure 1. Panel B shows cells stained with anti-AVP serum, whereas panels A and C were stained with anti-CPP serum. Commonly stained cells are numbered in all three panels. Star is in a common vessel. Calibration bar equals 20 μm.

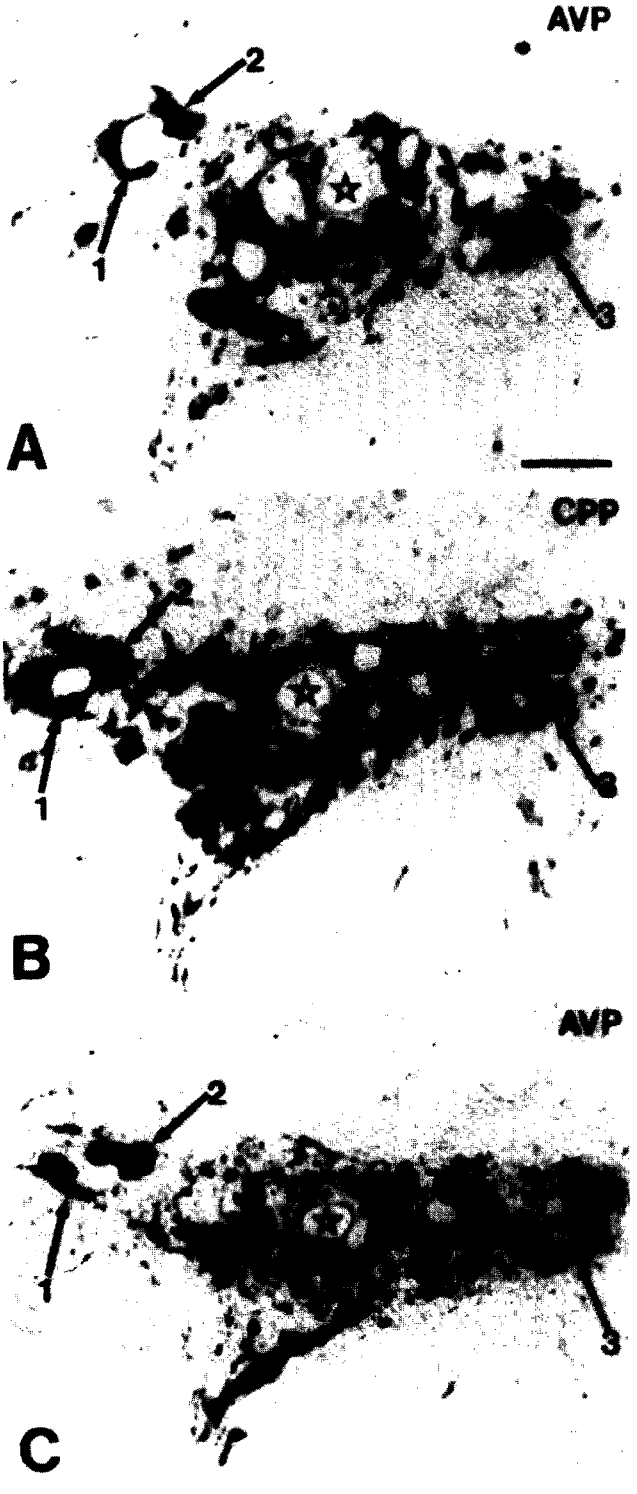

Figure 3. Nucleus circularis: This nucleus has cells which contain both AVP and CPP immunoreactivity. The same animal and methods were used as in Figure 1. Panel B shows cells stained with anti-CPP serum, whereas panels A and C show cells stained with anti-AVP serum. Commonly stained cells are numbered in all three panels. Star is in a common vessel. Calibration bar equals 20 μ m.

Figure 4. Suprachiasmatic nucleus: Cells of this nucleus contain CPP but not OXY immunoreactivity. The same animal and methods were used as in Figure 1. Arrows indicate two of the many CPP-stained cells. The inset photo is from an adjacent section and was exposed to anti-OXY serum. No OXY-positive cells appeared in this nucleus, whereas cells in other areas of the same animal were OXY-positive. Star is in a common vessel. Calibration bar equals 20 μm.

* * * *

the other. However, it should be noted that analysis of serial 5 μm sections cannot eliminate the possibility of two immunoreactive peptides occuring independently in separate cells. This type of analysis is conservative and can only provide evidence that two given immunoreactive substances occur in some or most cells of a particular region. Given these considerations, we conclude that CPP and AVP immunoreactivities are stored together extremely commonly, but cannot yet exclude the possibility of occasional separate localization.

The relationship between CPP and OXY is characterized in figures 5 through 7. The same nuclei, supraoptic (Fig. 5), paraventricular (Fig. 6) and circularis (Fig. 7), all contain OXY positive cells, but in no case were we able to detect cellular overlap between CPP and OXY. In all these cases, we were able to detect cells which had been stained with CPP but which were unstained in the adjacent section by OXY. The suprachiasmatic nucleus as reported above was unstained by OXY, yet was stained by CPP (Fig. 4). While it is very difficult to eliminate the possibility of two substances being co-stored in the same cells under the conditions used here, no example of OXY or CPP-like immunoreactivity within the same cells could be detected.

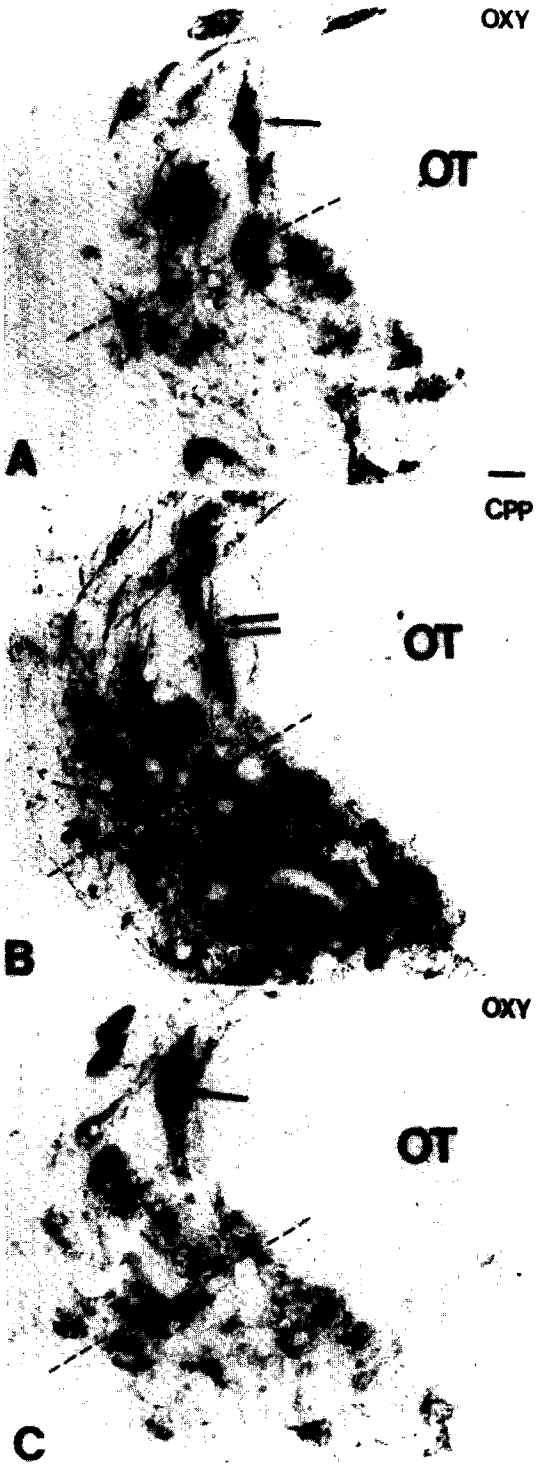

Figure 5. Supraoptic nucleus: OXY-positive cells do not stain with CPP antiserum. Serial 5 μ m sections were taken from a colchicine pretreated rat (50 μ g ICV, 48 hours prior to sacrifice). Anti-OXY serum was used to stain cells in panels A and C, whereas anti-CPP serum stained cells in panel B. Note that OXY-positive cells are sparsely and widely distributed at this level of the nucleus. In contrast, CPP-containing cells are predominantly located in the ventral portion of the nucleus (see dotted line). Single arrow in panels A and C shows an OXY-positive cell which is not seen in CPP-stained tissue in panel B (double arrow). Calibration bar equals 20 μ m.

Figure 6. Paraventricular nucleus: OXY-positive cells do not stain with CPP antiserum. The same animal and methods were used as in Figure 4. Anti-OXY serum was used to stain cells in panels A and C, whereas anti-CPP serum stained cells in panel B. Note that OXY-positive cells are more plentiful than CPP-positive cells. The single arrow in panels A and C and double-arrow in panel B point to the same cell, which is unstained with OXY antiserum, but is stained with CPP antiserum. Star is in a common vessel. Calibration bar equals 20 μ m.

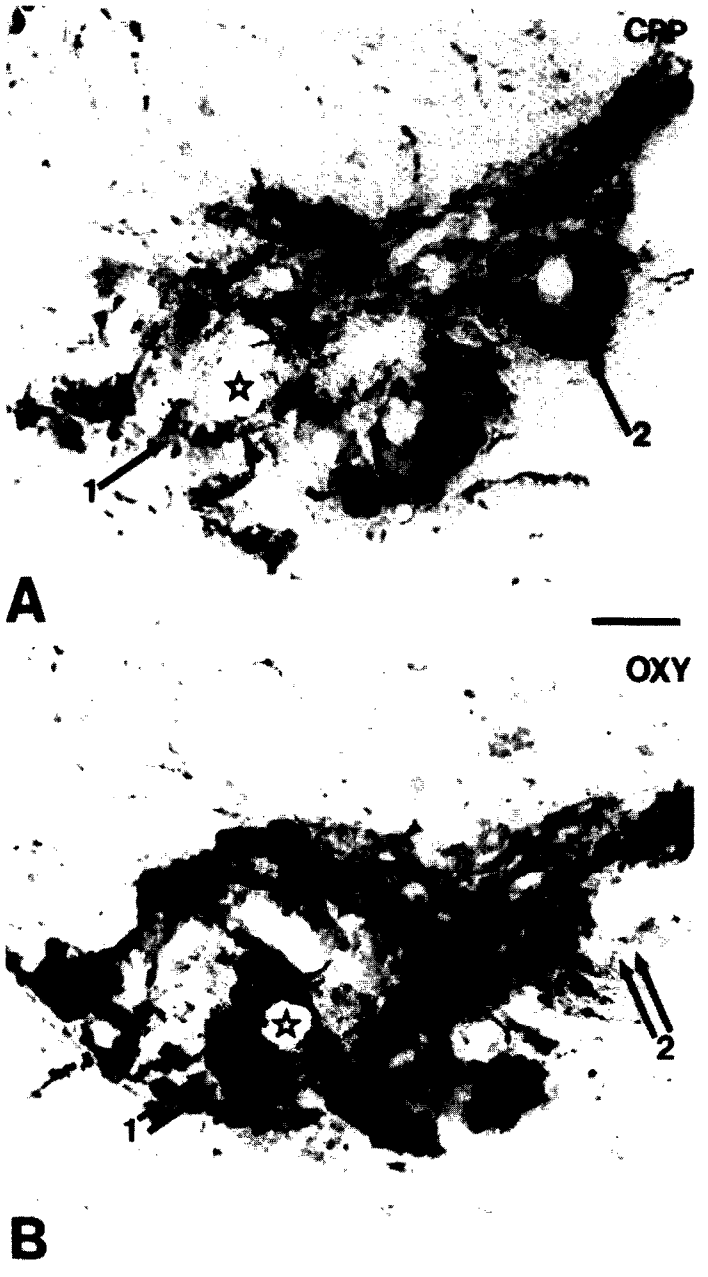

Figure 7. Nucleus circularis: OXY-positive cells do not stain with anti-CPP serum. The same animal and methods were used as in Figure 4. Anti-OXY (panel B) and anti-CPP (panel A) stain different cells. For example, note that cell number 1 (double arrow) is OXY-stained but not CPP-stained (single arrow), whereas cell number 2 (single arrow) is CPP-stained but not OXY-stained (double arrow). Star is in common vessel. Calibration bar equals 20 μm.

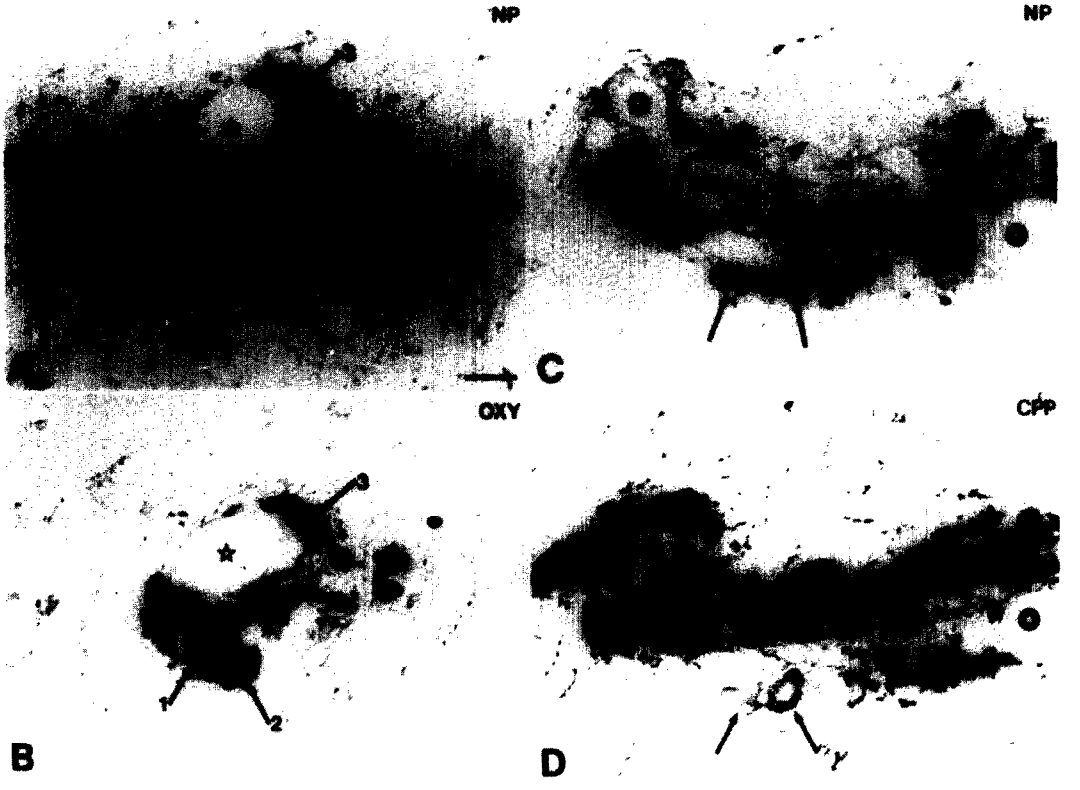

Figure 8. Nucleus circularis: The NP antiserum used in this study preferentially stains OXY cells and generally does not stain CPP cells. Cells in panels A and C were stained with the anti-NP serum, while cells in panel B were stained with anti-OXY serum; cells in panel D were stained with anti-CPP serum. Note that NP (panel A) and OXY (panel B) stain the same cells (see numbered cells in both panels). In contrast, a large number of CPP-stained cells (panel D) are unstained (or very lightly stained) by the anti-NP (panel C). The two cells (arrows) that are stained by both NP and CPP antisera, probably contain AVP-NP and are vasopressinergic. It is assumed that the staining of some AVP cells by the NP antiserum is a result of crossreactivity between both AVP-NP and OXY-NP. Stars indicate a common vessel in panels A, B, and panels C, D. Calibration bar equals 20 μm.

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The NP antiserum used in this study seems to contain antibodies against both OXY-NP and AVP-NP. At 1:20,000 dilution, all OXY-containing cells were stained with the NP antiserum (Fig. 8A, B). However, under the same conditions, and in the same animals, it was shown that CPP and OXY-NP appear not to co-localize to the same magnocellular neurons of supraoptic, paraventricular, and circularis nuclei (Fig. 8C, D). In general the NP antiserum tended not to stain the CPP positive cells, with few exceptions (see arrows Fig. 8C, D). We are unable to be more certain about this point because of the complex nature of the NP antiserum.

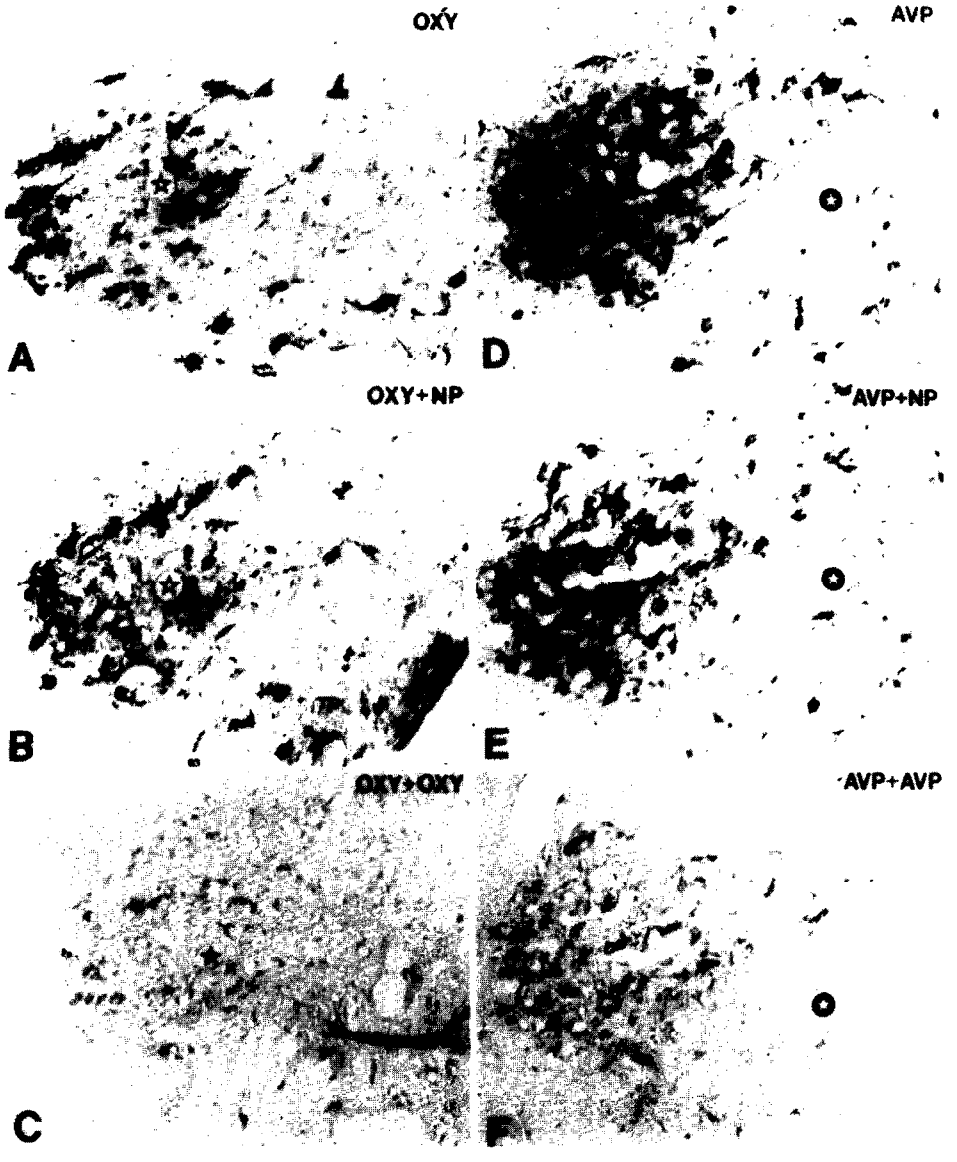

Figure 9. Paraventricular nucleus: Neither OXY nor AVP-stained cells are blocked by NP, but are blocked by OXY and AVP, respectively. As reported in the text, OXY antiserum was not blocked by AVP, and AVP antiserum was not blocked by OXY. Cells in panels A, B and C were stained with OXY antiserum. Cells in panel B were also exposed to 50 μ M NP (a mixture of OXY-NP and AVP-NP), while cells in panel C were exposed to 10μ M OXY. Note the complete blockade of staining with OXY. Star is in a common vessel in A, B, and C. Cells in panels D, E, and F were stained with AVP antiserum. Cells in panel E were exposed to 50 μM NP, and cells in panel F to 10 μM AVP. Note the blockade with AVP. Star is in a common vessel in D, E, and F. Calibration bar equals 20 μm.

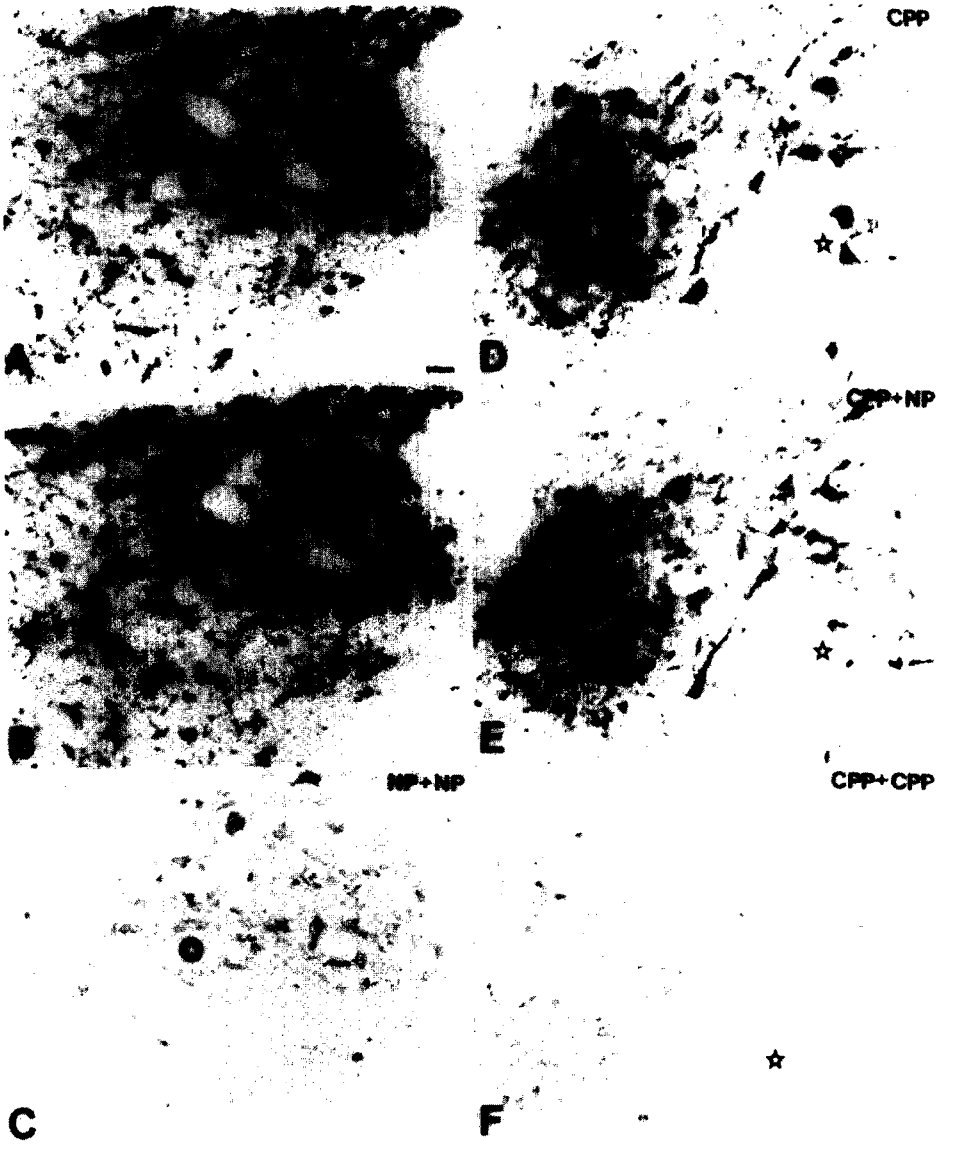

Figure 10. Paraventricular nucleus: NP antiserum was not blocked by CPP, but was blocked by NP itself. In contrast, CPP antiserum was not blocked by NP, but by CPP itself. Cells in panels A, B, and C were stained with NP antiserum. Cells in panel B were exposed to 50 μM CPP. Cells in panel C were blocked by 1 μM NP. Cells in panels D, E, and F were stained with CPP antiserum. Cells in panel E were exposed to 50 μM NP (OXY-NP and AVP-NP). Cells in panel F were blocked by 1 μM CPP. Star is in vessels common to A, B, C, and D, E, F. Calibration bar equals 20 μm.

Cross-blocking studies between OXY and NP (Fig. 9A, B, C), AVP and NP (Fig. 9D, E, F), as well as NP and CPP (Fig. 10) yielded no examples of cross-reactivity between the several antisera and the peptides tested. Thus it is not reasonable to attribute CPP staining to cross-reactivity with NP or any other tested peptides.

DISCUSSION

The immunocytochemical analysis of the localization of the CPP-like immunoreactivity is in very strong agreement with the distribution pattern of AVP in all the magnocellular nuclei of rat brain. Examples of co-localization of AVP and CPP in each of the hypothalamic nuclear areas have been presented. No examples of cellular co-localization of CPP immunoreactivity with OXY was detected, even though the same number of studies under blind conditions were carried out in parallel with CPP, AVP and OXY staining. It would appear that OXY has no clear relationship to CPP, and if OXY has a similarly constructed precursor which has yet to be proven, it may have a peptide at its C-terminus which has a different structure than CPP.

The relationship between AVP, its associated AVP-NP and CPP (4) raises significant questions about the native biology of each of the three peptide products. The biology of AVP, while complex, is well studied. It has many effects across a variety of target systems and is, in its own right, a very potent substance (cf. 11-13). The role of NP has been studied somewhat less and has been thought to be that of a carrier protein (cf. 14), but could well be without any important biological function. No biological effects in the administration of NP have been reported. Because of the highly conservative nature of the C-terminal glycopeptide of propressophysin, it is interesting to speculate what role it might play. Is it a carrier protein, an active peptide, or does it perhaps play a role in modulating or synergizing with AVP or NP? An increasingly important principle emerging from neuropeptide research is that of co-synthesis of several bioactive substances from the same precursor. In the case of pro-opiomelanocortin, ACTH, α-MSH, β-endorphin and a long N-terminal fragment are produced by the same precursor in endocrine and neuronal cells (15-19). Some groups have reported a synergistic effect in peripheral endocrine function and in central neural function of two or three peptides from the same pro-opiomelanocortin precursor (20-22). It is conceivable that a similar type of interaction might occur between AVP, NP and CPP.

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