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Substances resembling C-terminal vasopressin fragments are present in the brain but not in the pituitary gland

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In order to investigate the endogenous occurrence of vasopressin fragments that have previously been found to be generated in vitro by brain peptidases and to have highly potent central activity, extracts of hypothalamus, hippocampus and the pituitary gland were fractionated by high pressure liquid chromatography and analyzed by radioimmunoassay systems with different specificities. Substances that were immunologically and chromatographically similar to synthetic C-terminal vasopressin fragments were detected in brain tissue in different amounts, but were virtually absent in the pituitary gland. It is suggested that these components may represent endogenous vasopressin metabolites. The preferential presence in brain supports a selectively central function of these peptides.

Arginine-vasopressin (AVP_{1-9}) is a hormone in the peripheral circulation and neuropeptide in the brain affecting a number of central processes[1,5,6,10,11]. Unlike the exclusively hormonal activities of AVP_{1-9}, the central activities do not require the entire nonapeptide structure; several synthetic fragments of AVP_{1-9} have central activities by themselves[6]. Recently, a number of AVP fragments that were generated by in vitro proteolysis of AVP_{1-9} by synaptic membranes have been characterized[2,12]. One of these peptides, [pGlu^4,Cyt^6] AVP_{4-9}, and its des-glycinamide derivative were a thousand times more potent than AVP_{1-9} in affecting behavior, but lacked vasopressor activity[3,6]. These findings suggested that such AVP fragments possessing selectively central activity might have a role as neuropeptides in the brain. Here we report the presence of peptides that resemble AVP fragments in brain as characterized in high-pressure liquid chromatography in combination with two radioimmunoassays using antisera of different specificity. It is shown that the hippocampus, a terminal area of the central vasopressinergic system, is relatively rich in these materials, whereas the pituitary gland containing terminals for peripheral AVP is virtually devoid of them. It is suggested that these substances represent metabolites of AVP that are preferentially generated in brain. Such AVP metabolites might be the active principles for some of the central actions of AVP.

Tissue was used from male Wistar rats (180–200 g) which had been handled for 5 days prior to decapitation. The whole pituitary gland was taken and the hypothalamus and hippocampus were dissected from the brain[8]. Tissues were immediately frozen on dry ice and extracted after boiling in 1 M acetic acid and subsequent cooling to 0 °C by homogenization using a Branson sonifier. Particulate material was removed by centrifugation at 20,000 g_{av} for 30 min and the clear supernatants were lyophilized. Subsequently, samples were reconstituted in a small volume of 0.05 M acetic acid and made up to 1.0 ml with starting HPLC solvent. Fractionation of extracts was carried out by reverse phase HPLC with ammonium acetate and methanol in the mobile phase[2,12]. Fractions were collected and prepared for radioimmunoassay determination (for details see legend to Fig. 1). The radioimmunoassay systems employed antiserum W1A or antiserum W4E, [^{125}I]AVP_{1-9} as tracer and synthetic AVP_{1-9} as a standard. Procedures have been previously described[7]. Crossreaction of antiserum W1A

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in this system was 50% for [Cyt⁶]AVP_{2-9}, 31% for [Cyt⁶]AVP_{3-9}, 23% for [pGlu⁴,Cyt⁶]AVP_{4-9} and 13% for [Cyt⁶]AVP_{5-9}. The antiserum also recognized the reduced forms of these peptides. The C-terminal AVP fragments gave parallel and complete displacement of the tracer. Cross-reaction with AVP_{7-9}, AVP_{1-8} and oxytocin was 0.12%, 0.01%, and 0.02%, respectively. Crossreaction of antiserum W4E was 100% with AVP_{1-9} and 50% for AVP_{1-8}. [Cyt⁶]AVP_{3-9}, AVP_{1-7}, and oxytocin crossreacted 0.001%, 0.9%, and 0.02%, respectively. The data demonstrate that antiserum W1A has high specificity for the C-terminal portion of AVP_{1-9}, while antiserum W4E is directed to a portion of the N-terminal AVP sequence.

A reverse phase HPLC system that allowed separation of AVP_{1-9} and a number of C-terminal AVP fragments was used to investigate the presence of AVP related peptides in the brain. By reverse phase HPLC fractionation of hypothalamic and hippocampal extracts a number of components immunoreactive with the C-terminal AVP antiserum W1A were detected (Fig. 1A and 2A). The main component eluted in fractions 61 and 62. It co-eluted with synthetic AVP_{1-9} and was detected in a similar amount when using antiserum W4E (Fig. 1B and 2B) indicating its identity as being AVP_{1-9}. The additional W1A immunoreactive components eluted at shorter retention times. These components failed to react with antiserum W4E indicating that they represented peptides having the C-terminus of AVP_{1-9}. Upon HPLC component II and III co-migrated with synthetic [Cyt⁶]AVP_{2-9} and [Cyt⁶]AVP_{3-9}, respectively. Component IV had similar retention time as the reduced form of [pGlu⁴,Cyt⁶]AVP_{4-9}. Component V eluted at the position of [Cyt⁶]AVP_{4-9} and the reduced form of [Cyt⁶]AVP_{5-9}, while component VI eluted similar to [Cyt⁶]AVP_{5-9}. By extracting brain tissue in the presence of [³H-Tyr²]AVP_{1-9} it was excluded that these peptides were artifacts of the extraction procedure. The radioactive peptide was recovered intact without any degradation products as analysed by reverse phase HPLC after extraction in 1 M acetic acid or 0.1 M hydrochloric acid.

The levels of the immunoreactive components in brain tissue were well below that of AVP_{1-9}. In the hypothalamus they each represented approximately 1.5–3% of the AVP_{1-9} content (Table I). In the hip-

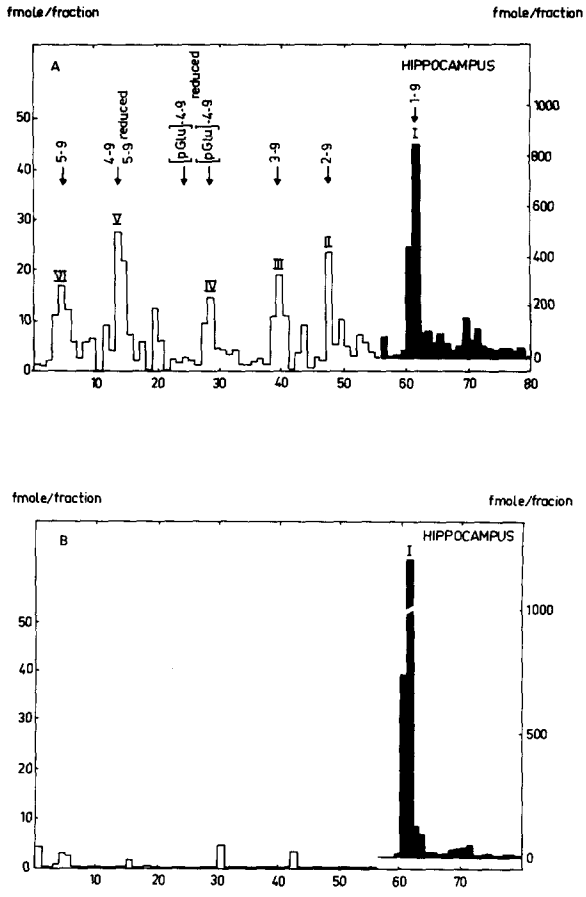

Fig. 1. HPLC profiles of AVP immunoreactive peptides in an extract of the rat hippocampus. An extract was made from 10 rat hippocampi and prepared for HPLC as described in the text. Samples for reverse phase HPLC were injected into a μBondapak C_{18} column (300 mm × 4 mm, Waters Assoc.) and eluted by a concave gradient (program 7 on a Waters Assoc. model 660 gradient mixing computer) of 10 mM ammonium acetate, pH 4.15 (solvent A) and 0.15% acetic acid in methanol (solvent B). The gradient ran from 0% B to 40% B in 30 min at a flow rate of 2.0 ml/min. The gradient lag time was 5 min. HPLC was performed on an apparatus which was only used for pmol quantities of peptides or below and wich was washed with 6 M nitric acid before a series of experiments to avoid any contamination. Fractions of 1 min were collected in 100 μl of 0.1% bovine serum albumin, methanol was evaporated in vacuo at 60 °C and samples were freeze dried. For radioimmunoassay determination residues were taken up in radioimmunoassay buffer in appropriate dilution. The radioimmunoassay for C-terminal AVP fragments (A) employed antiserum W1A (final dilution 1:160,000); antiserum W4E (final dilution 1:30,000) was used in the radioimmunoassay specific for the N-terminal region of AVP (B). In both radioimmunoassay systems [¹²⁵I]AVP_{1-9} was used as a tracer and synthetic AVP_{1-9} as standard. HPLC systems were calibrated within each experiment with a mixture containing approximately 1 pmol of synthetic peptide markers as indicated by the vertical arrows. The numbers refer to the structure of the peptides with intact disulfide bridge or with reduced disulfide bridge.

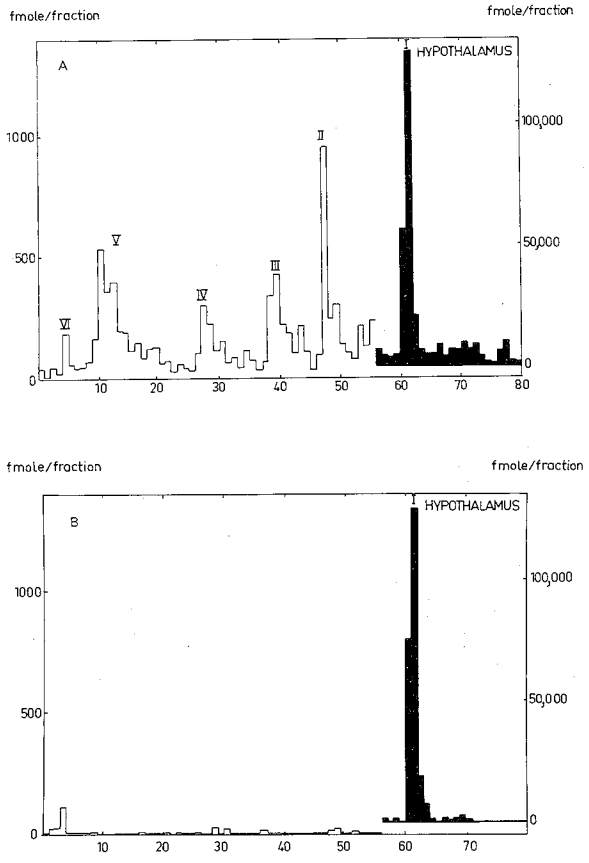

Fig. 2. Fractionation by reverse phase HPLC of peptides immunoreactive with the C-terminal AVP antiserum W1A (A) and the N-terminal AVP antiserum W4E (B) in an extract of 5 rat hypothalami. Details are described in the legend to Fig. 1 and in the text.

pocampus levels were relatively high being between 3.4% and 17.7% of the content of AVP_{1-9} (Table I). In contrast, in the pituitary gland virtually all immu-

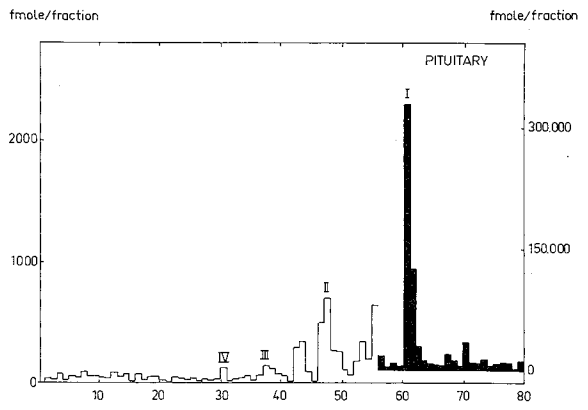

Fig. 3. Reverse phase HPLC fractionation of peptides immunoreactive with the C-terminal AVP antiserum W1A in an extract of the rat pituitary gland. The quantities shown represent those of a single pituitary gland.

noreactivity represented intact AVP_{1-9} (Fig. 3). Traces of component II, III and IV were detected. They represented only 0.61%, 0.09%, and 0.07%, respectively, of the AVP_{1-9} content (Table I).

From the experimental data it is concluded that a number of peptides with similarity to C-terminal AVP fragments is present in brain tissue. This conclusion is based on, firstly, the immunoreactivity of the peptides in a radioimmunoassay recognizing the C-terminal portion of AVP and lack of immunoreactivity for a N-terminal antiserum and, secondly, the co-elution of components with synthetic C-terminal AVP fragments in HPLC. The data indicate a close structural similarity to the C-terminal AVP fragments that have previously been identified after in vitro proteolysis of AVP_{1-9} by brain membranes[2,3,12]. It is tempting to speculate that these components rep-

TABLE I

Content of C-terminal AVP fragments in comparison with AVP_{1-9} in rat hypothalamus, hippocampus and pituitary gland

The quantity of components was determined by radioimmunoassay using antiserum W1A after HPLC fractionation of extracts, taking into account the crossreaction of various synthetic C-terminal AVP fragments (see text); b.d., below detection.

Component	*Hypothalamus*		*Hippocampus*		*Pituitary*	
	pmol/mg protein	*proportion of AVP_{1-9} content %*	*fmol/mg protein*	*proportion of AVP_{1-9} content %*	*pmol/mg protein*	*proportion of AVP_{1-9} content %*
I (AVP_{1-9})	18.27	100	37.80	100	1913.4	100
II	0.23	1.80	1.66	4.40	11.59	0.61
III	0.25	2.00	3.61	9.60	1.65	0.09
IV	0.24	1.90	3.61	9.60	1.27	0.07
V	0.37	2.90	6.45	17.1	b.d.	—
VI	0.19	1.50	8.67	22.90	b.d.	—

resent endogenous metabolites of AVP. However, definite identification of the components must await isolation and further chemical characterization. Experiments in this direction are being undertaken.

The potent and specifically central activities of some of the in vitro generated AVP metabolites, such as [pGlu^4,Cyt^6]AVP$_{4-9}$ that is at least a 1000-fold more potent than AVP$_{1-9}$ in affecting passive avoidance behavior[2,3], indicates the physiological relevance of endogenous AVP fragments in brain. The hippocampus, a central terminal of the vasopressinergic system and a site of behavioral action of AVP[9], is relatively rich in the C-terminal AVP-like components. The hypothalamus which contains AVP producing cell bodies[4], has significantly lower amounts of the components in proportion of AVP$_{1-9}$, suggesting that production of the components is favoured in terminal brain areas, possibly due to the presence of an appropriate enzyme system. However, the pituitary gland that is extremely rich in AVP$_{1-9}$ and the major storage site of AVP for peripheral functions, is virtually devoid of these components. This could be of relevance since the C-terminal AVP fragments lack the hormonal activities of AVP$_{1-9}$, indicating that they are unimportant as peripherally acting peptides.

The preferential occurence of substances resembling C-terminal AVP fragments in brain together with their previously reported central activity[3] suggests a role of AVP metabolites as endogenous neuropeptides of the vasopressinergic system in brain.

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1 Boer, G. J., Swaab, D. F., Uylings, H. B. M., Boer, K., Buijs, R. M. and Velis, D. N., Neuropeptides in Rat Brain Development. In P. S. McConnell, G. J. Boer, H. J. Romijn, N. E. van de Poll and M. A. Corner (Eds.), *Adaptive Capabilities of the Nervous System, Progress in Brain Research, Vol. 53*, 1980, pp. 207–227.

2 Burbach, J. P. H. and Lebouille, J. L. M., Proteolytic conversion of arginine-vasopressin and oxytocin by brain synaptic membranes: characterization of formed peptides and mechanism of proteolysis, *J. biol. Chem.*, 258 (1983) 1487–1494.

3 Burbach, J. P. H., Kovács, G. L., De Wied, D., Van Nispen, J. W. and Greven, H. M., A major metabolite of arginine-vasopressin in the brain is a highly potent neuropeptide, *Science*, 221 (1983) 1310–1312.

4 Buijs, R. M., Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat: pathways to the limbic system, medulla oblongata and spinal cord, *Cell Tiss. Res.*, 192 (1979) 423–435.

5 De Wied, D., Behavioural actions of neurohypophyseal peptides, *Proc. Roy. Soc.*, B 210 (1980) 183–195.

6 De Wied, D., Gaffori, O., Van Ree, J. M. and De Jong, W., Central target for the behavioural effects of vasopressin neuropeptides, *Nature (Lond.)*, 308 (1984) 276–278.

7 Dogterom, Snijdewindt, F. G. M. and Buijs, R. M., The distribution of oxytocin and vasopressin in the rat brain, *Neurosci. Lett.*, 9 (1978) 431–436.

8 Gispen, W. H., Schotman, P. and De Kloet, E. R., Brain RNA and hypophysectomy: a topographical study, *Neuroendocrinology*, 9 (1972) 285–296.

9 Kovács, G. L., Versteeg, D. H. G., Bohus, B., De Kloet, E. R. and De Wied, D., Effect of oxytocin and vasopressin on memory consolidation: sites of action and catecholaminergic correlates after microinjection into limbic midbrain structures, *Brain Research*, 175 (1978) 303–314.

10 Pedersen, C. A. and Prange, A. J., Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin, *Proc. nat. Acad. Sci. U.S.A.*, 76 (1979) 6661–6665.

11 Södersten, P., Henning, M., Melin, P. and Ludin, S., Vasopressin alters female sexual behaviour by acting on the brain independently of alterations in blood pressure, *Nature (Lond.)*, 310 (1983) 608–610.

12 Wang, X.-C., Burbach, J. P. H., Verhoef, J. and De Wied, D., Proteolytic conversion of arginine-vasotocin by synaptic membranes from rat and chicken brain, *Brain Research*, 275 (1983) 83–90.