Raz and A. Ben-Ze'ev, *Int. J. Cancer* **29**, 711 (1982).
4. J. Folkman and H. P. Greenspan, *Biochim. Biophys. Acta* **417**, 211 (1975); S. C. Wittelsberger *et al.*, *Cell* **24**, 859 (1981).
5. J. Folkman and A. Moscona, *Nature (London)* **273**, 345 (1978).
6. I. Yahara and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1579 (1975); A. Ben-Ze'ev, A. Duerr, F. Solomon, S. Penman, *Cell* **17**, 859 (1979).
7. V. Schirrmacher, *Immunobiology* **157**, 89 (1980); L. Weiss, *Am. J. Pathol.* **97**, 60 (1978).
8. A. Ben-Ze'ev, S. R. Farmer, S. Penman, *Cell* **21**, 365 (1980).
9. D. Gospodarowicz, G. Greenburg, C. R. Birdwell, *Cancer Res.* **30**, 4155 (1978).
10. P. D. Benya and J. D. Shaffer, *Cell* **30**, 215 (1982).
11. D. Brouty-Boye, R. W. Tucker, J. Folkman, *Int. J. Cancer* **26**, 501 (1980).
12. E. Klein, *Exp. Cell. Res.* **8**, 188 (1955).
13. P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007 (1975).
14. We thank M. Feldman and Y. Aloni for their careful review of the manuscript, R. Karkash and T. Kreizman for technical assistance, and M. Baer and P. Rubinstein for preparation of the manuscript. Supported in part by grants from the Israel Cancer Association (A.R.) and the United States–Israel Binational Foundation (A.B-Z.). A.R. is the incumbent of the Sophie M. T. and Richard S. Richards Career Development Chair in Cancer Research in perpetuity. Avri Ben-Ze'ev is the incumbent of the Ruth and Leonard Simon Career Development Chair in perpetuity.

2 February 1983; revised 11 May 1983

A Major Metabolite of Arginine Vasopressin in the Brain Is a Highly Potent Neuropeptide

Abstract. *A peptide that accumulated as the major product during the proteolysis of arginine vasopressin by rat brain synaptic membranes was isolated and its structure was shown to be the hexapeptide pGlu-Asn-Cys(Cys)-Pro-Arg-Gly-NH_2. When administered intracerebroventricularly in extremely low doses, this vasopressin fragment and its desglycinamide derivative facilitated memory consolidation in a passive avoidance situation. These vasopressin metabolites, which are devoid of pressor activity, constitute highly potent neuropeptides with selective effects on memory and related processes; they are activated via proteolytic processing of vasopressin by brain peptidases.*

The nonapeptide arginine vasopressin (AVP) regulates peripheral as well as central functions. The regulatory role of AVP in the central nervous system is evident in the influence of AVP on learning and memory, brain development, cardiovascular- and thermoregulation, development of tolerance, physical dependence on opiates and ethanol, and drug-seeking behavior (1). Studies on animal behavior and clinical trials point to a memory-improving influence of AVP. In experimental animals AVP improves learning, increases resistance to extinction of active avoidance behavior, facilitates passive avoidance behavior, and protects against retrograde amnesia (2). Several reports describe that in clinical trials AVP or derived peptides improve the performance of patients with certain memory disorders and increase cognitive responses of unimpaired subjects (3).

Structure-activity studies using synthetic AVP fragments have shown that the peripheral and central activities of AVP can be dissociated and that different short sequences derived from the AVP structure affect different aspects of memory (4). Based on these findings the existence of vasopressin fragments with more selective or potent activities has been presumed (4). We have approached the identification of such active AVP fragments by studying the conversion of AVP by proteolytic enzymes in the brain. We report here that during proteolysis of AVP the peptide pGlu-Asn-Cys(Cys)-Pro-Arg-Gly-NH_2 arises as a major metabolite and that this peptide and its desglycinamide derivative are more potent and selective in facilitating memory consolidation in rats than the parent molecule.

The metabolism of AVP was studied by in vitro incubation of AVP with isolated synaptic membranes from rat brain. Products of proteolysis were isolated by reversed-phase high-pressure liquid chromatography (HPLC) on a μBondapak C18 column using a gradient of methanol and ammonium acetate (*p*H 4.15) (see legend to Fig. 1). After incubation for 6 hours a major metabolite which eluted at 15 minutes in the HPLC system accumulated (Fig. 1). This component was collected from the HPLC effluent and subjected to amino acid analysis after performic acid oxidation (5) and determination of free NH_2-terminal groups (6). Analyses demonstrated that the fraction contained a single peptide. The amino acid composition of the isolated peptide was Cys(O_3H), 2.0; Asp, 1.0; Glu, 0.9; Arg, 1.0; and Gly, 1.0 (7). The amounts of Tyr and Phe were below the detection limit. Pro was not detected by this amino acid analysis technique (5). In the end-group analysis monodansyl-Cyt (dansyl-Cys-OH H-Cys-OH) was detected. After treatment of the isolated peptide with pyroglutamate-aminopeptidase (E.C. 3.4.11.8) according to procedures described in (8) dansyl-Asp was detected, demonstrating that the peptide contained pGlu at its NH_2-terminus. The analyses showed that the isolated peptide was the COOH-terminal hexapeptide of AVP having an intact disulfide bridge. The structure of this peptide, which we refer to as [pGlu^4, Cyt^6]AVP-(4–9), is

H-Cys-OH
|
pGlu-Asn-Cys-Pro-Arg-Gly-NH_2

This peptide and its des-Gly-NH_2 derivative [pGlu^4, Cyt^6]AVP-(4–8) were synthesized by the classical approach of fragment condensation. Synthetic and isolated [pGlu^4, Cyt^6]AVP-(4–9) were found to coelute in the reversed-phase HPLC system described in Fig. 1 and in paired-ion HPLC using heptanesulfonic acid as the ion-pairing reagent (9).

The activity of peptides on memory processes was tested in a step-through passive avoidance situation using a single learning trial in male Wistar rats (10). The apparatus consisted of an illuminated, elevated platform attached to a dark compartment. The animals, male Wistar rats weighing 180 to 200 g, were habituated to the dark compartment and were subsequently placed on the illuminated platform to enter the dark. On the following day three more trials were given. At the end of the third trial, upon entering the dark compartment animals received an unescapable foot shock (0.25 mA for 2 seconds) through the grid floor. Immediately after this learning trial animals were treated with 1 μl of peptide in saline or saline alone by intracerebroventricular injection through a stainless steel cannula. The cannula had been placed in the right lateral ventricle at least 5 days before the behavioral experiments started. The influence of substances on the storage of the aversive experience (memory consolidation) was assessed by testing the retention of the passive avoidance response 24 or 48 hours after the learning trial. Retention of passive avoidance behavior was measured by the latency to reenter the dark compartment up to a maximum of 300 seconds after placing the animals on the illuminated platform.

Arginine vasopressin caused a significant facilitation of passive avoidance behavior in doses of 100 pg and higher (Table 1). The activity of [pGlu^4, Cyt^6]-AVP-(4–9) was tested in a dose range of 0.01 to 1000 pg. The peptide displayed an inverted U–shaped dose-response relationship: in extremely low doses, 0.03 and 0.10 pg, the peptide facilitated passive avoidance behavior; the lowest effective dose at the 24-hour latency was

0.03 pg and 0.01 pg at the 48-hour latency. At the 24-hour latency activity was lost when doses of 0.3 pg and higher were given. At the 48-hour latency the magnitude of the effect was less than at the 24-hour latency; the effective dose was between 0.01 and 1 pg. The des-Gly-NH_2 derivative [pGlu^4, Cyt^6]AVP-(4–8), however, consistently facilitated passive avoidance over the tested dose range of 0.10 to 10 pg (Table 1); behavior was affected 24 hours as well as 48 hours after administration. After administration of 10 and 100 pg of [pGlu^4, Cyt^6]-AVP-(4–9) and -(4–8) 23 hours after training no significant effect on passive avoidance behavior was found. No aversive effects of the peptides were observed in experiments in which animals did not receive foot shock in the training session. The passive avoidance response of nonshocked rats treated intracerebroventricularly with 10 pg of [pGlu^4, Cyt^6]-AVP-(4–8) was minimal and not different from the response of nonshocked, saline-treated animals (Table 1). These observations all point to the potent, long-term activity of the [pGlu^4, Cyt^6]AVP-(4–9) and -(4–8) peptides on memory consolidation.

The inverted U–shaped dose-response relationship observed with [pGlu^4, Cyt^6]-AVP-(4–9), which was not apparent for the (4–8) derivative in the tested dose range, may indicate that a secondary activity site is retained by the presence of Gly-NH_2 in [pGlu^4, Cyt^6]AVP-(4–9). Indeed, the tripeptide H-Pro-Arg-Gly-NH_2 and the related dipeptide H-Lys-Gly-NH_2 are known to have behavioral activity (*4*).

In order to screen for peripheral activity of [pGlu^4, Cyt^6]AVP-(4–9) and [pGlu^4, Cyt^6]AVP-(4–8), the peptides were tested by intravenous injection in doses of 10 to 500 ng in the in vivo bioassay described by Dekanski (*11*). In this test administration of 10 ng of AVP caused a significant rise in blood pressure of 26 mmHg. The two peptides did not display any pressor activity. In addition, the action of AVP was not inhibited by treatment with [pGlu^4, Cyt^6]AVP-(4–9) or [pGlu^4, Cyt^6]AVP-(4–8).

Our results demonstrate that [pGlu^4, Cyt^6]AVP-(4–9) and -(4–8) have central activity after intracerebroventricular administration but lack the vasopressor activity after peripheral injection. In addition, the central effects of these peptides seem to be more selective than those of AVP, since, in contrast to AVP, [pGlu^4, Cyt^6]AVP-(4–9) failed to normalize endotoxin-induced hyperthermia in the rat.

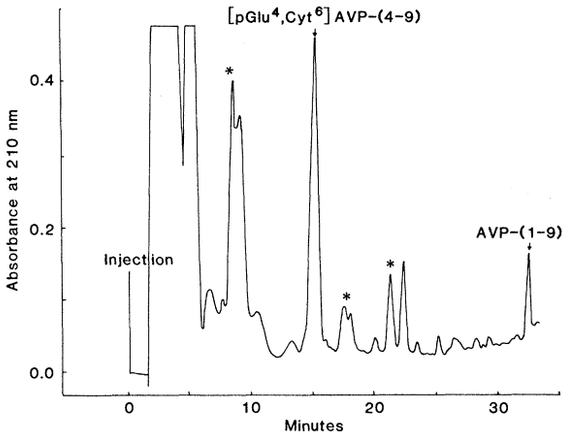

Fig. 1. Fractionation of metabolites of [Arg^8]vasopressin [AVP-(1–9)] by reversed-phase HPLC. AVP-(1–9) ($7.5 \times 10^{-5}M$) was incubated with a synaptic membrane preparation from rat brain tissue (*13*) (2.5 mg of protein per milliliter) in 4.0 ml of 40 m*M* sodium phosphate (*p*H 7.0) at 37°C for 6 hours. The reaction was stopped by addition of acetic acid to a final concentration of 1*M* and subsequent heating of the sample in a boiling water bath. After removal of membranes by centrifugation the supernatant was lyophilized and taken up in 1.0 ml of 10 m*M* ammonium acetate (*p*H 4.15). HPLC was performed on a μBondapak C18 reversed-phase column which was eluted with a concave gradient of 10 m*M* ammonium acetate (*p*H 4.15) (solvent A) and 0.15 percent acetic acid in methanol (solvent B). The gradient ran from 0 to 40 percent B in A during 30 minutes at a flow rate of 2.0 ml/min. The gradient lag time was 6 minutes. The components marked with an asterisk were derived from the synaptic membrane fraction as judged from a parallel incubation in the absence of AVP-(1–9). The metabolite eluting at a retention time of 15 minutes was collected, methanol was evaporated in vacuo at 70°C, and the sample was freeze-dried. The arrows indicate the elution position of synthetic [pGlu^4, Cyt^6]AVP-(4–9) and AVP-(1–9).

The effect of these AVP fragments on facilitating consolidation of passive avoidance behavior, however, is fully retained; in this paradigm they are even a thousand times more potent than AVP. Thus, the central activities of [pGlu^4, Cyt^6]AVP-(4–9) and [pGlu^4, Cyt^6]AVP-(4–8) seem to be directed toward the modulation of memory and related processes. The highly potent, selective, and long-term activity of the AVP metabolite [pGlu^4, Cyt^6]AVP-(4–9) and its derivative [pGlu^4, Cyt^6]AVP-(4–8) may be the basis for development of AVP-derived peptides for selective treatment of memory disorders.

These experiments were based on the hypothesis that AVP serves as a precursor peptide in the brain (*4*). This concept,

Table 1. Effect of [Arg^8] vasopressin [AVP-(1–9)], [pGlu^4, Cyt^6]AVP-(4–9), and [pGlu^4, Cyt^6]AVP-(4–8) on memory consolidation as measured by the retention of a passive avoidance response 24 and 48 hours after intracerebroventricular administration. Values are medians in seconds. Statistically significant differences between peptide-treated and saline-treated animals were assessed by the Mann-Whitney *U* test. The numbers in parentheses represent the number of experimental animals. Peptides were administered immediately after the learning trial and animals were tested for retention of passive avoidance behavior 24 or 48 hours later as described in the text and (*10*).

Dose (pg)	Retention latency (seconds)							
	AVP-(1–9)		[pGlu^4, Cyt^6]AVP-(4–9)		[pGlu^4, Cyt^6]AVP-(4–8)		Saline	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
0.01			76 (7)	85* (7)			64 (30)	59 (30)
0.03			96† (7)	75 (9)				
0.10			301‡ (7)	126‡ (8)	147‡ (7)	108‡ (7)		
0.30			73 (4)	71 (4)	118† (7)	94† (7)		
1	59 (5)	76 (5)	82 (8)	89* (8)	177‡ (5)	140‡ (5)		
10	90 (5)	64 (5)	33 (8)	45 (8)	172‡ (5)	126‡ (5)		
100	106† (5)	100† (5)	99 (6)	75 (6)				
1,000	210‡ (6)	98‡ (6)	60 (6)	30 (6)				
10,000	260‡ (6)	167† (6)						
10, no shock					5.5 (6)	2.5 (6)	5 (6)	2.5 (6)

*$P < 0.05$. †$P < 0.01$. ‡$P < 0.001$.

which was originally based on structure-activity studies of the memory effects of AVP, is elaborated by the present identification of a preferentially formed metabolite with potent, selective effects on the consolidation of passive avoidance behavior. This metabolite, [pGlu^4, Cyt^6]-AVP-(4–9) may be an active principle mediating central activities of AVP and it could be responsible for one or more of the effects observed after administration of AVP. Recently we found that the peptide is formed by stepwise aminopeptidase cleavage of AVP (*12*). Its accumulation is promoted by the internal cyclization of the NH_2-terminal Gln residue into pGlu, thus protecting the peptide against further aminopeptidase degradation. Preliminary experiments suggest the in vivo presence of vasopressin metabolites in the brain. Using HPLC in combination with radioimmunoassay systems recognizing the COOH-terminal portion of AVP, a peptide with properties of [pGlu^4, Cyt^6]AVP-(4–9) was detected amongst other fragments. The peptidases involved in the proteolytic processing of AVP into neuropeptides with potent and selective central activities may play a key role in the modulation of AVP activity in the brain.

J. PETER H. BURBACH
GABOR L. KOVÁCS*
DAVID DE WIED
Rudolf Magnus Institute for Pharmacology, University of Utrecht Medical Faculty, Vondellaan 6, 3521 GD Utrecht, Netherlands
JAN W. VAN NISPEN
HENK M. GREVEN
Scientific Development Group, Organon Int. B.V., 5340 BH Oss, Netherlands

References and Notes

1. D. de Wied, in *Frontiers in Neuroendocrinology*, W. F. Ganong and L. Martini, Eds. (Oxford Univ. Press, London, 1969), pp. 97–140; J. B. Flexner, L. B. Flexner, P. L. Hoffman, R. Walter, *Brain Res.* **134**, 139 (1977); D. de Wied, *Proc. R. Soc. London Ser. B* **210**, 183 (1980); G. J. de Boer *et al.*, *Prog. Brain Res.* **53**, 207 (1980); C. A. M. Versteeg, B. Bohus, W. de Jong, in *Approach to Hypertensive Diseases*, Y. Yamori, W. Lovenberg, E. D. Freis, Eds. (Raven, New York, 1979), pp. 329–335; K. E. Cooper, N. W. Kasting, K. Lederis, W. L. Veale, *J. Physiol. (London)* **295**, 33 (1981); J. M. van Ree and D. de Wied, in *Endogenous Peptides and Learning and Memory Processes*, J. L. Martinez *et al.*, Eds. (Academic Press, New York, 1981), pp. 397–412; P. L. Hoffman, R. F. Ritzmann, B. Tabakoff, *Pharmacol. Biochem. Behav.* **13**, 279 (1980); H. Rigter, C. Dortmans, J. C. Crabbe, Jr., *ibid.*, p. 285.
2. B. Bohus, W. H. Gispen, D. de Wied, *Neuroendocrinology* **11**, 137 (1973); R. Ader and D. de Wied, *Psychonom. Sci.* **29**, 46 (1972); D. de Wied, *Nature (London)* **232**, 58 (1971); D. de Wied and B. Bohus, *ibid.* **212**, 1484 (1966); W. D. Pfeiffer and H. B. Bookin, *Pharmacol. Biochem. Behav.* **9**, 261 (1978); H. Rigter, H. van Riezen, D. de Wied, *Physiol. Behav.* **13**, 381 (1974); J. F. Celestian, R. J. Carey, M. Miller, *Physiol. Behav.* **15**, 707 (1975); D. de Wied, B. Bohus, Tj. B. van Wimersma Greidanus, *Brain Res.* **85**, 152 (1975); J. B. Flexner, L. B. Flexner, R. Walter, P. L. Hoffman, *Pharmacol. Biochem. Behav.* **8**, 93 (1977).
3. J. C. Oliveros *et al.*, *Lancet* **1978-I**, 42 (1978); A. le Boeuf, J. Lodge, P. G. Eames, *ibid.* **1979-II**, 1370 (1979); J. J. Legros *et al.*, *ibid.* **1978-I**, 41 (1978); H. Weingartner *et al.*, *Science* **211**, 601 (1981); H. Weingartner, W. Kaye, P. Gold, S. A. Smallberg, R. Peterson, J. C. Gillin, M. Ebert, *Life Sci.* **29**, 2721 (1981); P. W. Gold, J. C. Ballenger, H. Weingartner, F. K. Goodwin, R. M. Post, *Lancet* **1979-I**, 992 (1979); F. Laczi *et al.*, *Psychoneuroendocrinology*, in press; J. Jolles, *Prog. Brain Res.*, in press.
4. D. de Wied, H. M. Greven, S. Lande, A. Witter, *Br. J. Pharmacol.* **45**, 118 (1972); R. Walter, L. B. Flexner, R. F. Ritzmann, H. N. Bhargava, P. L. Hoffman, in *Polypeptide Hormones*, R. E. Beers and G. Bassett, Eds. (Raven, New York, 1980), pp. 321–336; D. de Wied and B. Bohus, *Prog. Brain Res.* **48**, 327 (1978); J. P. H. Burbach and D. de Wied, in *Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides*, D. H. Schlesinger, Ed. (Elsevier/North-Holland, Amsterdam, 1981), pp. 69–87.
5. J. P. H. Burbach, A. Prins, J. L. M. Lebouille, J. Verhoef, A. Witter, *J. Chromatogr.* **237**, 339 (1982).
6. W. R. Gray and J. F. Smith, *Anal. Biochem.* **33**, 36 (1970); K. Narita, H. Matsuo, T. Nakajima, in *Molecular Biology, Biochemistry, and Biophysics: Protein Sequence Determination*, S. B. Needleman, Ed. (Springer Verlag, Berlin, 1975), pp. 30–103.
7. Abbreviations used are: Asp, aspartic acid; Arg, arginine; Cys, cysteine; Cys(O_3H), cysteic acid; Cyt, Cystine; Glu, glutamic acid; pGlu, pyroglutamic acid; Gly, glycine; Gly-NH_2, glycinamide; Phe, phenylalanine; and Tyr, tyrosine.
8. R. F. Doolittle and R. W. Armentrout, *Biochemistry* **7**, 516 (1968); D. N. Podell and G. N. Abraham, *Biochem. Biophys. Res. Commun.* **81**, 176 (1978).
9. A. Witter, H. Scholtens, J. Verhoef, *Neuroendocrinology* **30**, 377 (1980).
10. R. Ader and D. de Wied, *Psychonom. Sci.* **26**, 125 (1972).
11. J. Dekanski, *Br. J. Pharmacol.* **7**, 567 (1952).
12. J. P. H. Burbach and J. L. M. Lebouille, *J. Biol. Chem.* **258**, 1487 (1983).
13. J. P. H. Burbach, E. R. de Kloet, P. Schotman, D. de Wied, *ibid.* **256**, 12463 (1981).
14. We thank J. Cox-van Put for blood pressure measurements, H. de Vos Burchart-Lodewijks for expert cannulation, and O. Gaffori for some behavioral data.
* On leave from the Institute of Pathophysiology, University Medical School, Szeged, Hungary.

25 April 1983

Which Behavior Does the Lamprey Central Motor Program Mediate?

Abstract. *The isolated lamprey spinal cord, when bathed in 2 millimolar D-glutamic acid, will generate a pattern of motor neuron discharge that has generally been assumed to represent the central motor program for swimming. Motion pictures of behaving lampreys were analyzed by a computer algorithm to estimate undulatory movement parameters that could be directly compared with those generated during D-glutamate–induced undulations. The D-glutamate–induced movement parameters were significantly different from those observed during normal behaviors, including swimming, but accurately predicted the undulations produced by spinally transected adult lampreys.*

The lamprey occupies a strategic evolutionary position in terms of the organization of its nervous system (*1*), and serves as an important cellular model for recovery from spinal cord lesions (*2*) and for the analysis of motor pattern generation (*3*). The lamprey spinal cord has many of the technical advantages of simple invertebrate systems and contains identifiable classes of neurons (*4*). In addition, the lamprey spinal cord can generate a pharmacologically induced motor pattern, "fictive" locomotion, that has been assumed to represent the central motor program underlying swimming (*5*).

The simple motor apparatus of the lamprey allows few behaviors other than those produced by lateral axial undulations. One would therefore expect that the differences between behaviors can be quantified in terms of identifiable movement parameters. To quantify these undulatory behaviors, we developed a computer algorithm that can automatically analyze cinematographic images of freely behaving lampreys (*6*). This algorithm enabled us to estimate such parameters of undulatory movements as repetition frequency, intersegmental delay, phase lag, and curvature and to compare these features with D-glutamate–induced programs generated in situ. Using this algorithm, we were able to analyze the common behaviors of larval and adult lampreys as well as the behaviors of specimens recovering from lesions of the spinal cord (*6*). In addition, we analyzed the movements produced by specimens in which the spinal cord was exposed to a bath containing D-glutamic acid, the normal stimulus of fictive locomotion (*3*). We found that the dynamic organization of the D-glutamate–induced motor program does not closely resemble that of any normal behavior but is similar to the spinal undulations produced spontaneously by spinally transected adult lampreys (*7*).

Recently transformed specimens of the sea lamprey *Petromyzon marinus* were filmed from above with a super 8 movie camera and the films were projected frame-by-frame onto a magnetic digitizing tablet. The digitized images were then rotated to produce the normalized projection shown in Fig. 1A. The arrays of points that delineate each side