

Annetocin, an Annelid Oxytocin-Related Peptide, Induces Egg-Laying Behavior in the Earthworm, *Eisenia foetida*

T. OUMI, K. UKENA, O. MATSUSHIMA, T. IKEDA, T. FUJITA,
H. MINAKATA, AND K. NOMOTO

Biological Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima 739 (T.O., K.U., O.M.), and Suntory Institute for Bioorganic Research, Shimamoto, Mishima, Osaka 618 (T.I., T.F., H.M., K.N.), Japan

ABSTRACT Annetocin, an oxytocin-related peptide which we isolated from the earthworm *Eisenia foetida*, induced a series of egg-laying-related behaviors in the earthworms. These stereotyped behaviors consisted of well-defined rotatory movements, characteristic body-shape changes, and mucous secretion from the clitellum. Each of these behaviors is known to be associated with formation of the cocoon in which eggs are deposited. In fact, some of the earthworms injected with annetocin (>5 nmol) laid eggs. Such egg-laying-related behaviors except for oviposition were also induced by oxytocin, but not by Arg-vasopressin and some other bioactive peptides isolated from *E. foetida*. Furthermore, annetocin also induced these egg-laying-like behaviors in the leech *Whitmania pigra*, but not in the polychaete *Perinereis vancaurica*. These results suggest that annetocin plays some key role in triggering stereotyped egg-laying behaviors in terrestrial or freshwater annelids that have the clitella. © 1996 Wiley-Liss, Inc.

About fifteen species of peptides of the oxytocin-vasopressin superfamily have been identified in vertebrates and invertebrates. Although the peptides of this superfamily have been extensively studied in vertebrates, the invertebrate peptides have not been reported until quite recently. The invertebrate peptides of this superfamily so far reported are as follows: conopressin G and conopressin S isolated from the gastropod molluscs, *Conus geographus* and *C. striatus* (Cruz et al., '87), respectively, Arg-vasopressin-like diuretic hormone of the insect, *Locusta migratoria* (Proux et al., '87), and cephalotocin from the cephalopod mollusc, *Octopus vulgaris* (Reich, '92). However, little is known about the physiological role of these invertebrate oxytocin-vasopressin-related peptides except for the diuretic action of the antiparallel homodimer of the *Locusta* peptide (Picquot and Proux, '90) and modulatory effect of conopressin G on gill behaviors in *Aplysia californica* (Martínez-Padrón et al., '92).

In annelids, oxytocin-like immunoreactivity has been detected in all ganglia of the earthworm, *Pheretima hilgendorfi* (Kinoshita and Kawashima, '86), and in the sex segmental ganglia of the three species of leeches, *Theromyzon tessulatum*, *Hirudo medicinalis*, and *Erpobdella octoculata* (Salzet et al., '93). Recently, we isolated and chemically iden-

tified an oxytocin-related peptide, annetocin, from the oligochaete, *Eisenia foetida*, which stimulated the gut and nephridial movements in the earthworm (Oumi et al., '94; Ukena et al., '95a). Since annetocin had an excitatory effect on the nephridial pulsatory movement, we suggested at first that it might participate in the regulation of water balance (Oumi et al., '94). However, no direct evidence for the activity on water balance has been obtained at present. In some vertebrates, oxytocin and vasotocin are known for their participation in reproductive events such as parturition and egg laying.

It is well known that most species of earthworms and leeches exhibit stereotyped behaviors to form cocoons for egg laying (Barnes, '87; Grove and Cowley, '26; Nagao, '57), as schematically illustrated in Figure 1.

Here we report the possible involvement of annetocin in induction of egg-laying behaviors in *E. foetida* and the leech *Whitmania pigra*.

Received March 20, 1996; revision accepted June 11, 1996.
Address reprint requests to Dr. O. Matsushima, Biological Science, Faculty of Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739, Japan.

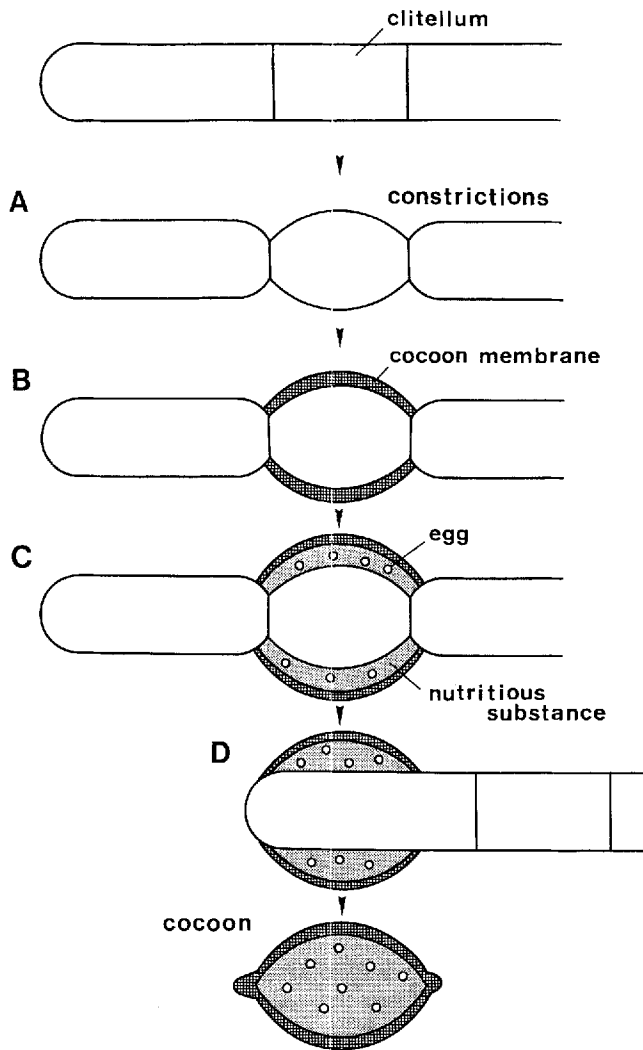


Fig. 1. Schematic illustration of egg-laying behavior of earthworms and leeches. (A) Both ends of the clitellum are constricted; (B) the cocoon membrane is secreted from the clitellar glands; (C) nutritious substance is secreted into the space between the cocoon membrane and the clitellum, and then eggs are deposited into the cocoon; (D) the cocoon is slipped forward over the anterior end of the animal (Barnes, '87; Grove and Cowley, '26; Nagao, '57).

MATERIALS AND METHODS

Animals

The lumbricid earthworms, *E. foetida*, were purchased from a fishing-bait store and kept in wet compost at 25°C. The animals weighing 0.35–0.55 g were grouped into two populations; one was a population of sexually active animals that had conspicuously swelling clitella, and the other was a population of sexually inactive animals that had no visible clitellum.

The fresh-water leeches, *W. pigra*, were collected at the paddy fields in Higashi-Hiroshima City, Japan (in June and July, 1994). They were kept in an aquarium with dechlorinated tap water at 25°C and fed with the fresh-water pulmonate snails, *Austropeplea ollula*. The leeches were grouped into three populations: sexually active adult animals (1.5–2.5 g) with conspicuous clitella (in early July, spawning season), sexually inactive adult animals (1.5–2.5 g) without visible clitella (in late September, non-breeding season), and immature animals (0.5–0.7 g) without clitella. Animals weighing 0.7–1.5 g were not used in the present experiments.

The polychaetes, *Perinereis vancaurica*, were purchased from a fishing-bait store and kept in artificial seawater (ASW) at 25°C. Animals weighing 1.3–1.6 g were used in the experiments.

Injection experiments

Different amounts of annetocin dissolved in 5 μ l each of physiological saline (see below) were injected into the coelom of *E. foetida* at the mediadorsal body wall. For comparison, the animals were also injected with oxytocin, Arg-vasopressin, and other bioactive peptides isolated from the earthworm *E. foetida* in our laboratory (Table 1). The earthworm peptides have excitatory (GGNG-2, ETP, S-Iamide) or inhibitory (EIPP-2) effects on spontaneous contractions of the earthworm gut. Ten microliters of the solution of annetocin was injected into *W. pigra* (5 μ l for immature animals) and *P. vancaurica* in the same manner as in *E. foetida*. Control animals received injection with the same volume of physiological saline. The animals were placed on plastic Petri dishes (filled with ASW in the case of *P. vancaurica*) at 25°C, and their behaviors were observed and recorded photographically. The latent period was defined as the time required for induction of each behavior after injection. Experiments on leeches were carried out during spawning season (early July) except for those on sexually inactive adult animals (late September). Compositions of physiological salines were: 102 mM NaCl, 1.6 mM KCl, 1.8 mM CaCl₂, 5 mM Hepes (pH 7.2) for *E. foetida*; 115 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 10 mM Hepes (pH 7.2) for *W. pigra* (Evans et al., '91); and 445 mM NaCl, 55 mM MgCl₂, 10 mM CaCl₂, 10 mM KCl, 10 mM Tris-HCl (pH 7.6) for *P. vancaurica* (ASW).

Peptides

Annetocin, GGNG-2, ETP, S-Iamide, and EIPP-2 (Table 1) were synthesized with a solid-phase peptide

TABLE 1. Primary structures of peptides used in this study¹

Name	Sequence	References
Annetocin	Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH ₂	Oumi et al. ('94)
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH ₂	
Arg-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH ₂	
GGNG-2	Gly-Lys-Cys-Ala-Gly-Gln-Trp-Ala-Ile-His-Ala-Cys-Ala-Gly-Gly-Asn-Gly	Oumi et al. ('95)
ETP	Gly-Phe-Lys-Asp-Gly-Ala-Ala-Asp-Arg-Ile-Ser-His-Gly-Phe-NH ₂	Ukena et al. ('95b)
S-Iamide	Pro-Ser-Lys-Tyr-Val-Arg-Ile-NH ₂	In preparation
EIPP-2	Ser-His-Leu-Phe-Val-NH ₂	Ukena et al. ('96)

¹Two cysteine residues of annetocin, oxytocin, Arg-vasopressin, and GGNG-2 form an S-S bond. ETP: *Eisenia* tetradecapeptide, EIPP-2: *Eisenia* inhibitory pentapeptide-2.

synthesizer (Applied Biosystems 430A, Foster City, CA, or Shimadzu PSSM-8, Kyoto, Japan) and purified by reversed-phase HPLC (Oumi et al., '94; Ukena et al., '95b). Oxytocin and Arg-vasopressin were purchased from Peptide Institute, Inc. (Osaka, Japan).

RESULTS

Earthworm, E. foetida

As shown in Table 2, annetocin (5 nmol or more) induced mucous secretion from the clitellum and constriction of the body-wall muscles at both ends of the clitellum in all sexually active earthworms, behaviors which are known to associate with cocoon formation. Further, secretion of the cocoon membrane and oviposition was observed in some cases (Figs. 1 and 2). Constriction of the clitellar region was accompanied by some other morphological changes in accordance with the description of Grove and Cowley ('26) such as elongation of several anterior segments of the animals, transverse expansion of the following segments down to the clitellum, and slight elongation of the residual segments posterior to the clitellum. Oviposition into the cocoon was observed in three out of ten earthworms injected with 10 nmol of annetocin,

which laid 1, 6, and 28 eggs, respectively. Further, one out of ten animals injected with 5 nmol laid 13 eggs. When eggs were deposited, the cocoon was slipped forward over the anterior end of the animals. Mucous secretion and clitellar constriction started a few minutes after the injection, and the cocoon was taken off about 25 minutes after the injection. Most of the sexually inactive animals injected with 5 nmol characteristically altered their body shape, but none of clitellum constriction, mucous secretion, cocoon membrane formation, or oviposition occurred. The injection of physiological saline did not induce any of these responses.

The injection of oxytocin also induced egg-laying-like behaviors excluding oviposition in the sexually active animals, but Arg-vasopressin did not (Table 3). Injection of GGNG-2, ETP, and EIPP-2 did not induce any response in the earthworm. The S-Iamide peptide induced only mucous-secreting response which was similar to that induced by injection with annetocin (Table 3).

Leech, W. pigra

As shown in Table 4, 1 nmol of annetocin induced a series of egg-laying-like behaviors in most

TABLE 2. Effects of annetocin injection on behaviors of the earthworm *E. foetida*

Earthworms	Annetocin (nmol)	Number of earthworms				
		Total	Mucous secretion	Constriction	Cocoon membrane	Oviposition
Sexually active	10	10	10	10	3	3
	5	10	10	10	3	1
	2.5	10	10	5	0	0
	0.5	10	9	0	0	0
	0.25	10	5	0	0	0
	Saline	10	0	0	0	0
Sexually inactive	5	10	0	8 ¹	0	0
	Saline	5	0	0	0	0
Latent period (min, mean \pm S.D.)			3.4 \pm 1.9 (n = 44)	3.4 \pm 2.0 (n = 33)	(Cocoon deposition) 23.3 \pm 0.9 (n=4)	

¹Characteristic alteration of body-shape except for clitellum constriction.

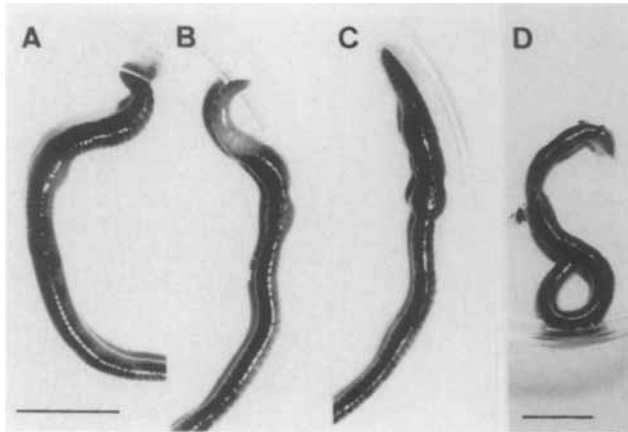


Fig. 2. Effect of annetocin injection (5 nmol) on behaviors of the earthworm *E. foetida*. A to C show the anterior half of the same animal. **A:** Control earthworm before the injection. **B:** Four minutes after the injection. **C:** Ten minutes after the injection. This animal did not lay eggs. **D:** The earthworm which laid eggs, and the cocoon was being slipped forward. Bar = 10 mm.

of sexually active or inactive adult leeches (Figs. 1 and 3), which corresponded with the description of Nagao ('57): remarkable constriction of both ends of the clitellum (about 15 minutes after the injection), accordion-like movement of the clitellum (about 20 minutes), secretion of transparent yellow-greenish mucus (cocoon membrane) from the clitellum (about 35 minutes), and secretion of opaque white mucus (nutritious substance) from the clitellum (about 50 minutes) (Fig. 3). However, no oviposition was observed in the leeches. When injected with 1 nmol of annetocin, all of the sexually inactive adult animals exhibited constriction and accordion-like movement of the clitella, and some of them secreted the mucus for the cocoon membrane. However, nutritious substance was never secreted. Most of the immature animals injected with 0.5 nmol exhibited the constrict-

tion and the accordion-like movement of the clitellar region, but neither cocoon membrane nor nutritious substance was secreted. The injection of physiological saline caused no such behavioral or morphological changes in any leeches.

Polychaete, Perinereis vancaurica

The polychaete seaworm, *P. vancaurica*, injected with 10 nmol of annetocin did not exhibit any behavioral changes at all.

DISCUSSION

Annetocin, an oxytocin-related peptide isolated from the earthworm *E. foetida*, induced a series of egg-laying behaviors in the animal, suggesting that the peptide plays some principal role in natural egg-laying behaviors in *E. foetida*. In annelids, bioactive substances inducing egg-laying behaviors have not been reported so far. In other invertebrate phyla, however, the molluscan peptides, egg-laying hormone (ELH) isolated from *Aplysia californica* (Chiu et al., '79) and caudodorsal cell hormone (CDCH) from *Lymnaea stagnalis* (Ebberink et al., '85), are reported to induce egg laying in respective animals. Atrial gland peptides isolated from *A. californica* also induce the egg laying by causing afterdischarge in the bag cells containing ELH (Heller et al., '80). However, these molluscan peptides have no structural relation to oxytocin-vasopressin superfamily peptides.

The egg-laying behaviors of these animals are expressed as stereotyped pattern, which include ovulation, egg packaging, oviposition, and related behaviors (Joosse and Geraerts, '83). However, it has been suggested that some neuropeptides in addition to ELH or CDCH are necessary for induction of the entire egg-laying behaviors (Scheller et al., '83; Vreugdenhil et al., '88). In this connection, such multiple neuropeptides involved in the stereotyped egg-laying behavior have been known

TABLE 3. Effects of injection of some peptides on behaviors of sexually active earthworm

Peptides	Quantity (nmol)	Number of earthworms				
		Total	Mucous secretion	Constriction	Cocoon membrane	Oviposition
Oxytocin	10	9	9	9	1	0
	5	10	9	9	0	0
	2.5	10	8	5	0	0
Arg-vasopressin	10	9	0	0	0	0
GGNG-2	5	5	0	0	0	0
ETP	5	5	0	0	0	0
S-Iamide	5	5	5	0	0	0
EIPP-2	5	5	0	0	0	0

TABLE 4. Effects of annetocin injection on behaviors of the leech *W. pigra*

Leeches	Annetocin (nmol)	Number of leeches				Nutritious substance
		Total	Constriction	Accordion movements	Cocoon membrane	
Sexually active	1	13	12	11	11	11
Adult	0.5	10	9	5	2	2
	0.1	10	1	0	0	0
	Saline	10	0	0	0	0
Sexually inactive	1	10	10	10	2	0
Adult	Saline	5	0	0	0	0
Immature	0.5	9	8	5	0	0
	Saline	5	0	0	0	0
Latent period (min, mean \pm S.D.)			15.5 \pm 7.9 (n = 40)	20.9 \pm 9.2 (n = 31)	36.1 \pm 10.1 (n = 15)	51.1 \pm 8.4 (n = 13)

to be encoded by single gene or homologous multiple genes (Scheller et al., '83; Vreugdenhil et al., '88).

In the present study, oviposition was induced in only a part of the earthworm population by injection of annetocin. In annelids, expression of complete egg-laying behavior may also require a combination of some peptides as molluscan egg-laying behavior. Besides, some prerequisite in addition to the swelling clitella may be essential for annetocin-induced oviposition. In mammals, for example, oxytocin-induced parturition requires the rapid increase in the number of oxytocin receptors in the uterus just before the parturition (Soloff et al., '79).

Oxytocin also induced egg-laying-like behaviors, but Arg-vasopressin did not. We have observed similar relations in activities on the earthworm

gut—annetocin, oxytocin, and vasotocin stimulated spontaneous contraction of the gut of *E. foetida*, while Arg-vasopressin did not (Ukena et al., '95a)—which we speculated would be important for exertion of the actions whether the residue at the third position is aliphatic or aromatic. The same viewpoint appears to be applicable to the activity on egg-laying behaviors in the present study. In mammals, the only difference in the amino acid residue at the third position between Arg-vasopressin (Phe) and vasotocin (Ile) specifies the action of the peptide, that is, hydromineral regulation or reproduction (Acher, '78, '80). Thus, regulation of gut movement and reproductive events like egg laying in the earthworms may require the aliphatic residue at the third position of the peptides as in annetocin, oxytocin, and vasotocin.

In the leech *W. pigra*, annetocin also induced a part of egg-laying-like behaviors. Although oxytocin-like peptides have not been isolated from leeches, oxytocin-like immunoreactivity has been detected in the sex segmental ganglia of the three species of leeches, *Theromyzon tessulatum*, *Hirudo medicinalis*, and *Erpobdella octoculata* (Salzet et al., '93). It is likely that some oxytocin-related peptide is also present in leeches and involved in induction of egg-laying behaviors.

In the present study, it was found that annetocin triggered a series of egg-laying-related behaviors in the earthworms and leeches. Further study is needed to understand physiological and biochemical aspects of the mechanisms underlying annetocin-induced egg-laying behavior in annelids. Especially it remains to be solved how such homologous behaviors (egg-laying and parturition) and structurally related peptides (annetocin and oxytocin) have been conserved between annelids and mammals in spite of their long evolutionary distance.

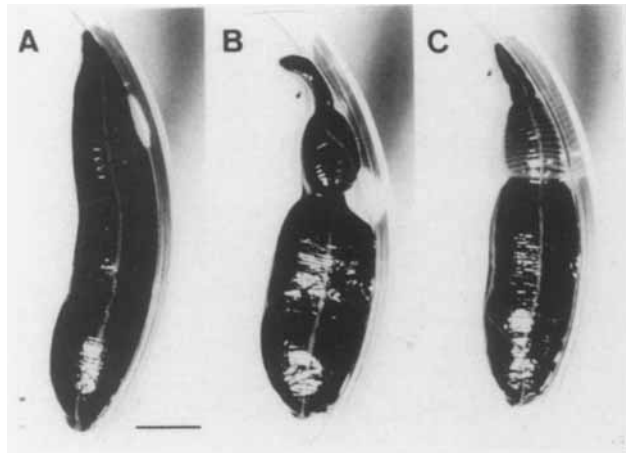


Fig. 3. Effect of annetocin injection (1 nmol) on behaviors of the leech *W. pigra*. A to C show the same animal. A: Control leech. B: Forty minutes after the injection. C: Sixty minutes after the injection. Cocoon membrane and white nutritious substance can be seen (C). Bar = 10 mm.

ACKNOWLEDGMENTS

We express our sincere thanks to Professor Y. Muneoka, Hiroshima University, for constant encouragement and valuable suggestions for this study. This study was partly supported by SUNBOR GRANT from Suntory Institute for Bioorganic Research, Osaka, Japan.

LITERATURE CITED

- Acher, R. (1978) Phylogenetic lines in polypeptide hormones. In: Evolution of Protein Molecules. H. Matsubara and T. Yamanaka, eds. Japan Scientific Societies Press, Tokyo, pp. 265–285.
- Acher, R. (1980) Molecular evolution of biologically active polypeptide. Proc. R. Soc. Lond. B, 210:21–43.
- Barnes, R.D. (1987) **The Annelids** In: Invertebrate Zoology, 5th ed. R.D. Barnes, ed. Saunders College Publishing, New York, pp. 263–341.
- Chiu, A.Y., M.W. Hunkapiller, E. Heller, D.K. Stuart, L.E. Hood, and F. Strumwasser (1979) Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. Proc. Natl. Acad. Sci. U.S.A., 76:6656–6660.
- Cruz, L.J., V. Santos, G.C. Zafaralla, C.A. Ramilo, R. Zeikus, W.R. Gray, and B.M. Olivera (1987) Invertebrate vasopressin/oxytocin homologs. J. Biol. Chem., 262:15821–15824.
- Ebberink, R.H.M., H. van Loeenhout, W.P.M. Geraerts, and J. Joosse (1985) Purification and amino acid sequence of the ovulation neurohormone of *Lymnaea stagnalis*. Proc. Natl. Acad. Sci. U.S.A., 82:7767–7771.
- Evans, B.D., J. Pohl, N.A. Kartsonis, and R.L. Calabrese (1991) Identification of RFamide neuropeptides in the medicinal leech. Peptides, 12:897–908.
- Grove, A.J., and L.F. Cowley (1926) On the reproductive processes of the brandling worm, *Eisenia foetida*. (Sav.). Quart. J. Micr. Sci., 70:559–581.
- Heller, E., L.K. Kaczmarek, M.W. Hunkapiller, L.E. Hood, and F. Strumwasser (1980) Purification and primary structure of two neuroactive peptides that cause bag cell after-discharge and egg-laying in *Aplysia*. Proc. Natl. Acad. Sci. U.S.A., 77:2328–2332.
- Joosse, J., and W.P.M. Geraerts (1983) **Endocrinology** In: Mollusca, vol. 4. A.S.M. Saleuddin and K.M. Wilbur, eds. Academic Press, New York, pp. 317–406.
- Kinoshita, K., and S. Kawashima (1986) Differential localization of vasopressin- and oxytocin-immunoreactive cells and conventional neurosecretory cells in the ganglia of the earthworm *Pheretima hilgendorfi*. J. Morphol., 187:343–351.
- Martínez-Padrón, M., W.R. Gray, and K. Lukowiak (1992) Conopressin G, a molluscan vasopressin-like peptide, alters gill behaviors in *Aplysia*. Can. J. Physiol. Pharmacol., 70:259–267.
- Nagao, Z. (1957) Observations on the breeding habits in a fresh-water leech, *Herpobdella lineata* O.F. Müller. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool., 13:192–196.
- Oumi, T., K. Ukena, O. Matsushima, T. Ikeda, T. Fujita, H. Minakata, and K. Nomoto (1994) Annetocin: An oxytocin-related peptide isolated from the earthworm, *Eisenia foetida*. Biochem. Biophys. Res. Commun., 198:393–399.
- Oumi, T., K. Ukena, O. Matsushima, T. Ikeda, T. Fujita, H. Minakata, and K. Nomoto (1995) The GGNG peptides: Novel myoactive peptides isolated from the gut and the whole body of the earthworms. Biochem. Biophys. Res. Commun., 216:1072–1078.
- Picquot, M., and J. Proux (1990) Biosynthesis and degradation of the arginine-vasopressin-like insect diuretic hormone, a neurohormone in the migratory locust. Regul. Peptides, 31:139–156.
- Proux, J.P., C.A. Miller, J.P. Li, R.L. Carney, A. Girardie, M. Delaage, and D.A. Schooley (1987) Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. Biochem. Biophys. Res. Commun., 149:180–186.
- Reich, G. (1992) A new peptide of the oxytocin/vasopressin family isolated from nerves of the cephalopod *Octopus vulgaris*. Neurosci. Lett., 134:191–194.
- Salzet, M., C. Watzet, M. Verger-Bocquet, J.C. Beauvillain, and J. Malecha (1993) Oxytocin-like peptide: A novel epitope colocalized with the FMRFamide-like peptide in the supernumerary neurons of the sex segmental ganglia of leeches: Morphological and biochemical characterization: putative anti-diuretic function. Brain Res., 601:173–184.
- Scheller, R.H., J.F. Jackson, L.B. McAllister, B.S. Rothman, E. Mayeri, and R. Axel (1983) A single gene encodes multiple neuropeptides mediating a stereotyped behavior. Cell, 32:7–22.
- Soloff, M.S., M. Alexandrova, and M.J. Fernstrom (1979) Oxytocin receptors: Triggers for parturition and lactation? Science, 204:1313–1315.
- Ukena, K., T. Oumi, O. Matsushima, T. Ikeda, T. Fujita, H. Minakata, and K. Nomoto (1995a) Effects of annetocin, an oxytocin-related peptide isolated from the earthworm *Eisenia foetida*, and some putative neurotransmitters on gut motility of the earthworm. J. Exp. Zool., 272:184–193.
- Ukena, K., T. Oumi, O. Matsushima, T. Ikeda, T. Fujita, H. Minakata, and K. Nomoto (1995b) A novel gut tetradecapeptide isolated from the earthworm, *Eisenia foetida*. Peptides, 16:995–999.
- Ukena, K., T. Oumi, O. Matsushima, T. Takahashi, Y. Muneoka, T. Fujita, H. Minakata, and K. Nomoto (1996) Inhibitory pentapeptides isolated from the gut of the earthworm, *Eisenia foetida*. Comp. Biochem. Physiol. 113A (in press).
- Vreugdenhil, E., J.F. Jackson, T. Bouwmeester, A.B. Smit, J. van Minnen, H. van Heerikhuizen, J. Klootwijk, and J. Joosse (1988) Isolation, characterization, and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the fresh-water snail *Lymnaea stagnalis*. J. Neurosci., 8:4184–4191.