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Comparative distribution of leucokinin and functionally related peptides in the nervous system of several insects

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The University of Arizona, 1993
Comparative distribution of leucokinin and functionally related peptides in the nervous system of several insects

by

Yuetian Chen

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Henry H. Hagedorn
Professor of Entomology

April 30, 1925
Date
Acknowledgment

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Abstract

Antisera against leucokinin IV were used to test for the presence of leucokinin-like peptides in the central nervous systems of *Nauphoeta cinerea*, *Acheta domesticus*, *Schistocerca americana*, *Aedes aegypti*, *Manduca sexta* and *Apis mellifera*. Leucokinin immunoreactive neurosecretory cells were found in the pars intercerebralis and pars lateralis of the brains of *N. cinerea*, *A. domesticus*, *A. aegypti*, but not in the brains of *S. americana*, *M. sexta* and *A. mellifera*. The neurohemal release sites were also very different among species. In contrast, the distribution patterns of leucokinin immunoreactive neurosecretory cells were very similar among abdominal ganglia of all six species. The identity of the leucokinin immunoreactive material in the brain, corpora cardiaca and abdominal ganglia of *N. cinerea* was shown by HPLC combined with ELISA. In *N. cinerea* and *A. domesticus*, leucokinin and vasopressin were found to co-localize in the same neurosecretory cells. In *M. sexta*, leucokinin and diuretic hormone co-localized in the same neurosecretory cells in abdominal ganglia, but not in the brain.
INTRODUCTION

The neuroendocrine system

The neuroendocrine system of animals serves as a mode of internal communication between the nervous system and the endocrine system. This system consists of neurosecretory cells secreting neuropeptides and other biogenic amines in the central and peripheral nervous systems. The same neuropeptide may work either as a neurotransmitter or neuromodulator affecting only neighboring neurons, or as a neurohormone, sending signals to the entire body for regulation of different physiological processes.

In vertebrates, neurosecretory cells (i.e., the cells in the hypothalamus) primarily communicate between the nervous and endocrine systems, as is the case in the anterior pituitary (Scharrer, 1967). A few neurohormones, such as vasopressin and oxytocin, originate from neurosecretory cells in the hypothalamus and are released from the posterior pituitary to act directly on non-endocrine target tissues. In contrast, in invertebrates, most neurosecretory products appear to act directly on non-endocrine target tissues (Gainer et al., 1977).

Neuropeptides in insects have been shown to control many processes including diapause (Williams 1946, 1947, Hasegawa 1957, Takeda and Girardie, 1985), molt initiation (Williams 1947, 1952), heart rate (Gersch 1958, Veenstra 1989), color change (Dupont-Raabe 1951, Hashiguchi et al., 1965, Suzuki et al., 1976, Matsumoto et al., 1985, 1986) and metabolism (Mayer & Candy, 1969). These peptides are synthesized by neurosecretory cells that are distributed in the
central nervous system (brain and segmental ganglia), corpus cardiaca-corpus allata system and peripheral nervous system of insects. Comparison between the corpus cardiaca-corpus allata system of the insect brain and the hypothalamic-hypophysial system of vertebrate brain reveals that these neuroendocrine systems have striking structural and functional similarities. The hypothalamic nuclei of the vertebrates seem to be equivalent to the pars intercerebralis of the insects. Neurosecretory cells are found in both centers, which send nerve fibers to endocrine organs: the pituitary gland and the corpora cardiaca-corpora allata respectively (Scharrer & Scharrer 1944). In addition to these similarities between vertebrate and insects, the neurosecretory cells in insects are present throughout the nervous system and are, therefore, more decentralized. The products of these cells play important roles in controlling the development, metabolism and reproduction of insects.

**Physiological approaches to neuroendocrinology**

Morphological and cytophysiological investigations have provided indirect evidence for the role of neurosecretory products in the regulation of developmental, reproductive and metabolic processes. Secretory activity of nerve cells was first reported in the teleost fish, *Phoxinus laevis* (Scharrer 1928). Later, the Scharrers examined a wide variety of vertebrate and invertebrate groups and confirmed the apparently universal occurrence of neurosecretory cells (Scharrer 1977). With the development of the electron microscope, major sites of synthesis and release of neurosecretory products could be identified, as secretions were packaged into membrane-bound, electron-dense granules (Bargmann & Scharrer 1951). Additional experiments established that
neurosecretory cells are functionally similar to other protein-secreting cells in
that the neurosecretory products are packaged at the Golgi apparatus and
released by exocytosis (Scharrer & Brown 1961). Since the products of
neurosecretory cells appeared to be released in the blood, these neuropeptides
were initially considered to function as hormones. The idea that these
neuropeptides could also function as neurotransmitter was introduced with the
discovery of peptidergic neurons by Bargmann (1967). The peptidergic neurons
include neurons showing the classical synaptic pattern and others that are
neither local (synaptic) or truly neurohormonal but have a relatively narrow zone
of extracellular stroma between the terminal and effect cells. Still other
peptidergic neurons have synaptic-like contacts in which the two neurons are in
close contact, thereby providing for a reciprocal exchange of neurotransmitter. It
was demonstrated that the peptide factor extracted from molluscan ganglia could
modulate bursting pacemaker activity, suggesting that neuropeptides could
function also as neuromodulators (Ifshin et al. 1975). Buijs and Swaab (1979)
demonstrated vasopressin and oxytocin synapses in the rat limbic system by
immuno-electron microscopy and suggested that these peptides function as
neurotransmitters. Thus it became clear that neuropeptides could function in
variety of roles, i.e. as neurotransmitters, neuromodulators and neurohormones,
and therefore that the same peptide could have multiple roles.

Different techniques such as organ culture, transplantation experiments
(Nikitovitch-Winer & Everett 1958, Oksche et al. 1964), electrical stimulation, and
lesion experiments of specific hypothalamic centers (Szentagothai et al. 1962)
provided further evidence for the physiological functions of various
neurosecretory cells. On the basis of histological evidence, Fukuda (1967)
identified a single pair of large neurosecretory cells in the subesophageal ganglion in *Bombyx mori* as the site of diapause hormone release. However, extracts of pupal brains also showed diapause hormone activity (Sonobe & Keino 1975). Pars intercerebralis and lateral neurosecretory cells were shown to produce prothoracicotropic hormone (PTTH) in the locust (Girardie 1974, Girardie & De Reggi 1978), using electrical stimulation followed by determination of hemolymph ecdysteroid titers. But the experiment was not sufficiently precise to define the location of the PTTH neurosecretory cells.

Synthesis sites of diuretic hormone were identified in *Rhodnius* by testing the diuretic activity of isolated neurons (Berlind & Maddrell 1979); but it was unproved that these were the only neurons that synthesized diuretic hormone.

In general, these early studies were limited by lack of knowledge of the nature of the compounds released, whether they are composed of peptides only, or whether they are several different compounds. In addition, the exact sites of release and location of receptors remained to be determined.

**Molecular approaches to neuroendocrinology**

As a result of advances in biochemistry and molecular biology, the exact structure and chemical character of the neurosecretory products and their physiological function could be better studied. To date, more than 100 neuropeptides have been isolated and identified. Proctolin was the first insect neuropeptide to be structurally characterized (Sterratt & Brown 1975). Since then, over 60 additional insect neuropeptides have been sequenced including the adipokinetic hormone/hyperglycemic hormone (AKH/HrGH) family (Stone et al. 1976), the bombyxin family (Nagasawa et al. 1979, 1984) and the leucokinin
family (Holman et al. 1986, 1987). Also, some of the specific genes which codes for certain peptides have been investigated.

Immunological techniques have greatly enhanced the further characterization and purification of neuropeptides from species with well-characterized peptides. An example is the use of radioimmunoassay to characterize and purify the thyrotropin-releasing factor from several species (Grimm-Jørgensen et al. 1975, Berson & Yalow 1973). Antisera raised against a number of mammalian neuropeptides were found to be reactive against nervous systems of diverse phyla, including several invertebrates (Rémy et al. 1977, Strambi et al. 1979, Veenstra et al. 1984), indicating that vertebrate-like peptides also exist in invertebrates. It remains to be seen whether they fulfill the same functions. An antiserum against vertebrate Arg-vasopressin was used to purify related oligo-peptides from the locust (Proux et al. 1987). FMRF amide peptides, with the structure (Phe-Met-Arg-Phe-NH₂), were also purified from Drosophila using similar techniques (Nambu et al. 1988). The cardio-accelerator peptide corazonin was first isolated from the American cockroach (Veenstra 1989). With this purified peptide, antisera against corazonin were used in a very sensitive enzyme-linked immunosorbent assay (ELISA) to quantify the peptide, and isolate it from Schistocerca americana, Nauphoeta cinerea and Manduca sexta (Veenstra 1991).

The structural and functional similarities in the neuroendocrine systems of insects and vertebrates have counterparts in the chemical similarity of peptide messenger substances used by the nervous system (Scharrer 1987). Some of these peptides share very high levels of similarity of their amino acid sequence
A better understanding of the evolutionary relationships between these peptides was obtained when genes of the peptides were isolated and described. cDNA-clones coding for insulin-related peptides have been identified in *Locusta migratoria* (Lagueux *et al.* 1990) and *Bombyx mori* (*Adachi et al.* 1989, *Iwami et al.* 1989). A cDNA-clone coding for FMRF-amide was identified from both *Drosophila virilis* and *D. melanogaster* (Schneider & Taghert 1988, 1990, *Nambu et al.* 1988). Structures of the precursors of AKH I and AKH II have also been elucidated (Schulz-Aellen 1989). Results from biochemical and molecular biological investigations have indicated that the primary and secondary structure of insulin and insulin-related peptides appear quite conserved and thus have long evolutionary histories (Steiner & Chan 1988), whereas others such as eclosion hormones have no known vertebrate homologues (*Nagasawa et al.* 1987).

**Immunological study of neuropeptides**

Antibodies, which are natural defense molecules produced by vertebrate immune systems in response to specific foreign antigens, can be used to purify neuropeptides, and study their distribution and physiological roles. Specific uses of neuropeptide antisera are:

*Interspecific comparisons.* Antisera against purified neuropeptides can be used to screen related peptides in other species. Antisera against insulin in vertebrates were used to screen insulin-related peptides in invertebrates (*Tager et al.* 1976, *Duve et al.* 1979, *El-Salhy et al.* 1984, *Hansen et al.* 1990). Proctolin was originally isolated from the American cockroach. Proctolin immunoreactivity
has been demonstrated in members of the Crustacea, Annelida, and Mammalia (Adams 1990). Crustacean cardioactive peptide (CCAP) is a peptide that was found to be a cardioacceleratory substance in extracts from the pericardial organs of *Carcinus maenas* (Price & Greenberg 1977) and has been biochemically characterized (Stangier *et al.* 1987). Radioimmunological studies demonstrated that peptides related to CCAP are also present in *Locust migratoria* (Stangier *et al.* 1989). Using immunocytochemical methods, CCAP immunoreactive neurons were also found in *Tenebrio molitor* (Breidbach & Dircksin 1991). FMRF-amide is a cardioaccelerator from molluscan heart which belongs to a large "RF" peptide family (for cholecystokinin, gastric and pancreatic polypeptide, the last two amino acids are Arg-Phe-NH$_2$). Different orders of insects have been found to produce FMRF-amide-related peptides using appropriate antisera (Boer *et al.* 1980, Veenstra 1984, Remy *et al.* 1988, Tsang *et al.* 1991, Homberg *et al.* 1991, Eichmuller *et al.* 1991)

*Identifying synthesis and releasing sites.* Using immunocytochemical staining, the neurosecretory cells and nerve pathway of specific peptides can be mapped in both vertebrates and invertebrates. In vertebrates, the synthetic sites of vasopressin have been investigated and their receptors localized by immunocytochemistry (Leclerc & Pelletier 1974). The neurosecretory cells producing vasopressin-related peptides in insects and their release sites were also investigated in different insects (Remy & Girardie, 1980, Veenstra, 1984, Nässel *et al.* 1989, Davis *et al.* 1992). Many other insect neurosecretory cells which produce different neuropeptides were also mapped in more than one insect nervous system such as FMRF-amide-related peptides, CCAP peptide, vasopressin-like peptides, and proctolin peptides.
**Monitoring hemolymph titers.** Using an antibody against a specific peptide, the developmental fluctuations of this peptide can be monitored which may help to understand the physiological function of the peptide. Recently, an radioimmunoassay was developed to test the titer of bombyxin in the hemolymph of Silkmoth, *Bombyx mori*, during larval-larval, larval pupal and pupal-adult development using monoclonal antibody against this peptide (Saegusa et al. 1992).

The distribution, site of synthesis and titer changes in the hemolymph of neuropeptides are very important to understanding the neuroendocrine system. Some groups of peptides are distributed in different insects such as AKH, PTTH and vasopressin. PTTH shows variation in the sites of synthesis among *Locusta*, (median neurosecretory cells and lateral neurosecretory cells; Girardie 1967, 1974, Girardie & De Reggi 1978), *Rhodnius* (median neurosecretory cells; Wigglesworth, 1940), *Bombyx* (median neurosecretory cells; Mizoguchi et al. 1987) and *Manduca* (lateral neurosecretory cells only; Agui et al. 1979). In contrast, For vasopressin the distribution patterns of neurosecretory cells were very similar among different insects (Davis 1992, Davis, unpublished data, Veenstra, 1984). It is possible that vasopressin has a longer evolutionary history or a more conserved function in insects. By comparing the distribution of the same group of peptides within the nervous system of different insects, the evolutionary history of the peptides, and their underlying communication systems, can be partially revealed. A complication in understanding evolution is that one physiological process may be controlled by more than one peptide, and so the whole suite of controlling peptides may have to be studied to understand the evolutionary history of the dependent physiological processes.
Diuretic neuropeptides in insects

Insect diuresis can be divided into two parts, secretion by Malpighian tubules and reabsorption by the rectum. The Malpighian tubules absorb a liquid from the hemolymph and the rectum reabsorbs various amounts of water and amino acids, sugars, lipids and certain ions to the hemolymph. The volume of fluid excreted by Malpighian tubules, and selective re-absorption by rectum varies depending on physiological need, and appears to be regulated by the neuroendocrine systems in insects. The change of diuretic activity has been determined by measuring the amount of urine secreted (Maddrell, 1963), modifications in the transepithelial voltage (Maddrell & Klunsuwan 1973, Williams & Beyenbanch 1983,1984), and the intracellular level of cAMP (Morgan & Mordue 1985). When Malpighian tubules are stimulated by diuretic neuropeptides, the fluid secretion can be accelerated more than a thousand times (Maddrell et al. 1992).

In earlier studies, the investigation of insect diuretic peptides was based on histological evidence. A relationship between neurosecretion in the type A neurosecretory cells in the nervous system and osmoregulation in the female blow fly (Calliphora erythrocephala) was established in histophysiological studies (Thomsen 1952). These type A cells also produce anti-diuretic hormones as later demonstrated by immunocytochemistry (Tamarelle & Girardie 1989). Histological studies of Oryctes rhinoceros (Kannan & Prabhu 1985), Locusta migratoria (Girardie 1970), Aeshna cyanea (Charlet 1974) and Leucophaea maderae (De Besse 1978) indicated that storage of type A neurosecretory cell products in the brain and corpora cardiaca was variable but could be correlated with
humidity. Differences in the amount of products in neurosecretory cells of ventral ganglia of *R. prolixus* were also correlated with environmental humidity (Baudry 1968, 1969). These histophysiological studies indicated that diuresis is controlled by neurosecretory cells and that these neurosecretory cells are present in brain, corpora cardiaca and abdominal ganglia.

Bioassay methods more directly demonstrated the presence of a diuretic hormone in the brain and abdominal ganglia of several insects. By sectioning nerves of *S. gregaria* (Mordue & Goldsworthy 1969) and activating different parts of the brain of the stick insect, *Carausius morosus*, (Pilcher 1970), the central region of the protocerebrum has been shown to control diuretic activity. By selectively removing the pars intercerebralis of the brain, the regions in which a diuretic hormone occurs in the dragonfly *A. cyanea* (Charlet 1974) and in *L. migratoria* (Proux 1979) were localized. Diuretic hormone was also found in the abdominal ganglia of *R. prolixus* by incubating isolated neurons with Malpighian tubules and measuring secretory activity (Berlind & Maddrell 1979).

Distinct diuretic and antidiuretic factors have been isolated from the storage and glandular lobes of the corpora cardiaca of various insects (Morgan & Mordue 1983, Proux *et al.* 1984, Herault *et al.* 1985). Several factors that control the diuresis of the mosquito *A. aegypti* have been isolated from head extracts (Petzel *et al.* 1985). However, the structures of some of these diuretic neurohormones in different insects has only been recently determined (see Table 2). Vasopressin-like factors with diuretic activity were isolated from the corpora cardiaca of *L. migratoria* (Morgan 1987). Several diuretic hormones that resemble mammalian corticotropin releasing factor (CRF) have been sequenced from *L.*
*migratoria* (Kay et al. 1991, Lehmberg et al. 1991), *M. sexta* (Kataoka et al. 1989, Blackburn et al. 1991), *A domesticus* (Kay 1991) and *P. americana* (Kay et al. 1992) and these CRF-related diuretic peptides have 30-46 amino acid residues (Table 1). A cDNA clone encoding a precursor of the *M. sexta* diuretic hormone has been isolated (Digan et al. 1992). In addition to diuretic hormone, some of the myotropic peptides such as leucokinins (Holman et al. 1986), achatakinins (Coast et al. 1990) and culekinin depolarizing peptides isolated from mosquito *Culex salinarius* (Hayes, unpublished data) also have diuretic effects (Hayes et al. 1989, Coast et al. 1990). Thus diuresis appears to be controlled by more than one peptide.

The mechanism by which these peptides control diuresis in insects is not well understood. It was demonstrated that diuretic hormone can affect cAMP levels and membrane potential of the Malpighian tubules and rectum. However, the patterns of the effects were different among these peptides during bioassays. Some peptides stimulate fluid secretion by influencing membrane potential but not changing the levels of cAMP (Coast et al. 1991, Hayes et al. 1989). In contrast, some peptides stimulate fluid secretion by changing cAMP levels (Morgan & Mordue 1985, Rafaeli et al. 1987, Lehmberg et al. 1991, Kay et al. 1991, 1992). Spring and Clark (1990) have developed a model to account for the actions of these diuretic peptides I, II and an antidiuretic peptide in the cricket. The model suggests that diuretic peptide-I could elevate intracellular cAMP levels, presumably by activating a G-protein, which would in turn activate cAMP-dependent protein kinases. The result of this cascade would be the opening of sodium channels in the basolateral membrane, increasing intracellular sodium concentration and activating the apical cation pump. Diuretic peptide-II could
have multiple effects. First, it could activate an inhibitory G-protein, blocking adenylate cyclase, and activate a phosphodiesterase to lower the cAMP concentrations. Second, it could act to lower intracellular calcium concentrations, closing the potassium-channel and increasing potassium transport by the apical cation pump. Antidiuretic hormone, on the other hand, may act by elevating intracellular calcium concentrations, possibly via the phosphatidyl inositol (PI3) pathway, opening the potassium channel that would hyperpolarize the basolateral membrane and decrease the potassium available to the cation pump. However, how diuretic peptides control diuresis in insects still needs to be investigated.

Finding the synthesis and releasing sites of diuretic neuropeptides is another approach to understanding physiological function. The synthesis sites of a diuretic hormone in *M. sexta* have been localized (Veenstra & Hagedorn 1990). Intra and interspecific comparisons of the distribution of various diuretic peptides can help us further understand the mechanism of diuretic regulation.

**Leucokinins: myotropic neuropeptides possibly involved in diuresis**

Leucokinins are a class of myotropic neuropeptides isolated from head extracts of the cockroach, *Leucophaea maderae* (Holman *et al.* 1986a, 1986b, 1987a, 1987b). They consist of a group of eight neuropeptides, all of which contain a similar C-terminal core sequence of 5 amino acids [Phe-X-Ser-Trp-Gly-NH2] (Table 2), which has been demonstrated to be essential for maintenance of their physiological effects (Hayes *et al.* 1989). The physiological roles of these peptides are not well understood. The other series of five myotropic peptides, the achetakinins, have been isolated and structurally characterized from head
extracts of the cricket, *A. domesticus*, using a purification system similar to the method applied to the leucokininins (Holman, *et al.* 1990).

The structures of these five myotropic peptides also contain a C-terminal core that can stimulate hindgut contraction of *L. maderae*, and which has a very similar sequence to that of the leucokinins (Table 2). In addition to the stimulation of hindgut contraction of the cockroach, achetakinins can stimulate fluid secretion by the Malpighian tubules of *A. domesticus* (Coast *et al.* 1991). Leucokinins and achetakinins clearly belong to the same neuropeptide family. Recently, a related peptide was isolated from *L. migratoria* that had the same C-terminal sequence as that of the leucokinins (Schoofs *et al.* 1992; Table 2). Later, two leucokinin peptides which were called culekinin depolarizing peptides were isolated from the mosquitoes, *Culex salinarius* (Hayes *et al.* 1992, from Clements, 1992, Table 2). Using immunocytochemical staining, leucokinin-like peptides have been found in the blow fly, *Phormia terraenovae* (Nässel 1991) and turnip moth, *Agrotis segetum* (Cartera 1992). Experiments examining the effect of leucokinins on the Malpighian tubules of the mosquito, *A. aegypti*, indicated that leucokinins can stimulate fluid secretion and depolarize transepithelial membrane potential even in these distantly related insects. These results were very similar to the effects of the factors extracted from the mosquitoes themselves. Thus it seems that leucokinins are a class of neuropeptides that exist in all insects and are important to diuretic activity.

In this thesis I report an immunocytochemical study of the distribution of leucokinin-like peptides in the nervous systems of *Nauphoeta cinerea* and five other species representing four different orders of insects: *Schistocerca americana,*
Manduca sexta, A. domesticus, A. aegypti and Apis mellifera. The presence of leucokinins in the nervous system of N. cinerea was confirmed by separation of the peptides from the nervous system with HPLC and testing the immunoreactivity to anti-leucokinin antiserum using a ELISA. The distribution of leucokinins in the nervous systems of the insects examined was compared with the distributions of Lys-vasopressin immunoreactive cells in N. cinerea and A. domesticus, and the distribution of diuretic hormone in M. sexta. The results indicated that Lys-vasopressin immunoreactive cells, which may also be involved in diuresis in some insects, co-localized in the same neurosecretory cells in the abdominal ganglia with the leucokinins in N. cinerea, A. domesticus and M. sexta. M. sexta diuretic hormone co-localized with leucokinin in the same neurosecretory cells in the abdominal ganglia of M. sexta.
<table>
<thead>
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<th>Peptides</th>
<th>Sequences</th>
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<tr>
<td>P-DP</td>
<td>TGSGPSLSTVNPLDVLROPILLETARRMRQSQDQIOANREILQT-I-NH₂</td>
</tr>
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<td>M-DPI</td>
<td>RPMPSLDLPSVLRKLSLEKERVKHALRAANRNFLND-I-NH₂</td>
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<tr>
<td>M-DPII</td>
<td>SFSVNPADVILDILQHRYMEKVAO NR N FL NR I-NH₂</td>
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Table 2. Known amino acid sequence of leucokininns and related neuropeptides

<table>
<thead>
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<th>Peptides *</th>
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<tbody>
<tr>
<td>Lk-I</td>
<td>Asp-Pro-Ala-Phe-Asn-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-II</td>
<td>Asp-Pro-Gly-Phe-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-III</td>
<td>Asp-Gln-Gly-Phe-Asn-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-IV</td>
<td>Asp-Ala-Ser-Phe-His-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-V</td>
<td>Gly-Ser-Gly-Phe-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-VI</td>
<td>FGlu-Ser-Ser-Phe-His-Ser-Trp-GlyNH2</td>
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<tr>
<td>Lk-VII</td>
<td>Asp-Pro-Ala-Phe-Ser-Trp-GlyNH2</td>
</tr>
<tr>
<td>Lk-VIII</td>
<td>Gly-Ala-Asp-Phe-Tyr-Ser-Trp-GlyNH2</td>
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<tr>
<td>A-I</td>
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</tr>
<tr>
<td>A-II</td>
<td>Ala-Tyr-Phe-Ser-Pro-Trp-GlyNH2</td>
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<tr>
<td>A-III</td>
<td>Asn-Phe-Lys-Phe-Asn-Pro-Trp-GlyNH2</td>
</tr>
<tr>
<td>Lc-I</td>
<td>Ala-Phe-Ser-Ser-Trp-GlyNH2</td>
</tr>
<tr>
<td>CDP-I</td>
<td>Asn-Pro-Phe-His-Ser-Trp-GlyNH2</td>
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<tr>
<td>CDP-II</td>
<td>Asn-Asn-Ala-Asn-Val-Phe-Tyr-Pro-Trp-GlyNH2</td>
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</tbody>
</table>

* Lk=leucokinin; A=achetakinin; Lc=locustakinin; CDP=Culekinin depolarizing peptide. See text for references.
MATERIALS AND METHODS

Experimental animals

Since leucokinins were originally isolated from a cockroach (L. maderae), another cockroach (N. cinerea) was tested for the presence of leucokinin-like peptides. In order to see whether the leucokinin IV which is the peptide has high effect on diuresis antiserum would recognize leucokinin-related peptides in other insect species and how widely the leucokinin-like peptides were distributed in the insects, five additional insect species representing several orders were also investigated: Acheta domesticus (Orthoptera), Schistocerca americana (Orthoptera), Manduca sexta (Lepidoptera), Aedes aegypti (Diptera) and Apis mellifera (Hymenoptera)

Cockroaches (N. cinerea) were reared on dog chow and water in the laboratory at room temperature. Mosquitoes (A. aegypti of the Rock strain) were reared in the laboratory according to Shapiro and Hagedorn (1982). Locusts (S. americana), reared on wheat leaves were obtained from Dr. E. A. Bernays laboratory (University of Arizona, Tucson). Tobacco hornworm (M. sexta) were obtained from the laboratory in Division of Neurobiology (University of Arizona, Tucson). Adult honey bees (A. mellifera) were obtained from the Carl Hayden Bee Research Center in Tucson. Crickets (A. domesticus) were obtained from Flukers Cricket Farm, Baton Rouge, LA.
**Antiserum preparation**

*Conjugate preparation.* Since leucokinin is a very short peptide (only 8 amino acids), it is too small to induce antibody production. Such peptides need to be conjugated to a carrier protein that is immunogenic in order to generate antisera. Using glutaraldehyde (Harrow and Lane 1990), leucokinin IV (Peninsula Laboratories, Belmont, CA. USA) was conjugated to bovine thyroglobulin (St. Louis, MO. USA) as the carrier protein. Thyroglobulin (3.5 mg) was dissolved in 1 ml phosphate buffered saline (PBS: 0.15M, pH 7.5). Leucokinin IV was dissolved in 50 µl H2O and added to the thyroglobulin solution. Glutaraldehyde (2 ml of 0.25%) was then slowly added into the solution containing thyroglobulin and leucokinin IV. After one hour at room temperature, 10 ml of 1 M glycine in PBS (0.15M, pH 7.5) were added in to stop the reaction. After 1 hour, the conjugate mixture was dialyzed against PBS (0.15M, pH 7.2) for 2 days at 4°C with 4 solution changes. The conjugate was stored at -20°C.

*Rabbit injection and bleeding.* Two rabbits (New Zealand strain, female) were used to raise the antisera. Before injection, 220µg of conjugate (1 ml conjugate solution) was mixed with 1 ml PBS and added to a pre-warmed (37°C water bath for 10 min.) vial of RIBI adjuvant (Hamilton, MN, USA) containing 0.5 mg of monophosphoryl lipid A, 0.5 mg synthetic trehalose dicorynomycolate, 0.5 mg cell wall skeleton, 0.04 ml squalene and 0.004 ml monooleate (Tween 80) and mixed. RIBI function as Freund's complete but it is not harmful to the rabbits. Each rabbit was injected with 1 ml of the mixture at multiple sites along the back bone, representing 150-200 µl per injection site. Six weeks later, 130µg (about
600µl of conjugate were mixed with 1 ml of Freund's incomplete adjuvant (lacking killed mycobacterium) and 0.4 ml of PBS and mixed. Each rabbit was injected with 1 ml of the homogenate.

The first bleeding was done one week after the second injection. The blood was taken from the central ear vein with a cannulated needle and a 50 ml plastic syringe. A light bulb was used to increase the temperature of the ear to stimulate the blood circulation. Booster shots were given four weeks after each bleeding.

**Antisera purification.** About 25-30 ml of blood was obtained from the first rabbit and 10 ml of blood was obtained from the second rabbit after each bleeding. Blood was allowed to coagulate at room temperature for about 5 hours. After centrifugation at 10,000 rpm/min., the serum was collected, divided into aliquots and stored at -70°C. A total of eight bleedings was obtained from one rabbit, and six from the other rabbit.

**Antisera labeling.** Two kinds of labels were used in our experiment, a peroxidase-antiperoxidase (PAP) label and a fluorescent label. In order to test whether two different peptides (leucokinin and vasopressin or leucokinin and diuretic hormone of *M. sexta*) co-localize in the same neurosecretory cells, the peptides were labeled differently. In double labeling experiments, one peptide was labeled indirectly [the peptide was first recognized by its antiserum (raised in rabbits) and the IgG of the antiserum was recognized by anti-rabbit immunoglobulins IgG labeled with fluorescent rhodamine]. The second peptide was recognized directly by labeling the IgG with fluorescein isothiocyanate. Anti-leucokinin IgG and anti-C-terminal diuretic hormone IgG were partially
purified and conjugated with fluorescein isothiocyanate (FITC) using a kit from Boehringer Mannheim (Indianapolis, IN, USA) and kindly provided by Jan Veenstra. The FITC to anti-leucokinin IgG protein ratio was 2.56 and to antidiuretic hormone IgG protein ratio is 2.43.

Tissue section staining

The central nervous system was dissected from the insects under saline (0.9% NaCl) and fixed in GPA (1 ml 25% glutaraldehyde, 40 μl glacial acetic acid, 3 ml saturated aqueous picric acid) or Bouin's mixture (1 ml paraformaldehyde, 1 ml glacial acetic acid and 3 ml saturated aqueous picric acid) for 4 to 5 hours. The tissues were then washed with 70% ethanol until the yellowish color disappeared and dehydrated in increasing concentrations of ethanol, (80%, 90%, 95%, 100% and 100%) for 15 min. each, then cleared in toluene once for 30 min. and xylene twice for 30 min. Tissues were incubated at 60 °C in a mixture of 1:1 for 15 min. and then in 100% paraffin for 15 min. before being oriented and allowed to cool in embedding paraffin.

The embedded tissues were sectioned at 7 μm with a microtome and then spread on a water bath at 45° C and affixed to slides that coated with one drop of 10 μl 1% poly-L-lysine spread on a slide and held at room temperature for at least 24 hours before use. The coated slides were warmed on a slide warmer to 60° C and 1 ml of H2O was applied to the slide creating a small water bath to which the sections were transferred and incubated overnight on a slide warmer before staining.
Peroxidase-antiperoxidase (PAP) section staining. The staining procedures were according to Schooneveld and Veenstra (1989). The slides with tissue sections were deparaffinized in xylene twice, for 3 min. each time and hydrated through decreasing concentrations of ethanol by 3 min. in each (100%, 100%, 95%, 90%, 80% and 70%). The slides were then washed in water for 10 min., twice in PBS (0.15 M, pH 7.4) for 5 min. and finally in PBS for 15 min. Slides were then transferred to petri dishes lined with wet paper to maintain humidity. Sections were blocked by 10% normal goat serum for 10 min. Anti-leucokinin IV antibody (diluted 1:4000 in PBS) was then applied to the slides and incubated overnight at 4 °C. The slides were washed twice for 10 min. in PBS and transferred back to the humidifier. After a second blocking step (10% NGS for 10 min.), the anti-rabbit immunoglobulins IgG (Sigma, Louis, MO, USA) (diluted 1:50 in PBS) were incubated with sections for 60 min. at room temperature. Slides were washed in PBS for 10 min., and incubated with rabbit PAP complex (Sigma, Louis, MO, USA) (1:200) for 60 min. at room temperature. Slides were washed in PBS for 10 min. and Tris buffer (Tris-HCl 0.05 M, pH 7.6) for 5 min. The substrate of 5 mg of 3, 3'-di-amino-benzidine-tetra-hydrochloride (DAB) was dissolved in 10 ml Tris buffer, and hydrogen peroxide was added to a final concentration of 0.01% just before use, then applied to the slide for 5 min. After a single rinse in water, stained tissues were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 95%, 100%, 100%) for 3 min. each, cleared in xylene twice for 3 min. each, mounted with DPX mountant (Fluka chemie AG, Switzerland) and covered with a cover slip. The slides were dried in a 37 °C incubator for 20 hours.
**Fluorescent labeled section staining.** The nervous tissues were fixed in Bouin's mixture as described above. Tissues were first incubated with antisera against leucokinin-IV overnight, then they were washed in a PBS bath twice for 10 min. each and the anti-rabbit immunoglobulins IgG L- Rhodamine conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) (1:200) were applied and incubated for two hours. The slides were washed in a PBS bath twice (10 min. each) and cleared in 55% glycerin diluted with carbonate buffer (0.05 M carbonate-bicarbonate, pH 9.5) for 30 min., mounted with 80% glycerin, covered with a cover slip and observed under a Nikon optiphoto fluorescent microscope. To determine co-localization, the glycerin was washed away after the first observation and FITC-labeled anti-leucokinins or anti-C-terminal diuretic hormone IgG (1:100) was applied to sections and incubated for 4 hours at room temperature. Washing and further treatment was then repeated as above.

**Whole mount staining**

The staining procedures were those used by Davis (1989). The central nervous system was dissected as described above and fixed in 4% paraformaldehyde in PO4 buffer [(paraformaldehyde (10 g) was first dissolved in distilled H2O (110 ml), heated to 60° C with stirring for 10 min. Drops of NaOH (1N) were added until the solution became clear and distilled H2O was added to 125 ml and mixed with PO4 buffer (125 ml, 0.2 M, pH 7.4).] Tissues were fixed overnight at 4°C, then washed at room temperature with PBSAT (0.15M PBS, pH 7.4, 0.1% sodium azide and 0.5% Triton X-100) 6 times, 1 hour each with agitation. Tissue remained in the final wash overnight at 4 °C. Tissue were blocked with 2% (NGS) in PBSAT at room temperature for 5 hours. The tissues
were soaked in primary antisera (anti-leucokinin IV antibody) which was diluted in PBSAT/2% NGS with agitation at room temperature overnight. The preparation was washed 6 times with PBSAT for 1 hour each time and incubated with fluorescent-labeled secondary antibody (anti-rabbit IgG 1:200) overnight at 4°C. Tissue was washed 6 times and then cleared in a glycerin series of 40%, 60% and 80%, covered with a cover slip and observed under a fluorescent microscope. If co-localization was to be tested, the glycerin was washed out with PBS and the tissues were incubated with the FITC labeled leucokinin antibodies or C-terminal antibodies (1:100) over night at room temperature. After washing with PBSAT 6 times for 1 hour each, tissue was cleared with a glycerin series of 40%, 60% and 80%, covered with cover slide and observed under a fluorescent microscope.

Two types of controls were performed.

CI. Diluted leucokinin antisera (1:1000) was incubated with 10 nmol leucokinin overnight before incubation with tissue; and

CII. During the staining process, the first antiserum was omitted. If the staining was abolished, then the primary antibody was binding specifically.

- **Enzyme-linked immunosorbent assay (ELISA) of leucokinins**

ELISA was used to test the specificity of the antiserum against leucokinins and to determine the concentrations of leucokinins in the nervous tissues.

Leucokinin IV was conjugated to bovine serum albumin (BSA) using iodoacetic acid anhydride as described by Wetzel et al. (1990). In this system,
peptides are first reacted with iodoacetic acid, which occurs preferentially at the N-terminal of the peptide, and then the iodoacetyl-peptide is reacted with a thiol group. Iodoacetic acid anhydride (250 mg) was dissolved in 2 ml of dry tetrahydrofuran (THF) to a concentration of 350 mM and aliquots (200μl each) were placed at -20 °C in Eppendorf tubes, which were stored in 50-ml plastic screw-top conical tubes containing Drierite. Stored in this way, reagents have been reported to be stable for at least 9 months. Leucokinin (0.2 mg) was dissolved in 0.4 ml of 2-(n-morpholino)ethanesulfonic acid (MES) buffer (0.1 M MES, pH 6.0). Iodacetic acid anhydride (4 μl) in tetrahydrofuran was added and vortexed for 3 times for each for 3 min. BSA (5 mg) in 7 ml TRIS-HCl buffer [(1 M Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM DL-dithiothreitol (DTT) (pH 7.5)] was added and incubated at room temperature for 1 hour. The mixture was dialyzed against 200 ml buffer (10 mM NaOAc, 0.25 mM EDTA, pH 5.4) for 24 hours at 4°C. A mixture of 4 ml was obtained after dialysis was completed. 475 μl of a buffer (1M TRIS-HCl, 100 mM EDTA, pH 8.0) was added and incubated at 37 °C for 20 hours. The final volume of the conjugate was about 5 ml and stored at -70 °C.

Testing the conjugate. The conjugate was diluted in the following series: 250, 125, 50, 25, 10, 5, and 2 ng/50 μl. The different concentration of conjugate was loaded in a 96 well-ELISA plate at 50 μl/well and incubated for 3 hours with agitation in a 37 °C incubator. After being washed with 0.05% Tween-20 in PBS for 5 min, 200μl of 1% NGS in PBS was added to the wells, the plates were incubated for 1 hour at room temperature to block the non-specific binding. Antisera against leucokinin were diluted 10,000 to 500,000 fold with 1% NGS. Diluted antiserum (100 μl) were added to each well to form different
35 combinations of concentrations between antiserum and conjugate. Plates were incubated at 4 °C overnight with agitation. The plates were then washed with 0.05% Tween-20 in PBS three times for 5 min each with agitation at room temperature. Anti-rabbit IgG (Kpl, MD, USA) diluted to 1:1000 (70 µl) was added in each well and incubated for one hour. The plates were washed with 0.05% Tween-20 in PBS four times for 5 min each with agitation at room temperature. The developer (Kpl, MD, USA) (a mixture of 50% tetramethylbenzidine (TMB) peroxidase solution and 50% peroxidase solution B) (75 µl) was added in each well and incubated for 30 min, then stopped with of 75 µl of 1 M H₃PO₄. The optic densities were determined on the Biotek ELISA reader at 450 nm.

Establishing a standard curve for leucokinins. The optimum concentrations for the antiserum was 1:250,000 and the conjugate was 10 ng/well. The competitive ELISA was then used to establish a standard curve. Conjugate in 50 µl PBS volume (10 ng) was coated to each well of the ELISA plate as described above. Standard concentrations of leucokinin IV or the test samples extracted from nervous tissue were added in order to the well (50 µl/well). 50 µl of 1:500,000 anti-leucokinin antiserum was then added to each well and incubated at 4 °C overnight. The subsequent procedures were followed as above. By comparing the optical density of samples with the optical density of the standard curve; the concentrations of leucokinins in the samples were calculated as fmol per animal equivalent according to the standard curve.
HPLC fractionation of *N. cinerea* neuropeptides

*Tissue preparation.* The central nervous system of *N. cinerea* was dissected in 0.9% saline and separated into brain, abdominal ganglia, optical lobes, and combined corpora cardiaca and corpora allata complex and stored in Eppendorf tubes separately on dry ice. Tissues were homogenized with a glass homogenizer in 1 ml of Bennett's mixture (1% NaCl, 5% formic acid, 1% trifluoroacetic acid (TFA) and 1 M HCl in water) with the addition of 50 µl of 25% thiodiglycol (Veenstra 1989). The extract was centrifuged and the pellet re-extracted in 0.5 ml of Bennett's mixture. The combined supernatants were loaded on a previously activated and equilibrated C-18 Sep-Pak (Waters Associates, MA, USA). The Sep-Pak was washed with 5 ml water and 5 ml of 13% acetonitrile, and the peptides were eluted with 5 ml of 32% acetonitrile (all eluants contained 0.1% TFA). The eluant was reduced in volume to about 1 ml by vacuum centrifugation, diluted with 2 ml 0.1% TFA and further separated by high-performance liquid chromatography (HPLC).

*HPLC.* The HPLC apparatus consisted of a Beckman system Gold programmable solvent module 126 and a Scanning Detector Module 167. Separations were performed on an analytical Beckman 4.6 mm x 25 cm C18 column and pre-column. Solvents consisted of 0.1% TFA in water (A) and 0.1 TFA in 65% acetonitrile (B). The column was eluted with 20% B for 10 min at a flow rate of 1 ml/min. Material from Sep-Pak was injected and eluted using 20% B for 10 min, followed by a linear gradient to 70% B for 50 min and then a linear gradient to 100% B for 10 min. The elution rate was at 1 ml/min. Fractions were collected every minute and aliquots were analyzed by ELISA for leucokinin
immunoreactivity. The fraction of immunoreactive peaks were diluted in series and test their affinity with the anti-leucokinin IV antisera by ELISA.
RESULTS

Specificity of antisera

The specificity of leucokinin antiserum was tested by immunocytochemistry and the leucokinin IV ELISA. Pre-immunization sera did not react with the sections of the brain. Weak immunoreactivity was observed using antiserum from the first bleeding (i.e., the antiserum could only be diluted to 1:250). At the second bleeding, the antibody titer increased such that the antiserum could be diluted to 1:2000. Preabsorption of the antisera with leucokinin always abolished immunoreactivity.

There was no apparent difference between the antisera of the two rabbits in immunocytochemical staining. When antisera were tested with ELISA, there were differences in titer between the two rabbits and between different bleedings. The antiserum from the eighth bleeding of one rabbit could be diluted to 1:250,000 and was used in the most sensitive ELISA, enabling detection of leucokinin IV concentrations down to 10 fmol/well. Competitive ELISA showed that antisera bound to leucokinin IV and a single, presumably homologous, HPLC fraction from the corpora cardica-corpora allata complex of N. cinerea. Neither corazonin or M. sexta allatotropin reacted in this ELISA (Fig. 1).
Fig. 1. Anti-leucokinin IV affinities to leucokinin IV, HPLC immunoreactive peak fraction from corpora cardiaca of *Nauphoeta cinerea*, allatotropin and corazonin. Shown are the ODs for leucokinin IV competitive ELISA with various concentrations of the substances shown. See Materials and Methods for details.
Leucokinin homogeneity in *N. cinerea*

Different parts of the nervous systems of *N. cinerea* were extracted and separated by HPLC. Fractions collected at 1 min intervals (see Materials and Methods above) were tested for leucokinin-IV immunoreactivity by competitive ELISA. Brain, corpora cardiaca and abdominal ganglia all showed a single immunoreactive peak at an elution time of 35 min, indicating that the same neuropeptide was detected by the antiserum (Fig. 2). The immunoreactive peaks at 35 min from the brain, corpora cardiaca and abdominal ganglia were diluted serially (concentration is calculated to tissue equivalent per well) to test their affinity with anti-leucokinin antisera and the results showed that they all specifically bound the antisera. Three fractions have the same shapes (Fig. 3) further indicating that the same peptide was recognized by antiserum.
Fig. 2. Leucokinin IV immunoreactivity of HPLC fractions from brains, corpora cardiaca and abdominal ganglia of adult *Nauphoeta cinerea*. See text for details. ODs have been transformed using the inverse function for greater clarity. The major peak in all tissues was from fraction 35.
Fig. 3. Anti-leucokinin IV affinities with immunoreactive peaks in 35 min from brains, abdominal ganglia and corpora cardiaca. Shown are the ODs for leucokinin IV competitive ELISA with various concentrations of the leucokinin IV or tissue equivalent of brain, corpora cardiaca and abdominal ganglia. See detail for text.
Distribution of leucokinin and vasopressin immunoreactive patterns in *N. cinerea*

The central nervous system of *N. cinerea* consists of the brain and ten segmental ganglia; including the subesophageal ganglion, three thoracic ganglia and six abdominal ganglia (Fig. 4). The retrocerebral complex includes the paired corpora cardiaca and corpora allata. The cardiac nervous system contains paired lateral cardiac nerve and segmental nerves. Anti-Lys-vasopressin and anti-leucokinin-IV bind to identical neurosecretory cells in the brain and abdominal ganglia.

**Brain and retrocerebral complex.** There were several groups of leucokinin-positive cells and immunoreactive processes in the brain:

1. a group of 100-120 median neurosecretory cells in the pars intercerebralis (MNSC Figs. 4, 5A) and their projection axons to the retrocerebral complex through the nervi corporis cardiaca I (NCCI Fig. 4);
2. a group of four lateral neurosecretory cells (LNSC) in each par laterum and projection axons in the nervi corporis cardiaca II (NCCII) (Figs. 4, 5B);
3. a group of 10-15 inter-neurons at the base of each optic lobe (Fig. 5A);
4. three pairs of neurons in each protocerebral hemisphere, two of which were close to the optic lobes (Figs. 4, 5A) and one pair in the posterior protocerebrum which had the same location as cells in *L. maderae* as described by Nässel et al. (1992), who called them dorsal neurons. Their axons appeared to descend through all the neuromeres to the terminal ganglion;
5. numerous processes in the central body complex, posterior deutocerebrum, tritocerebrum and optical lobes (Fig. 4);
(6) the immunoreactive processes in the frontal ganglion;
(7) the axon terminals and some intrinsic cells in the corpora cardiaca (Fig. 4).

*Subesophageal ganglion.* There were no leucokinin-immunoreactive cell bodies in the subesophageal ganglion but processes within the ganglion were stained.

*Thoracic ganglia.* In the prothoracic ganglion, three pairs of intensely stained neurons were present in the anterior part of the ganglion, and a group of lightly stained cells were present in the posterior (Fig. 5B). In the mesothoracic ganglion, only four neurons were leucokinin-positive. The metathoracic ganglion is fused with the first abdominal ganglion. In addition to the four neurons which stained at the same position of the neurons as in the mesothoracic ganglion, there were two pairs of leucokinin-positive neurosecretory cells in the posterior ganglion (one pair in each side), which corresponds ontogenetically to the first abdominal ganglion (Figs. 4, 5D). The branches of the descending axons also stained in all thoracic ganglia (Fig. 4).

*Abdominal ganglia and perivisceral organs.* In each of the abdominal ganglia, one posterior pair of leucokinin-positive neurosecretory cells were found in each side of the ganglion (Figs. 4, 5E). Like the neurosecretory cells in metathoracic ganglion, the axons of these cells projected from the ganglion through ventral nerves to the transverse nerve, where they bifurcated. One branch entered the transverse nerve and then faded away, while the other extended to the lateral cardiac nerve, which also stained strongly (Fig. 6). The terminal abdominal ganglion is fused with the penultimate abdominal ganglion, and had three
neurosecretory cells in each side of the ganglion (Figs. 4, 5F).
Fig. 4. Distribution of leucokinin positive cells and processes in *Nauphoeta cinerea*. AG 1-9: abdominal ganglia. MNSC: median neurosecretory cells. MSG: mesothoracic ganglion. MTG: metathoracic ganglion. LNSC: lateral neurosecretory cells. OLIN: neurons in optic lobes. PDN: posterior dorsal neurons. TAG: terminal abdominal ganglion.
Fig 5. Leucokinin positive neurosecretory cells and processes in *Nauphoeta cinerea*. (A) Brain tissue showing median neurosecretory cells (MNSC), lateral neurosecretory cells (LNSC) and various processes. (B) First thoracic ganglion showing six strongly immunoreactive neurons (arrows) and a group of weakly reactive neurons in the posterior of the ganglion. (C) Second thoracic ganglion showing a similar pattern. (D) Fused third thoracic and first abdominal ganglia showing patterns similar to other thoracic ganglia and posteriorly, similar to other abdominal ganglia. (E) Abdominal ganglion showing two pairs of neurosecretory cells in each side of the ganglion and their axons projecting along the ventral nerve (arrows). (F) Last abdominal ganglion showing three neurosecretory cells at each side of the ganglion and immunoreactive processes. Scale bars: all of them are 100 μm.
Fig. 6. Distribution of leucokinin positive cells and processes of abdominal ganglia and their neurohemal release sites of *Nauphoeta cinerea*. AG: abdominal ganglion; LCN: lateral cardiac nerve.
Distribution of leucokinin and vasopressin immunoreactive patterns in
*A. domesticus*

The central nervous system of *A. domesticus* is very similar to *N. cinerea* except that *A. domesticus* has seven ganglia in the abdomen.

**Brain and retrocerebral complex.** In the brain, the distribution of leucokinin-positive neurons was similar to that for *N. cinerea*. There was a group of 40-60 median neurosecretory cells stained in the pars intercerebralis (Figs. 7, 8A) and four lateral neurosecretory cells in each pars lateralis (Fig. 8A). The median neurosecretory cells did not stain as intensely as lateral cells. There were two pairs of neurons in each side of the posterior protocerebrum. Two of them projected their axons to the subesophageal ganglion and descended through the neuromeres to the terminal abdominal ganglion located as observed in *N. cinerea*. A pair of interneurons and their axon branches close to the base of each optic lobe were positive. Immunoreactive processes were also observed in the central bodies and tritocerebrum of the brain. However, there were no leucokinin-immunoreactive neurons observed in the optic lobes. Processes in the frontal ganglion and corpora cardiaca also stained (Fig. 8F) but none were seen in the corpora allata (Fig. 8G).

**Segmental ganglia and their perivisceral organs.** In the subesophageal ganglion, several groups of neurons and their axons stained (Fig. 7). Six neurons stained lightly in the prothoracic ganglion. The neuropiles and descending axons stained heavily in all thoracic ganglia (Fig. 7). In the metathoracic ganglion, which is fused with the first two abdominal ganglia, four pairs of immunoreactive neurosecretory cells were observed in the posterior part of the
ganglion (Fig. 7), which corresponds ontogenetically to the first two abdominal ganglia. In the abdominal ganglia, one pair of leucokinin-positive neurosecretory cells were observed in each posterior part of the ganglion (Figs. 7, 8E). These cells projected axons out of the ganglion through ventral nerves to the link nerve, where they bifurcated, one branch entering the transverse nerve, and the other extended toward the dorsal nerve in the direction of the lateral cardiac nerve (Figs. 8E, 9). In the first five abdominal ganglia, the cardiac nerves were only lightly stained. In the last five abdominal ganglia, the axon branch to the dorsal nerve extended to the cardiac nerve producing varicose branches that form a neurohemal meshwork on each side of the heart (Figs. 8B, 9).

Antiserum against Lys-vasopressin recognized the same neurosecretory cells as those stained by the antiserum against leucokinin (Figs. 8C, D).
Fig 7. Distribution of leucokinin positive neurosecretory cells and processes in *Acheta domesticus*. SEG: subesophageal ganglion. Other abbreviations as in Fig 4.
Fig. 8. Leucokinin and vasopressin positive cells and processes in *Acheta domesticus*. (A) Anti-leucokinin reactive median and lateral neurosecretory cells of the brain. (B) Processes in cardiac nerves. (C) Anti-leucokinin and (D) anti-vasopressin reactive cells in the same tissue preparation from the third abdominal ganglion. (E) Anti-leucokinin neurosecretory cells and processes of abdominal ganglion. (F) Processes in the frontal ganglion. (G) Processes in the corpora cardiaca. Scale bars: all of them are 100μm.
Fig. 9. Distribution of leucokinin positive neurosecretory cells in abdominal ganglia and their neurohemal release sites of *Acheta domesticus*. Abbreviations as in Fig 6.
Distribution of leucokinin immunoreactive pattern in S. americana

Central nervous system. The central nervous system of S. americana is very similar morphologically to that of N. cinerea. But, the distribution of leucokinin-positive neurons in the brain was quite different. Only one pair of immunoreactive neurons on each side of the pars intercerebralis was observed, and their axons could not be followed (Figs. 10, 11A). No immunoreactive neurons were found in the pars intercerebralis. There was one pair of positive neurons in each lateral part of procerebrum. A group of interneurons close to the base of the optic lobes, and processes of these neurons also stained (Fig. 10). Another group of three interneurons and their axons stained in the tritocerebrum (Fig. 10). Immunoreactive processes were also observed in the central body. In the subesophageal ganglion, three groups of positive neurons were observed (Figs. 10, 11C). Two descending axons were found in the subesophageal ganglion, however, the origin of these axons could not be determined. In the first two thoracic ganglia, a pair of positive bilateral neurosecretory cells in the anterior part of the ganglion and a group of positive neurons were present in the middle of the ganglion (Fig. 10). The metathoracic ganglion is fused with the first three abdominal ganglia. This fused ganglion has three bilateral triplets of positive neurosecretory cells in the posterior region (Figs. 10, 11B). In each abdominal ganglion, a pair of leucokinin-positive neurosecretory cells was stained in posterior region of each side of the ganglion (Figs. 10, 11D). In the terminal abdominal ganglion, there were no leucokinin immunoreactive neurons. Neuropils and descending axons stained in all segmental ganglia.

Peripheral nervous system. The first bilateral triplet of cells in the metathoracic ganglion projected their axons through the first ventral nerve to the
link nerve and returned via the transverse nerve, terminating in varicose branches within the transverse nerve between the metathoracic ganglion and the first abdominal ganglion. The whole structure has a shape like an inverted "Y" (Fig. 12). The second bilateral triplet of cells project their axons through the second ventral nerves to the link nerve and again return and form a ipsilateral, dendritic-like arborization near the first abdominal ganglia (Fig. 12). The third group of bilateral triplet neurons projected their axons through the third ventral nerve and terminated in varicose branches within the third transverse nerve which was located between the first and second abdominal ganglion (Figs. 11E, 12). In each abdominal ganglion, leucokinin-positive neurons projected their axons via the ventral nerves to the link nerve and terminated in the transverse nerve, which is located in the anterior part of the next posterior abdominal ganglion (Fig. 12).
Fig. 10. Distribution of leucokinin positive cells and processes in *Schistocerca americana*. Abbreviations as in Fig. 4.
Fig. 11. Leucokinin positive cells and processes of *Schistocerca americana.*

(A) Brain showing two pairs of strongly immunoreactive neurons which are not the median neurosecretory cells, and two pairs of neurons in the lateral part of the brain. (B) The last fused thoracic ganglion showing three groups of immunoreactive neurosecretory cells and processes. (C) Subesophageal ganglion showing immunoreactive processes. (D) Third abdominal ganglion showing two pairs of immunoreactive neurosecretory cells and processes. (E) Neurohemal release sites on the fifth transverse nerve. Scale bars: (A), (B), (C) and (D), 50μm; (E), 100μm.
Fig. 12. Distribution of leucokinin positive neurosecretory cells in abdominal ganglia and their neurohemal release sites of *Schistocerca americana*. NHO: neurohemal organ. Other abbreviations as in Fig 4.
Distribution of leucokinin immunoreactive pattern in *A. aegypti*

The distribution of leucokinin-immunoreactive neurons was very similar between the larvae and adult in *A. aegypti*. In the brain, about ten neurosecretory cells stained in the pars intercerebralis (Fig. 14A) and three neurosecretory cells stained in each side of the pars lateralis (Fig. 13A). Immunoreactive processes were observed in the central bodies and posterior part of the procerebrum, but no immunoreactive interneurons were observed. The median neurosecretory cells did not stain as strongly as the lateral neurosecretory cells. This pattern was very similar to *A. domesticus*. There were no leucokinin-immunoreactive neurons in the subesophageal ganglion. In the posterior part of the fused thoracic ganglia of the larvae, two staining axons ascended from the first abdominal ganglion but no cell bodies were stained (Fig. 13B). Some immunoreactive processes were observed in the thoracic ganglia of the larvae (Fig. 13B). In the thoracic ganglia of the adult, there were two groups of neurosecretory cells stained in the posterior part of the ganglion, which corresponded to the first abdominal ganglion. The immunoreactive processes were also observed in the thoracic ganglion of the adult. Three neurosecretory cells stained in each side of the first abdominal ganglion of larvae; but in the adult, two cells disappeared so that only four staining neurosecretory cells were present in the first abdominal ganglion which fused to the thoracic ganglion mass (Fig. 13A). For both larvae and adults, each abdominal ganglion had two pairs of stained neurosecretory cells in the posterior part (Figs. 13, 14B). In the terminal abdominal ganglion of the adult, the location of the four positive neurons differed slightly, one bilateral neuron was present in the anterior part the ganglion and another median pair of neurons in the posterior part (Fig. 14C).
Axons of all immunoreactive neurons in each abdominal ganglion projected out via ventral nerves to form a diffuse pattern.

**Distribution of leucokinin immunoreactive pattern A. mellifera**

No leucokinin immunoreactive neurons were found in the brain, subesophageal ganglion and thoracic ganglion in adult *A. mellifera*. In each abdominal ganglion, four positive neurosecretory cells were observed in each side of the ganglion (Figs. 14E, 15). The axons of these neurons projected out via the ventral nerve and terminated in a bulbous-like neurohemal site which was very close to the ganglion. Leucokinin-positive material accumulated there (Figs. 14D, 15).
FIG. 13. Distribution of leucokinin positive cells and processes in *Aedes aegypti*. (A) Adult. (B) Larva.
FIG 14. Leucokinin positive cells and processes in *Aedes aegypti* and *Apis mellifera*. (A) Brain of adult *A. aegypti* showing a group of immunoreactive median neurosecretory cells and later neurosecretory cells. (B) 1st abdominal ganglion of *A. aegypti* larva showing 6 immunoreactive neurosecretory cells. (C) last abdominal ganglion of adult mosquito showing 2 pairs of immunoreactive neurosecretory cells and neurohemal release sites. (D) Neurohemal release site of leucokinin in *A. mellifera*. (E) 2nd abdominal ganglion of *A. mellifera* showing 8 leucokinin immunoreactive neurosecretory cells and location of neurohemal site. Scale bars: (A), 100|μm; (B), 50|μm; (C), 100|μm; (D), 25 |μm; (E), 50|μm.
FIG 15. Distribution of leucokinin positive cells and processes in the abdominal ganglia of *Apis mellifera*. Abbreviations as in Fig 12.
Distribution of leucokinin and diuretic hormone immunoreactive neurons in *M. sexta*

In the larvae, a group of four cells stained lightly in the pars intercerebralis (Fig. 16A). The location of DH-positive cells was very similar to the cells found previously by Veenstra and Hagedorn (1991). However, the anti-leucokinin staining was inconsistent, making it difficult to decide whether both peptides were localized in the same four cells or not. The intrinsic cells of the corpora cardiaca showed leucokinin immunoreactivity (Fig. 16G). No leucokinin-positive neurons were found in the subesophageal ganglion, but immunoreactive processes were observed. In each of the thoracic ganglia and first abdominal ganglion, one of the leucokinin positive neurons in each side of the ganglion was observed. From the second to the sixth abdominal ganglia, two pairs of leucokinin positive neurosecretory cells were observed in the posterior part of each ganglion (Figs. 16D, 17). The seventh abdominal ganglion is fused to the terminal abdominal ganglion and each part had two pairs of bilateral leucokinin-positive neurosecretory cells. The location of leucokinin-positive neurons in the terminal abdominal ganglion was very similar to that of *A. aegypti*: one bilateral pair was present in the anterior region of the ganglion and another pair in the posterior region (Figs. 16B, 17). The peripheral nervous system also stained with leucokinin antiserum. The positive neurons in the abdominal ganglia projected their axons via the ventral nerves to the link nerve and then bifurcated with one branch going to the dorsal region before fading away and another branch projecting to the transverse nerve that stained heavily (Fig 18).
In the adult, a group of median neurosecretory cells in the pars intercerebralis also stained lightly and inconsistently as in the larvae. The intrinsic cells and some axons stained heavily in the corpora cardiaca. No immunoreactive processes were observed in the subesophageal and thoracic ganglia. In each abdominal ganglion, two pairs of leucokinin-positive neurons were observed in the posterior ganglion. In the terminal abdominal ganglion, four pairs of leucokinin positive neurons were observed. The peripheral nervous system was not investigated in the adult.

The distribution of the diuretic hormone-positive neurons in *M. sexta* was compared with leucokinin-positive neurons in the nervous system using double-labeling methods. Because staining of leucokinin antiserum in the brain of both larvae and adult was inconsistent, co-localization of these two peptides could not be determined in the brain. Both antisera stained the corpora cardiaca but occupied different sites: Antiserum against leucokinin stained intrinsic cells of corpora cardiaca and their axons, antiserum against diuretic hormone stained the terminal of NCCI (Figs. 16G, H). In the abdominal ganglia, the antiserum against diuretic hormone stained the same cells as the antiserum against leucokinin except in the terminal abdominal ganglion (Figs. 16I, J). However, one pair of cells that was only lightly stained with anti-diuretic hormone was not easily observed in larvae (Fig. 16J) or adults. This staining difference could be observed in the staining of the transverse nerve of the larvae (Fig. 16K, L). In terminal abdominal ganglion, only two cells in anterior part of the ganglion stained both leucokinin and diuretic hormones. Two peptides localized different cells in posterior part of the ganglion (Figs. 16C, D).
Fig. 16. Leucokinin and *Manduca*-diuretic-hormone immunoreactive neurons of *Manduca*-sexta. (A) Anti-leucokinin reactive neurons in the brain of a third instar larva showing three of four median neurosecretory cells. (B) Fourth abdominal ganglion of larva with two pairs of neurosecretory cells and transverse nerves. (C) Terminal abdominal ganglion of larva with leucokinin positive neurons and (D) Terminal abdominal ganglion of larva showing diuretic hormone immunoreactive neurons at different locations. (E) Rhodamine anti-leucokinin staining and (F) fluorescein anti-diuretic hormone staining of the brain of the pharate adult. (G) Rhodamine anti-leucokinin staining and (H) Fluorescein anti-diuretic hormone staining of the corpora cardiaca of the pharate adult. (I) Rhodamine anti-leucokinin staining and (J) fluorescein anti-diuretic hormone staining of the fourth abdominal ganglion of the pharate adult. (K) Rhodamine anti-leucokinin staining and (L) Fluorescein anti-diuretic hormone staining of the third transverse nerve of larva. Scale bars: (A), 50\(\mu\)m; (B), (C) and (D), 100 \(\mu\)m; (E), (F), (G), (H), (I), (J), (K) and (L), 100 \(\mu\)m.
Fig. 17. Distribution of leucokinin positive neurosecretory cells in abdominal ganglia and their neurohemal release sites of *Manduca sexta* larva. Abbreviations as in Fig. 11.
DISCUSSION

Conservation of the leucokinin system

The significant differences and similarities among the species examine are summarized in Figure 18. All six species had leucokinin-positive neurosecretory cells. The distributions of two other peptides, Lys-vasopressin and the Manduca diuretic hormone, were also compared to the distribution of leucokinin positive neurons using double staining. The two peptides co-localized in neurons of some species.

The leucokinins are a group of peptides originally isolated from the cockroach L. maderae (Holman et al. 1986a, 1986b, 1987a, 1987b). Some of these structurally related peptides also have been isolated from the cricket A. domesticus (Holman et al. 1991), the locust L. migratoria (Schoofs et al. 1992) and the mosquito, A. aegypti (Hayes et al. unpublished data). Antiserum against leucokinin I showed the same immunoreactive patterns in L. maderae (Nässel et al. 1992) as in N. cinerea (Results).

The anti-leucokinin IV antiserum used in these experiments was found to be highly specific in a competitive ELISA, to bind the same HPLC fractions from several tissues of one species (N. cinerea), and to stain similar cells in abdominal ganglia of all species tested. From these results, it seems reasonable that the peptides that have been detected by anti-leucokinin IV all belong in the same functional class, to which I will refer simply as "leucokinin," rather than "leucokinin-like."
Fig. 18. Major similarities in the locations of leucokinin positive cells in the six species examined. Also shown are the different patterns of leucokinin release sites from the abdominal ganglia. Leucokinin and vasopressin positive cells are shown as ●. Vasopressin reactivity was examined only in *N. cinerea*, *S. americana* and *A. domesticus*. For *M. sexta* only: cells positive for leucokinin and *M. sexta* diuretic hormone are shown by ●; for diuretic hormone only by +, and for leucokinin only by ◎. Inconsistent staining for leucokinin is shown by △. Cells positive for leucokinin only (with vasopressin and DH reactivity untested) are shown by *. Fig. 18. Major similarities in the locations of leucokinin positive cells in the six species examined. Also shown are the different patterns of leucokinin release sites from the abdominal ganglia. Leucokinin and vasopressin positive cells are shown as ●. Vasopressin reactivity was examined only in *N. cinerea*, *S. americana* and *A. domesticus*. For *M. sexta* only: cells positive for leucokinin and *M. sexta* diuretic hormone are shown by ●; for diuretic hormone only by +, and for leucokinin only by ◎. Inconsistent staining for leucokinin is shown by △. Cells positive for leucokinin only (with vasopressin and DH reactivity untested) are shown by *. 
Interspecific comparisons of the anatomy of leucokinin secretion, transport and release

In the six insects examined, there were similarities and differences in the distribution patterns of neurosecretory cells. The whole distribution pattern of leucokinin in nervous system of *N. cinerea* is very similar to the *L. maderae* (Nässel *et al.* 1992). In the brain of *N. cinerea, A. domesticus, A. aegypti*, there are median neurosecretory cells and lateral neurosecretory cells showed immunoreactivity. The distribution patterns among these immunoreactive neurosecretory cells were very similar, except that in *A. domesticus and A. aegypti*, the median neurosecretory cells stained not as strongly as lateral neurosecretory cells. In the brain, the immunoreactive process in central bodies, posterior deutocerebrum and tritocerebrum were observed and indicated that leucokinin peptides could function as both neurotransmitter and neuromodulator in these species. The neurosecretory cells in the brain are one of the major synthesis sites of these three species. In *S. americana*, there were groups of cells staining in the brain but given their location, it was difficult to decide if they were neurosecretory cells. In *M. sexta*, a group of median neurosecretory cells showed immunoreactivity. However, the staining was inconsistent. In *A. mellifera*, no immunoreactivity was observed in the brain. So, it is difficult to decide if the brain is the major source of leucokinin in these three species.

Although immunoreactivity was observed in corpora cardiaca in *N. cinerea, A. domesticus* and *M. sexta*, immunoreactive intrinsic cells were only observed in *N. cinerea* and *M. sexta*. So, corpora cardiaca function as the site of synthesis as well as neurohemal release sites of the leucokinins in *N. cinerea* and
The corpora cardiaca of *A. domesticus* only function as a neurohemal release site for leucokinin.

No immunoreactive neurons were observed in the subesophageal ganglia and thoracic ganglia of *A. aegypti*, *M. sexta* and *A. mellifera*. Some immunoreactive neurons and processes were observed in *N. cinerea*, *A. domesticus* and *S. americana*. However, the distribution patterns were different. The descending axons were observed in all segmental ganglia in these three species. In *N. cinerea* and *A. domesticus*, the descending axons come from the dorsal neurons, but in *S. americana*, the origin of these neurons could not be traced.

In the abdominal ganglia, the distribution patterns of neurosecretory cells were very similar among the six species. It is likely that leucokinin are synthesized in homologous neurosecretory cells in the abdominal ganglia.

The neurohemal release sites of leucokinin peptides in abdominal ganglia were very different from one another. In *N. cinerea*, lateral cardiac nerves appear to be the major release site of leucokinins because the lateral cardiac nerves stained very heavily whereas the neurohemal organ in the transverse nerves did not. In *A. domesticus*, the neurohemal release site also seems to be the lateral cardiac nerve in the last five abdominal segments. In *S. americana*, the release site seems to be the neurohemal organ in the transverse nerves in the abdominal ganglia. In *M. sexta*, the neurohemal release site is also in the transverse nerve in the abdominal ganglia. In *A. aegypti*, the neurohemal release site had a diffuse pattern in the peripheral nervous system of each abdominal ganglion. In *A. mellifera*, the neurohemal release sites formed a bulbous shape region near each abdominal ganglion. Although the neurohemal release sites were different in
different species, the peptides were all transferred via ventral nerves to these different release sites in abdominal areas. Given this distribution, leucokinin IV seems mainly to function as a neurohormone with the difference of releasing sites possibly reflecting their evolutionary history. Leucokinins were suggested to be related to diuresis (Hayes et al. 1989, Coast et al. 1991). Early studies also demonstrated that the diuretic factors are released from nerves in the abdominal ganglia of Calliphora erythrocephala (Schwartz & Reynolds 1979), Rhodnius prolixus and Glossina morsitans (Maddrell 1966; Maddrell & Gee 1974). Diuretic factors present in abdominal ganglia have been investigated in P. americana (Mills 1967). It is possible that groups of ubiquitous neurosecretory cells in the abdominal ganglia are involved in diuresis. Since leucokinin immunoreactive neurosecretory cells in the abdominal ganglia seem to be homologous cells, it is possible that these cells are original source of diuretic peptides. The observation that both vasopressin peptide and diuretic hormone of M. sexta localized the same abdominal ganglion cells as leucokinin further strengthen this argument.

Significance of leucokinin and vasopressin co-localization

The leucokinins were suggested to be related to the diuresis of insects based on bioassays (Hayes et al. 1989, 1992, Coast et al. 1991). Vasopressin-like peptides were also demonstrated to be involved in diuresis of A. domesticus and L. migratoria (Strambi et al. 1978, Morgan 1987). Both leucokinin and vasopressin was present in the different species tested. The results of my experiments indicate that the distribution patterns of leucokinins were very similar to those of Lys-vasopressin in the American cockroach, P. americana (Davis et al. 1992) and the locust, S. americana (Davis person comun.). The co-localization experiment
presented here indicated that both peptides localized in the same neurosecretory cells in abdominal ganglia of *N. cinerea* and *A. domesticus*.

In the locust, the leucokinin-like peptides are mainly synthesized in the neurosecretory cells of the abdominal ganglia. The immunoreactive neurosecretory cells in abdominal ganglia in *S. americana* also immunoreact to Lys-vasopressin (Davis, personal comm.). Again these results indicate that leucokinins and vasopressins are produced by the same cells in the abdominal ganglia. Neurosecretory cells in abdominal ganglia related to diuresis were also found in *S. gregaria* by Delphin (1965) and *L. migratoria* by Proux (1978). The position of leucokinin-immunoreactive cells in abdominal ganglia of *N. cinerea* is very similar to the cells described in *S. gregaria*.

In the cricket, *A. domesticus*, the neurosecretory cells in the brain and abdominal ganglia are the major sources of the leucokinin-like peptides. The achetakinins are structurally related to leucokinins and were also isolated from the cricket brain and corpora cardiaca which have been shown to be involved in stimulating hindgut contraction of cockroach (Holman *et al.* 1990) and fluid secretion by Malpighian tubules of crickets (Coast *et al.* 1991); thus the antiserum might have recognized this group of peptides in these experiments. A vasopressin-like factor has been demonstrated to cause diuresis in *A. domesticus* (Strambi *et al.* 1978). The result of the co-localizing experiment presented here suggests that both vasopressin-like peptide and leucokinins are present in the same neurosecretory cells in the brain and abdominal ganglia. These neurons might also be involved in secreting other diuretic factors.
The distribution patterns of leucokinins and vasopressins have been investigated in grasshoppers, cockroaches, blowflies, and crickets. The results indicate that both peptides are produced by the same group of neurosecretory cells in each insect species. The neurosecretory cells involved in diuretic hormone secretion suggested by Baudry (1968), and Berlind and Maddrell (1979) were also immunoreactive to leucokinin antiserum (Veenstra, unpublished data). According to the location of these neurosecretory cells in the abdominal ganglia, these cells appear to be homologous. Since both leucokinins and vasopressins affect diuresis (Hays et al. 1989, Proux & Rougon-Rapuzzi, 1980), it is possible that this group of neurosecretory cells secretes leucokinins and vasopressin-like peptides as diuretic hormones for different specific functions related to diuresis.

**Leucokinin and diuretic hormone neurosecretion in *M. sexta***

Leucokinins are present in both larvae and adults of *M. sexta*. The distribution pattern of neurosecretory cells and axons was very similar between larvae and adults. The major source of leucokinin in *M. sexta* appears to be the intrinsic cells of the corpora cardiaca and neurosecretory cells in the abdominal ganglia.

The diuretic hormones of *M. sexta* have been isolated (Kataoka et al. 1989, Blackburn et al. 1991), and their sources have been localized by antibodies raised to these peptides (Veenstra & Hagedorn 1991). The results of the co-localization experiments indicate that the diuretic hormone of *M. sexta* colocalized with leucokinin in the abdominal ganglia, but one of the cells contained less diuretic hormone than leucokinin. The results of co-localization further suggested that these groups of neurosecretory cells in abdominal ganglia are involved in
The poor staining of the brains by leucokinin antiserum made it difficult to determine whether or not leucokinin co-localizes with diuretic hormone in the brain. Both peptides were present in the corpora cardiaca. However, they localized to different places. The intrinsic cells stained for leucokinin only. The functional significance of the differences in distribution has yet to be determined.

Comparison of the distribution pattern of leucokinin-like peptides with FMRFamide-like peptides in insects

Leucokinin-like peptides have now been investigated in two cockroaches (L. maderae, and N. cinerea, locust (S. americana), cricket (A. domesticus), blowfly (Phormia terraenovae, Nässel & Lundquist 1991) turnip moth (Agrotis segetum, Cantera et al. 1992), mosquitoes (A. aegypti) and honey bees (A. mellifera). They may be commonly present in insects as is the case with other groups of peptides such as FMRFamide-like peptides, proctolin and cardioactive peptides. Comparing the distribution patterns of FMRFamide-like peptide in the different insects including the American cockroach P. americana (Endo et al. 1982), the blowfly, Calliphora erythrocephala and C. vomitoria (Duve & Thorpe 1980,1982), the locusts, S. gregaria (Myers & Evans 1985) and the locust L. migratoria (Ferber & Pflüger 1992), the honey bee, A. mellifera (Eichmüller et al. 1991), blood-feeding bug R. prolixus (Tsang & Orchard, 1990), tobacco hornworm M. sexta (Homberg et al. 1991), mosquitoes (Brown & Lea 1988) and Colorado potato beetle Leptinotarsa decemlineata (Veenstra 1984), it is very interesting that the distribution of immunoreactive neurons of FMRFamide was not unique as was found for the leucokinins. This may be because the structure of the leucokinins is more
conserved, so immunoreactive neurons more closely reflect the physiological differences.
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