Immunocytochemical studies on peptidergic neurons in the Colorado potato beetle and some other insect species

Promotor : dr. L.M. Schoonhoven, hoogleraar in de algemene dierfysiologie en de vergelijkende dierfysiologie.

Co-promotor: dr. H. Schooneveld, wetenschappelijk hoofdmedewerker bij de vakgroep entomologie.

J.A. VEENSTRA

IMMUNOCYTOCHEMICAL STUDIES ON PEPTIDERGIC NEURONS IN THE COLORADO POTATO BEETLE AND SOME OTHER INSECT SPECIES

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 19 oktober 1984
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISBN=154536_03

STELLINGEN

1. Het voorkomen van vertebratenpeptiden in insekten kan niet door immunocytochemisch onderzoek alleen worden aangetoond.

   Dit proefschrift.

2. De gevonden co-localisatie van gastrine- en pancreaspolypeptide-achtige immunoreactiviteit in bepaalde neuronen van de bromvlieg wordt niet veroorzaakt door een co-localisatie van twee peptiden in deze neuronen, maar door kruisreacties van de gebruikte antisera met een FMRFamide-achtig peptide.

   Dit proefschrift.
   H. Duve en A. Thorpe, 1984. Cell Tissue Res 237: 309-320.

3. Het is geenszins aangetoond, dat het uit de bromvlieg geïsoleerde pancreaspolypeptide-achtige molecuul uit het centrale zenuwstelsel van dit insekt afkomstig is.

   H. Duve en A. Thorpe, 1980. Cell Tissue Res 210: 101-109.
   H. Duve en A. Thorpe, 1982. Cell Tissue Res 227: 67-77.
   H. Duve, A. Thorpe, N.R. Lazarus en P.J. Lowry, 1982. Biochem J 201: 429-432.

4. De in hondehypofyse immunocytochemisch aangetoonde pancreaspolypeptide-achtige substantie is γl-MSH.

   S. Fujii, S. Baba en T. Fujita, 1982. Biomed Res 3: 525-535.

5. In de verscheidenheid aan effekten die het teweeg brengt en in het voorkomen in verschillende moleculaire vormen lijkt het adipokinetische hormoon op enkephaline, hetgeen erop zou kunnen duiden, dat ook het adipokinetisch hormoon een stress-hormoon is.

6. Soms lijken insektenfysiologen op marsmannetjes die proberen de werking van auto's te doorgronden door sierstrips te bestuderen.

7. In het Turks ware de e consequenter als ä te schrijven.

8. De ten gevolge van het gebruik van felle verlichting optredende pupilvernauwing van de tandarts en zijn assistente kan in hoge mate bijdragen aan het onbehaaglijkheidsgevoel van de patiënt.

9. Stellingen treft men in de regel aan bij werken die nog onvoltooid zijn of hersteld dienen te worden, het geeft daarom te denken dat ze bij een academisch proefschrift verplicht zijn.

J.A. Veenstra
Immunocytochemical studies on peptidergic neurons in the Colorado potato beetle and some other insect species.
Wageningen, 19 oktober 1984.

CONTENTS

GENERAL INTRODUCTION

The more complex the organismal differentiation of an animal, the stronger it needs an efficient control system for communication between different parts of the body. Such control is exerted through neural, neuroendocrine and endocrine systems. The nervous system uses "short-lasting" neurotransmitters, which act directly on the target via synapses. Endocrine glands produce "long-lasting" hormones which are released into the blood stream and may essentially reach all parts of the body. Nerve cells which synthesize and release neurohormones are called neurosecretory cells. Most neurohormones are peptides. The three control and communication systems have been studied extensively in mammals, but the underlying principles are applicable to all metazoan animals and some of them, e.g. the mechanisms of conduction of nerve impulses (Hodgkin 1964), have been first discovered in an invertebrate.

These systems are also found in insects. The major endocrine glands, the corpus allatum and the prothoracic gland, produce juvenile and moulting hormone respectively, which regulate postembryonic development and may be involved in the regulation of reproduction and diapause. After elucidation of the chemical structures of these hormones, a sesquiterpene and a steroid, much effort has been directed towards the development of chemically related substances in order to try to control insect pests by interference with their physiological functions. In the case of juvenile hormone these efforts have been partially successful (Staal 1982), thus illustrating Wigglesworth's words (1965): "The physiology of insects is to some the handmaid of Economic Entomology".

Other insect hormones, including a diuretic and hypoglycaemic hormone have been found in the corpus cardiacum (Goldsworthy and Mordue 1974), a neurohaemal organ connected to the brain. Most of these hormones are synthesized by neurosecretory cells in the brain, but the corpus cardiacum also contains intrinsic secretory cells. In locusts these cells synthesize the adipokinetic hormones, which mobilize lipids for energy during prolonged flight. Most of these hormones are peptides, but so far the chemical structure of only two insect peptides has been elucidated, the adipokinetic hormone (Stone et al. 1976) and the pentapeptide neurotransmitter called proctolin from cockroaches (Starratt and Brown 1975).

It seems likely that at least some regulatory peptides are evolutionarily very old. Thus the *Hydra* head activator neuropeptide, which induces the growth and differentiation of heads in *Hydra*, has also been extracted from vertebrates (Bodenmüller and Schaller 1981). Furthermore an insulin-like and a pancreatic

polypeptide-like peptide, very similar to vertebrate homologues, have been immunocytochemically detected (Duve and Thorpe 1979, 1980), and were isolated and characterized in blowflies (Duve et al. 1979, 1982).

In the Department of Entomology at Wageningen interest has since 25 years been focussed on endocrine control mechanisms in the Colorado potato beetle. Most work has been concentrated on regulation of adult diapause, which is induced by short day conditions. Diapause is caused partly by slow rate of juvenile hormone production by the corpora allata (de Wilde et al. 1968; de Kort 1981). The physiological regulation of corpus allatum activity appears to be based on two mechanisms, a humoral stimulation and a neural inhibition by the lateral neurosecretory cells in the brain (Khan et al. 1983, 1984). These neurosecretory cells may well be peptidergic. In addition, regulation of energy metabolism in the flight muscles of the Colorado potato beetle seems to be mediated by a peptide closely related to locust adipokinetic hormone (Weeda 1981). In the past the neurosecretory system has been extensively studied with the aid of selective neurosecretory stains and electron microscopy (Schooneveld 1970, 1974a,b). However, these methods gave little information concerning the nature of the substances produced by these cells. Immunocytochemical methods are more specific and give us some idea on the chemical structure of the peptide present in the cells. It is plausible that, in addition to insulin and pancreatic polypeptide, other vertebrate regulatory peptides may also be present in insects. It was therefore considered appropriate to re-examine the neuroendocrine system of the Colorado potato beetle immunocytochemically with antisera to vertebrate peptides, the insect peptides adipokinetic hormone and proctolin, and antisera to other regulatory peptides. Most observations were carried out on the Colorado potato beetle, but for comparative and confirmatory purposes other insect species were also studied. It is expected that the data presented here contribute to the understanding of basic neural and neuroendocrine communication mechanisms.

REFERENCES

Bodenmüller H, Schaller HC (1981) Conserved amino acid sequence of a neuropeptide, the head activator, from coelenterates to humans. Nature 293: 579-580.

Duve H, Thorpe A (1979) Immunofluorescent localization of insulin-like material in the median neurosecretory cells of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tissue Res 200: 187-191.

Duve H, Thorpe A (1980) Localization of pancreatic polypeptide (PP)-like immunoreactive material in neurones of the brain of the blowfly, *Calliphora*

erythrocephala (Diptera). Cell Tissue Res 210: 101-109.

Duve H, Thorpe A, Lazarus NR (1979) The isolation of material displaying insulin-like immunological and biological activity from the brain of the blowfly *Calliphora vomitoria*. Biochem J 184: 221-227.

Duve H, Thorpe A, Lazarus R, Lowry PJ (1982) A neuropeptide of the blowfly *Calliphora vomitoria* with an amino acid composition homologous with vertebrate pancreatic polypeptide. Biochem J 201: 429-432.

Goldsworthy GJ, Mordue W (1974) Neurosecretory hormones in insects. J Endocr 60: 529-558.

Hodgkin AL (1964) The conduction of the nervous impulse. Liverpool University press, Liverpool.

Khan MA, Koopmanschap AB, de Kort CAD (1983) The relative importance of nervous and humoral pathways for the control of corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Gen Comp Endocrinol 52: 214-221.

Khan, MA, Romberg-Privee HM, Schooneveld H (1984) Innervation of the corpus allatum in the Colorado potato beetle as revealed by retrograde diffusion with horse radish peroxidase. Gen Comp Endocrinol 55: 66-73.

de Kort, CAD (1981) Hormonal and metabolic regulation of adult diapause in the Colorado beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) Ent Gen 7: 261-271.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say) as a function of daylength. Neth J Zool 20: 151-237.

Schooneveld (1974a) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) I. Characterization of the protocerebral neurosecretory cells. Cell Tissue Res 154: 275-288.

Schooneveld (1974b) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) II. Pathways of axonal secretion transport and innervation of neurosecretory cells. Cell Tissue Res 154: 289-301.

Staal GB (1982) Insect control with growth regulators interfering with the endocrine system. Ent exp appl 31: 15-23.

Starratt AN, Brown BE (1975) Structure of the pentapeptide proctolin, a proposed neurotransmitter in insects. Life Sci 17: 1253-1256.

Stone JV, Mordue W, Battley KE, Morris HR (1976) Structure of locust adipokinetic hormone that regulates lipid utilisation during flight. Nature 263: 207-211.

Weeda E (1981) Hormonal regulation of proline synthesis and glucose release in the fat body of the Colorado potato beetle, *Leptinotarsa decemlineata*. J Insect Physiol 27: 411-417.

Wigglesworth VB (1965) The principles of insect physiology. 6th edition, Methuen & Co. Ltd, London and E.P. Dutton & Co Inc, New York.

de Wilde J, Staal GB, de Kort CAD, de Loof A, Baard G (1968) Juvenile hormone titre in the haemolymph as a function of photoperiodic treatment in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Proc K Ned Acad Wet C71: 321-326.

IMMUNOCYTOCHEMICAL LOCALIZATION OF NEURONS IN THE NERVOUS SYSTEM OF THE COLORADO POTATO BEETLE WITH ANTISERA AGAINST FMRFAMIDE AND BOVINE PANCREATIC POLYPEPTIDE

SUMMARY

Particular neurons in the nervous system of the Colorado potato beetle, *Leptinotarsa decemlineata*, are recognized by antisera against bovine pancreatic polypeptide and FMRFamide. Both antisera react with the same neurons. Solid phase absorptions showed that antiserum against bovine pancreatic polypeptide cross-reacts with FMRFamide, whereas antiserum against FMRFamide cross-reacts with bovine pancreatic polypeptide. Some of the immunoreactive neurons have axons branching extensively within the neuropil, which suggests that the peptide is used as transmitter. In the corpus cardiacum, a neurohaemal organ in insects, numerous immunoreactive axon terminals are present. Here, the peptide material is presumably released as a hormone.

INTRODUCTION

By means of selective neurosecretory stains and electron microscopy several types of peptidergic neurons can be distinguished in the brain of the Colorado potato beetle, *Leptinotarsa decemlineata* (Schooneveld 1970, 1974) and in that of a number of other insect species (Raabe 1982). In recent years many more peptidergic neurons have been demonstrated by immunocytochemistry with antisera against biologically active vertebrate peptides (e.g., El-Salhy et al. 1980; Yui et al. 1980). An antiserum against the molluscan cardioexcitatory peptide FMRFamide showed some hitherto undetected peptidergic neurons in the Colorado potato beetle (Boer et al. 1980).

Here we present a detailed study on the distribution of FMRFamide-like immunoreactivity in the brain and other ganglia of the central and stomatogastric nervous system in the head of this insect. The resemblance of the locations of the immunoreactive neurons in its brain with those in the brain of *Calliphora erythrocephala* recently reported to contain bovine pancreatic polypeptide (BPP)-like substances (Duve and Thorpe 1980) led us to test anti-BPP serum also in the Colorado potato beetle. We here report that both the anti-FMRFamide and the anti-BPP serum recognize the same cells.

MATERIALS AND METHODS

We used 5- to 8-day-old reproducing female beetles that had been reared in the laboratory. For light microscopy the brain, suboesophageal ganglion, retrocerebral glands, and stomatogastric nervous system of head and oesophagus were dissected out and fixed in a mixture of glutaraldehyde, picric acid and acetic acid (GPA, Boer et al. 1979), for 4 to 15 h at room temperature. Paraplast sections were cut at 7 µm. For electron microscopy tissues were fixed for 1 to 2 h in icecold 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 1 mM $CaCl_2$, rinsed in the same buffer with 0.2 M sucrose, and embedded in the Epon 812 substitute LX-112 (Ladd Industries). Postfixation with OsO_4 was omitted, because it reduced immunostaining. Consecutive ultrathin and semithin (0.5 µm) sections were cut. The former were contrasted with uranyl acetate and lead citrate and used for ultrastructural study of the immunoreactive elements which were identified in the semithin sections at the light microscopic level with immunocytochemistry. The resin was removed from the semithin sections with a saturated solution of NaOH in methanol (15 min).

Bovine pancreatic polypeptide (BPP) (lot number 615-D63-166-7) and antiserum against BPP (lot number 615-R110-146-16) were kindly provided by Dr. R.E. Chance (Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, U.S.A.). FMRFamide (Phe-Met-Arg-Phe-NH_2) was purchased from Peninsula Laboratories (San Carlos, California, U.S.A.). The antiserum against FMRFamide (number 646) was a kind gift from Dr. L.P.C. Schot (Free University, Amsterdam, The Netherlands).

Sections were stained immunocytochemically with the indirect peroxidase-anti-peroxidase (PAP) method (Sternberger 1974). After removal of Paraplast, endogenous peroxidase activity was inhibited by 0.0125% H_2O_2 in methanol for 20 min. Incubations with the primary antisera were performed overnight at $4^{o}C$ in dilutions of 1:2000 for the Colorado potato beetle. For rabbit pancreas, which was used as a control tissue, the anti-BPP serum was diluted 1:40000 and the anti-FMRFamide serum 1:500. As a substrate for the peroxidase 3,3´diamino- benzidine (Sigma) was used. The possibility that both antisera reacted with the same neurons was tested by staining alternating serial sections with the two antisera.

The specificity of the primary antisera was checked by preabsorbing the antisera (anti-FMRFamide diluted 1:250, anti BPP 1:1000) 3 times with the antigens coupled to Sepharose 4B beads according to Pharmacia instructions. Cross absorptions between the two antisera and their antigens were performed in the same way. Paraldehyde-fuchsine staining (Schooneveld 1970) was used to locate the known neurosecretory cells in the brain.

RESULTS

FMRFamide and BPP antisera both reveal populations of neurons which seem to occupy similar positions. The alternate sections show that neurons indeed contain substances immunoreactive towards both antisera (Figs. 3, 4). These neurons are referred to as BPP-FMRFamide-like neurons. Immunoreactive neurons were found in the central nervous system as well as the stomatogastric nervous system (Fig. 1). The optic lobe contains two groups of 3-4 small neurons (ca. 10-15 µm in diameter) with variable reaction intensity. The axons of these cells appear to penetrate into the medulla externa in a plane parallel to its outer surface. Some axons run along the medulla to the external chiasma, while others seem to enter the adjacent neuropil (Fig. 8). Each cerebral lobe contains two moderately immunoreactive cells (ca. 25 µm in diameter) just in front of the median group of neurosecretory cells of the pars intercerebralis. The cells are possibly identical with the C-type cells described by Schooneveld (1970). Ventrally to the lateral neurosecretory cells another

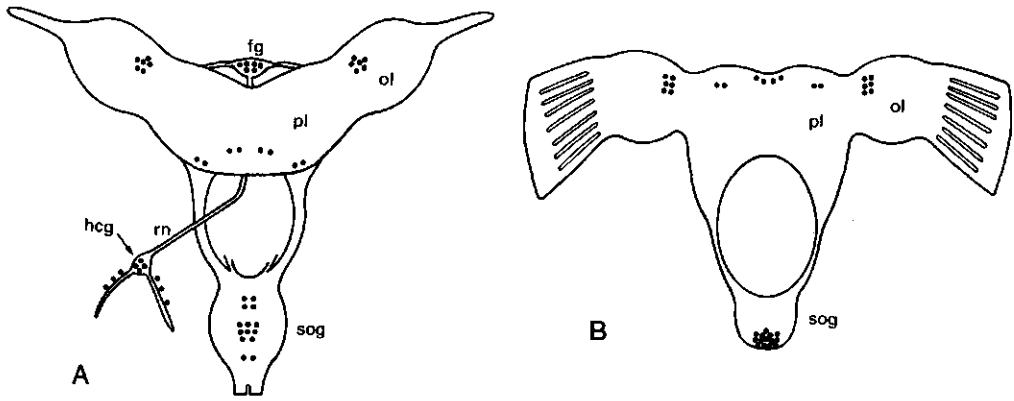


Fig. 1 **A,B**. Diagram of nervous system in head of Colorado potato beetle showing positions of BPP-FMRFamide-like neurons **A** projection in transversal plane; **B** projection in horizontal plane. Recurrent nerve enlarged for clarity. fg Frontal ganglion; hcg hypocerebral ganglion; ol optic lobe; pl protocerebral lobe; rn recurrent nerve; sog suboesophageal ganglion.

pair of neurons (ca. 15-20 µm in diameter) is located (Fig. 7).

The suboesophageal ganglion contains 14 immunoreactive neurons arranged in three ventro-medial groups (Fig. 5). A group of 4 neurons (ca. 20 µm in diameter) is located in front of the ganglion. A group of 8 neurons (ca. 20 µm) in diameter) lies more caudally, and just above both paraldehyde-fuchsine positive neurosecretory A-cells (Schooneveld 1970). Two more BPP-FMRFamide-like cells (ca. 25 µm in diameter) lie in the posterior part of the ganglion. Weakly immunoreactive neurons have sometimes been found in the deutocerebrum.

The axons of the neurons in the brain and suboesophageal ganglion are well visualized by the antisera. They run into the neuropil where they branch frequently. Virtually all neuropil areas in the brain and suboesophageal ganglion are invaded by these fibres (Fig. 5, 7, 8).

As to the stomatogastric nervous system, six small immunoreactive neurons (ca. 15 µm in diameter) and one larger neuron (ca. 25 µm in diameter) are usually present in the frontal ganglion (Fig. 9). The hypocerebral ganglion (Fig. 10) contains immunoreactive neurons with variable immunoreactivity. A few neurons staining very intensely are situated along the stomodaeal nerves (Fig. 6). The axons of these cells run along the paired nerves in the direction of the oesophagus.

The corpus cardiacum also contains fibres which react with both FMRFamide and BPP antisera as follows from staining of semi-thin alternate epon sections (Fig. 2). The fibres may reach the corpora cardiaca via the nervi corporis cardiaci

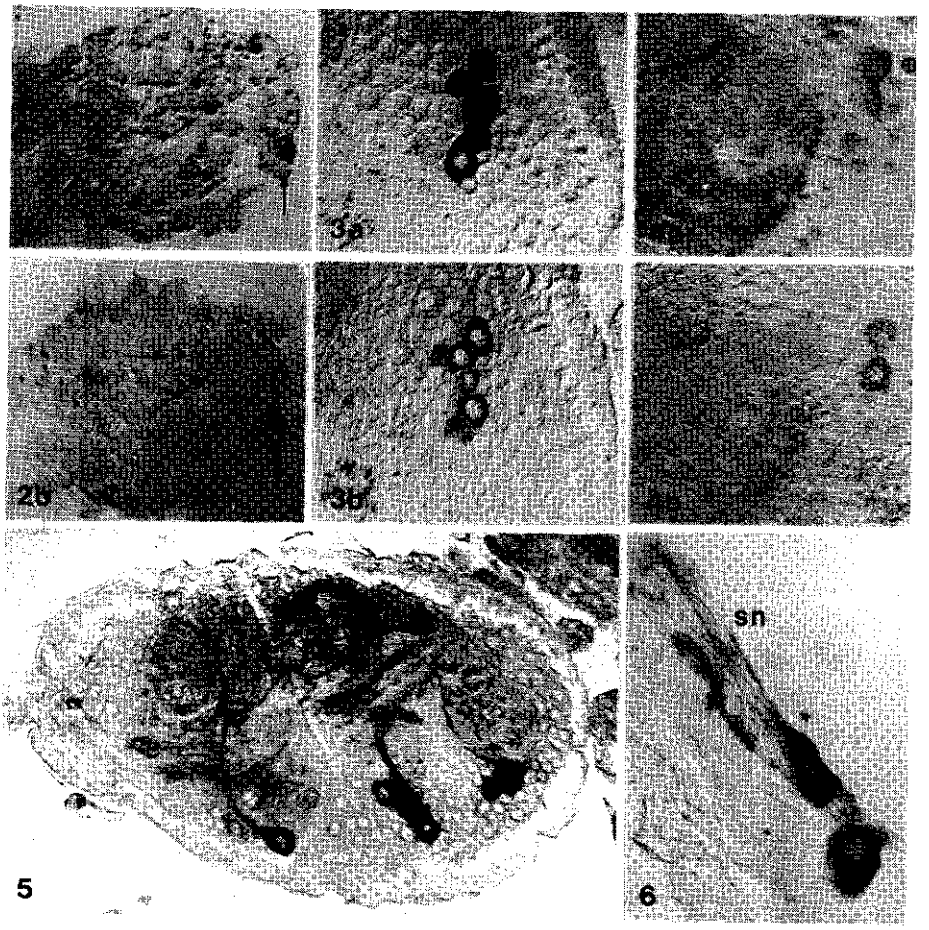


Fig. 2 **a, b.** Adjacent semi-thin Epon sections through corpus cardiacum showing immunoreactive axon terminals. **a** Localization of BPP-like immunoreactivity; **b** Localization of FMRFamide-like immunoreactivity. Note larger axon terminals revealed by both antisera (arrows). x308.
Fig. 3 **a, b.** Adjacent sections through suboesophageal ganglion. **a** Treated with BPP antiserum; **b** Treated with FMRFamide antiserum. Note immunoreactive cell bodies revealed by both antisera. x308.
Fig. 4 **a, b.** Adjacent sections through protocerebral lobe. **a** Treated with BPP antiserum; **b** Treated with FMRFamide antiserum. Note immunoreactive cell bodies revealed by both antisera. x308.
Fig. 5. Sagittal section through suboesophageal ganglion showing all three groups of BPP-FMRFamide-like neurons with axons and extensive distribution of immunoreactive fibres in neuropil. Frontal part of ganglion at right. x202.
Fig. 6. BPP-FMRFamide-like neurons associated with stomodaeal nerve sn. x481.

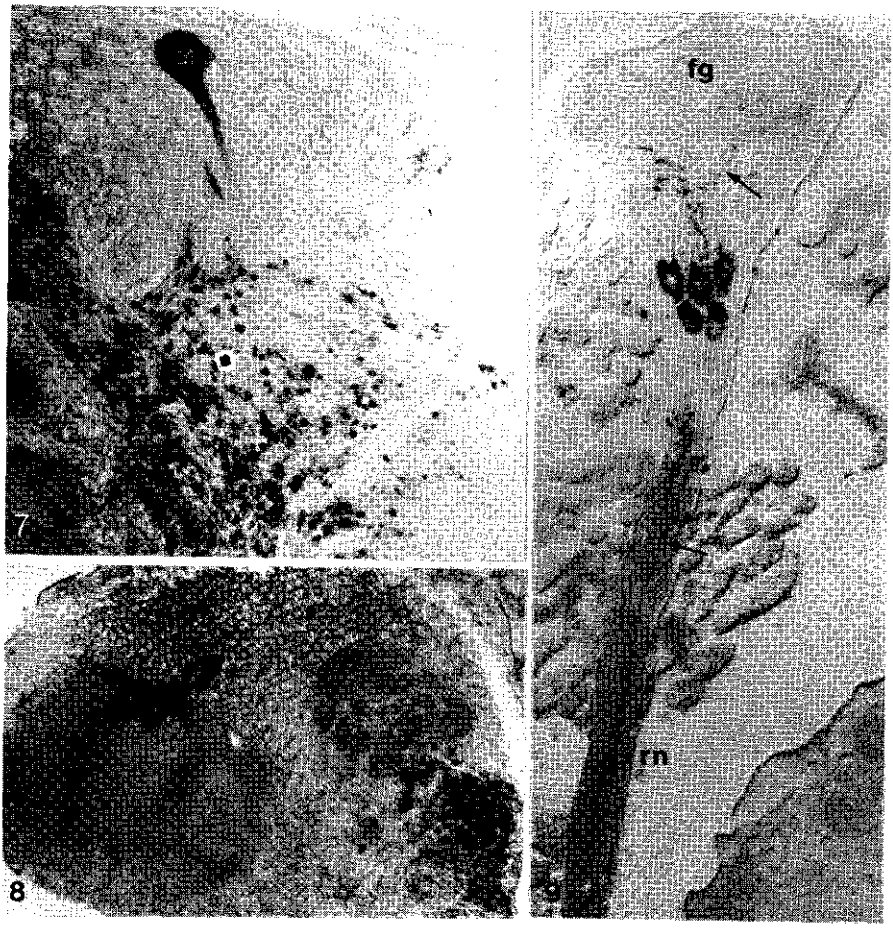


Fig. 7. BPP-FMRFamide-like neurons in protocerebral lobe with axon and extensive axon ramifications in neuropil. x432.
Fig. 8. Oblique horizontal section through optic lobe showing BPP-FMRF-amide-like neurons and their fibres. x202.
Fig. 9. BPP-FMRFamide-like neurons in frontal ganglion fg. Note axons (arrows) in ganglion and recurrent nerve (rn). x308.

(which connect the corpora cardiaca with the brain) as well as via the nervi corporis cardiaci suboesophageales, which connect the corpora cardiaca with the suboesophageal ganglion. To investigate which route carries most of the material, both nerve pairs were severed. After a week the cut ends of the nervi corporis cardiaci generate "de novo" neurohaemal organs, as found before in this insect (de Wilde and Boer 1969). We also found a bulbous structure with possible neurohaemal function at

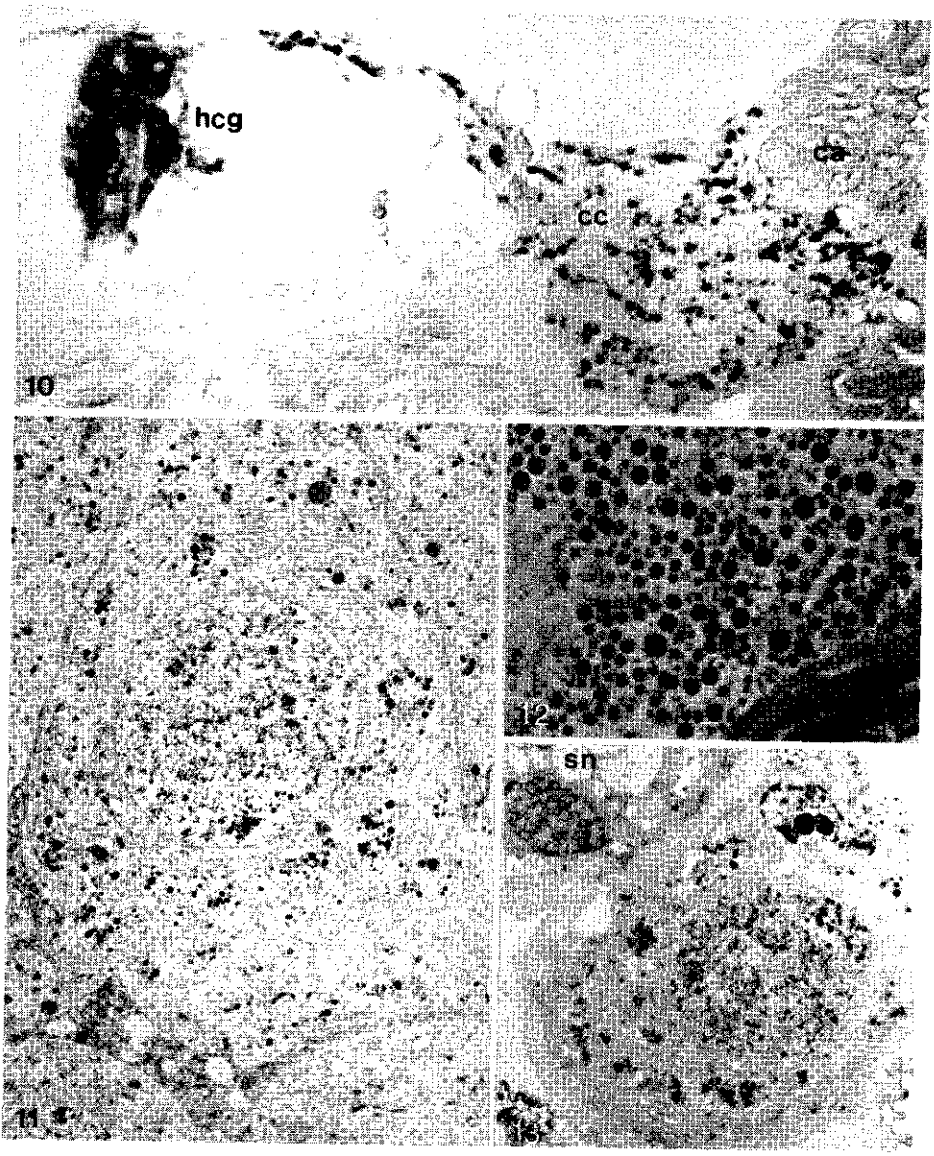


Fig. 10. Horizontal section through hypocerebral ganglion (hcg) containing BPP-FMRFamide-like neurons, corpus cardiacum (cc) with many BPP-FMRFamide-like neurosecretory axon terminals, and corpus allatum (ca). x263.

Fig. 11. Ultrastructure of BPP-FMRFamide-like neuron in suboesophageal ganglion. Note electron-dense granules in cytoplasm. x4800.

Fig. 12. Ultrastructure of BPP-FMRFamide-like axon terminal in corpus cardiacum. x12000.

Fig. 13. Ultrastructure of BPP-FMRFamide-like neuron on stomodaeal nerve sn. x4800.

the cut nervi corporis cardiaci suboesophageales, which contained an important accumulation of immunoreactive material. It seems therefore likely that most of the fibres in the corpora cardiaca are derived from neurons in the suboesophageal ganglion.

Ultrastructural studies of cells showing positive reactions in semi-thin sections show that the BPP-FMRFamide-like neurons in the suboesophageal ganglion (Fig. 11) and the neurons associated with the stomodaeal nerves (Fig. 13) all contain similar electron-dense granules, 100 to 200 nm in diameter. The positive fibres in the corpus cardiacum form neurosecretory axon terminals. They contain abundant electron-dense granules of the same size (Fig. 12).

The FMRFamide antiserum also recognized cells in rabbit pancreas. The same cells also react with anti-BPP serum as shown by staining of alternate sections. The immunoreactivity of the antisera was completely suppressed by absorption with the homologous antigens coupled to Sepharose 4B beads. The antisera had also lost their immunoreactivity in the beetle after cross-absorptions, but the immunoreactivity of the BPP-antiserum, when tested on rabbit pancreas, remained unchanged after preabsorption with FMRFamide coupled to Sepharose 4B.

DISCUSSION

This study shows that certain neurons in the nervous system of the head of the Colorado potato beetle contain a substance or substances which are recognized by both anti-FMRFamide and anti-BPP serum. As the specificity of anti-FMRF-amide sera may be restricted to an antigenic determinant of only a few amino acids in the peptide involved, there is a good chance that such antisera will cross-react with peptides containing similar amino acid sequences but no biological FMRF-amide-like activity (Price 1983). Anti-FMRFamide serum has been shown before to recognize particular cells in chicken pancreas (Dockray et al. 1981a). This reaction was later shown to be due to avian pancreatic polypeptide (Dockray and Williams 1983). Similar results have been reported from dog ileum, where FMRF-amide-like immunoreactivity occurs in cells containing a pancreatic polypeptide-like substance (Dockray et al. 1981b). It thus seems that antisera against FMRF-amide easily cross-react with peptides belonging to the pancreatic polypeptide family. We therefore consider it likely that the FMRFamide-like immunoreactivity in rabbit pancreas is due to the presence of pancreatic polypeptide.

Research in progress shows that some but not all of the BPP-FMRFamide-like neurons are also immunoreactive to an antiserum against vasopressin. This strongly

indicates the presence of more than one BPP-FMRFamide-like substance in the Colorado potato beetle. Similarly, the nervous system of the snail *Lymnaea stagnalis* was reported to contain several FMRFamide-like substances (Schot and Boer 1982).

There is a resemblance in the positions of the BPP-FMRFamide-like neurons in the Colorado potato beetle and the BPP-like neurons in the nervous system of two blowfly species, *Calliphora erythrocephala* and *C. vomitoria* (Duve and Thorpe 1980, 1982). However, immunoreactive fibres occur in low numbers in the neuropil and are absent in the corpus cardiacum of these flies. Such fibres are abundant in the Colorado potato beetle. Yet, considering their numbers and positions, the neurons in these holometabolous species seem to be homologous, which perhaps reflects their common ancestral origin. The numbers and positions of BPP-like neurons in the brain of the hemimetabolous cockroach *Periplaneta americana* (Endo et al. 1982) are different from those in the holometabolous species indicating the absence of homology. The Colorado potato beetle and the blowfly are phylogenetically more closely related to each other than to the more primitive cockroach. BPP-like cells have been reported from two other holometabolous insects, the silkworm *Bombyx mori* (Yui et al. 1980) and the hoverfly, *Eristalis aeneus* (El-Salhy et al. 1980). From both species only larval brains were studied, which makes a comparison with the blowfly and the beetle impossible; in holometabolous insects the structure and organization of the brain changes dramatically during metamorphosis.

Duve et al. (1981, 1982) isolated a peptide from blowfly heads which is similar to BPP, both in molecular size and in amino acid composition. With regard to the homology in localization of BPP-like substances in the brains of flies and beetles, the presence of a similar peptide in the Colorado potato beetle is indicated but remains to be proven.

We can only speculate on the function of the immunoreactive neurons. FMRFamide was originally isolated from a clam as a cardioexcitatory substance and was later shown to be effective on muscles of several molluscan species (Price and Greenberg 1977; Greenberg and Price 1979). The close association of BPP-FMRFamide-like neurons with the stomatogastric nervous system and the extensive distribution of immunoreactive neurons in the neuropil of the central nervous system suggest that the peptide is an important neurochemical mediator. The innervation of the gut musculature of a cockroach by BPP-like nerves (Iwanaga et al. 1981) also points to a possible role of the BPP-like substance as a neurotransmitter. The neurons on the stomodaeal nerves resemble "peripheral" neurosecretory cells as described in other insect species, e.g., the link-nerve neurons of *Carausius morosus* (Fifield and Finlayson 1978) and the peripheral neurosecretory cell on

the proctodaeal nerve of *Oryctes nasicornis* (Nagy 1978). On the other hand, some neurons may have a neurohormonal function. The immunoreactive cells in the sub-oesophageal ganglion probably release their peptide into the blood by means of axons terminating in the corpus cardiacum. It thus seems that the Colorado potato beetle provides a good parallel to the situation in vertebrates where biologically active peptides are not exclusively used as hormones, but have a transmitter function within the nervous system as well (cf. Hökfelt et al. 1980; Snyder 1980). The migratory locust, *Locusta migratoria*, represents a similar parallel in that its adipokinetic hormone or related immunoreactive substances are distributed throughout the central nervous system as well as the corpus cardiacum (Schooneveld et al. 1983).

REFERENCES

Boer HH, Schot LPC, Roubos EW, Maat A ter, Lodder JC, Reichelt D (1979) ACTH-like immunoreactivity in two electronically coupled giant neurons in the pond snail *Lymnaea stagnalis*. Cell Tissue Res 202: 231-240.

Boer HH, Schot LPC, Veenstra JA, Reichelt D (1980) Immunocytochemical identification of neural elements in the central nervous systems of a snail, some insects, a fish and a mammal with an antiserum to the molluscan cardio-excitatory tetrapeptide FMRF-amide. Cell Tissue Res 213: 21-27.

Dockray GJ, Williams RG (1983) FMRFamide-like immunoreactivity in rat brain: development of a radioimmunoassay and its application in studies of distribution and chromatographic properties. Brain Res 266: 295-303.

Dockray GJ, Vaillant C, Williams RG (1981a) New vertebrate brain-gut peptide related to a molluscan neuropeptide and an opioid peptide. Nature 293: 655-657.

Dockray GJ, Vaillant C, Williams RG, Gayton RJ, Osborne NN (1981b) Vertebrate brain-gut peptides related to FMRF-amide and met-enkephalin-Arg^{6}Phe^{7}. Peptides 2 (suppl. 2): 25-30.

Duve H, Thorpe A (1980) Localisation of pancreatic polypeptide (PP)-like immuno-reactive material in neurones of the brain of the blowfly, *Calliphora erythrocephala* (Diptera). Cell Tissue Res 210: 101-109.

Duve H, Thorpe A (1982) The distribution of pancreatic polypeptide in the nervous system and gut of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tisse Res 227: 67-77.

Duve H, Thorpe A, Neville R, Lazarus NR (1981) Isolation and partial characterization of pancreatic polypeptide-like material in the brain of the blowfly *Calliphora vomitoria*. Biochem J 197: 767-770.

Duve H, Thorpe A, Lazarus NR, Lowry PJ (1982) A neuropeptide of the blowfly *Calliphora vomitoria* with an amino acid composition homologous with vertebrate pancreatic polypeptide. Biochem J 201: 429-432.

El-Salhy M, Abou-El-Ela R, Falkmer S, Grimelius L, Wilander E (1980) Immunohistochemical evidence of gastro-entero-pancreatic neurohormonal peptides of vertebrate type in the nervous system of a dipteran insect, the hoverfly, *Eristalis aeneus*. Regul Pept 1: 187-204.

Endo Y, Iwagana T, Fujita T, Nishiitsutsuji-Uwo J (1982) Localization of pancreatic polypeptide (PP)-like immunoreactivity in the central and visceral nervous systems of the cockroach *Periplaneta*. Cell Tissue Res 227: 1-9.

Fifield SM, Finlayson LH (1978) Peripheral neurons and peripheral neurosecretion in the stick insect, *Carausius morosus*. Proc R Soc Lond Ser B 200: 63-85.

Greenberg MJ, Price DA (1979) FMRF-amide, a cardioexcitatory neuropeptide of molluscs: An agent in search of a mission. Am Zool 19: 163-174.

Hökfelt T, Johansson O, Ljungdahl A, Lundberg LM, Schultzberg M (1980) Peptidergic neurones. Nature 284: 515-520.

Iwanaga T, Fujita T, Nishiitsutsuji-Uwo J, Endo Y (1981) Immunohistochemical demonstration of PP-, somatostatin-, entero-glucagon- and VIP-like immunoreactivities in the cockroach midgut. Biomed Res 2: 202-207.

Nagy F (1978) Ultrastructure of a peripheral neurosecretory cell in the proctodaeal nerve of the larva of *Oryctes nasicornis* L. (Coleoptera: Scarabaeidae). Int J Insect Morphol Embryol 7: 325-336.

Price DA (1983) FMRF-amide: assays and artifacts. In: Lever J, Boer HH (eds) Molluscan neuroendocrinology. North-Holland Publishing Company, Amsterdam, Oxford, New York, pp 184-190.

Price DA, Greenberg MJ (1977) Purification and characterization of a cardioexcitatory neuropeptide from the central ganglia of a bivalve mollusc. Prep Biochem 7: 50-62.

Raabe M (1982) Neurohormones. Plenum Press, New York, London.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado beetle, *Leptinotarsa decemlineata* (Say), as a function of daylength. Neth J Zool 20: 151-237.

Schooneveld H (1974) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) I. Characterization of the protocerebral neurosecretory cells. Cell Tissue Res 154: 275-288.

Schooneveld H, Tesser GI, Veenstra JA, Romberg-Privee H (1983) Adipokinetic hormone and AKH-like peptide demonstrated in the corpora cardiaca and nervous system of *Locusta migratoria* by immunocytochemistry. Cell Tissue Res 230: 67-76.

Schot LPC, Boer HH (1982) Immunocytochemical demonstration of peptidergic cells in the pond snail *Lymnaea stagnalis* with an antiserum to the molluscan cardioexcitatory tetrapeptide FMRF-amide. Cell Tissue Res 225: 347-354.

Snyder SH (1980) Brain peptides as neurotransmitters. Science 209: 976-983.

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall Inc New Yersey.

Wilde J de, Boer JA de (1969) Humoral and nervous pathways in photoperiodic induction of diapause in *Leptinotarsa decemlineata*. J Insect Physiol 15: 661-67.

Yui R, Fujita T, Ito S (1980) Insulin-, gastrin-, pancreatic polypeptide-like immunoreactive neurons in the brain of the silkworm, *Bombyx mori*. Biomed Res 1: 42-46.

IMMUNOCYTOCHEMICAL LOCALIZATION OF PEPTIDERGIC CELLS IN THE NEURO–ENDOCRINE SYSTEM OF THE COLORADO POTATO BEETLE, Leptinotarsa decemlineata, WITH ANTISERA AGAINST VASOPRESSIN, VASOTOCIN AND OXYTOCIN

SUMMARY

Antisera against vasopressin, vasotocin, oxytocin, neurophysin-1 and neurophysin-2 were used to investigate immunocytochemically the presence of neurons containing substances antigenically related to these peptides in the nervous system of the Colorado potato beetle. Ten different antisera were used, four against vasopressin, three against oxytocin and one against vasotocin, neurophysin-1, and neurophysin-2. Immunoreactivity was shown by all antisera except those against the neurophysins. The vasopressin antisera all gave different results. One antiserum revealed only a single neuron pair, whereas others revealed in addition one or two other different cell groups. The oxytocin antisera likewise revealed different neurons. The fixation procedure influenced the outcome of the immunocytochemical reaction. Immunoreactivity as revealed by vasopressin, vasotocin and oxytocin antisera is often co-localized in the same neurons; solid phase adsorptions showed that this is due to cross-reactivity of the antisera. Some of the immunoreactive neurons are identical to those recently described to contain a bovine pancreatic polypeptide/FMRFamide-like peptide. This co-localization is probably not due to a cross-reaction. These findings indicate the presence of several vasopressin-like and oxytocin-like substances which in the Colorado potato beetle all have a different degree of immunocytochemical resemblance to vasopressin and oxytocin.

INTRODUCTION

By means of selective neurosecretory stains and electron microscopy several types of peptidergic neurons have been distinguished in the brain of the Colorado potato beetle, *Leptinotarsa decemlineata* (Schooneveld 1970, 1974) and in several other insect species (Raabe 1982). Later on, other peptidergic neurons have been demonstrated by immunocytochemistry with antisera to regulatory vertebrate peptides (e.g. El-Salhy et al. 1980, 1983; Yui et al. 1980). Recently in the Colorado potato beetle peptidergic neurons were immunocytochemically identified with antisera to bovine pancreatic polypeptide (BPP) and the molluscan cardioexcitatory tetrapeptide FMRFamide (Veenstra and Schooneveld 1984). The two antisera cross-react in this species. This confirms that specificity in immunocytochemistry may be limited (e.g. Swaab et al. 1977), as BPP and FMRFamide are chemically rather different.

It appears that peptides immunochemically related to vasopressin, vasotocin and oxytocin are widely distributed in the animal kingdom. Thus vasopressin- and oxytocin-like substances have been detected in several insect species (Rémy et al. 1977, 1979; Strambi et al. 1979; Rémy and Girardie 1980; cf. Proux and Rougon-Rapuzzi 1980; Hansen et al. 1982), in a prawn (van Herp and Bellon-Humbert 1982), in a cephalopod (Martin et al. 1980), in a snail (Schot et al. 1981), in a seahare (Moore et al. 1981) and in *Hydra* (Grimmelikhuizen et al. 1982). In addition neurophysin-like substances have been found in the locust *Locusta migratoria* (Rémy et al. 1979; Camier et al. 1980) and in the prawn *Palaemon serratus* (van Herp and Bellon-Humbert 1982).

This paper deals with peptidergic neurons in the Colorado potato beetle which contain substances that are immunocytochemically related to vasopressin, vasotocin and oxytocin. Special attention was paid to specificity problems by comparing the immunocytochemical results of different antisera against the same peptide. Furthermore changes in immunoreactions caused by variation in the fixation procedure were studied.

MATERIALS AND METHODS

Seven to twelve-day-old reproducing beetles (more than 300 specimens) were used. They were reared in the laboratory under long-day conditions. Some were injected with 1.5 to 5 µg of colchicine (Sigma) two or three days before use. For light microscopy the brain, retrocerebral complex and suboesophageal ganglion were dissected out in Ringer and fixed for 4 to 20 hrs in GPA (a mixture of one

volume 25% glutaraldehyde, three volumes of a saturated aquaeous solution of picric acid with 1% acetic acid), or BHS (Bouin Hollande, without acetic acid and with 10% of saturated mercuric chloride), or for 1 to 2 hrs in icecold GAB (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 1 mM $CaCl_2$). Paraplast sections were cut at 7 µm. For electron microscopy tissues were fixed for 1 to 2 hrs in icecold GAB and embedded in the epon 812 substitute LX-112 (Ladd Industries). Consecutive ultra-thin and semi-thin (0.5 µm) sections were cut. The ultra-thin sections were contrasted with uranyl acetate and lead citrate and used for ultrastructural study, after the identification of immunoreactive elements in the semi-thin sections with the light microscope. The resin was removed from the semi-thin sections with a saturated solution of NaOH in methanol (15 min).

Four different antisera against vasopressin were used (VP1, VP2, VP3 and VP4), three against oxytocin (OT1, OT2 and OT3), one against vasotocin (VT), one against neurophysin-1 (NP1) and one against neurophysin-2 (NP2). The antisera VP1 (nr. 125), VP2 (nr. 126), OT1 (nr. 02D) and VT (nr. 82) were a kind gift from Prof. Dr. D.F. Swaab, Amsterdam; antisera VP3 (nr. 134-III), VP4 (nr. 135-III), OT2 (nr. 132-II), and OT4 (nr. 133-IV) were a kind gift from Dr. C.J.P. Grimmelikhuijzen, Heidelberg; and antisera NP1 and NP2 were kindly supplied by Prof.Dr. K. Dierickx, Gent. For comparison with the recently described BPP/FMRF-amide-like neurons (Veenstra and Schooneveld 1984), an antiserum against bovine pancreatic polypeptide (nr. 615-R110-146-16), a kind gift from Dr. R.E. Chance, Indianapolis, or an antiserum against FMRFamide (nr. 646), a kind gift from Dr. L.P.C. Schot, Amsterdam, was used. Vasopressin and vasotocin were purchased from Sanbio, oxytocin from Sigma and FMRFamide from Peninsula Laboratories. Bovine pancreatic polypeptide was a kind gift from Dr. R.E. Chance, Indianapolis.

Sections were immunocytochemically stained with the indirect peroxidase-anti-peroxidase (PAP) method (Sternberger, 1974). Endogenous peroxidase was inhibited by 0.01% H_2O_2 in water for 20 minutes. Incubations with the primary antisera were performed overnight at 4°C. The optimal dilutions of the primary antisera were as follows: VP1 1:2000, VP2: 1:2000, VP3: 1:1000, VP4: 1:1000, VT 1:2000, OT2 1:4000, OT3 1:1000, FMRFamide 1:2000, BPP 1:2000. As a substrate for the peroxidase 3.3'diaminobenzidine (Sigma) was used. The possibility that two antisera reacted with the same neurons was tested by staining alternate 5µm sections. The specificity of the primary antisera was checked by preadsorbing the diluted antisera 3 times with 10 nmol of the antigens coupled to Sepharose 4B beads according to Pharmacia instructions. Cross-adsorptions between antisera and antigens were performed in the same way.

Paraldehyde fuchsin staining (Schooneveld 1970) was used for the identification of classical neurosecretory cells.

RESULTS

Five different groups of cells were revealed by the antisera to vasopressin, vasotocin and oxytocin (Fig. 1). Fixation appeared to be important for immunoreactivity. Not all these neurons are immunoreactive with all antisera. Details of the immunoreactivities of the different neuron groups are summarized in Table 1.

(1) In the optic lobe a group of 5 to 7 small neurons contains vasopressin-like immunoreactivity. These neurons are also immunoreactive with BPP and FMRF-amide antisera, as was demonstrated by staining alternating sections (Fig. 2). Their axons surround and innervate the medulla externa.

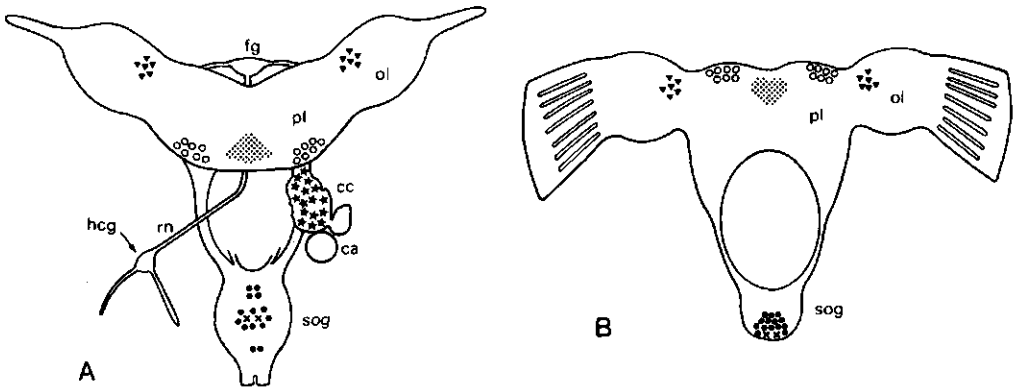


Fig. 1. Diagram of nervous system in head of Colorado potato beetle showing positions of immunoreactive neurons, paraldehyde fuchsin positive cells in the suboesophageal ganglion: crosses; neurons in the suboesophageal ganglion which are also BPP/FMRFamide-immunoreactive: solid circles; neurons in the optic lobe: triangles; intrinsic secretory cells of the corpus cardiacum: asterisks; lateral neurosecretory cells: open circles. A, projection in transversal plane; B, projection in horizontal plane. For clarity the recurrent nerve has been enlarged and the corpus cardiacum and corpus allatum on the left side removed. Stippled area in the brain indicates the location of the median neurosecretory cells. *ca*, corpus allatum; *cc*, corpus cardiacum; *fg*, frontal ganglion; *hcg*, hypocerebral ganglion; *ol*, optic lobe; *pl*, protocerebral lobe; *rn*, recurrent nerve; *sog*, suboesophageal ganglion.

Table 1. Vasopressin-, vasotocin- and oxytocin-like immunoreactive neurons; their location and their identification by the antisera after the different fixation procedures.

cells	fixation(s) governing immunoreactivity	antisera recognizing neuropeptide
(1) BPP/FMRFamide-like neurons in optic lobe	GPA BHS	VP2, VP4
(2) Pair of paraldehyde fuchsin positive neurons in suboesophageal ganglion	GAB GPA BHS	VP1, VP2, VP3, VP4, VT, OT1, OT2, OT3
(3) Three groups of BPP/FMRFamide-like neurons in suboesophageal ganglion	GPA BHS	VP1, VT, OT1
(4) Lateral neurosecretory cells in protocerebrum	BHS	OT2
(5) Intrinsic secretory cells of corpus cardiacum	GPA BHS	VP4

(2) A pair of neurosecretory cells in the suboesophageal ganglion is immunoreactive with all antisera and after all three fixation methods; the cells also react with paraldehyde fuchsin. Axons of these neurons reach the brain via the contralateral circumoesophageal connectives. Large arborizations are present in the deutocerebrum and a few dendrites are sometimes found in the optic lobe. Other branches of the same neurons leave the suboesophageal ganglion to the thoracic ganglia; their final destinations have not been determined. No axons of these cells have been observed to enter the corpora cardiaca.

(3) An other group of immunoreactive neurons is located in the ventro-median part of the suboesophageal ganglion. It consists of three subgroups, containing 4, 8 and 2 neurons. The subgroup of 8 neurons is located just above the paraldehyde fuchsin positive neurons. The subgroup of 4 is located more frontally, and that of 2 neurons more caudally in the ganglion. These neurons are also BPP/FMRF-amide-like immunoreactive, as could be demonstrated by staining alternating sections (Fig. 3). Axons of these cells leave the suboesophageal ganglion via the nervi corporis cardiaci suboesophageales to the corpora cardiaca, where they branch frequently; axon terminals are found throughout the corpora cardiaca (Fig. 4b). Other axons leave the ganglion via the circumoesophageal connectives to the brain, where they end around the median neurosecretory cells (Fig. 5).

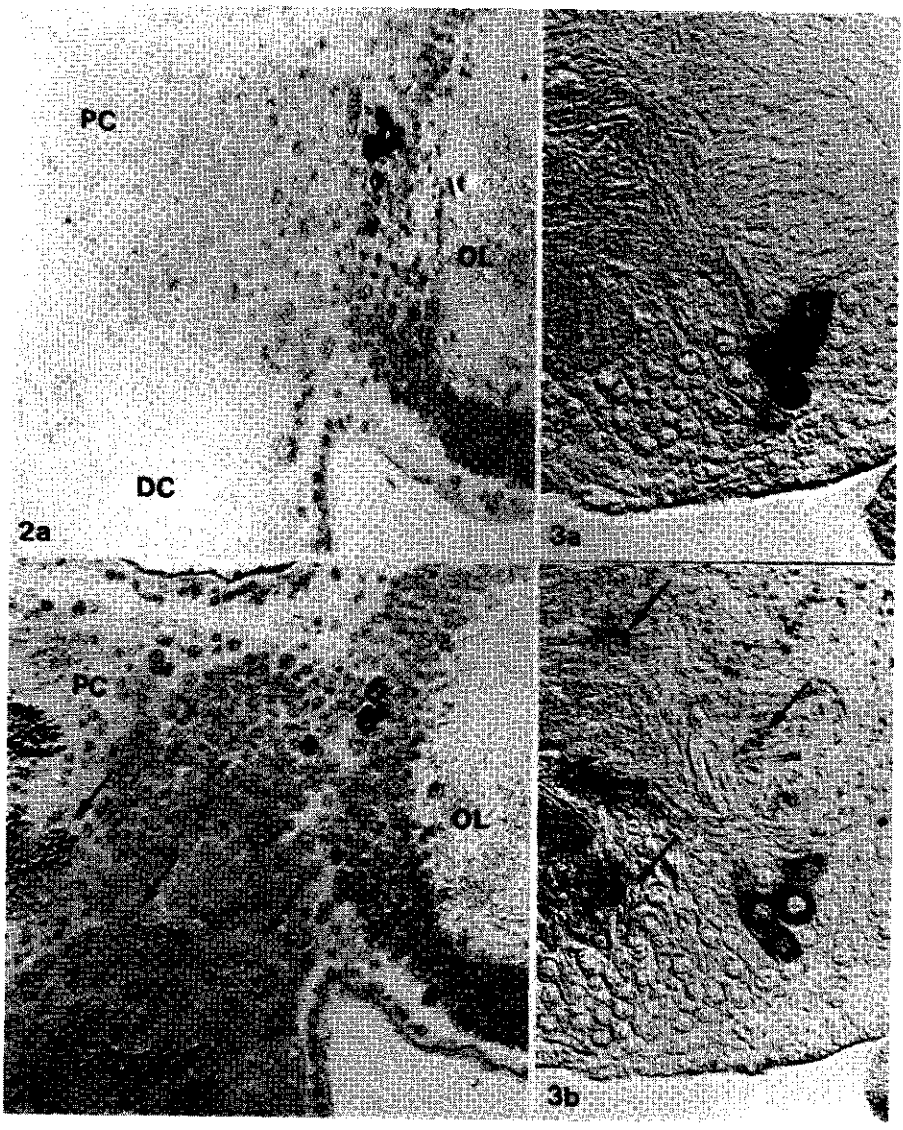


Fig. 2a, b. Adjacent sections through brain, GPA fixation. a, treated with VP4 antiserum; b, treated with BPP antiserum. Note immunoreactive cell bodies in the optic lobe (*ol*) revealed by both antisera, while in protocerebrum (*pc*) and deutocerebrum (*dc*) immunoreactive fibres (arrows) from other neurons are present that react only with BPP antiserum. x300.
Fig. 3a, b. Adjacent sections through suboesophageal ganglion, GPA fixation. a, treated with VP1 antiserum; b, treated with FMRFamide antiserum. Note immunoreactive cell bodies revealed by both antisera and immunoreactive fibres (arrows) in neuropil, derived from other neurons, revealed only by FMRFamide antiserum. x300.

(4) Several lateral neurosecretory cells react with antiserum OT2, but only after BHS fixation (Fig. 6). Axons of these cells run to the corpora allata, where they branch; numerous immunoreactive axon terminals are present between the corpus allatum cells (Fig. 4c). The cell bodies of this group were only revealed in colchicine injected animals, but their axons and axon terminals in the corpora allata were also immunoreactive in untreated animals.

(5) In the corpus cardiacum about fifty intrinsic secretory cells, scattered in small groups all over the glands, were immunoreactive with antiserum VP4 (Fig. 4a). The extrinsic secretory cells of the corpus cardiacum, which are arranged in a cluster close to the corpus allatum, were not immunoreactive.

None of the antisera to vasopressin, vasotocin and oxytocin reacted immunocytochemically with the median neurosecretory cells of the pars intercerebralis.

With antisera NP1 and NP2 in concentrations of 1:1000 or lower, no neurophysin-like immunoreactive neurons were observed. Higher concentrations of NP2 gave a strong background staining. The NP1 antiserum gave only some positive reaction with concentrations as high as 1:150 to 1:300. As the same antiserum can be used in dilutions of 1:2000 for other insect species, we considered this reaction unspecific.

Ultrastructural studies were necessarily limited to the paraldehyde fuchsin positive neurons of the suboesophageal ganglion, as they were the only immunoreactive neurons after appropriate fixation for electron microscopy (GAB). Electron-dense granules (150-250 nm in diameter) can be distinguished in the cytoplasm of these cells (Fig. 7).

The results of the adsorption controls have been summarized in Table 2.

DISCUSSION

This study shows that five groups of peptidergic cells can be distinguished on the basis of immunocytochemical identification with antisera to vasopressin, vasotocin and oxytocin. All immunoreactivities were specific, since staining was blocked after adsorptions of the antisera with the homologous peptides. However this does not mean that vasopressin, vasotocin and oxytocin are indeed present in these cells. Neither are we sure whether one or more immunoreactive molecules are involved. Polyvalent antisera as used in this study contain populations of different antibodies, which have affinities to different parts of the antigen. Vasopressin, vasotocin and oxytocin differ in only two amino acids. Antisera to one of these three peptides therefore usually also contain antibodies which react with the invariant parts of these peptides and are therefore likely to react also with the

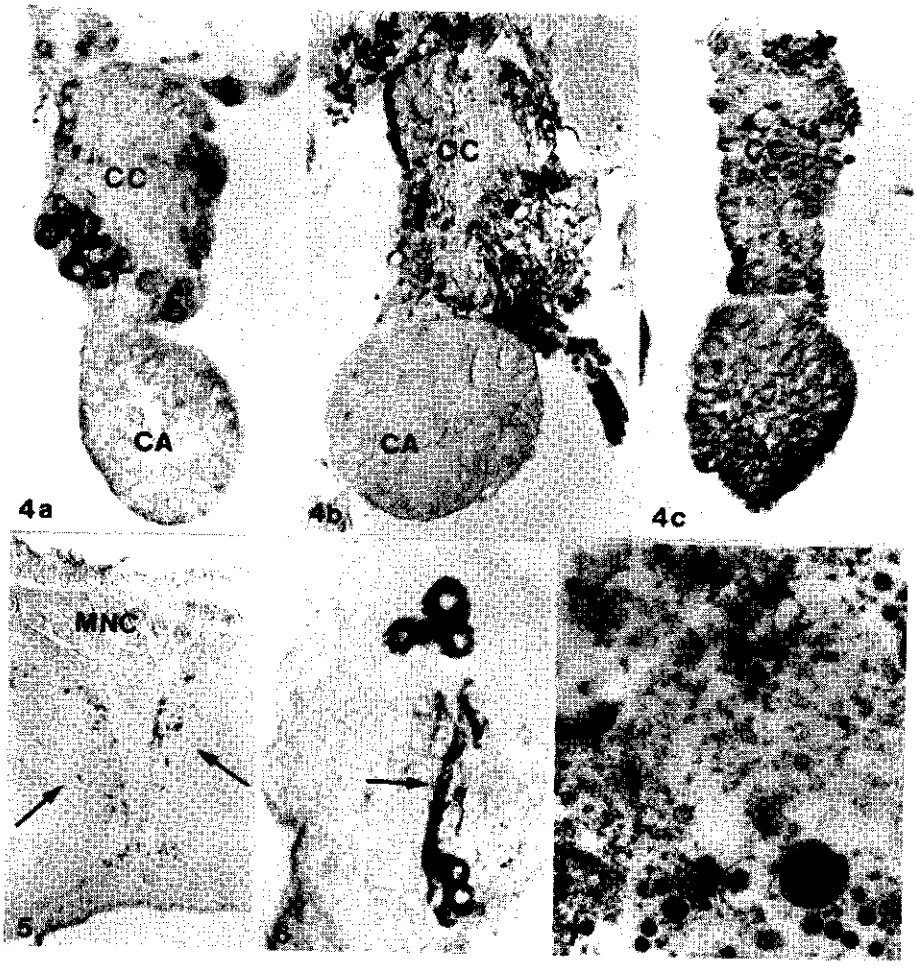


Fig. 4a, b, c. Sections through the corpus cardiacum *(cc)* and corpus allatum *(ca)*. a, treated with VP4 antiserum after GPA fixation, x230 ; b, treated with VP1 antiserum after GPA fixation, x250 ; c, treated with OT2 antiserum after BHS fixation, x210. Note immunoreactive glandular cells in corpus cardiacum in (a), immunoreactive axon terminals in the corpus cardiacum in (b) and (c) and that immunoreactive axon terminals in the corpus allatum are absent in (a) and (b), while they are present in (c).

Fig. 5. Immunoreactive fibres (arrows) from neurons of the suboesophageal ganglion terminating in the *neuropil* around the median neurosecretory cells *(mnc)*, as demonstrated with antiserum VP1. x150.

Fig. 6. Immunoreactive lateral neurosecretory cells and their axons (arrows) demonstrated with OT2 antiserum after BHS fixation. x300.

Fig. 7. Ultrastructure of cytoplasm of paraldehyde fuchsine positive vasopressin-like neuron in the suboesophageal ganglion. Note presence of electron dense granules. x14 000.

Table 2. Solid phase-adsorption experiments. Results are expressed as positive (+) if the antisera showed no residual immunoreactivity and negative (-) if reactivity was completely retained.

	adsorbens				
antiserum	Vasopressin	Vasotocin	Oxytocin	FMRFamide	BPP
VP1	+	+	+	nt	nt
VP2	+	+	+	-	-
VP3	+	+	+	-	-
VP4	+	+	+	-	-
VT	+	+	+	-	-
OT1	+	+	+	-	-
OT2	+	+	+	-	-
OT3	+	+	+	-	-
a-FMRFamide	-	-	-	+*)	+*)
a-BPP	-	-	-	+*)	+*)

nt: not tested
*) data from Veenstra and Schooneveld (1984)

other two peptides. For a specific localization of e.g. vasopressin in mammals, the vasopressin antiserum is differentially adsorbed with oxytocin on solid phase to remove antibodies which cross-react with oxytocin (e.g. Vandesande and Dierickx 1975; van Leeuwen et al. 1979). Sometimes rather unexpected cross-reactions may occur. Thus it has been reported that the vasopressin antisera VP1 and VP2 used in this study cross-react with α-MSH cells in the pituitary (van Leeuwen et al. 1979). For these reason the chemical nature of the immunocytochemically revealed substances remain unresolved (e.g. Swaab et al. 1977; van Leeuwen 1982). Yet, all antisera to vasopressin, vasotocin and oxytocin react with some substance in the paraldehyde fuchsin positive cells in the suboesophageal ganglion. It seems unlikely, that this is coincidental and that the immunoreactivity can be explained only as a cross-reaction with a peptide entirely unrelated to vasopressin, vasotocin and oxytocin. We assume that the chemical structure of the peptide present in those cells is very similar to these vertebrate peptides. A similar vasopressin-like peptide has been demonstrated in *Locusta migratoria* (Proux and Rougon-Rapuzzi 1980), although this peptide has a larger molecular size than vasopressin (Cupo and Proux 1983). The vasopressin/vasotocin/oxytocin-like substances in other types of neurons did not react with some of the antisera and may therefore be different from the above substance, and be less similar to the vertebrate peptides.

It was demonstrated that the method of fixation greatly affects the immunoreactivity of the peptides present in the various neurons. Similar observations were made on the FMRFamide-like immunoreactivity of the pond snail *Lymnaea stagnalis* (Schot and Boer 1982). This is another argument for the assumption that the Colorado potato beetle contains several distinct vasopressin/vasotocin/oxytocin-like peptides.

The co-localization of vasopressin-, vasotocin- and oxytocin-immunoreactivities in the same neurons suggests that the antisera reacted with the same substance. This was confirmed by cross-adsorptions between these peptides and their antisera. We also found a co-localization of BPP/FMRFamide-like and vasopressin-like material in the optic lobe and with vasopressin/oxytocin/vasotocin-like material in the suboesophageal ganglion. Cross-adsorptions were not effective in abolishing immunoreactivity in this case, suggesting that two separate antigenic determinants are present in the same neurons. This does not necessarily mean that these neurons contain two peptides, as both antigenic determinants might be present on the same peptide (Boer and Schot 1983).

Immunocytochemically detectable vasopressin-like material has been reported from several insect species. In *Clitumnus extradentatus* (Rémy et al. 1977), *Acheta domesticus* (Strambi et al. 1979) and *Locusta migratoria* (Rémy et al. 1979; Rémy and Girardie 1980) the suboesophageal ganglion contains a pair of ventro-median cells, which are immunoreactive with an antiserum against vasopressin. These neurons are also strongly paraldehyde fuchsin positive. Strambi and coworkers (cited by Proux and Rougon-Rapuzzi 1980) found similar results for species of *Periplaneta, Blabera, Gryllus* and *Polistes*. It seems that these neurons in all these species are homologous with the paraldehyde fuchsin positive neurons in the Colorado potato beetle: (1) they have the same histochemical characteristics (anti-vasopressin positive, paraldehyde fuchsin positive) and (2) they are located in the same area of the ganglion. So it is clear that immunocytochemistry may be an useful tool for establishing homologies between peptidergic neurons of different insect species. However it is also clear that such homologies may be easily masked as antisera against the same peptide may have different specificities. In the species investigated by the above authors vasopressin-like axon terminals have not been found in the corpora cardiaca. On the other hand ocytocin- and vasopressin-like material has been reported to be present in the release area of the corpus cardiacum of *Leucophaea maderae* by Hansen et al. (1982). In the Colorado potato beetle the demonstration of material immunoreactive to antisera against vasopressin and oxytocin in the corpus cardiacum is dependent on the specificity of the antiserum

as well as the fixation method. So it seems well possible that the discrepancy between the results of Hansen et al. (1982) and the other workers is in fact due to the use of antisera with different specificities.

Whereas neurophysin-2-like immunoreactivity has been found in two insect species, i.e. *Clitumnus extradentatus* (Rémy et al. 1977) and *Locusta migratoria* (Rémy et al. 1979; Rémy and Girardie 1980), no such immunoreactivity was found in the Colorado potato beetle. Probably the antiserum used in the present study recognizes another part of the neurophysin-2 molecule than the antiserum used by Rémy and coworkers. This hypothesis is sustained by the observation that the neurophysin-2 antiserum used in this study did not react with the neurophysin-2-like cells in the suboesophageal ganglion of *Locusta migratoria* after BHS fixation, whereas it did react with neurons in the brain of this insect species (unpubl. observations).

Only speculations on the functions of the immunoreactive cells (and the peptides they contain) can be made, as there are no experimental data on their possible functions. As mentioned earlier a vasopressin-like peptide has been isolated from *Locusta migratoria* (Proux and Rougon-Rapuzzi 1980; Cupo and Proux 1983). There is good evidence that this peptide acts as a diuretic hormone (Proux et al. 1982). As the vasopressin-like cells in the suboesophageal ganglion of the Colorado potato beetle and *Locusta migratoria* are homologous (Veenstra 1984), a vasopressin-like peptide with a similar function in the Colorado potato beetle may be expected.

The oxytocin-like lateral neurosecretory cells are obviously the same neurons that in horse radish peroxidase backfilling experiments have been shown to innervate the corpora allata (Khan et al. 1984). These neurons are possibly involved in the control of corpus allatum function. The peptides produced by the intrinsic secretory cells of the corpus cardiacum and the vasopressin/vasotocin/oxytocin/BPP/FMRF-amide-like neurons in the suboesophageal ganglion are probably released from the corpora cardiaca into the blood. The immunoreactive neurons in the optic lobe have extensive axon ramifications within the neuropile: their peptide therefore possibly functions as a neurotransmitter/neuromodulator.

REFERENCES

Boer HH, Schot LPC (1983) Phylogenetic aspects of peptidergic systems. In: Lever J, Boer HH (eds) Molluscan neuroendocrinology. North-Holland Publishing Company, Amsterdam, Oxford, New York, pp. 9-14.

Camier M, Girardie J, Rémy C, Girardie A, Cohen P (1980) Identification of immunoreactive neurophysin-like proteins in the central nervous system of an insect: *Locusta migratoria*. Biochem Biophys Res Comm 93: 792-796.

Cupo A, Proux J (1983) Biochemical characterization of a vasopressin-like neuropeptide in *Locusta migratoria*. Evidence of a high molecular weight protein encoding vasopressin sequence. Neuropeptides 3: 309-318.

El-Salhy M, Abou-El-Ela R, Falkmer S, Grimelius L, Wilander E (1980) Immunohistochemical evidence of gastro-entero-pancreatic neurohormonal peptides of vertebrate type in the nervous system of a dipteran insect, the hoverfly, *Eristalis aeneus*. Regul Peptides 1: 187-204.

El-Salhy M, Falkmer S, Kramer KJ, Speirs RD (1983) Immunohistochemical investigations of neuropeptides in the brain, corpora cardiaca, and corpora allata of an adult lepidopteran insect, *Manduca sexta* (L) Cell Tissue Res 232: 295-317.

Grimmelikhuijzen CJP, Dierickx K, Boer GJ (1982) Oxytocin/vasopressin-like immunoreactivity is present in the nervous system of *Hydra*. Neuroscience 7: 3191-3199.

Hansen BL, Hansen GN, Scharrer B (1982) Immunoreactive material resembling vertebrate neuropeptides in the corpus cardiacum and corpus allatum of the insect *Leucophaea maderae*. Cell Tissue Res 225: 319-329.

Herp F van, Bellon-Humbert C (1982) Localisation immunocytochemique de substances apparentées à la neurophysine et à la vasopressine dans le pédoncule oculaire de *Palaemon serratus* Pennant (Crustacé Décapode Natantia). C R Acad Sc Paris 295 (III): 97-102.

Khan MA, Romberg-Privee HM, Schooneveld H (1984) Innervation of the corpus allatum in the Colorado potato beetle as revealed by retrograde diffusion with horse radish peroxidase. Gen Comp Endocrinol 55: 66-73.

Leeuwen F van (1982) Specific immunocytochemical localization of neuropeptides: A utopian goal? In: Bullock GR, Petrusz P (ed) Techniques in immunocytochemistry. Vol 1, Academic Press, London, pp. 283-299.

Leeuwen FW van, Raay C de, Swaab DF, Fisser B (1979) The localization of oxytocin, vasopressin, somatostatin and luteinizing hormone releasing hormone in the rat neurohypophysis. Cell Tissue Res 202: 189-201.

Martin R, Frösch D, Voigt KH (1980) Immunocytochemical evidence for vasopressin-like material in a cephalopod neurohaemal organ. Gen Comp Endocrinol 42: 235-243.

Moore GJ, Thornhill JA, Gill V, Lederis K, Lukowiak K (1981) An arginine vasotocin-like neuropeptide is present in the nervous system of the marine mollusc *Aplysia californica*. Brain Res 206: 213-218.

Proux J, Rougon-Rapuzzi G (1980) Evidence for vasopressin-like molecule in migratory locust. Radioimmunological measurements in different tissues: correlations with various states of hydration. Gen Comp Endocrinol 42: 378-383.

Proux J, Rougon G, Cupo A (1982) Enhancement of excretion across locust malpighian tubules by a diuretic vasopressin-like hormone. Gen Comp Endocrinol 47: 449-457.

Raabe M (1982) Neurohormones. Plenum Press, New York, London.

Rémy C, Girardie J, Dubois MP (1977) Exploration immunocytologique des ganglions cérébroides et sous-oesophagien du phasme *Clitumnus extradentatus*: existence d'une neurosécrétion apparenté à la vasopressine-neurophysine. C R Acad Sc Paris 285D: 1495-1497.

Rémy C, Girardie J, Dubois MP (1979) Vertebrate neuropeptide-like substances in the suboesophageal ganglion of two insects: *Locusta migratoria* R and F (Orthoptera) and *Bombyx mori* L. (Lepidoptera). Immunocytological investigation. Gen Comp Endocrinol 37: 93-100.

Rémy C, Girardie J (1980) Anatomical organization of two vasopressin-neurophysin-like neurosecretory cells throughout the central nervous system of the migratory locust. Gen Comp Endocrinol 40: 27-35.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado beetle, *Leptinotarsa decemlineata* (Say), as a function of daylength. Neth J Zool 20: 151-237.

Schooneveld H (1974) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) I. Characterization of the protocerebral neurosecretory cells. Cell Tissue Res 154: 275-288.

Schot LPC, Boer HH, Swaab DF, Noorden S van (1981) Immunocytochemical demonstration of peptidergic neurons in the central nervous system of the pond snail *Lymnaea stagnalis* with antisera raised to biologically active peptides of vertebrates. Cell Tissue Res 216: 273-291.

Schot LPC, Boer HH (1982) Immunocytochemical demonstration of peptidergic cells in the pond snail *Lymnaea stagnalis* with an antiserum to the molluscan cardioexcitatory tetrapeptide FMRFamide. Cell Tissue Res 225: 347-354.

Sternberger LA (1974) Immunocytochemistry. Prentice Hall Inc. New Yersey.

Strambi C, Rougon-Rapuzzi G, Cupo A, Martin N, Strambi A (1979) Mise en évidence immunocytologique d'un composé apparentée à la vasopressine dans le système nerveux du grillon *Acheta domesticus*. C R Acad Sc Paris 288D: 131-133.

Swaab DF, Pool CW, Leeuwen FW van (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-391.

Vandesande F, Dierickx K (1975) Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic magnocellular neurosecretory system of the rat. Cell Tissue Res 164: 153-162.

Veenstra JA (1984) Immunocytochemical demonstration of a homology in peptidergic neurosecretory cells in the suboesophageal ganglion of a beetle and a locust with antisera to bovine pancreatic polypeptide, FMRFamide and α-MSH. Neurosci Lett 48: 185-190.

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of peptidergic neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235: 303-308.

Yui R, Fujita T, Ito S (1980) Insulin-, gastrin-, pancreatic polypeptide-like immunoreactive neurons in the brain of the silkworm, *Bombyx mori*. Biomed Res 1: 42-46.

IMMUNOCYTOCHEMICAL DEMONSTRATION OF A HOMOLOGY IN PEPTIDERGIC NEUROSECRETORY CELLS IN THE SUBOESOPHAGEAL GANGLION OF A BEETLE AND A LOCUST WITH ANTISERA TO BOVINE PANCREATIC POLYPEPTIDE, FMRFAMIDE, VASOPRESSIN AND α–MSH

SUMMARY

In the suboesophageal ganglion of the Colorado potato beetle and the migratory locust three types of peptidergic neurosecretory cells were identified immunocytochemically with antisera to bovine pancreatic polypeptide, FMRFamide, vasopressin and α-MSH. Their locations and immunocytochemical reactions are similar, which suggests, that these peptidergic cells in both insect species are homologous and perhaps have similar functions.

INTRODUCTION

Many peptidergic neurons have been demonstrated in insects by immunocytochemistry with antisera to biologically active vertebrate peptides (Duve and Thorpe 1979, 1980; Rémy et al. 1979; Rémy and Girardie 1980; Yui et al. 1980; El-Salhy et al. 1980, 1983; Hansen et al. 1982), but so far only a few peptides have been isolated (Duve et al. 1979, 1982; Proux and Rougon-Rapuzzi 1980; Cupo and Proux 1983). As antiserum specificity in immunocytochemistry is difficult to prove and often limited (Swaab et al. 1977; van Leeuwen 1982), it remains to be seen whether all the immunocytochemically localized vertebrate peptides in insects are indeed related to their vertebrate analogues. Nevertheless, if the part of the insect peptide recognized by the antiserum has been relatively stable during evolution, homologous neurons containing that peptide may be revealed in different species. I therefore tested antisera reacting with neurosecretory cells in the suboesophageal ganglion of the Colorado potato beetle, *Leptinotarsa decemlineata* (Veenstra and Schooneveld 1984; Veenstra et al. 1984), on the suboesophageal ganglion of another insect species, the migratory locust, *Locusta migratoria*, to see whether such a homology could be revealed. I report here on the evidence supporting the concept of homologous neurosecretory cells in these species.

MATERIALS AND METHODS

Suboesophageal ganglia of adult Colorado potato beetles and migratory locusts were dissected under Ringer and fixed for 4 hrs in GPA (Veenstra et al. 1984). Serial paraffin sections of 7 µm were incubated overnight at 4°C with one of the following antisera: anti-bovine pancreatic polypeptide (BPP) (nr. 615-R110-146-16, from Dr. R.E. Chance, Indianapolis), anti-FMRFamide (nr 646, from Dr. L.P.C. Schot, Amsterdam), anti-vasopressin (VP)1 (nr. 134-III, from Dr. C.J.P. Grimmelikhuijzen, Heidelberg), anti-VP2 and anti-α-MSH (nrs. 125 and 4394-23-4 respectively, from Prof. D.F. Swaab, Amsterdam), in dilutions of 1:2000, 1:2000, 1:1000, 1:2000 and 1:2000 respectively. The immunocytochemical reactions were visualized with the peroxidase-anti-peroxidase (PAP)-method (Sternberger 1979) with 3,3'diamino-benzidine (Sigma) as a substrate for the peroxidase. Alternate serial sections of 6 µm were used to demonstrate the co-localization of immunoreactivities in the same neurons. Paraldehyde fuchsin staining (Schooneveld 1970) was used for comparison with the described neurosecretory cells in the locust (Chalaye 1967). Antisera preabsorbed with peptide coupled to Sepharose-4B-beads

were used to test antiserum specificity. FMRFamide was purchased from Peninsula Laboratories, vasopressin from Sanbio and BPP was a kind gift of Dr. R.E. Chance.

RESULTS AND DISCUSSION

A comparison between the types of immunoreactive neurosecretory cells in the suboesophageal ganglion of both species (Fig. 1, Table I) shows that they are strikingly similar, both in location within the ganglion and in the immunocytochemical reactions with the various antisera. These findings show, that neurosecretory cells located in comparable places are synthesizing similar substances. These facts are taken as evidence for their common ancestral origin and thus illustrate that they are homologous. Given the large evolutionary separation between the holometabolous Colorado potato beetle and the hemimetabolous migratory locust, it is not unlikely that homologous neurosecretory cells are also present in other insect species. In addition to the neurosecretory cells smaller "ordinary" neurons were revealed by anti-BPP, anti-FMRFamide, and anti-VP2. As such neurons can rarely be distinguished in the suboesophageal ganglion of the Colorado potato beetle, they will not be further discussed.

The immunoreactivity of all antisera was blocked after preabsorption with the homologous peptides. BPP- and FMRFamide-like immunoreactivity was always co-localized in the same neurosecretory cells (Table I, Fig. 2). Cross-absorptions of FMRFamide antiserum with BPP and BPP antiserum with FMRFamide blocked the immunoreactivity of these antisera in the Colorado potato beetle (Veenstra

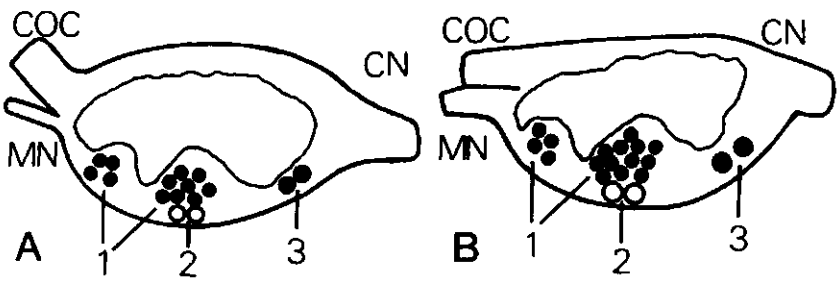


Fig. 1. Schematized drawings in the sagittal plane of projection of the suboesophageal ganglion of the Colorado potato beetle (A) and the migratory locust (B), showing the locations of immunoreactive neurosecretory cells; types 1 and 3, closed circles, type 2, open circles. *COC*, circumoesophageal connective; *CON*, connective to prothoracic ganglion; *MN*, mandibular nerve.

Table 1. Characterization of the different types of immunoreactive neurosecretory cells in the suboesophageal ganglion of the Colorado potato beetle and the migratory locust.

	reaction with					
type	PAF	anti-VP1	anti-VP2	anti-BPP	anti-FMRF	anti-α-MSH
beetle 1	-	-	+	+	+	-
locust 1	-	-	+	+	+	-
beetle 2	+	+	+	-	-	-
locust 2	+	+	+	-	-	-
beetle 3	-	-	+	+	+	+
locust 3	-	-	+	+	+	+

PAF, paraldehyde fuchsin; +, positive reaction; -, no reaction.

and Schooneveld 1984) and the migratory locust. So it appears that the cross-reaction of these antisera reported in an earlier paper (Veenstra and Schooneveld 1984) is not restricted to the Colorado potato beetle. VP2-like immunoreactivity is often co-localized with the BPP-FMRFamide-like immunoreactivity (Table 1, Fig. 3). Cross-absorptions of VP-antisera with BPP or FMRFamide and vice versa did not affect the immunoreactivity of the antisera. This suggests the presence of two antigenic determinants in cell types 1 and 3. However, it does not necessarily imply that these cells contain two separate peptides, as the two determinants may be present on the same peptide. Only a few of the tested preparations were satisfactorily stained with anti-α-MSH (Fig. 4b, 5), but usually this antiserum gave such a strong background staining, that no positive cells could be identified. The reason for this background staining is unknown, but made effective absorption controls impossible. As α-MSH-immunoreactive neurosecretory cells occur in both species, the findings further support the homology concept. The difference in α-MSH-immunoreactivity of type 1 and 3 makes it possible to differentiate between both types of neurosecretory cells.

The immunoreactive neurosecretory cells of the migratory locust demonstrated in this paper have been described before. Thus types 1 and 3 should be considered identical to the neurosecretory C-cells described by Chalaye (1967). Type 2 cells are identical to the A-cells reported earlier to contain VP-like immunoreactivity (Rémy et al. 1979; Rémy and Girardie 1980). A VP-like peptide was later isolated from the suboesophageal ganglion of the migratory locust (Proux and Rougon-Rapuzzi

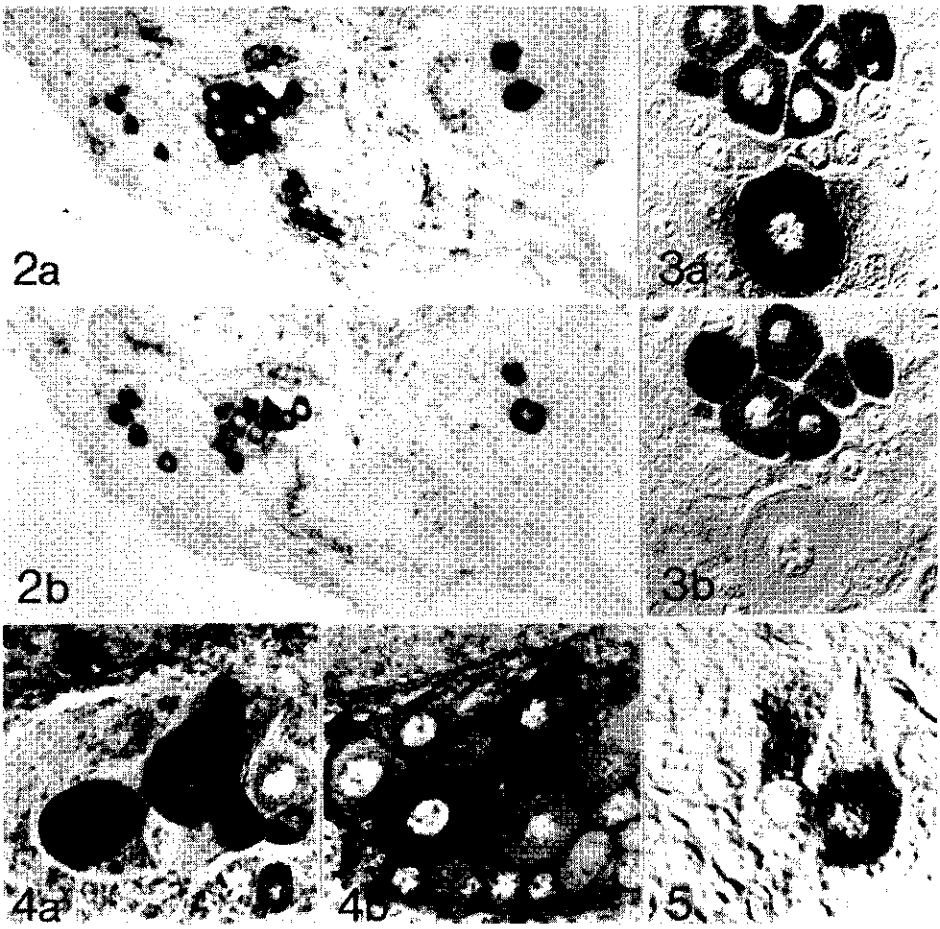


Fig. 2a, b. Adjacent oblique sagittal sections through the suboesophageal ganglion of the migratory locust, showing types 1 and 3 of neurosecretory cells, as revealed by anti-FMRFamide (a) and anti-BPP (b). Note that the same cells react with both antisera. x123.
Fig. 3a, b. Adjacent transversal sections through the suboesophageal ganglion of the migratory locust, showing immunocytochemical reactions with anti-VP2 (a) and anti-FMRFamide (b). Note that type 1 neurosecretory cells, at the top of the micrographs, react with both antisera, while type 2, the large cell at the bottom, reacts with anti-VP2 and not with anti-FMRFamide. x308.
Fig. 4a, b. Adjacent sections through type 3 neurosecretory cells in the suboesophageal ganglion of the migratory locust as revealed by anti-BPP (a) and anti-α-MSH (b). x308.
Fig. 5. Type 3 neurosecretory cells in the suboesophageal ganglion of the Colorado potato beetle, as revealed by anti-α-MSH. x693.

1980), but it was shown to be not identical to VP (Cupo and Proux 1983). The peptide was originally supposed to be synthesized in type 2 neurosecretory cells. However, from the results presented here it appears that the peptide might be synthesized in type 1 or 3 neurosecretory cells, as they also contain VP-like immunoreactivity.

So far it was impossible to detect homologies between neurosecretory cells of unrelated insect species on the basis of application of selective neurosecretory stains. These staining methods differ widely among researchers, which makes comparisons between species difficult (Rowell 1977). However, it was shown, that immunocytochemical methods, due to their high specificity, revealed such homologies between insect species as distantly related as the Colorado potato beetle and the migratory locust. Apparently those parts of the insect peptides recognized by the antisera have been relatively stable during evolution. Perhaps because those parts are essential for the functioning of these insect peptides, whatever their function may be. If these functions have also been relatively stable during evolution, the discovery of the function of a peptide in one species may lead to the elucidation of the function of its homologue in other species. Evidence has been presented that the VP-like peptide from the suboesophageal ganglion of the migratory locust may function as a diuretic hormone (Proux and Rougon-Rapuzzi 1980; Proux et al. 1982). It is therefore tempting to speculate, that homologous vasopressin-like peptides in other insect species may have the same function.

REFERENCES

Chalaye D (1967) Neurosécrétion de la chaine nerveuse ventrale de *Locusta migratoria migratorioides* R. et F. (Orthoptère acridien). Bull Soc Zool Fr 92: 87-108.

Cupo A, Proux J (1983) Biochemical characterization of a vasopressin-like neuropeptide in *Locusta migratoria*. Evidence of a high molecular weight protein encoding vasopressin sequence. Neuropeptides 3: 309-318.

Duve H, Thorpe A (1979) Immunofluorescent localization of insulin-like material in the median neurosecretory cells of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tissue Res 200: 187-191.

Duve H, Thorpe A (1980) Localization of pancreatic polypeptide (PP)-like immunoreactive material in neurones of the brain of the blowfly, *Calliphora erythrocephala* (Diptera). Cell Tissue Res 210: 101-109.

Duve H, Thorpe A, Lazarus N (1979) The isolation of material displaying insulin-like immunological and biological activity from the brain of the blowfly, *Calliphora*

vomitoria. Biochem J 181: 221-227.

Duve H, Thorpe A, Lazarus NR, Lowry PJ (1982) A neuropeptide of the blowfly *Calliphora vomitoria* with an amino acid composition homologous with vertebrate pancreatic polypeptide. Biochem J 201: 429-432.

El-Salhy M, Abou-El-Ela R, Falkmer S, Grimelius L, Wilander E (1980) Immunohistochemical evidence of gastro-entero-pancreatic neurohormonal peptides of vertebrate type in the nervous system of a dipteran insect, the hoverfly, *Eristalis aeneus*. Regul Peptides 1: 187-204.

El-Salhy M, Falkmer S, Kramer KJ, Speirs RD (1983) Immunohistochemical investigations of neuropeptides in the brain, corpora cardiaca, and corpora allata of an adult lepidopteran insect, *Manduca sexta* (L) Cell Tissue Res 232: 295-317.

Hansen BL, Hansen GN, Scharrer B (1982) Immunoreactive material resembling vertebrate neuropeptides in the corpus cardiacum and corpus allatum of the insect *Leucophaea maderae*. Cell Tissue Res 225: 319-329.

Leeuwen F van (1982) Specific immunocytochemical localization of neuropeptides: A utopian goal? In: Bullock GR, Petrusz P (eds) Techniques in immunocytochemistry, Vol 1, Academic Press, London, pp. 283-299.

Proux J, Rougon-Rapuzzi G (1980) Evidence for vasopressin-like molecule in migratory locust. Radioimmunological measurements in different tissues: correlations with various states of hydration. Gen Comp Endocrinol 42: 378-383.

Proux J, Rougon G, Cupo A (1982) Enhancement of excretion across locust malpighian tubules by a diuretic vasopressin-like hormone. Gen Comp Endocrinol 47: 449-457.

Rémy C, Girardie J (1980) Anatomical organization of two vasopressin-neurophysin-like neurosecretory cells throughout the central nervous system of the migratory locust. Gen Comp Endocrinol 40: 27-35.

Rémy C, Girardie J, Dubois MP (1979) Vertebrate neuropeptide-like substances in the suboesophageal ganglion of two insects: *Locusta migratoria* R. and F. (Orthoptera) and *Bombyx mori* L. (Lepidoptera). Immunocytochemical investigation. Gen Comp Endocrinol 37: 93-100.

Rowell HF (1977) The cells of the insect neurosecretory system: constancy, variability, and the concept of the unique identifiable neuron. Advan Insect Physiol 12: 63-123.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* Say, as a function of daylength. Neth J Zool 20: 151-237.

Sternberger L (1979) Immunocytochemistry (2nd ed), John Wiley and Sons, New York.

Swaab DF, Pool CW, Leeuwen FW van (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-391.

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235: 303-308.

Veenstra JA, Romberg-Privee HM, Schooneveld H (1984) Immunocytochemical localization of peptidergic cells in the neuroendocrine system of the Colorado potato beetle, *Leptinotarsa decemlineata*, with antisera against vasopressin, vasotocin and oxytocin. Histochemistry 81: 29-35.

Yui R, Fujita T, Ito S (1980) Insulin-, gastrin-, pancreatic polypeptide-like immunoreactive neurons in the brain of the silk-worm, *Bombyx mori*. Biomed Res 1: 42-46.

IMMUNOCYTOCHEMICAL LOCALIZATION OF GASTRIN–RELEASING PEPTIDE/ BOMBESIN–LIKE IMMUNOREACTIVE NEURONS IN INSECTS

SUMMARY

GRP/bombesin-like immunoreactive material was immunocytochemically detected in neurons of seven insect species belonging to seven orders, while such neurons were not found in three insect species belonging to two other orders. In some insect species certain neurons were found in corresponding places and approximately the same numbers. It seems likely that such neurons have a common evolutionary origin and are homologous. The fact that the GRP antiserum reveals such homologous neurons in species belonging to different orders, suggests that the part of the GRP/bombesin-like peptide recognized by the antiserum has been relatively stable during evolution. As the GRP antiserum had to be used in much higher concentrations on insect tissue than for GRP endocrine cells in chicken proventriculus, the chemical resemblance of the insect peptide(s) to GRP and bombesin may be limited.

INTRODUCTION

Several substances reacting with antisera to biologically active vertebrate peptides have been demonstrated immunocytochemically in invertebrates (e.g. Yui et al. 1980; Schot et al. 1981; El-Salhy et al. 1980, 1983; Hansen et al. 1982; Veenstra and Schooneveld 1984; Veenstra 1984). The best documented examples in insects are an insulin- and pancreatic polypeptide-like peptide from blowflies, which are not only immunoreactive to antisera against the vertebrate homologues, but also show a marked similarity to vertebrate insulin and pancreatic polypeptide in amino acid composition (Duve and Thorpe 1979, 1980; Duve et al. 1979, 1982). These and other findings suggest that at least some of the biologically active vertebrate peptides are evolutionary very old (Scharrer 1978).

Bombesin, a tetradecapeptide, and the related peptides ranatensin, alytensin, litorin and the phyllolitorins were originally isolated from the skin of amphibian species (Anastasi et al. 1971; Erspammer and Melchiori, 1973; Melchiori 1978; Yasuhara et al. 1983). Later the immunochemical demonstration of a bombesin-like peptide in mammalian gut led to the isolation and characterization of gastrin-releasing peptide (GRP), a heptacosapeptide with a high degree of homology to bombesin in its ten C-terminal amino acids (McDonald et al. 1979). This peptide proved to be present also in mammalian central nervous system (e.g. Yanaihara et al. 1981; Roth et al. 1982). I here report on the immunocytochemical localization in a number of insect species of neurons, which are immunoreactive to an antiserum to GRP.

MATERIALS AND METHODS

Ten insect species belonging to nine insect orders (see Results) were used in this study. All specimens were adults and taken from laboratory cultures. From each species at least six specimens were studied. The brain, suboesophageal ganglion, corpora cardiaca and corpora allata were dissected out under Ringer and fixed for 4 hrs in GPA (a mixture of 1 volume of 25% glutaraldehyde, 3 volumes of a saturated aqueous solution of picric acid with 1% acetic acid), embedded in paraplast and sectioned at 7 µm. Some specimens of *Locusta migratoria* and *Leptinotarsa decemlineata* were injected with respectively 15 and 1.5 µg colchicine two days before use. Chicken proventriculus, known to contain numerous bombesin-immunoreactive endocrine cells (Timson et al. 1979), was used as control tissue and was also fixed in GPA.

Sections were stained immunocytochemically with the indirect peroxidase-anti-

peroxidase (PAP) method (Sternberger 1974), using 3,3'-diaminobenzidine (Sigma) as a substrate for the peroxidase. The GRP antiserum (number R-6903, a kind gift of Prof. N. Yanaihara, Shizuoka, Japan) was used overnight in a dilution of 1:2000 for insects and 1:2000 to 1:40 000 for chicken proventriculus. This antiserum has been well characterized by radio immuno assay and is known to be directed against the C-terminus of GRP, being fully cross-reactive with bombesin (Yanaihara et al. 1981). The specificity was checked by preadsorbing the diluted antiserum overnight with bombesin in concentrations of 0.1 to 10 nmol per ml (liquid phase adsorptions). Cross-reactivity with substance P, which shares the dipeptide-amide (-Leu-Met-NH_2) with GRP and bombesin, was checked in the same way. Antibodies from the antiserum which cross-react with substance P, were removed by solid phase adsorptions, using Sepharose-4B beads (Pharmacia). Antiserum was adsorbed three times with beads equivalent to 10 nmol substance P per ml diluted antiserum. After immunocytochemical staining sections were counterstained with Mayer's haematoxylin.

RESULTS

Immunoreactive neurons were found in the following species:

Firebrat, *Thermobia domestica* (Zygentoma) (Fig. 1a)

Forty to 60 immunoreactive neurons are located in the protocerebrum, on the roof of the oesophageal foramen; their axons branch extensively in the neuropil. Single immunoreactive neurons are present in the protocerebrum at the base of the optic lobes and in the dorsomedial lobes of the protocerebrum (Fig. 2). One immunoreactive neuron is present on each side in the ventro-lateral area of the suboesophageal ganglion. Many immunoreactive axon profiles were found in the neuropil areas of the protocerebrum, tritocerebrum and suboesophageal ganglion.

American cockroach, *Periplaneta americana* (Dictyoptera) (Fig. 1b)

One pair of strongly immunoreactive neurons is present on each side in the protocerebrum on the roof of the oesophageal foramen, close to the midline of the brain (Fig. 4). Their axons run via the median bundle to the contra-lateral circumoesophageal connective into the suboesophageal ganglion. In this ganglion and in the tritocerebrum several immunoreactive axon profiles were found. Weakly to moderately immunoreactive neurons were sometimes found in the lateral tritocerebrum, whereas very fine immunoreactive fibres were occasionally found in the neuropil of the protocerebrum.

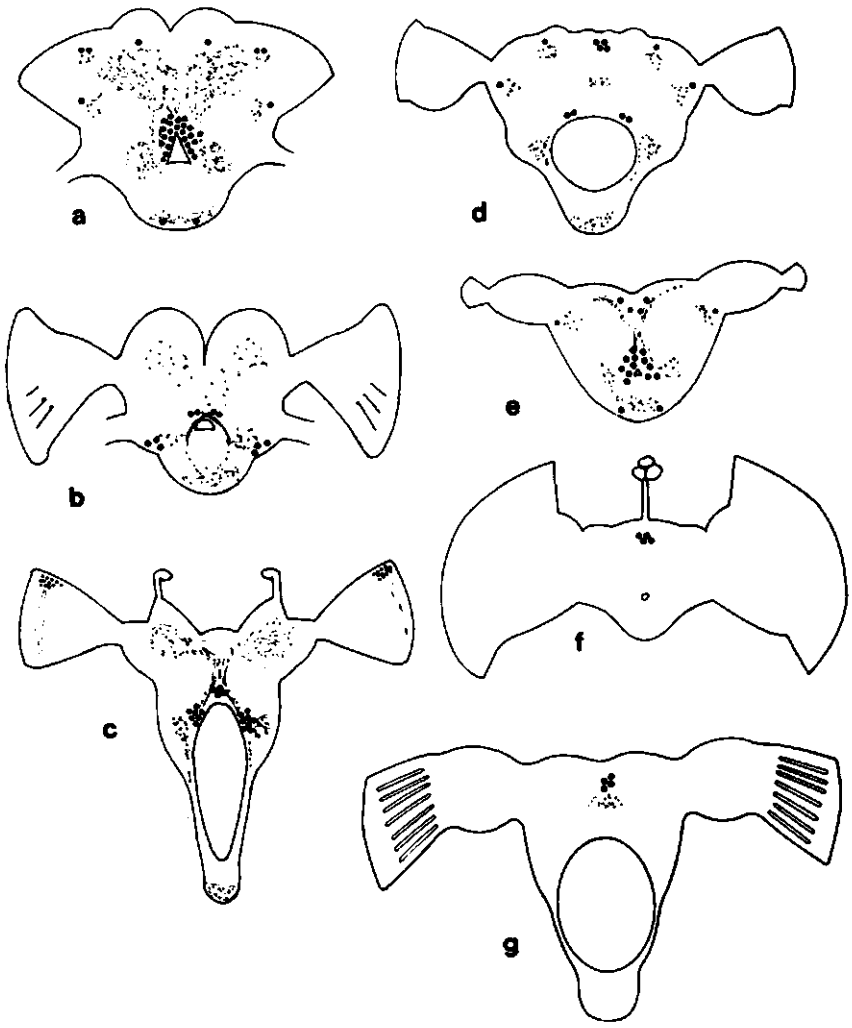


Fig. 1. Diagram of the nervous system of the head of: a, *Thermobia domestica*; b, *Periplaneta americana*; c, *Locusta migratoria*; d, *Extatosoma tiaratum*; e, *Dysdercus fulvoniger*; f, *Musca domestica*; g, *Leptinotarsa decemlineata*, showing positions of GRP/bombesin-like neurons (closed circles) and axon fibres (lines).

Migratory locust, *Locusta migratoria* (Orthoptera) (Fig. 1c)

Four immunoreactive neurons are located in the protocerebrum on the roof of the oesophageal foramen (Fig. 6). Their axons could not be followed for any distance, as they were not discernible from the axons of the immunoreactive neurons in the tritocerebrum. Thirty to 40 immunoreactive neurons are present in each

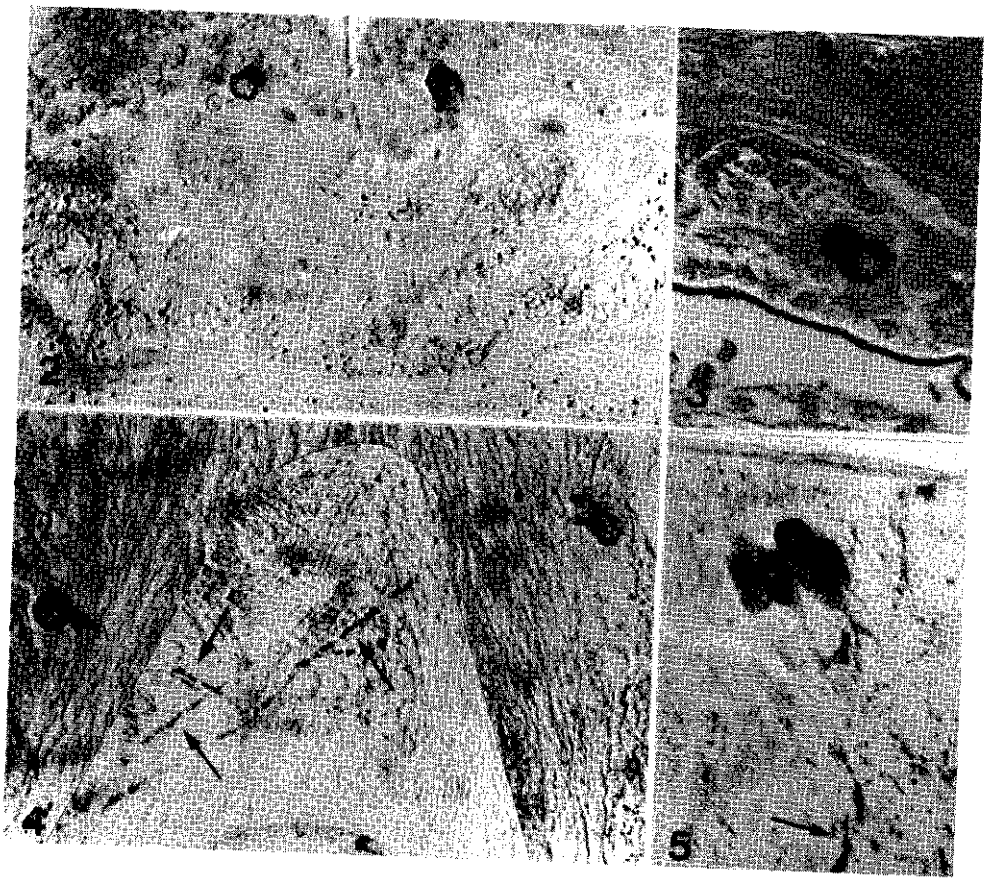


Fig. 2. Horizontal section through the protocerebrum of *Thermobia domestica*, showing immunoreactive neurons in the dorso-medial lobes and numerous immunoreactive axon profiles in the neuropil. X300.
Fig. 3. Transversal section through the brain of *Extatosoma tiaratum*, showing the left pair of immunoreactive neurons above the oesophageal foramen. X300.
Fig. 4. A montage photo of two non-adjacent sections through the brain of *Periplaneta americana*, showing the two pairs of immunoreactive neurons and their crossing axons (arrows) above the roof of the oesophageal foramen. X300.
Fig. 5. Immunoreactive neurons and axon profiles (arrows) in the tritocerebrum of *Locusta migratoria*. X300.

tritocerebrum (Fig. 5). Numerous fine axons of these neurons run via the median bundle to the contra-lateral neuropil areas. Other immunoreactive fibres are present in the tritocerebrum. In the suboesophageal ganglion immunoreactive fibres, but no cell bodies, were found in the ventral neuropil areas of the ganglion. In each optic lobe about 250 small neurons (ca 7-10 µm) are immunoreactive. They are located in the cortex dorsal from the lamina ganglionaris and intermingled with

non-immunoreactive neurons of the same size (Fig. 8). They have very fine axons entering the lamina, where parallel plexuses are formed.

Stick insect, *Extatosoma tiaratum* (Phasmida) (Fig. 1d)

Four immunoreactive neurons are present on the roof of the oesophageal foramen, two on each side (Fig. 3). Their axons could be followed for only a small distance and seem to run to the contra-lateral circumoesophageal connective. In the lateral protocerebrum close to the optic lobe, beneath the optic tract, one immunoreactive neuron is located on each side of the brain. Axons were also revealed in the adjacent neuropil. Furthermore one immunoreactive neuron lateral to the corpora pedunculata was revealed. In three out of seven specimens four large median neurosecretory cells were weakly stained. In these animals the ellipsoid body of the central body was also found to contain fine axon fibres. Such fibres were always found in the tritocerebrum and the suboesophageal ganglion.

Cotton stainer, *Dysdercus fulvoniger* (Hemiptera) (Fig. 1e)

Two immunoreactive neurons were found in each brain half caudally in the group of median neurosecretory cells in the pars intercerebralis. Another immunoreactive neuron was found slightly more ventrally and caudally. A small and weakly immunoreactive neuron was usually present in the lateral protocerebrum, close to the base of the optic lobe. The most intensely stained neurons are located in the medium tritocerebrum near the oesophageal foramen, where on each side 6 to 12 neurons were found. Their axons follow the medium bundle into the contralateral protocerebral neuropil, where they branch and end. Axon branches are also present in the tritocerebrum. In the suboesophageal ganglion one ventro-lateral pair of neurons is located in the posterior half of the ganglion. Immunoreactive axons of these neurons are confined to the ventral neuropil area of the ganglion.

Housefly, *Musca domestica* (Diptera) (Fig. 1f)

Four immunoreactive neurons are present within the group of median neurosecretory cells (Fig. 7). Their axons were not visualized and no other neurons were found in this species.

Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera) (Fig. 1g)

A group of four immunoreactive neurons was found medio-ventrally in the group of median neurosecretory cells (Fig. 9). These neurons are probably identical to the E-type neurosecretory cells described by Schooneveld (1970). Their axons were found to ramify in the ellipsoid body of the central body. No immunoreactive

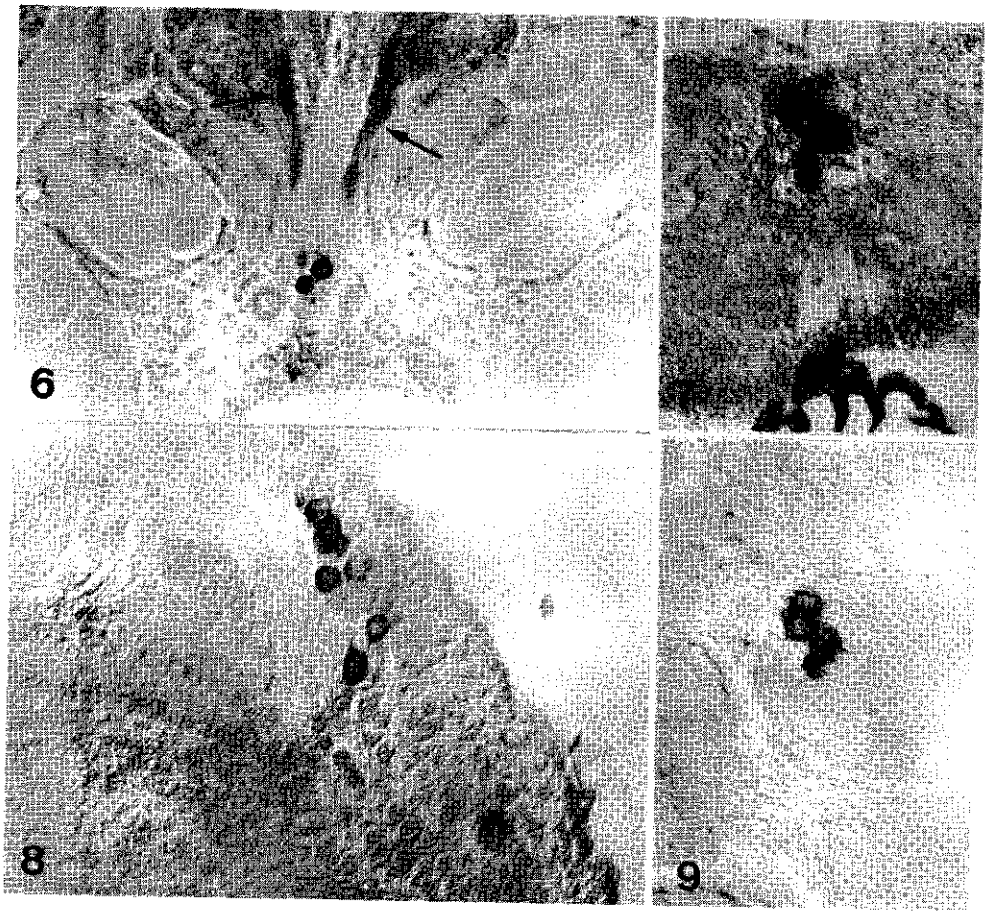


Fig. 6. Section through the brain of *Locusta migratoria*, showing immunoreactive neurons above the oesophageal foramen and immunoreactive axons of the trito-cerebral neurons, following the median bundle (arrows). X120.
Fig. 7. Immunoreactive neurons in the pars intercerebralis of *Musca domestica*. X300.
Fig. 8. Immunoreactive neurons innervating the lamina ganglionaris in the optic lobe of *Locusta migratoria*. X300.
Fig. 9. Immunoreactive neurosecretory cells in the pars intercerebralis of *Leptinotarsa decemlineata*. X300.

material was found in the suboesophageal ganglion, corpora cardiaca, corpora allata or frontal ganglion.

The locations of the immunoreactive neurons of these species are summarized in Fig. 1. No immunoreactive neurons were found in the small white butterfly, *Pieris rapae*, and the greater wax moth, *Galleria mellonella* (both Lepidoptera) and in the honey bee, *Apis mellifera* (Hymenoptera).

All immunoreactivity was abolished after liquid phase preadsorption with 0.3 nmol bombesin per ml diluted antiserum. The immunoreactivity on both insects and chicken proventriculus was also abolished after liquid phase preadsorption with substance P, although a concentration of 10 nmol per ml was needed to obtain that effect. The antiserum from which antibodies cross-reactive with substance P had been removed by solid phase adsorption appeared to be no longer immunoreactive on insects; staining of GRP endocrine cells in chicken proventriculus was achieved only after the concentration of the antiserum had been increased from 1:40 000 to 1:4000.

DISCUSSION

An important question is to which extent the immunocytochemically localized substance present in insect neurons is chemically related to GRP and bombesin. The immunocytochemical reaction of the GRP antiserum was completely abolished after liquid phase preadsorption of the antiserum with very small amounts of bombesin, suggesting that the insect substance bears some resemblance to that peptide. However, the fact that we had to use the GRP antiserum in a much higher concentration (1:2000) on insect tissue than on chicken proventriculus (1:40 000) suggests that only few antibodies in the serum react with the insect substance. This presumed peptide therefore may have only a small chemical resemblance to GRP and bombesin.

It is well known that GRP/bombesin antisera easily cross-react with substance P, due to the common C-terminal dipeptide-amide (-Leu-Met-NH_2) of GRP/bombesin and substance P (e.g. Roth et al. 1982). Although the cross-reactivity of our GRP antiserum with substance P is negligible in radio immuno assay (Yanaihara et al. 1982), such a cross-reactivity might be considerable in immunocytochemistry on insects, because we used a relatively high serum concentration. In principle, such antiserum cross-reactivity may arise in two different ways. First, a relatively small proportion of the antibodies present in the antiserum may recognize only a small antigenic determinant, in this case perhaps the dipeptide-amide -Leu-Met-NH_2. Second, a large proportion of the antibodies present in the antiserum might also react with another peptide, perhaps substance P, but with much lower affinity than it would react with the peptide used as antigen. I therefore used solid phase adsorptions to remove antibodies which cross-react with substance P. The results showed that the immunoreactivity was not only abolished on insects, but also greatly reduced on chicken proventriculus. This shows that a considerable proportion of the antibodies present in the GRP antiserum is cross-reactive with substance P,

but presumably with a very low affinity. More detailed conclusions as to the specificity of the immunocytochemical reactions of the GRP antiserum on insects can therefore not be drawn at this stage. In this study no substance P antiserum has been used, but it seems unlikely that the insect GRP-like substance has much in common with substance P. Substance P-like immunoreactive neurons were described in the brain of *Locusta migratoria* (Benedeczky et al. 1982); these are different from our GRP/bombesin-like immunoreactive neurons as they are located in other areas of the brain. Furthermore no substance P-like immunoreactive neurons were found in the brain of *Leptinotarsa decemlineata* (unpubl. observations).

It is perhaps significant that only a limited number of the hundreds of thousands of neurons present in insect brain are recognized by the GRP-antiserum. It shows that the labelling of these neurons is highly selective. Some of the immunocytochemically localized neurons seem to be present in different species in comparable numbers and places. I consider it very likely that they are homologous in the sense that they have a common evolutionary origin. Such presumed homologous neurons are the four immunoreactive cells present in the part of the brain constituting the roof of the oesophageal foramen in *Locusta migratoria*, *Periplaneta americana* and *Extatosoma tiaratum*. The tritocerebral immunoreactive neurons in several hemimetabolous species might be homologous as well, although here also differences between the species are present. The number of neurons as well as their immunocytochemical stainability varies largely among these species. It seems likely that the four GRP-like neurons in the partes intercerebrales of *Leptinotarsa decemlineata* and *Musca domestica* are also homologous. In contrast, some neurons seem to be unique and present in only one species, e.g. those in the optic lobe of *Locusta migratoria*.

As the GRP/bombesin-like immunoreactive substances occur in homologous neurons of unrelated insect species, it seems that those parts of these substances recognized by the serum have been relatively stable during evolution. Those parts are thus likely to be essential for the physiological functions of the substances. These functions are unknown as yet, but it is possible that the homologous neurons in the different species have similar functions. None of these GRP/bombesin-like immunoreactive neurons were found in all insect species studied. This suggests that either during evolution the site of the synthesis of the GRP/bombesin-like immunoreactive peptide has changed several times, or that several different GRP/bombesin-like immunoreactive peptides are present in insects. If the chemical resemblance of the insect GRP/bombesin-like peptides to GRP and bombesin is limited, as suggested by the high antiserum concentration needed for insects in

comparison to the chicken GRP endocrine cells of the proventriculus, the latter possibility seems more likely.

Cells reacting with bombesin-antisera have been demonstrated immunocytochemically in the cockroach *Leucophaea maderae* (Hansen et al. 1982), whereas they appeared to be absent in the hoverfly *Eristalis aeneus* (El-Salhy et al. 1980) and the tobacco hornworm moth *Manduca sexta* (El-Salhy et al. 1983). Furthermore, bombesin-like neurons have been found in the snails *Achatina fulica* (van Noorden et al. 1980) and *Helix aspersa* (Osborne and Dockray 1982), as well as in the coelenterate *Hydra attenuata* (Grimmelikhuijzen et al. 1981). Thus there seems to be evidence that bombesin-like peptides are widely distributed within the animal kingdom, but in none of these invertebrates the bombesin-like substance has been fully characterized. No definite conclusions as to the identity of a peptide can be drawn on the basis of immunocytochemistry alone (e.g. Swaab et al. 1977) and it has been suggested that the bombesin-like peptide in *Hydra attenuata* might be identical to the substance P-like peptide in the same species (Grimmelikhuijzen 1984). Further research is therefore needed to confirm the speculation that bombesin has a very early evolutionary origin, like it has been shown likely for insulin, pancreatic polypeptide (Duve et al. 1979, 1982) and Hydra head activator neuropeptide (Bodenmüller and Schaller 1981).

REFERENCES

Anastasi A, Erspammer V, Bucci M (1971) Isolation and structure of bombesin and alytensin, two analogous active peptides from the skin of the european amphibians *Bombina* and *Alytes*. Experientia 26: 166-167.

Benedeczky I, Kiss JZ, Somogyi P (1982) Light and electron microscopic localization of substance P-like immunoreactivity in the cerebral ganglion of locusts with a monoclonal antibody. Histochemistry 75: 123-131.

Bodenmüller H, Schaller HC (1981) Conserved amino acid sequence of a neuropeptide, the head activator, from coelenterates to humans. Nature 293: 579-580.

Duve H, Thorpe A (1979) Immunofluorescent localization of insulin-like material in the median neurosecretory cells of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tissue Res 200: 187-191.

Duve H, Thorpe A (1980) Localisation of Pancreatic Polypeptide (PP)-like immunoreactive material in neurones of the brain of the blowfly, *Calliphora erythrocephala* (Diptera). Cell Tissue Res 210: 101-109.

Duve H, Thorpe A, Lazarus N (1979) The isolation of material displaying insulin-like immunological and biological activity from the brain of the blowfly, *Calliphora*

vomitoria. Biochem J 181: 221-227.

Duve H, Thorpe A, Lazarus NR, Lowry PJ (1982) A neuropeptide of the blowfly *Calliphora vomitoria* with an amino acid composition homologous with vertebrate pancreatic polypeptide. Biochem J 201: 429-432.

El-Salhy M, Abou-El-Ela R, Falkmer S, Grimelius L, Wilander E (1980) Immunohistochemical evidence of gastro-entero-pancreatic neurohormonal peptides of vertebrate type in the nervous system of a dipteran insect, the hoverfly, *Eristalis aeneus*. Regul Peptides 1: 187-204.

El-Salhy M, Falkmer S, Kramer KJ, Speirs RD (1983) Immunohistochemical investigations of neuropeptides in the brain, corpora cardiaca, and corpora allata of an adult lepidopteran insect, *Manduca sexta* (L). Cell Tissue Res 232: 295-317.

Erspammer V, Melchiori P (1973) Active polypeptides of amphibian skin and their synthetic analogues. Pure Appl Chem 35: 463-494.

Grimmelikhuijzen CJP (1984) Peptides in the nervous system of coelenterates. In: Falkmer S (ed) Evolution and tumor pathology of the neuroendocrine system. Elsevier, Amsterdam, pp. 39-58.

Grimmelikhuijzen CJP, Dockray GJ, Yanaihara N (1981) Bombesin-like immunoreactivity in the nervous system of *Hydra*. Histochemistry 73: 171-180.

Hansen BL, Hansen GN, Scharrer B (1982) Immunoreactive material resembling vertebrate neuropeptides in the corpus cardiacum and corpus allatum of the insect *Leucophaea maderae*. Cell Tissue Res 225: 319-329.

McDonald TJ, Jörnvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, Mutt V (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochem Biophys Res Commun 90: 227-233.

Melchiori P (1978) Bombesin and bombesin-like peptides of amphibian skin. In: Bloom SR (ed) Gut hormones. Churchill Livingstone, Edinburgh, pp 540-543.

Noorden S van, Fritsch HAR, Grillo TAI, Polak JM, Pearse AGE (1980) Immunocytochemical staining for vertebrate peptides in the nervous system of a gastropod mollusc. Gen Comp Endocrinol 40: 375-376.

Osborne NN, Dockray GJ (1982) Bombesin-like immunoreactivity in specific neurones of the snail *Helix aspersa* and an example of coexistence of substance P and serotonin in an invertebrate neuron. Neurochem Int 4: 175-180.

Roth KA, Weber E, Barchas JD (1982) Distribution of gastrin releasing-peptide-bombesin-like immunostaining in rat brain. Brain Res 251: 277-282.

Scharrer B (1978) Peptidergic neurons: Facts and trends. Gen Comp Endocrinol 34: 50-62.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say), as a function of daylength. Neth J Zool 20: 151-237.

Schot LPC, Boer HH, Swaab DF, Noorden S van (1981) Immunocytochemical demonstration of peptidergic neurons in the central nervous system of the pond snail *Lymnaea stagnalis* with antisera raised to biologically active peptides of vertebrates. Cell Tissue Res 216: 273-291.

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall Inc. New Yersey.

Swaab DF, Pool CW, Leeuwen FW van (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-391.

Veenstra JA (1984) Immunocytochemical demonstration of a homology in peptidergic neurosecretory cells in the suboesophageal ganglion of a beetle and a locust with antisera to bovine pancreatic polypeptide, FMRFamide, vasopressin and α-MSH. Neurosci Lett 48: 185-190.

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of peptidergic neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235: 303-308.

Timson CM, Polak JM, Wharton J, Ghatei MA, Bloom SR, Usellini L, Capella C, Solcia E, Brown MR, Pearse AGE (1979) Bombesin-like immunoreactivity in the avian gut and its localization to a distinct cell type. Histochemistry 61: 213-221.

Yanaihara N, Yanaihara C, Mochizuki T, Iwahara K, Fujita T, Iwanaga T (1981) Immunoreactive GRP. Peptides 2 supp. 2: 185-191.

Yasuhara T, Nakajima T, Nakihara K, Yanaihara C, Yanaihara N, Erspammer V, Erspammer GF (1983) Two new frog skin peptides, phyllolitorins, of the bombesin-ranatensin family from *Phyollomedusa sauvagei*. Biomed Res 4: 407-412.

Yui R, Fujita T, Ito S (1980) Insulin-, gastrin-, pancreatic polypeptide-like immunoreactive neurons in the brain of the silkworm, *Bombyx mori*. Biomed Res 1: 42-46.

IMMUNOCYTOCHEMICAL LOCALIZATION OF PEPTIDERGIC NEURONS AND NEUROSECRETORY CELLS IN THE NEURO–ENDOCRINE SYSTEM OF THE COLORADO POTATO BEETLE WITH ANTISERA TO VERTEBRATE REGULATORY PEPTIDES

SUMMARY

A large number of antisera to regulatory vertebrate peptides was tested immunocytochemically on the nervous system of the Colorado potato beetle to further characterize the peptidergic cells of the neuro-endocrine system and to reveal cells participating in endocrine control mechanisms. Neurons, neurosecretory cells, axons and axon terminals were revealed by antisera to ACTH, gastrin, CCK, α-endorphin, β-endorphin, γ1-MSH, insulin, motilin, human calcitonin, growth hormone, somatostatin, CRF, ovine prolactin and rat prolactin. Together with previously described results these findings demonstrate that at least 19 different peptidergic cell types are present in the Colorado potato beetle. Several of these cell types are identical with the known neurosecretory cells, while others have not been identified before.

The functions of the immunoreactive neurons are as yet unclear, although in two cases the localization of these cells gives some clues. Thus the lateral neurosecretory cells, which are immunoreactive with antisera to β-endorphin and ovine prolactin, may regulate corpus allatum activity, whereas a CRF immunoreactive substance seems to be used as neurotransmitter by antennal receptors. These immunocytochemical findings do not imply that the immunoreactive substances are evolutionarily related to the vertebrate peptides to which the antisera were raised. It is postulated that if the part of the substance recognized by a certain

antiserum is functionally important for the insect, which should be so if the insect peptide is evolutionarily related to its vertebrate homologue, that then the antiserum should reveal homologous cells in different insect species. The consequence of this hypothesis is, that if an antiserum does not reveal homologous neurons in different insect species, the immunologically demonstrated substance is probably of little physiological importance, and will not be related evolutionarily to the vertebrate analogue. The positive immunocytochemical results in the Colorado potato beetle are discussed in relation to these considerations.

INTRODUCTION

From vertebrates a large number of peptides has been isolated which perform essential roles in the regulation of various physiological processes. Although the first identified biologically active peptides were hormones, it is now clear that peptides may also function as neurotransmitters or neuromodulators (e.g. Hökfelt et al. 1980; Snyder 1980). The chemical structure of many of these peptides has been established. In insects, peptides are also known to regulate many physiological processes (Goldsworthy and Mordue 1974). However, so far the chemical structure of only two insect neuropeptides has been established: adipokinetic hormone from locusts, a decapeptide (Stone et al. 1976) and proctolin from cockroaches, a pentapeptide neurotransmitter (Starratt and Brown 1975). Little more is known of many insect hormones than their peptidergic nature (Stone and Mordue 1980). In recent years immunochemical assays for biologically active vertebrate peptides proved useful for the demonstration of peptides in insects and other invertebrate species (e.g. Duve et al. 1979, 1982; Yui et al. 1980; El-Salhy et al. 1980, 1983; Schot et al. 1981; Hansen et al. 1982; Proux et al. 1982). This has led to the isolation and characterization of invertebrate peptides which are identical to or very similar to their vertebrate homologues. Thus a pancreatic polypeptide-like and an insulin-like peptide were isolated from blowflies and proved to be remarkably similar to the vertebrate homologues (Duve et al. 1979, 1982). Furthermore two opioid peptides which proved to be identical with leu- and met-enkephalin were recently isolated from a mollusc (Leung and Stefano 1984).

We are since a long time interested in the neuro-endocrine system of the Colorado potato beetle and its role in the control of reproduction and diapause. The neuro-endocrine system of this insect has been studied before in detail, using selective neurosecretory stains and electron microscopy (Schooneveld 1970, 1974a,b). We started an immunocytochemical study of this system using antisera to biologically

active vertebrate peptides and made a description of the localization of various types of peptidergic cells, occurring inside and outside the known neuro-endocrine centres. Results regarding the characterization of immunoreactive substances in several types of cell with a few selected antisera to some vertebrate and invertebrate peptides have been described previously (Veenstra 1984; Veenstra and Schooneveld 1984; Veenstra and Yanaihara 1984; Veenstra et al. 1984a,b). Here we report on the distribution of various neurons which were identified by antisera to various other regulatory vertebrate peptides.

It is well known that the specificity of immunocytochemical reactions is limited (Swaab et al. 1977; Schot and Boer 1984; Veenstra and Schooneveld 1984). In a study like this where antisera to vertebrate peptides are tested on insect tissue, there is a real possibility of immunocytochemical reactions being caused by cross-reactions with substances having only a remote resemblance to the vertebrate antigen. Previous immunocytochemical studies suggested that certain antisera can be used to identify homologous cells in different species, indicating that the substances demonstrated immunocytochemically are of physiological importance (Veenstra 1984; Veenstra and Schooneveld 1984; Veenstra and Yanaihara 1984; Veenstra et al. 1984a). The results of the present study are therefore compared with literature data on other insect species to see whether more examples of homologous neurons can be found, thus confirming the relevance of the observations.

MATERIALS AND METHODS

About 600 adult, reproducing beetles, both males and females, were used in this study. They were bred in the laboratory under long-day conditions and were injected with 1.5 to 5 µg colchicine two days before use. The brain, suboesophageal ganglion and retrocerebral glands were dissected out in Ringer and fixed in GPA (a mixture of one volume 25% glutaraldehyde and three volumes of a saturated aqueous solution of picric acid with 1% acetic acid (Boer et al. 1979)) for 4 to 6 hrs at room temperature. Some antisera were also tested on material fixed in Bouin or Bouin Hollande sublimé without acetic acid (BHS); the fixations used for the different antisera are listed in Table 1.

Serial 7µm paraffin sections were stained immunocytochemically with the indirect peroxidase-anti-peroxidase (PAP) method (Sternberger 1974), or, if the primary antiserum had been raised in guinea pig, with peroxidase-labeled goat anti-guinea pig serum. Sections were incubated with the primary antiserum for 17 hrs at 4°C, the peroxidase was developed with 3,3'diaminobenzidine (DAB). One antiserum had been conjugated with FITC and was evaluated in the fluorescence

Table 1.
List of antisera tested

antiserum raised against	working concentrations or highest concentration tested	code	used fixations	source
ACTH	1:200	133	GPA	J.M.Polak
CLIP	1:300	429	GPA	J.M.Polak
human-Calcitonin	1:2000	5	GPA	J.M.Polak
CCK (9-20)	1:400	280	GPA	J.M.Polak
CCK	1:500	61007	GPA,Bouin	H.J.T.Goos
Gastrin	1:1200	655	GPA	J.M.Polak
Gastrin	1:2000	144	GPA	J.M.Polak
Gastrin	1:1000	138	GPA	F.T. Bosman
Gastrin	1:500	L111	GPA,Bouin	G.J.Dockray
α-endorphin	1:200	R-3404	GPA,BHS	N.Yanaihara
β-endorphin	1:2000 to 1:4000	R-5803	GPA,BHS	N.Yanaihara
β-endorphin	1:800	388	GPA	J.M.Polak
leu-enkephalin	1:500	774	GPA	J.M.Polak
leu-enkephalin	1:2000	493	GPA	J.M.Polak
met-enkephalin	1:500	773	GPA	J.M.Polak
met-enkephalin	1:1600	497	GPA	J.M.Polak
FMRFamide[a]	1:2000	646	GPA	H.H.Boer
CRF	1:500 to 1:2000	02B83	GPA	F.Vandesande
Bombesin	1:3000	625	GPA	J.M.Polak
Glucagon	1:500	K1711/130576	GPA,Bouin,BHS	W.J.Nooijen
Glucagon	1:500	Otto	GPA,Bouin	H.Duve and A. Thorpe
Glucagon	1:1000	499	GPA	J.M.Polak
Insulin[b]	1:100	436	GPA,Bouin,BHS	F.T.Bosman
Insulin[b]	1:800	413	GPA,Bouin	J.M.Polak
Insulin[b]	1:60		GPA,Bouin	J.M.Polak
Insulin[b] (FITC)	1:20	GpAB/Ins/FITC	GPA,Bouin	Nordic
Insulin[b]	1:20	K5982	GPA,Bouin	L.Heding
Insulin	1:20	LAA	GPA,Bouin	L.Heding
GIP	1:600	378	GPA	J.M.Polak
HCG	1:1000	226	GPA	J.M.Polak
γ1-MSH	1:3000	823	GPA	J.M.Polak

Motilin	1:1000	407	GPA	J.M.Polak
Motilin	1:200	123	GPA	J.M.Polak
Neurotensin	1:800	810	GPA	J.M.Polak
Growth hormone	1:7500	134	GPA	J.M.Polak
human prolactin	1:1800	556	GPA	J.M.Polak
ovine prolactin	1:1000	M3	GPA	J.A.M.Mattheij
ovine prolactin	1:500	M4	GPA	J.A.M.Mattheij
rat prolactin	1:500	M5	GPA	J.A.M.Mattheij
rat prolactin	1:500	M6	GPA	J.A.M.Mattheij
rat prolactin	1:500	M7	GPA	J.A.M.Mattheij
rat prolactin	1:500	M8	GPA	J.A.M.Mattheij
PTH[b)]	1:100	102	GPA	J.M.Polak
Secretin	1:2000	53	GPA	J.M.Polak
Somatostatin-14	1:1000	744	GPA	J.M.Polak
VIP	1:2000	652	GPA	J.M.Polak
TRH	1:2000		GPA	J.M.Polak
Substance P	1:1000	455	GPA	J.M.Polak

a)Results obtained with this antiserum have been published (Veenstra and Schooneveld 1984)

b)Antisera raised in guinea pig

microscope after 1 hr incubation at room temperature. This antiserum was purchased from Nordic, Tilburg, The Netherlands. Gifts of antisera from the following persons are gratefully acknowledged: Prof. H.H. Boer, Amsterdam; Prof. F.T. Bosman, Maastricht; Prof. G.J. Dockray, Liverpool; Dr. H.J.T. Goos, Utrecht; Dr. L. Heding, Bagsvaerd; Dr. J.A.M. Mattheij, Wageningen; Dr. W.J. Nooyen, Leiden; Prof. F. Vandesande, Leuven; Prof. N. Yanaihara, Shizuoka. Details of the different antisera and the dilutions used are listed in Table 1.

Paraldehyde fuchsin staining (Schooneveld 1970) was used to localize the known neurosecretory cells in the brain. In a few cases alternating serial sections were stained with two different antisera to see whether certain neurons were stained by more than one antiserum.

Adsorption controls were performed by preincubating the diluted primary antisera with the homologous peptide for 24 hrs at 4°C. The amounts of peptide needed to block the immunoreaction were as follows: motilin, ACTH, gastrin, β-endorphin, γ1-MSH and somatostatin 10 nmol per ml diluted antiserum and prolactin 10 µg per ml diluted antiserum. For CRF antiserum solid phase adsorptions were used as described before (Verhaert et al. 1984). Cross-adsorption controls were performed only for gastrin and FMRFamide antisera and the homologous peptides. For these experiments the diluted antisera were adsorbed three times for 1 h with 10 nmol of peptide coupled to Sepharose-4B-beads (Pharmacia).

The ovine prolactin antiserum nr M3 was also tested on some other insect species, which were all laboratory bred and fixed in GPA. These species include: *Epilachna varivestis, Tenebrio molitor* (both Coleoptera), *Musca domestica* (Diptera), *Locusta migratoria* (Orthoptera) and *Periplaneta americana* (Dictyoptera).

All antisera used in this study were tested on appropriate mammalian tissues to confirm the specificity and to assess their potency.

RESULTS

anti-ACTH (Fig. 1)

This antiserum revealed 2 to 3 small neurons in each brain half just lateral to the large lateral neurosecretory cells. Their axons branch in the neuropile surrounding the median neurosecretory cells (Fig. 7). The immunoreactivity was abolished after preadsorbing the antiserum with ACTH.

anti-human calcitonin (Fig. 2)

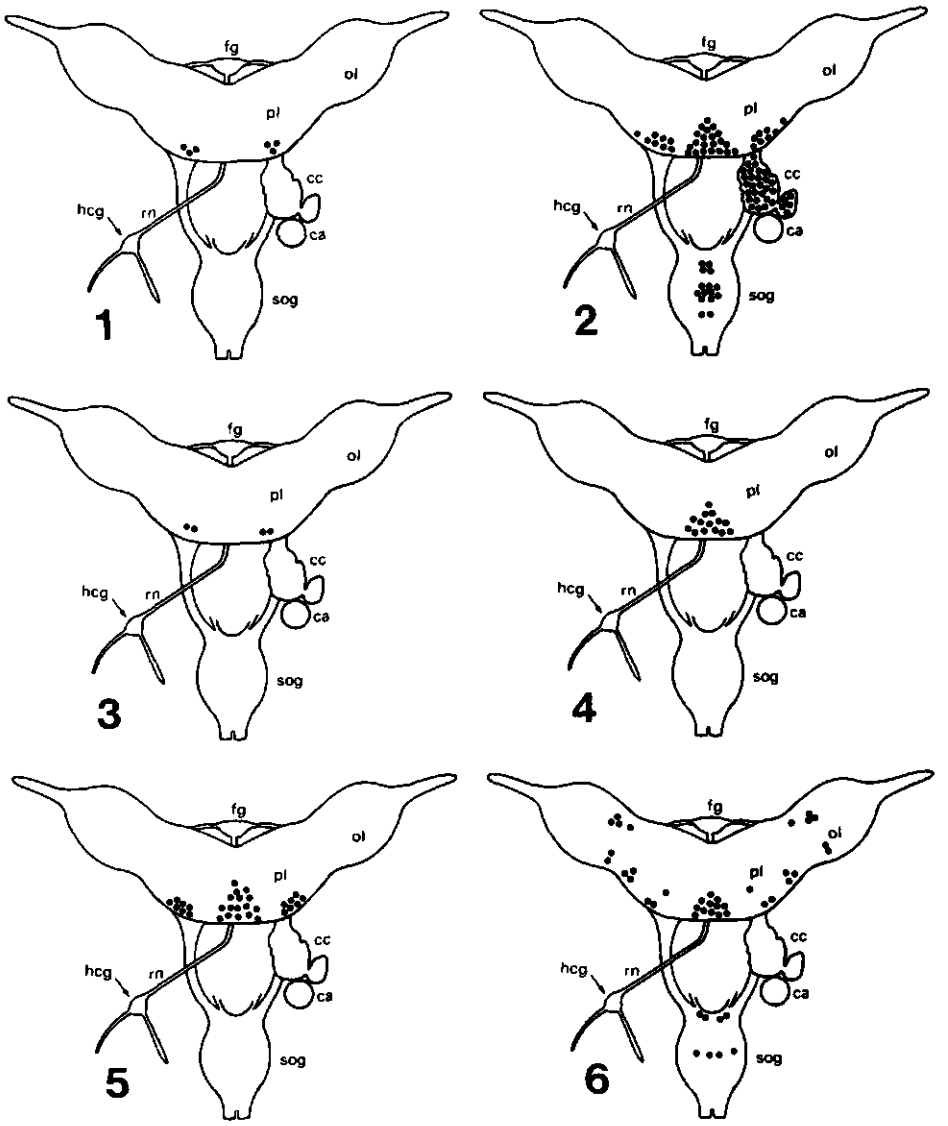
This antiserum revealed several types of neurosecretory cell. The cells include (i) at least one cell type of the median neurosecretory cells in the pars intercerebralis (probably the A or A-1 cells), (ii) the large lateral neurosecretory cells, (iii) several smaller cells located slightly lateral to the lateral neurosecretory cells, (iv) the neurosecretory cells in the suboesophageal ganglion and (v) the glandular cells of the corpus cardiacum. Axons and axon terminals in the corpus cardiacum and corpus allatum were also heavily labeled by this antiserum. The great majority of 'ordinary' neurons however was not labeled.

anti-CCK/gastrin (Fig. 3)

All antisera to gastrin and the CCK antiserum nr 67001 revealed the same immunoreactive neurons. The CCK antiserum nr 280, which recognizes the mid portion of CCK (9-20), was not immunoreactive. Only one pair of immunoreactive neurons occurs in each half of the dorsolateral protocerebrum. Axons of these neurons project to contralateral protocerebrum and to the tritocerebrum, where they branch extensively. These neurons appeared to contain also anti-FMRFamide immunoreactivity, as was shown by staining alternating serial sections. After adsorbing gastrin antiserum nr L111 with FMRFamide and FMRFamide antiserum nr 646 with tetragastrin, the immunoreactivity of both antisera was abolished, demonstrating that the co-localization of the two immunoreactivities is due to a cross-reaction. The immunoreactivity of the CCK antiserum nr 67001 and the gastrin antisera was abolished after pre-adsorption with tetragastrin.

anti-α-endorphin (Fig. 4)

This antiserum revealed some of the median neurosecretory cells in the pars intercerebralis and their axon terminals in the corpus cardiacum, but only after BHS fixation. Paraldehyde fuchsin staining showed that these cells belong to the A-1 type. The immunoreactivity obtained with this antiserum was quite variable in the Colorado potato beetle, whereas staining results with this antiserum on pituitary sections were consistent.



Figs. 1-6. Diagrams of the cephalic neuro-endocrine system of the Colorado potato beetle showing localization of immunoreactive neurons. Fig. 1. anti-ACTH; Fig. 2. anti-human calcitonin; Fig. 3. anti-gastrin/CCK; Fig. 4. anti-α-endorphin; Fig. 5. anti-β-endorphin; Fig. 6. anti-CRF. *ca*, corpus allatum; *cc*, corpus cardiacum; *fg*, frontal ganglion; *hcg*, hypocerebral ganglion; *ol*, optic lobe; *pl*, protocerebral lobe; *rn*, recurrent nerve; *sog*, suboesophageal ganglion.

anti-β-endorphin (Fig. 5)

Two antisera to β-endorphin were used in this study. One of them, nr 388, wat not immunoreactive, while the other, nr R5803, gave positive results. After GPA fixation some of the median neurosecretory cells (Fig. 8) and their axons and axon terminals in the corpus cardiacum were revealed. Paraldehyde fuchsin staining showed that these cells belong to the A-1 type of median neurosecretory cells. After BHS fixation the lateral neurosecretory cells and their axon terminals in the corpus allatum were also immunoreactive. The immunoreactivity was abolished after preadsorption of the antiserum with β-endorphin.

anti-corticotropin-releasing factor (Fig. 6)

Immunoreactive neurons were revealed with this antiserum in several areas of the brain. In the pars intercerebralis the B type neurosecretory cells were immunoreactive (Fig. 9). Their axons can be followed to the corpus cardiacum, where many immunoreactive axon terminals were found. Two groups of immunoreactive neurons were found in the optic lobe. Furthermore single neurons were sometimes found in the dorsal protocerebrum and optic lobe. Staining of alternating serial sections with this antiserum and FMRFamide antiserum showed that the two antisera reacted with different neurons.

Large numbers of immunoreactive axons enter the brain via the antennal nerve (Fig. 10). The cell bodies of these neurons appear to be located peripherally, i.e. in the antenna. The axons run to a certain neuropile area in the deutocerebrum. Many of these axons branch extensively in this area (Fig. 10), while some of them continue via the circumoesophageal connective to the ventral neuropile of the suboesophageal ganglion.

Six immunoreactive neurons were found in the suboesophageal ganglion. Two were located in the anterior part of the ganglion, four in the ventromedian area.

Immunoreactivity was abolished after preadsorption of the antiserum with corticotropin-releasing factor.

anti-insulin (Fig. 11)

Although all seven insulin antisera reacted specifically with pancreatic insular B cells, only one (without code) reacted more or less specifically with cells in the Colorado potato beetle. However, even the reaction of this antiserum was rather variable and in only a few specimens, all fixed in Bouin, a satisfactory immuno-

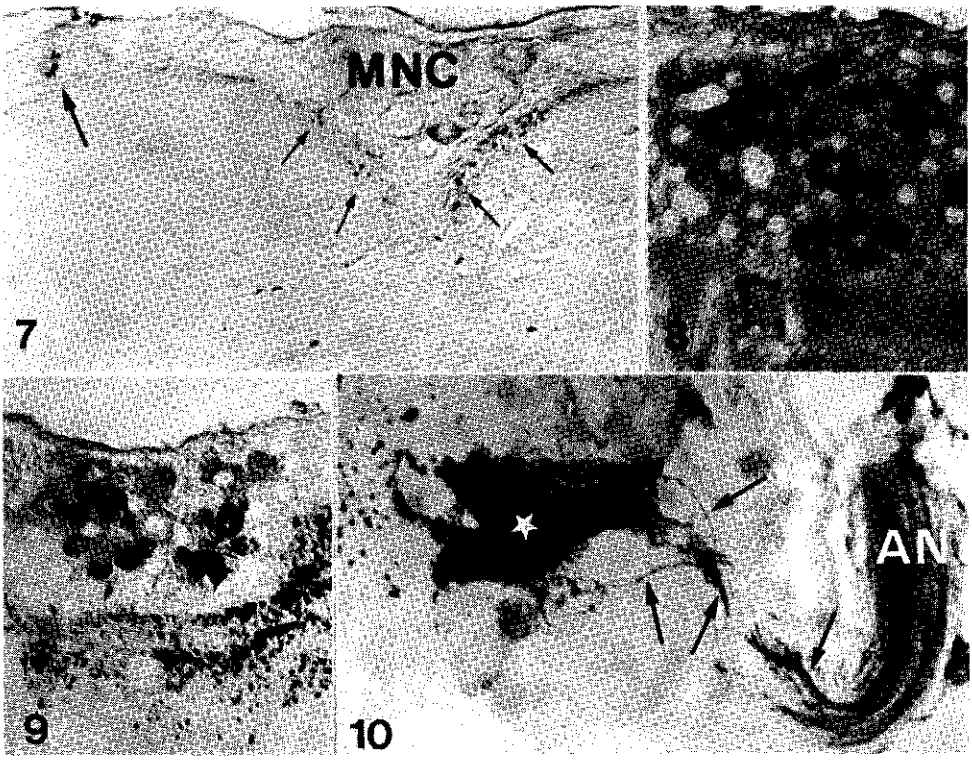


Fig. 7. Section through the protocerebrum showing ACTH-like immunoreactive neuron (*large arrow*) and axon fibres (*small arrows*) around the group of median neurosecretory cells (*MNC*). X300.
Fig. 8. β-endorphin immunoreactive A-1 type of neurosecretory cells in the pars intercerebralis. X300.
Fig. 9. B type neurosecretory cells in the pars intercerebralis as revealed by antiserum to cortocotropin-releasing factor. X300.
Fig. 10. Immunoreactive axons (*arrows*) in the antennal nerve (*AN*) and their branches invading a deutocerebral neuropile area (*asterisk*) as revealed by antiserum to corticotropin-releasing factor. X300.

reaction was found in some of the median neurosecretory cells.

anti-γ1-MSH

This antiserum revealed several neurons in the brain, suboesophageal and frontal ganglion. The neurons are identical with those which contain FMRFamide immunoreactivity.

anti-motilin (Fig. 12)

Two motilin antisera were used in this study. Antiserum nr 123 is N-terminal specific and revealed no immunoreactive neurons. The C-terminal specific antiserum nr 407, however, revealed 3 to 4 small neurons located between the lateral neurosecretory cells in the protocerebrum, as well as a group of 5 to 7 small neurons in the optic lobe with axons running to the lamina ganglionaris where they branch and end. In the corpus cardiacum the cluster of large extrinsic glandular cells and their axons and axon terminals were immunoreactive (Fig. 17), whereas the more numerous and smaller intrinsic secretory cells were not revealed. All immunoreactivity was abolished after preadsorption of the antiserum with motilin.

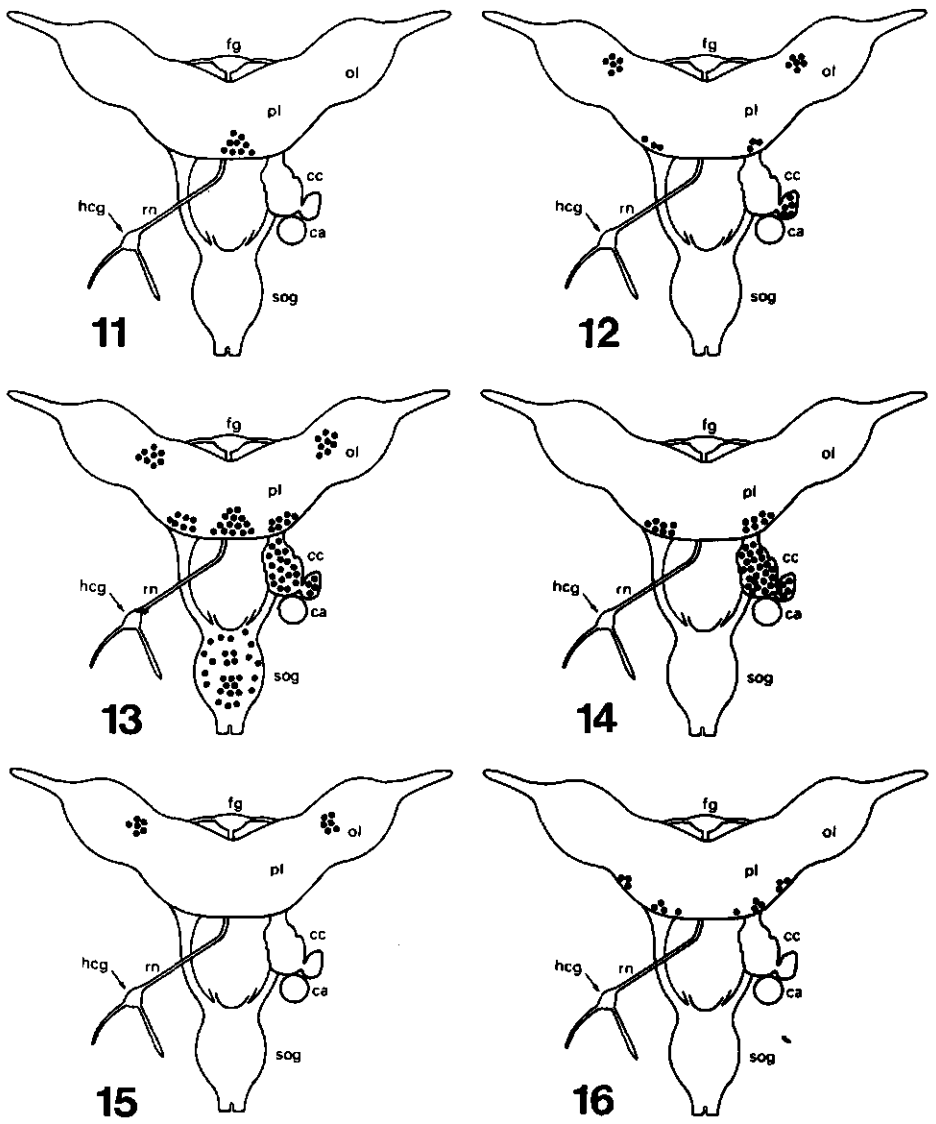
anti-growth hormone (Fig. 13)

This antiserum was immunoreactive with numerous neurons and neurosecretory cells. Both the lateral and most if not all median neurosecretory cells were immunoreactive (Fig. 18), as well as several neurons in the optic lobe, and suboesophageal ganglion. In the corpus cardiacum both types of glandular cell and axon terminals were labeled. Although many different cell types were immunoreactive, the immunoreaction itself was very selective. The great majority of neurons was not labeled, whereas axons of immunoreactive cells (e.g. those of the lateral neurosecretory cells) were well visualized (Fig. 18).

anti-ovine prolactin (Fig. 14)

One, nr M3, of the two ovine prolactin antisera tested was immunoreactive. It revealed three different groups of cells. One group comprised the lateral neurosecretory cells in the protocerebrum (Fig. 20). The reaction was only visible after injecting the beetles with colchicine; otherwise only one or two lateral neurosecretory cells were lightly stained. Their axons follow the NCC (nervi corporis cardiaci) to the corpus allatum, where numerous immunoreactive axon terminals were found (Fig. 21). Furthermore, both intrinsic and extrinsic glandular cells of the corpus cardiacum were immunoreactive (Fig. 21).

In *Epilachna varivestis* the glandular cells in the corpus cardiacum were revealed likewise by this antiserum, but not the lateral neurosecretory cells or axon terminals in the corpus allatum. No immunoreactive material could be demonstrated in *Musca domestica, Tenebrio molitor, Locusta migratoria* or *Periplaneta americana.*



Figs. 11-16. Diagrams of the cephalic neuro-endocrine system of the Colorado potato beetle showing localization of immunoreactive neurons. Fig. 11. anti-insulin; Fig. 12. anti-motilin; Fig. 13. anti-growth hormone; Fig. 14. anti-ovine prolactin; Fig. 15. anti-rat prolactin; Fig. 16. anti-somatostatin. *ca*, corpus allatum; *cc*, corpus cardiacum; *fg*, frontal ganglion; *hcg*, hypocerebral ganglion; *ol*, optic lobe; *pl*, protocerebral lobe; *rn*, recurrent nerve; *sog*, suboesophageal ganglion.

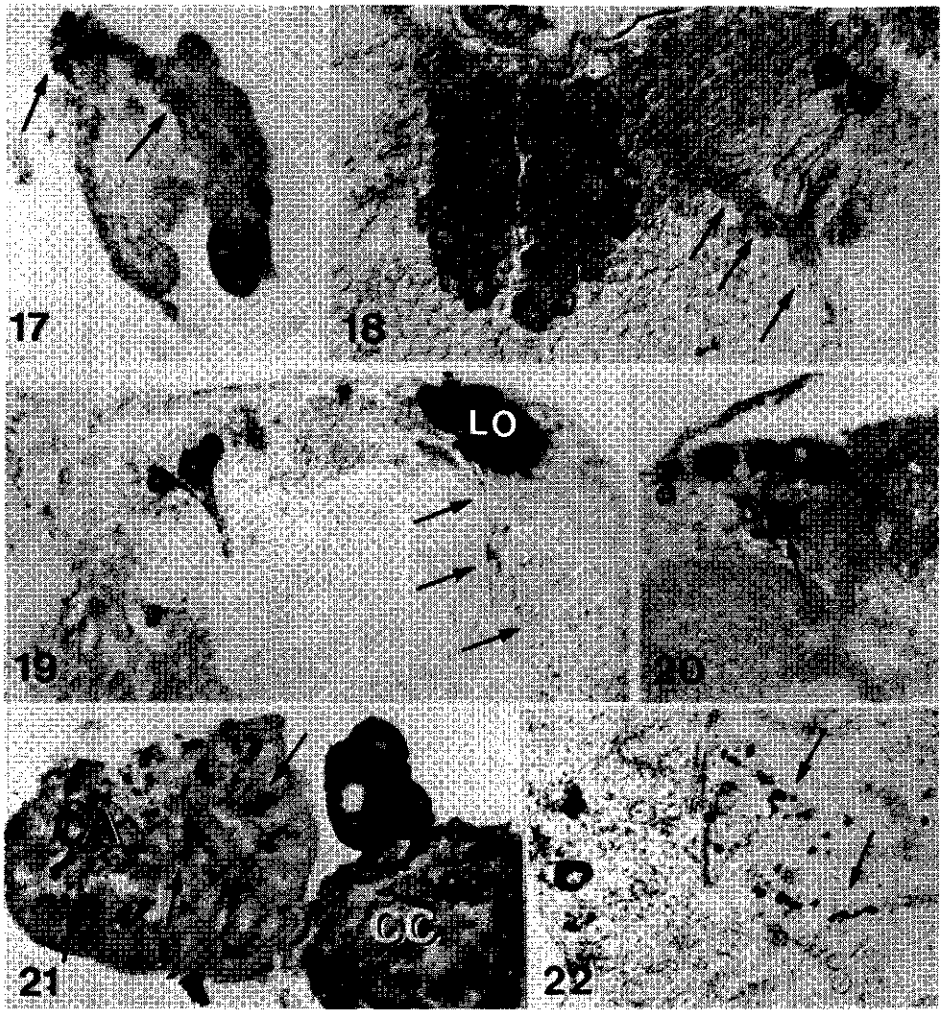


Fig. 17. Corpus cardiacum with motilin-like immunoreactive glandular cells and their processes (*arrows*). X275.
Fig. 18. Section through the protocerebrum showing growth hormone-like immunoreactive median neurosecretory cells (groups at the left) and lateral neurosecretory cells (the two cells at the top right). Note branching axons of the lateral neurosecretory cells (*arrows*). X300.
Fig. 19. Section through the optic lobe with rat prolactin immunoreactive neurons and their axon fibres in the lamina ganglionaris (*arrows*). Note that the black area at the upper right represents pigmental remnants of larval ocelli (*LO*).
Fig. 20. Lateral neurosecretory cells revealed with anti-ovine prolactin. X300.
Fig. 21. Immunoreactive glandular cells and axon terminals in the corpus cardiacum (CC) and immunoreactive axon terminals in the corpus allatum (CA) revealed with ovine prolactin antiserum. X480.
Fig. 22. Somatostatin-like immunoreactive neurons and axon fibres in the dorsocaudal protocerebrum. X480.

The immunoreactivity was abolished after preadsorption of the antiserum with ovine prolactin.

anti-rat prolactin (Fig. 15)

Only one, nr M6, out of four rat prolactin antisera reacted with neurons, which were located in two groups in the optic lobe. One group consists of 3 to 8 neurons and was located in the dorsomedium part of the optic lobe. The other group consists of 3 to 6 neurons and is located slightly more ventrally to the first group. Axons of some of these neurons run around the medulla externa and innervate the lamina ganglionaris (Fig. 19). The localization of these neurons reminds to the neurons containing FMRFamide immunoreactive material. However, staining alternating serial sections showed that the neurons are not identical. All immunoreactivity was abolished after preadsorption of the antiserum with rat prolactin, but not with ovine prolactin.

anti-somatostatin (Fig. 16)

Fibres containing somatostatin immunoreactive material were more easily revealed than the immunoreactive perikarya. The fibres are present in large numbers in the protocerebral, tritocerebral and suboesophageal neuropile. Somatostatin immunoreactive neurons are very small and often only weakly immunoreactive, even in colchicine injected animals. Three to 4 such neurons were generally found between the lateral neurosecretory cells. Their axons were seen in the neuropile surrounding the median and lateral neurosecretory cells. Four to 5 immunoreactive neurons were found in the caudolateral surface of the protocerebrum. The immunoreactivity was abolished after preadsorption of the antiserum with somatostatin.

Other antisera

No positive immunoreactions were obtained with antisera against CLIP (nr 429), leu-enkephalin (nrs 493, 774), met-enkephalin (nrs 497, 773), bombesin (nr 625), glucagon (nrs 499, Otto, K1711/130576), GIP (nr 378), HCG (nr 226), neurotensin (nr 810), human prolactin (nr 556), PTH (nr 102), secretin (nr 53), VIP (nr 652), TRH and substance P (nr 455). All these antisera, and those mentioned above to be not immunoreactive on the Colorado potato beetle, were immunoreactive on appropriate vertebrate tissues in the concentrations used.

DISCUSSION

Characterization of peptidergic cells

The present study demonstrates that a wide variety of peptides is present in the neuro-endocrine and central nervous system of the Colorado potato beetle. With antisera to various biologically active vertebrate peptides we identified here at least 10 different types of cell, which occur in constant places and numbers. In previous studies we found cells immunoreactive with antisera to bovine pancreatic polypeptide, FMRFamide (Veenstra and Schooneveld 1984), vasopressin, vasotocin, oxytocin (Veenstra et al. 1984a), α-MSH (Veenstra 1984), gastrin-releasing peptide (Veenstra and Yanaihara 1984), and proctolin (Veenstra et al. 1984b). Taking all results together, we can now distinguish 19 different types of cell with the aid of immunocytochemistry, which is considerably more than by conventional histological or electron microscopic techniques (Schooneveld 1970, 1974a).

Several cell types in known neuro-endocrine centers have been identified by more than one method. The comparison of present and earlier data indicates that the A-1-cells in the pars intercerebralis contain an endorphin immunoreactive substance and the B-cells a CRF immunoreactive substance. Furthermore the E-cells were revealed earlier by an antiserum to gastrin releasing peptide (Veenstra and Yanaihara 1984), the C-cells by antisera to bovine pancreatic polypeptide and FMRF-amide (Veenstra and Schooneveld 1984), and now also by an antiserum to γ1-MSH. The lateral neurosecretory cells had been found previously to contain an oxytocin immunoreactive substance (Veenstra et al. 1984a), and were now shown to be immuno-reactive with antisera to ovine prolactin and β-endorphin. The glandular cells of the corpus cardiacum were also immunoreactive. The so-called extrinsic cells were revealed by an antiserum to motilin, the intrinsic cells by a particular antiserum to vasopressin (Veenstra et al. 1984a); both types were immunoreactive with anti-serum to ovine prolactin. However, none of the antisera used so far gave a positive immunoreaction with the type A neurosecretory cell, to which much attention was paid in the past and which is strongly stained with the Gomori staining method for neurosecretory material (Schooneveld 1970).

Some neurons are immunoreactive with antisera to different peptides. Such co-localizations may be due to a cross-reaction between two antisera. Thus bovine pancreatic polypeptide and FMRFamide antisera were found to react with the same neurons of the Colorado potato beetle (Veenstra and Schooneveld 1984). We now found that the same neurons are also γ1-MSH immunoreactive. This is not surprising,

as FMRFamide and γ1-MSH share the C-terminal dipeptide amide (-Arg-Phe-NH_2) and their antisera are known to cross-react in mammals (Ali-Rachedi et al. 1983). Four of the neurons containing bovine pancreatic polypeptide/FMRFamide/γ1-MSH immunoreactive material were also immunoreactive with gastrin antisera. As we could abolish the gastrin immunoreactivity with FMRFamide and vice versa, the co-localization of FMRFamide and gastrin immunoreactivity is due to a cross-reaction as well. Thus the co-localization of gastrin and bovine pancreatic polypeptide in the same neurons, which *seems* to be no cross-reaction, as bovine pancreatic polypeptide antiserum does not react with gastrin and vice versa, *is* in fact a cross-reaction of antisera to gastrin and bovine pancreatic polypeptide with some unknown insect peptide, presumably resembling FMRFamide. This demonstrates that the utmost caution is needed in the interpretation of co-localizations of different peptides, as long as these peptides have not been demonstrated by other means in the same tissue. The danger of relying on immunocytochemistry alone to identify a peptide (Swaab et al. 1977) is further illustrated by our observations that the antisera to human calcitonin and growth hormone recognize many different cell types. It seems very unlikely that these antisera react with specific regulatory peptides.

Functions of immunoreactive cells

Although the nature of the presumed peptides is unknown so far, the locations of the immunoreactive cells may give a clue to the function of these cells. For instance, the lateral neurosecretory cells which were immunoreactive with antisera to ovine prolactin and β-endorphin, were seen to terminate in the corpora allata. They are probably identical with the cells which were back-filled via the corpora allata with horse radish peroxidase in attempts to locate the nervous center for control of corpus allatum activity (Khan et al. 1984). Furthermore, the CRF immunoreactive axons from the antennal nerve terminate in a deutocerebral neuropile area which seems to be homologous to the mechanosensory neuropile of *Musca domestica* described by Strausfeld (1976). They are therefore possibly derived from mechanosensory neurons.

It has been demonstrated that insect insulin-like peptides can lower haemolymph levels of sugar and stimulate glycogen synthesis by the fat body (e.g. Tager et al. 1976; Duve et al. 1979; Kramer et al. 1980, 1982). It seems therefore tempting to speculate on the functions of other vertebrate peptides in insects in analogy to their function in vertebrates. However such speculations are premature, as

demonstrated by the vasopressin immunoreactive peptide from the locust *Locusta migratoria* which seems to function as a diuretic hormone and not as an antidiuretic hormone as in vertebrates (Proux et al. 1982). Furthermore somatostatin immunoreactive material in the pond snail *Lymnaea stagnalis* was shown to be present in neurosecretory cells producing a growth hormone (Schot et al. 1981). Therefore much remains to be done to find the functions for these peptides in insects.

Homologous cells

There is good evidence that particular regulatory peptides, e.g. insulin, pancreatic polypeptide and Hydra head activator, are evolutionary very old (Duve et al. 1979, 1982; Bodenmüller and Schaller 1981), and are now present in both protostomians and deuterostomians. The cells producing these peptides in vertebrates and invertebrates can in principle be traced back through a genealogical series to a common ancestral cell, or cell group, producing the ancestral peptide and may therefore be called homologous (cf Campbell and Hodos 1970). The cells producing such a peptide in different insect species may be called homologous for the same reason.

If an antiserum reveals homologous cells in different insect species, it may be assumed that the part of the peptide recognized by the antiserum has been stable during evolution. If these insect species are not closely related to each other, it may also be assumed that this part of the substance is physiologically relevant, whatever its function may be.

Cases of homologous cells can hardly be proven by immunocytochemistry. They can only be made plausible if positive immunoreactions in different species are obtained in cells, which are located in similar places. Immunoreactions obtained only occasionally in one, or a few related species indicate that a non-significant part of a peptide is revealed. In such a case it seems very unlikely that the demonstrated substance is evolutionarily related to the vertebrate peptide used as antigen. We have suggested a homology between pancreatic polypeptide immunoreactive neurons in the Colorado potato beetle and the blowfly (Veenstra and Schooneveld 1984), between immunoreactive neurosecretory cells of the suboesophageal ganglion of the Colorado potato beetle and the migratory locust (Veenstra 1984), and between certain groups of gastrin-releasing peptide immunoreactive neurons in several insect species (Veenstra and Yanaihara 1984). We will compare some of our present data with data from other species to detect other possible cases of homologous cells.

Search for homologous immunoreactive cells

Insulin-like substances have been detected in several insect species by bioassay and radioimmunoassay (Seecof and Dewhurst 1974; Tager et al. 1976; Duve et al. 1979; LeRoith et al. 1981). Furthermore the amino acid composition of the insulin immunoreactive peptide from the blowfly *Calliphora vomitoria* is similar to those of vertebrate insulins (Duve and Thorpe 1984), suggesting that the insect peptide is evolutionarily related to insulin. Insulin immunoreactivity has been localized in some of the median neurosecretory cells of the Colorado potato beetle, *Calliphora erythrocephala* (Duve and Thorpe 1979), *Locusta migratoria* (Orchard and Loughton 1980), *Bombyx mori* (Yui et al. 1980), *Eristalis aeneus* (El-Salhy et al. 1980) and *Manduca sexta* (El-Salhy et al. 1983). These neurons are considered to be homologous.

An example of non-homologous localization of immunoreactive neurons in insects is provided by the ovine prolactin antiserum M3, which reacted with the lateral neurosecretory cells which innervate the corpus allatum in the Colorado potato beetle. This suggested that a prolactin immunoreactive peptide might be involved in the regulation of corpus allatum activity in this insect. As the corpus allatum synthesizes the juvenile hormone, a hormone of primary importance to all insect species, it seemed of interest to investigate whether the same antiserum would reveal a similar pathway in other insect species. No data on prolactin immunoreactive insect neurons exist in the literature. However, none of the other species tested in this study had lateral neurosecretory cells or axon terminals in the corpus allatum that were revealed by the antiserum. The closely related beetle *Epilachna varivestis* contained some immunoreactive material, but this was located in the glandular cells of the corpus cardiacum. The ovine prolactin immunoreactivity of the lateral neurosecretory cells of the Colorado potato beetle is therefore probably due to a casual immunological resemblance between prolactin and the immunoreactive material present in these cells.

Somatostatin immunoreactivity has been found in some median neurosecretory cells in *Locusta migratoria, Eristalis aeneus* and *Manduca sexta* (Doerr-Schott et al. 1978; El-Salhy et al. 1980, 1983), whereas *Periplaneta americana* also contains lateral neurosecretory cells that were somatostatin immunoreactive (Fujita et al. 1981). In the Colorado potato beetle such neurons were found elsewhere in the brain, not among the median neurosecretory cells. Somatostatin immunoreactive material was found in some of the glandular cells of the corpus cardiacum in *Leucophaea maderae* (Hansen et al. 1982) and was reported absent from *Thaumatopoea pityocampa* and *Clitumnus extradentatus* (Rémy et al. 1977, 1978). It thus seems that the

somatostatin immunoreactive median neurosecretory cells in at least some insect species might be homologous.

Neurons which were immunoreactive with β-endorphin antisera were found among the median neurosecretory cells in the brain of the Colorado potato beetle. Similar cells were found in *Manduca sexta* and *Eristalis aeneus* (El-Salhy et al. 1980, 1983), which also indicates a homology. Moreover, some of the other median neurosecretory cells are immunoreactive with antiserum to CRF in the Colorado potato beetle as well as in *Periplaneta americana* (Verhaert et al. 1984).

Several reports have appeared on the occurrence of gastrin/cholecystokinin immunoreactive material in insects (Kramer et al. 1977; Yui et al. 1980; El-Salhy et al. 1980, 1983; Duve and Thorpe 1981; Dockray et al. 1981), but we find no evidence for a homology between gastrin immunoreactive neurons in the Colorado potato beetle and other insect species. Although the antiserum and fixation method used by us were the same as those used by Duve and Thorpe (1981) for the blowfly, the localization of gastrin immunoreactive neurons in the two species proved to be different. The present work shows that there is a cross-reaction of the antisera to FMRFamide and gastrin with the same peptide. If the peptide present in these neurons is indeed similar to FMRFamide, as suggested earlier (Veenstra and Schooneveld 1984), this cross-reaction might be explained by the sequence -Met-ooo-Phe-NH_2, which is present in gastrin and cholecystokinin, as well as in FMRFamide.

It is clear from the above examples, that so far only in a few cases evidence is available to support the idea that particular immunoreactive neurons in different insect species are homologous. As the majority of the comparisons of insect species were based on literature data from different research groups using different antisera and fixation procedures, some homologies may have been masked by the use of different immunocytochemical procedures. The effect of differences in immunocytochemical procedures was systematically investigated in a study on vasopressin immunoreactive neurons in the Colorado potato beetle, using three different fixation procedures and four different antisera to vasopressin. The results showed that a positive reaction of particular groups of immunoreactive cells depended strongly on both the fixation procedure and the antiserum used (Veenstra et al. 1984a). These differences in immunocytochemical procedures may explain why antisera to some peptides, such as the enkephalins, substance P, glucagon, secretin, neurotensin and VIP, were not immunoreactive on the Colorado potato beetle, whereas similar antisera were immunoreactive on other insect species (El-Salhy et al. 1980, 1983; Rémy and Dubois 1981; Fujita et al. 1981; Jan and Jan 1982; Benedeczky et al. 1982;

Hansen et al. 1982; Pagés et al. 1983; Duve and Thorpe 1984).

Homologous immunoreactive insect neurons may therefore be more easily detected if for different insect species identical antisera and fixation methods are used. Such a study was recently performed using an antiserum to gastrin-releasing peptide, which indicated that there are several examples of homologous immunoreactive neurons (Veenstra and Yanaihara 1984).

REFERENCES

Ali-Rachedi A, Ferri G-L, Varndell IM, Noorden S van, Schot LPC, Ling NL, Bloom SR, Polak JM (1983) Immunocytochemical evidence for the presence of γ1-MSH-like immunoreactivity in pituitary corticotrophs and ACTH-producing tumours. Neuroendocrinology 37: 427-433.

Benedeczky I, Kiss JZ, Somogyi P (1982) Light and electron microscopic localization of substance P-like immunoreactivity in the cerebral ganglion of locust with a monoclonal antibody. Histochemistry 75: 123-131.

Bodenmüller H, Schaller HC (1981) Conserved amino acid sequence of a neuropeptide, the head activator, from coelenterates to humans. Nature 293: 579-580.

Boer HH, Schot LPC, Roubos EW, Maat A ter, Lodder JC, Reichelt D (1979) ACTH-like immunoreactivity in two electronically coupled giant neurons in the pond snail *Lymnaea stagnalis*. Cell Tissue Res 202: 231-240.

Campbell CBG, Hodos W (1970) The concept of homology and the evolution of the nervous system. Brain Behav Evol 3: 353-367.

Dockray GJ, Duve H, Thorpe A (1981) Immunochemical characterization of gastrin/cholecystokinin-like peptides in the brain of the blowfly, *Calliphora vomitoria*. Gen Comp Endocrinol 45: 491-496.

Doerr-Schott J, Joly L, Dubois MP (1978) Sur l'existence dans la pars intercerebralis d'un insecte (*Locusta migratoria* R et F) de cellules neurosécrétrices fixant un antisérum antisomatostatine. C R Acad Sc Paris Sér D 286: 93-95.

Duve H, Thorpe A (1979) Immunofluorescent localization of insulin-like material in the median neurosecretory cells of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tissue Res 200: 187-191.

Duve H, Thorpe A (1981) Gastrin/cholecystokinin (CCK)-like immunoreactive neurones in the brain of the blowfly, *Calliphora erythrocephala* (Diptera). Gen Comp Endocrinol 43: 381-391.

Duve H, Thorpe A (1984) Comparative aspects of insect vertebrate neurohormones. In: Bořkovec AB, Kelly TJ (eds) Insect neurochemistry and neurophysiology.

Plenum Press, New York, London, pp 171-196.

Duve H, Thorpe A, Lazarus NR (1979) The isolation of material displaying insulin-like immunological and biological activity from the brain of the blowfly, *Calliphora vomitoria*. Biochem J 184: 221-227.

Duve H, Thorpe A, Lazarus R, Lowry PJ (1982) A neuropeptide of the blowfly *Calliphora vomitoria* with an amino acid composition homologous with vertebrate pancreatic polypeptide. Biochem J 201: 429-432.

El-Salhy M, Abou-El-Ela R, Falkmer S, Grimelius L, Wilander E (1980) Immuno-histochemical evidence of gastro-entero-pancreatic neurohormonal peptides of vertebrate type in the nervous system of the larva of a dipteran insect, the hoverfly, *Eristalis aeneus*. Regul Pept 1: 187-204.

El-Salhy M, Falkmer S, Kramer KJ, Speirs RD (1983) Immunohistochemical investigations of neuropeptides in the brain, corpora cardiaca, and corpora allata of an adult lepidopteran insect, *Manduca sexta* (L). Cell Tissue Res 232: 295-317.

Fujita T, Yui R, Iwanaga T, Nishiitsutsuji-Uwo J, Endo Y, Yanaihara N (1981) Evolutionary aspects of "Brain-gut peptides": an immunohistochemical study. Peptides 2 suppl 2: 123-131.

Goldsworthy GJ, Mordue W (1974) Neurosecretory hormones in insects. J Endocr 60: 529-558.

Hansen BL, Hansen GN, Scharrer B (1982) Immunoreactive material resembling vertebrate neuropeptides in the corpus cardiacum and corpus allatum of the insect *Leucophaea maderae*. Cell Tissue Res 225: 319-329.

Hökfelt T, Johansson O, Ljundahl Å, Lundberg LM, Schultzberg M (1980) Peptidergic neurones. Nature 284: 515-520.

Jan YN, Jan LY (1982) Genetic and immunological studies of the nervous system of *Drosophila melanogaster*. In: Neuropharmacology of insects, Pitman, London, pp 221-239.

Khan MA, Romberg-Privee HM, Schooneveld H (1984) Innervation of the corpus allatum in the Colorado potato beetle as revealed by retrograde diffusion with horse radish peroxidase. Gen Comp Endocrinol 55: 66-73.

Kramer KJ, Speirs RD, Childs CN (1977) Immunochemical evidence for a gastrin-like peptide in insect neuroendocrine system. Gen Com Endocrinol 32: 423-426.

Kramer KJ, Tager HS, Childs CN (1980) Insulin-like and glucagon-like peptides in insect hemolymph. Insect Biochem 10: 179-182.

Kramer KJ, Childs CN, Speirs RD, Jacobs RM (1982) Purification of insulin-like peptides from insect haemolymph and royal jelly. Insect Biochem 12: 91-98.

LeRoith D, Lesniak MA, Roth J (1981) Insulin in insects and annelids. Diabetes 30: 70-76.

Leung MK, Stefano GB (1984) Isolation and identification of enkephalins in pedal ganglia of *Mytilus edulis* (Mollusca). Proc Natl Acad Sci USA 81: 955-958.

Orchard I, Loughton BG (1980) A hypolipaemic factor from the corpus cardiacum of locusts. Nature 286: 494-496.

Pagés M, Jeménez F, Ferrús A, Peralta E, Ramirez G, Gelpi E (1983) Enkephalin-like immunoreactivity in *Drosophila melanogaster*. Neuropeptides 4: 87-98.

Proux J, Rougon G, Cupo A (1982) Enhancement of excretion across locust malpighian tubules by a diuretic vasopressin-like hormone. Gen Comp Endocrinol 47: 449-457.

Rémy C, Dubois MP (1981) Immunohistological evidence of methionine-enkephalin-like material in the brain of the migratory locust. Cell Tissue Res 218: 271-278.

Rémy C, Girardie J, Dubois MP (1977) Exploration immunocytologique des ganglions cérébroïdes et sous-oesophagien du phasme *Clitumnus extradentatus* : existence d'une neurosécrétion apparentée à la vasopressine-neurophysine. C R Acad Sci Sér D 285: 1495-1497.

Rémy C, Girardie J, Dubois MP (1978) Présence dans le ganglion sous-oesophagien de le chenille processionaire du pin (*Thaumatopoea pityocampa* Schiff) de cellules révélées en immunofluorescence par un anticorps anti-α-endorphine. C R Acad Sc Paris Sér D 286: 651-653.

Schooneveld H (1970) Structural aspects of neurosecretory and corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say), as a function of daylength. Neth J Zool 20: 151-237.

Schooneveld H (1974a) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) I. Characterization of the protocerebral neurosecretory cells. Cell Tissue Res 154: 275-288.

Schooneveld H (1974b) Ultrastructure of the neurosecretory system of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) II. Pathways of axonal secretion transport and innervation of neurosecretory cells. Cell Tissue Res 154: 289-301.

Schot LPC, Boer HH (1984) Characterization of multiple immunoreactive neurons in the central nervous system of the pond snail *Lymnaea stagnalis* with different fixatives and antisera adsorbed with the homologous and heterologous antigens. *submitted.*

Schot LPC, Boer HH, Swaab DF, Noorden S van (1981) Immunocytochemical demonstration of peptidergic neurons in the central nervous system of the pond snail *Lymnaea stagnalis* with antisera raised to biologically active peptides of vertebrates. Cell Tissue Res 216: 273-291.

Seecof RL, Dewhurst S (1974) Insulin is a *Drosophila* hormone and acts to enhance the differentiation of embryonic *Drosophila* cells. Cell Differ 3: 63-70.

Snyder SH (1980) Brain peptides as neurotransmitters. Science 209: 976-983.

Starratt AN, Brown BE (1975) Structure of the pentapeptide proctolin, a proposed neurotransmitter in insects. Life Sci 17: 1253-1256.

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall Inc, New Yersey.

Stone JV, Mordue W (1980) Isolation of insect neuropeptides. Insect Biochem 10: 229-239.

Stone JV, Mordue W, Battley KE, Morris HR (1976) Structure of locust adipokinetic hormone that regulates lipid utilisation during flight. Nature 263: 207-211.

Strausfeld NJ (1976) Atlas of an insect brain. Springer-Verlag, Berlin, Heidelberg, New York.

Swaab DF, Pool CW, Leeuwen FW van (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-391.

Tager HS, Markese J, Kramer KJ, Speirs RD, Childs CN (1976) Glucagon-like and insulin-like hormones of the insect neurosecretory system. Biochem J 156: 515-520.

Veenstra JA (1984) Immunocytochemical demonstration of a homology in peptidergic neurosecretory cells in the suboesophageal ganglion of a beetle and a locust with antisera to bovine pancreatic polypeptide, FMRFamide, vasopressin and α-MSH. Neurosci Lett 48: 185-190.

Veenstra JA, Yanaihara N (1984) Immunocytochemical localization of gastrin-releasing peptide/bombesin-like immunoreactive neurons in insects. Histochemistry, *in press.*

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of peptidergic neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235: 303-308.

Veenstra JA, Romberg-Privee HM, Schooneveld H (1984a) Immunocytochemical localization of peptidergic cells in the neuro-endocrine system of the Colorado potato beetle, *Leptinotarsa decemlineata*, with antisera against vasopressin, vasotocin and oxytocin. Histochemistry 81: 29-35.

Veenstra JA, Romberg-Privee HM, Schooneveld H (1984b) A proctolin-like peptide and its immunocytochemical localization in the Colorado potato beetle, *Leptinotarsa decemlineata. submitted.*

Verhaert P, Marivoet S, Vandesande F, Loof A de (1984) Localization of CRF immunoreactivity in the central nervous system of three vertebrate and one insect species. Cell Tissue Res, *in press.*

Yui R, Fujita T, Ito S (1980) Insulin-, gastrin-, pancreatic polypeptide-like immunoreactive neurons in the brain of the silkworm, *Bombyx mori.* Biomed Res 1: 42-46.

A PROCTOLIN–LIKE PEPTIDE AND ITS IMMUNOCYTOCHEMICAL LOCALIZATION IN THE COLORADO POTATO BEETLE, Leptinotarsa decemlineata

SUMMARY

Evidence is presented that neurons in the adult Colorado potato beetle contain a proctolin-like substance. Using immunocytochemical methods the location of immunoreactive neurons in the central and stomatogastric nervous system is described. No neurons were found in the proto- and deutocerebrum or optic lobe. Few immunoreactive neurons are present in the tritocerebrum and numerous neurons occur in all ventral ganglia and the frontal ganglion. Two groups of neurosecretory cells in the suboesophageal ganglion contain a proctolin immunoreactive substance. In these cells this material is co-localized with a bovine pancreatic polypeptide/FMRF-amide-like substance and with a vasopressin/vasotocin/oxytocin-like substance. Proctolin immunoreactive axon terminals were found on the musculature of the fore- and hind-gut, of the vas deferens, and on some segmental muscles. Furthermore proctolin immunoreactive neurosecretory axon terminals were found in the corpus cardiacum. The proctolin-like substance may therefore function both as a neurotransmitter/neuromodulator and as a neurohormone. The presence of a proctolin-like substance was also demonstrated with a sensitive bioassay. On fractionation of extracts of nervous systems of the Colorado potato beetle with high performance liquid chromatography most of the proctolin-like bioactive material co-migrated with authentic proctolin. This shows that a proctolin-like substance of the Colorado potato beetle is very similar, if not identical, to the pentapeptide proctolin.

INTRODUCTION

Proctolin (Arg-Tyr-Leu-Pro-Thr) is an insect neurotransmitter, isolated from the cockroach *Periplaneta americana* (Brown 1975; Brown and Starratt 1975; Starratt and Brown 1975). It appears that proctolin or proctolin-related peptides are widely distributed through the arthropods, as similar peptides have been demonstrated in several insects (Brown 1977), as well as in crustaceans (Sullivan 1979; Kingan and Titmus 1980, 1983) and in xiphosurans (Benson et al. 1981b; Watson et al. 1983). The peptide was originally discovered by its ability to stimulate hind- and fore-gut contractions, but it is now known to be active on other muscles as well. Proctolin also causes contractions of skeletal muscles in locusts (Piek and Mantel 1977), it increases tonus of oviduct musculature in a horsefly (Cook and Meola 1978), and enhances contractions of cockroach and *Limulus* cardiac muscles (Miller 1979; Benson et al. 1981a). Except for these peripheral effects, it is active within the nervous system, where it has excitatory effects on cockroach neurons (Walker et al. 1980). Immunocytochemical studies revealed numerous putative proctolinergic neurons in the central nervous system of the cockroach *Periplaneta americana* (Eckert et al. 1981; Bishop and O'Shea 1982).

In an extensive study on the nervous and endocrine system of the Colorado potato beetle with immunocytochemistry using antisera to vertebrate regulatory peptides we have described several types of peptidergic neurons (Veenstra 1984; Veenstra and Schooneveld 1984; Veenstra and Yanaihara 1984; Veenstra et al. 1984a; Veenstra et al. 1984b). We here report on the presence of proctolin-like immunoreactive neurons and a proctolin-like bioactive peptide in the central nervous and peripheral nervous system of this species.

MATERIALS AND METHODS

Immunocytochemistry

Seven to 20-day-old reproducing beetles were used. They were reared in the laboratory under long day conditions. Some were injected with 1.5 µg of colchicine (Sigma) two days before fixation. The complete nervous system was dissected out under Ringer and fixed for 4 hrs in GPA (a mixture of one volume of 25% glutaraldehyde, three volumes of a saturated aqueous solution of picric acid with 1% acetic acid) (Boer et al. 1979), or 4% phosphate-buffered formaldehyde, pH 7.4. The former fixation was routinely used, as it gave a better preservation of the

immunoreactivity in the tissues. Furthermore a few fore- and hind-guts, some segmental muscles and male and female reproductive organs fixed in GPA were used. Sections were stained immunocytochemically with the indirect peroxidase-anti-peroxidase (PAP) method (Sternberger 1974). Endogenous peroxidase was inhibited by 0.01% H_2O_2 in water for 20 minutes. Proctolin antiserum (nr 9) (Bishop et al. 1981) was used in a dilution of 1:500 for 40 hrs at 4°C. Specificity of the primary antiserum was checked by preadsorbing it with 10 nmol proctolin (Peninsula) per ml diluted antiserum. Antisera to the molluscan cardioexcitatory tetrapeptide FMRF-amide (nr 646, a gift from Prof. H.H. Boer, Amsterdam), vasotocin (nr 82), and oxytocin (nr 02D, both gifts from Prof. D.F. Swaab, Amsterdam) were diluted 1:2000 and used overnight at 4°C. Possible co-existence of proctolin with other peptides in the same neuron was checked by immunostaining of alternate serial sections, or by using the double staining method of Nakane (1968). Solid-phase adsorptions were used to see whether the co-localization of proctolin with BPP/FMRFamide-like and vasopressin/vasotocin/oxytocin-like immunoreactive material might be due to a cross-reaction of the antisera. FMRFamide (Peninsula), vasopressin (Sanbio), vasotocin (Sanbio) and oxytocin (Sigma) were coupled to Sepharose-4B beads (Pharmacia). Diluted proctolin antiserum was adsorbed three times with beads equivalent to 50 nmol of peptide. Similarly the antisera to FMRFamide, vasotocin and oxytocin were adsorbed three times with 50 nmol of proctolin coupled to Sepharose-4B beads.

Extraction and bioassay of proctolin-like peptide

The extraction method is based on procedures described by O'Shea et al. (1984). Briefly, entire central nervous systems, frontal ganglia and retrocerebral glands of 100 (50 ♂♂ and 50 ♀♀) adult reproducing beetles were dissected out under Ringer, and transferred to a plastic vial containing an ice-cold mixture of methanol, water and acetic acid (90:9:1). After sonification and centrifugation the supernatant was dried under reduced pressure at 60°C. The crude sample was then partially purified using a C_{18} Sep-Pak cartridge (Waters Associates). One quarter of this Sep-Pak purified extract was used directly for bioassay, while the rest was fractionated with HPLC on a C_{18} column (Waters) using a linear gradient of 5-50% acetonitrile in 50 mM ammonium acetate pH 4.5. Thirty-five 50-drop-fractions (each approximately 1 ml) were collected, dried under reduced pressure and redissolved in physiological saline for bioassay. The column had been marked previously with ^{3}H-proctolin under identical conditions.

Bioassays were performed on the extensor tibiae muscle of *Schistocerca gregaria* (O'Shea et al. 1984). This muscle contains a small proximal bundle, which in the absence of neural stimulation possesses a myogenic rhythm. By cutting a small window in the femur of an isolated hind leg this bundle is exposed and 1 µl aliquots of test substances can be directly applied to it. The response is followed by monitoring the frequency of contractions of the tibia. Proctolin, which increases the frequency in a dose-dependent way, is used as standard. All bioassay data were converted into an equivalent amount of proctolin.

RESULTS

Immunocytochemical localization

The proctolin antiserum revealed numerous immunoreactive neurons in the nervous system. They occur in all but the hypocerebral ganglion. Their positions are indicated schematically in Fig. 1. The intensity of the immunoreaction was highly variable. Some neurons were stained strongly, others only weakly. The variations in immunocytochemical stainability among different individuals was also large. The overall intensity of the immunocytochemical staining was greatly enhanced by colchicine, injected routinely.

Proctolin immunoreactive neurons are scarce in the cerebral ganglion, but abundant in the ventral ganglia. In the protocerebrum weakly immunoreactive neurons were found only occasionally. In the optic lobe and deutocerebrum immunoreactive neurons were never found. The tritocerebrum contains 8 to 10 immunoreactive neurons on each side. They are located in the frontal part of the tritocerebrum, just under the deutocerebrum. Axons of these neurons run into the adjacent neuropile (Fig. 2). Immunoreactive axons were also found in the frontal connectives and in the antennal nerves.

Over 100 immunoreactive neurons are present in the suboesophageal ganglion. Some 80 neurons lie in the ventro-lateral areas of the ganglion. They could not be identified individually, since their numbers are large and their position variable. Some 8 to 10 immunoreactive (identifiable) neurons lie in the most frontal part of the ganglion, between the roots of the circumoesophageal connectives. About 6 to 8 immunoreactive neurons lie in the dorso-medial area, and two groups of 4 and 8 immunoreactive neurons in the ventral midline of the ganglion. Especially in the ventral neuropile areas of the suboesophageal ganglion several immunoreactive axon fibres were found.

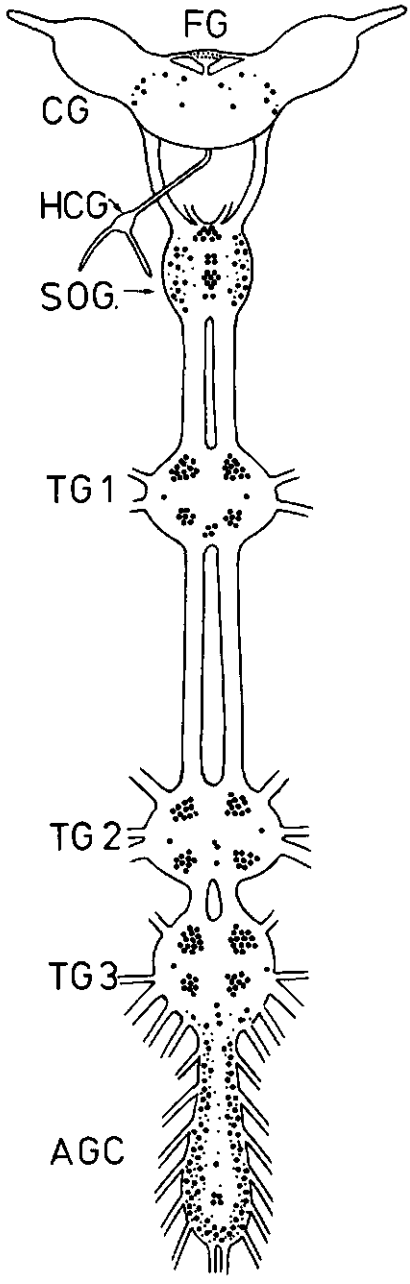


Fig. 1. Diagram of the nervous system of the Colorado potato beetle, showing schematically the distribution of proctolin immunoreactive neurons. *AGC*, abdominal ganglionic complex; *CG*, cerebral ganglion; *FG*, frontal ganglion; *HCG*, hypocerebral ganglion; *SOG*, suboesophageal ganglion; *TG1*, prothoracic ganglion; *TG2*, mesothoracic ganglion; *TG3*, metathoracic ganglion.

The general distribution of immunoreactive neurons in the three thoracic ganglia is rather similar. The frontal part of the ganglia contains a paired group of about 60 immunoreactive neurons (Fig. 5). A group of about 20 neurons is located ventrolaterally between the posterior connectives and the nerve roots innervating the legs. Three to 4 immunoreactive neurons lie on the dorsal surface of the ganglia above the ventral group of 60 neurons, while more caudally 2 to 3 immunoreactive neurons were found. One of the latter is larger and often stained more strongly than the other neurons. Often also 3 to 8 dorsal median neurons were immunoreactive. These neurons were localized most consistently in the posterior part of the prothoracic ganglion. Immunoreactive axons were found in several nerves leaving the thoracic ganglia, e.g. in those innervating the legs.

The abdominal ganglionic complex consists of the fused abdominal ganglia and contains a large number of in general small immunoreactive neurons, which could not be identified individually. Most of these neurons are located in the ventral and lateral areas. In the posterior part of the abdominal ganglionic complex a few dorsal median neurons were immunoreactive (Fig. 6).

Aboût 25 to 30 immunoreactive neurons were found in the frontal part of the frontal ganglion (Fig. 7).

Immunoreactive axons and axon terminals were found associated with the muscles of the hind- and fore-gut (Fig. 9) and with some unidentified segmental muscles of the abdomen. Furthermore, a proctolin immunoreactive innervation was found in the vas deferens (Fig. 10).

Special attention was paid to the possibility that proctolin immunoreactive material was co-localized with regulatory peptides detected by immunocytochemistry before. A comparison of the locations of the various immunoreactive neurons indicated the co-localization of proctolin with either bovine pancreatic polypeptide/FMRFamide-like material or vasopressin/vasotocin/oxytocin-like immunoreactive material, or both in neurons in the suboesophageal ganglion. The proctolin immunoreactive material in the frontal ganglion might be co-localized with a bovine pancreatic polypeptide immunoreactive substance. Staining serial alternating sections showed that the immunoreactive neurons in the ventral midline of the suboesophageal ganglion also contain bovine pancreatic polypeptide/FMRFamide and vasopressin/vasotocin/oxytocin immunoreactive material (Fig. 3,4). The best-stained preparations showed proctolin immunoreactive axons terminating in the corpora cardiaca. Comparison of serial alternating sections stained with proctolin and vasotocin antisera showed that the two paraldehyde fuchsin positive neurosecretory cells in the suboesophageal ganglion do not react with the proctolin antiserum. In the frontal

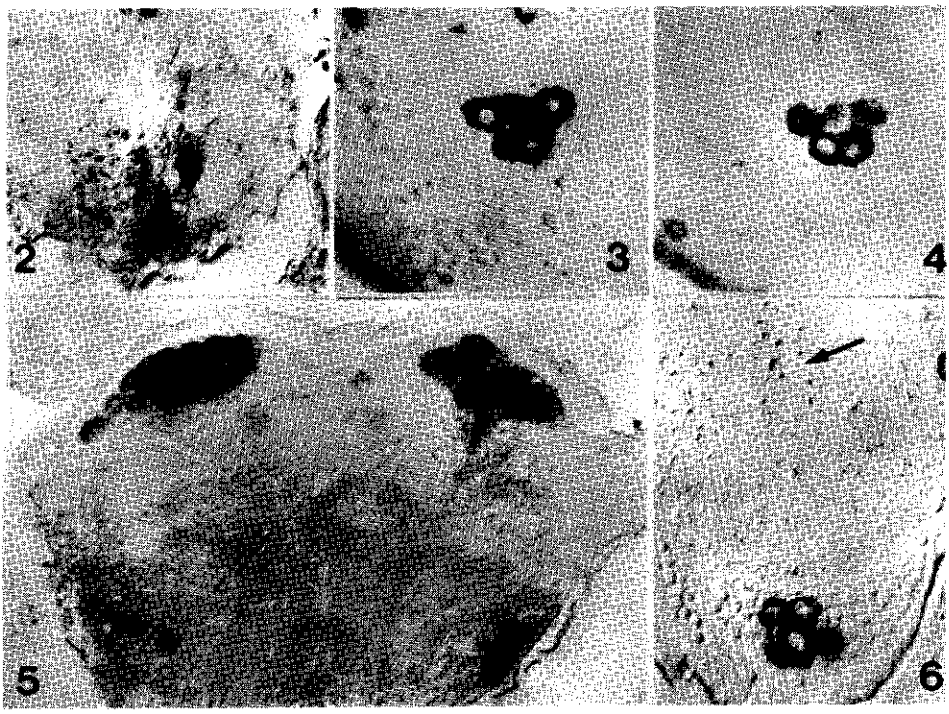


Fig. 2. Horizontal section through the tritocerebrum showing immunorea neurons and axon fibres. X215.
Fig. 3,4. Adjacent horizontal sections through the suboesophageal ganglior. Fig. 3, stained with FMRFamide antiserum, Fig 4, stained with proctolin antiserum. Note that the same neurons are labelled by the two antisera. X300.
Fig. 5. Horizontal section through the ventral part of the prothoracic ganglion demonstrating four groups of immunoreactive neurons. Frontal part of the ganglion at the top. X215.
Fig. 6. Horizontal section through the posterior part of the abdominal ganglionic complex, demonstrating immunoreactive dorsal medium neurons (at the bottom) and immunoreactive axon fibres (*arrow*). X300.

ganglion the proctolin and bovine pancreatic polypeptide/FMRFamide immunoreactive substances are located in separate neuron populations (Figs. 7,8).

All proctolin immunoreactivity was abolished by preadsorption of the antiserum with proctolin. The immunoreactivity of the proctolin antiserum was not affected by solid phase adsorptions with FMRFamide, vasopressin, vasotocin or oxytocin. Similarly, the immunoreactivity of the antisera to FMRFamide, vasotocin and oxytocin was not affected by solid adsorptions with proctolin.

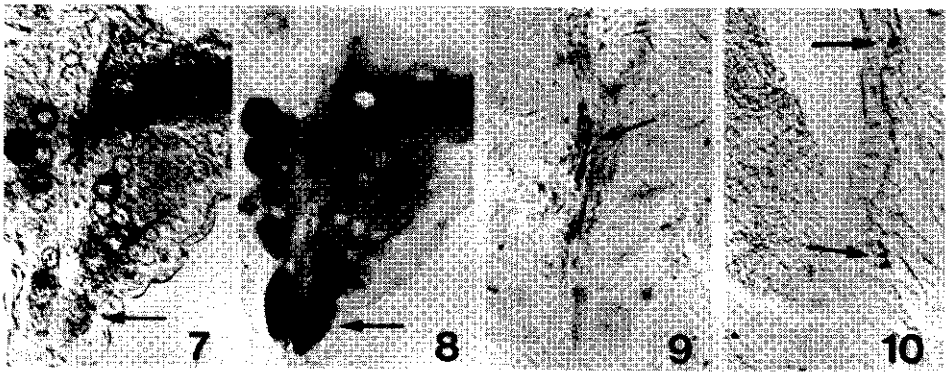


Fig. 7,8. Photomicrographs of the same oblique horizontal section through the frontal ganglion, stained first with proctolin antiserum and after photography (Fig. 7) stained in addition with FMRFamide antiserum (Fig. 8). Note that the FMRF-amide immunoreactive neurons (*arrows*) in Fig. 8 were not stained by the proctolin antiserum in Fig. 7. X340.
Fig. 9. Section through the fore-gut showing its innervation by proctolin immuno-reactive fibres (*arrows*). X480.
Fig. 10. Section through the testis showing proctolin immunoreactive fibres (*arrows*) in the vas deferens. X300.

Bioassay and partial purification of proctolin-like substance

The extract from pooled homogenates of 100 nervous systems and retrocerebral glands was pre-purified with a Sep-Pak cartridge. Part of the pre-purified material was bioassayed directly, yielding an amount of bioactive material equivalent to 40 fmol proctolin per beetle. The remainder was fractionated by high performance liquid chromatography and 1 ml fractions were bioassayed. After high performance liquid chromatography a total amount equivalent to 25 fmol proctolin per beetle was found. Most of the bioactive material co-migrated with ^{3}H-proctolin, the rest was found in two minor peaks (Fig. 11).

DISCUSSION

We demonstrated that a proctolin-like bioactive substance is present in the nervous system of the Colorado potato beetle. Since most of this proctolin-like bioactivity co-eluted with ^{3}H-proctolin on HPLC it is probably identical with, or very similar to authentic proctolin (Arg-Tyr-Leu-Pro-Thr). It is therefore likely that at least some of the proctolin immunoreactive neurons contain proctolin.

Two small peaks containing proctolin-like bioactivity eluted on approximately

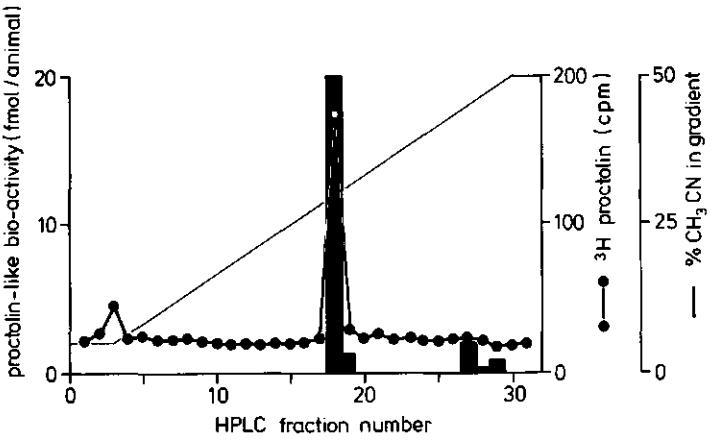


Fig. 11. Proctolin-like bioactivity detected in HPLC fractions of Sep-Pak pre-purified extracts of nervous tissue of *Leptinotarsa decemlineata*. The major proctolin-like bioactive peak co-migrates with ^{3}H-proctolin.

the same place as the myoactive peptides M1 and M2, which were recently isolated from the corpora cardiaca of *Periplaneta americana* (O'Shea et al. 1984). The peptides M1 and M2 are related to the crustacean red pigment concentrating hormone (RPCH) and the locust adipokinetic hormone (AKH). AKH- and RPCH-related peptides have been demonstrated by bioassay in a number of insect species, including the Colorado potato beetle (Mordue 1981). The small peaks of proctolin-like bioactivity therefore presumably contain the Colorado potato beetle's members of this peptide family.

It has been demonstrated that in *Periplaneta americana* proctolin may be used as a co-transmitter in an identified motorneuron, the slow coxal depressor (Adams and O'Shea 1983), while in another cell type, the lateral white cells, proctolin is probably co-localized with a biogenic amine (O'Shea and Adams 1981). It seemed therefore of interest to see whether proctolin immunoreactivity in the Colorado potato beetle might be co-localized with any of the peptides immunocytochemically detected as described before (Veenstra 1984; Veenstra and Schooneveld 1984; Veenstra and Yanaihara 1984; Veenstra et al. 1984a; Veenstra et al. 1984b). We indeed found evidence for a co-localization of proctolin immunoreactivity with substances showing bovine pancreatic polypeptide/FMRFamide and vasopressin/ vasotocin/oxytocin immunoreactivity in certain neurosecretory cells of the

suboesophageal ganglion. For neither immunocytochemically localized substances is the physiological function known at the present.

It has been suggested that proctolin might be used also as a transmitter of peripheral sensory neurons (Bishop and O'Shea 1982). We found immunoreactive axon terminals in the corpus cardiacum of the Colorado potato beetle, which suggests that a proctolin-like immunoreactive substance is released as a hormone into the haemolymph. However, as specificity in immunocytochemistry is limited (e.g. Swaab et al. 1977), it can not be excluded that this particular proctolin immunoreactive material is not identical with proctolin. It seems that most of the proctolin immunoreactive material is used as a neuromuscular transmitter. Thus proctolin immunoreactive axons were found to innervate the muscles of fore- and hind-gut. The fore-gut of insects is innervated by motorneurons located in the frontal ganglion and the tritocerebrum (Aubele and Klemm 1977). It seems therefore likely, that at least some of the proctolin-like immunoreactive neurons found by us in these parts of the nervous system of the Colorado potato beetle are motorneurons innervating the fore-gut. As we also found proctolin-like immunoreactive axons in the antennal nerve, other immunoreactive neurons in the tritocerebrum are therefore possibly motorneurons of the antennal musculature. We also found proctolin-like immunoreactive axons on some unidentified dorsal segmental muscles of the abdomen. In the American cockroach the slow coxal depressor motorneuron has been found to contain proctolin (O'Shea and Bishop 1982). The presence of immunoreactive neurons in the thoracic ganglia and of their axons in the nerve roots innervating the legs, suggests that proctolinergic leg motorneurons may also exist in the Colorado potato beetle. Furthermore evidence was found for a proctolinergic innervation of the reproductive system. Although physiological effects of proctolin on gonadal muscles have been described before (Cook and Meola 1978), the presence of a proctolin-like substance in the reproductive organs has not been reported before.

Proctolin immunoreactive neurons have also been reported from other insect species. These include *Locusta migratoria*, *Musca domestica*, *Calliphora erythrocephala* and *Dixippus morosus* (Eckert and Ude 1983). But detailed descriptions of the localization of proctolin immunoreactive neurons have only been given so far for the American cockroach, *Periplaneta americana* (Eckert et al. 1980; Bishop and O'Shea 1982), a hemimetabolous species. Although the Colorado potato beetle is a holometabolous species, the general localization of proctolin immunoreactive neurons in the two species seems to be rather similar. In both species very few or no immunoreactive neurons were found in the brain, except in the tritocerebrum,

whereas numerous proctolin immunoreactive neurons were found in the ventral ganglia. Neurons in the thoracic ganglia are mainly located in the ventral areas, more or less grouped into two bilateral clusters. The darkly staining dorsal neurons in the posterior parts of the thoracic ganglia of the Colorado potato beetle might be homologous to the giant dorsal bilateral (GDB) of the American cockroach. The immunoreactive neurons in the thoracic ganglia are more numerous in the Colorado potato beetle (about 180 per ganglion) than in the American cockroach (about 60-80 per ganglion). Part of this difference may be attributed to the use of GPA as fixation mixture instead of the 4% phosphate-buffered paraformaldehyde used by Bishop and O'Shea (1982). It has been reported, that the latter fixation reveals fewer immunoreactive neurons than GPA fixation (Eckert and Ude 1983). The abdominal ganglia in both species contain numerous proctolin immunoreactive neurons. The large structural differences of the abdominal ganglia in the two species make a further comparison impossible.

REFERENCES

Adams ME, O'Shea M (1983) Peptide co-transmitter at a neuromuscular junction. Science 221: 286-289.

Aubele E, Klemm N (1977) Origin, destination and mapping of tritocerebral neurons of locust. Cell Tissue Res 178: 199-219.

Benson JA, Sullivan RE, Watson WH, Augustine GJ (1981a) The neuropeptide proctolin acts directly on *Limulus* cardiac muscle to increase the amplitude of contraction. Brain Res 213: 449-454.

Benson J, Watson W, Augustine G, Sullivan R (1981b) A proctolin-like peptide from cardiac ganglion increases heart muscle contraction in *Limulus*. Experientia 37: 606.

Bishop CA, O'Shea M (1982) Neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH): immunocytochemical mapping of neurons in the central nervous system of the cockroach. J Comp Neurol 207: 223-238.

Bishop CA, O'Shea M, Miller RJ (1981) Neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH): immunological detection and neuronal localization in insect central nervous system. Proc Natl Acad Sci USA 78: 5899-5902.

Boer HH, Schot LPC, Roubos EW, Maat A ter, Lodder JC, Reichelt D (1979) ACTH-like immunoreactivity in two electronically coupled giant neurones in the pond snail *Lymnaea stagnalis*. Cell Tissue Res 202: 231-240.

Brown BE (1975) Proctolin: a peptide transmitter candidate in insects. Life Sci 17: 1241-1252.

Brown BE (1977) Occurrence of proctolin in six orders of insects. J Insect Physiol 23: 861-864.

Brown BE, Starratt AN (1975) Isolation of proctolin, a myotropic peptide, from *Periplaneta americana*. J Insect Physiol 21: 1879-1881.

Cook BJ, Meola S (1978) The oviduct musculature of the horsefly, *Tabanus sulcifrons*, and its response to 5-hydroxytryptamine and proctolin. Physiol Entomol 3: 273-280.

Eckert M, Ude J (1983) Immunocytochemical techniques for the identification of peptidergic neurons. In: Strausfeld NJ (ed) Functional neuroanatomy. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp 267-301.

Eckert M, Agricola H, Penzlin H (1981) Immunocytochemical identification of proctolin-like immunoreactivity in the terminal ganglion and hindgut of the cockroach *Periplaneta americana*. Cell Tissue Res 217: 633-645.

Kingan TG, Titmus M (1980) Proctolin radioimmunoassay in arthropod nervous system. Soc Neurosci Abstr 6: 619.

Kingan T, Titmus M (1983) Radioimmunological detection of proctolin in arthropods. Comp Biochem Physiol 74C: 75-78.

Miller T (1979) Nervous versus humoral control of insect heartbeat. Am Zool 19: 77-86.

Mordue W (1981) Adipokinetic hormone and related peptides. In: Farner DS, Lederis K (eds) Neurosecretion, molecules, cells, systems. Plenum Press, New York, London, pp 391-401.

Nakane PK (1968) Simultaneous localization of multiple tissue antigens using peroxidase labeled antibody method: A study on pituitary glands of the rat. J Histochem Cytochem 16: 560-577.

O'Shea M, Adams ME (1981) Pentapeptide (proctolin) associated with an identified neuron. Science 213: 567-569.

O'Shea M, Bishop CA (1982) Neuropeptide proctolin associated with an identified skeletal motoneuron. J Neuroscience 2: 1242-1251.

O'Shea M, Witten J, Schaffer M (1984) Isolation and characterization of two myoactive neuropeptides: further evidence of an invertebrate peptide family. J Neuroscience 4: 521-529.

Piek T, Mantel P (1977) Myogenic contractions in locust muscle induced by proctolin and by wasp *Philanthus triangulum* venom. J Insect Physiol 23: 321-325.

Starratt AN, Brown BE (1975) Structure of the pentapeptide proctolin, a proposed neurotransmitter in insects. Life Sci 17: 1253-1256.

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall Inc, New Jersey.

Sullivan RE (1979) A proctolin-like peptide in crab pericardial organs. J Exp Zool 210: 543-552.

Swaab DF, Pool CW, Leeuwen FW van (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-391.

Veenstra JA (1984) Immunocytochemical demonstration of a homology in peptidergic neurosecretory cells in the suboesophageal ganglion of a beetle and a locust with antisera to bovine pancreatic polypeptide, FMRFamide, vasopressin and α-MSH. Neurosci Lett 48: 185-190.

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of peptidergic neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235: 303-308.

Veenstra JA, Yanaihara N (1984) Immunocytochemical localization of gastrin-releasing peptide/bombesin-like immunoreactive neurons in insects. Histochemistry, in press.

Veenstra JA, Romberg-Privee HM, Schooneveld H (1984a) Immunocytochemical localization of peptidergic cells in the neuro-endocrine system of the Colorado potato beetle, *Leptinotarsa decemlineata*, with antisera against vasopressin, vasotocin and oxytocin. Histochemistry 81: 29-35.

Veenstra JA, Romberg-Privee HM, Schooneveld H, Polak JM (1984b) Immunocytochemical localization of peptidergic neurons and neurosecretory cells in the neuro-endocrine system of the Colorado potato beetle with antisera to vertebrate regulatory peptides. Histochemistry, in press.

Walker RJ, James VA, Roberts CJ, Kerkut GA (1980) Neurotransmitter receptors in invertebrates. In: Satelle DB, Hall LM (eds) Receptors for neurotransmitters, hormones and pheromones in insects. Elsevier/North Holland Biomedical Press, Amsterdam, pp 41-57.

Watson WH, Augustine GJ, Benson JA, Sullivan RE (1983) Proctolin and an endogenous proctolin-like peptide enhance the contractility of the *Limulus* heart. J Exp Biol 103: 55-73.

SUMMARY

This thesis describes the distribution, numbers, and morphology of peptidergic neurons and neurosecretory cells in the Colorado potato beetle, as detected with immunocytochemistry with antisera to various regulatory peptides from vertebrates, as well as to the molluscan cardioexcitatory peptide FMRFamide, and to the insect neuropeptide proctolin.

In chapter one, a description is given of peptidergic neurons in the Colorado potato beetle which are immunoreactive with antisera to FMRFamide and bovine pancreatic polypeptide. Results from cross-adsorption experiments suggest that the antisera to FMRFamide and bovine pancreatic polypeptide cross-react and reveal the same substance(s) in the Colorado potato beetle. Some of the immunoreactive neurons branch extensively in the neuropile, suggesting that the immunoreactive substance is used as a transmitter or modulator. The corpus cardiacum contains numerous immunoreactive axon terminals from neurosecretory cells located in the suboesophageal ganglion. These cells probably use the peptide as a hormone.

In chapter two, ten different antisera were used to differentiate between neurons in the neuroendocrine system of the Colorado potato beetle. Four antisera to vasopressin, three to oxytocin and one to vasotocin, neurophysin-1 and neurophysin-2 each were used. All antisera revealed immunoreactive cells, except for the two neurophysin antisera. The antisera to vasopressin all gave different results. Whereas one antiserum revealed only a single neuron pair, the other three antisera revealed additional groups of immunoreactive cells. The three oxytocin antisera also gave different results. It was further found that the fixation procedure also greatly influenced the immunocytochemical results. It was concluded that several vasopressin and oxytocin immunoreactive peptides are present in the Colorado potato beetle, all with a different degree of resemblance to vasopressin and oxytocin.

In chapter three, it was investigated whether immunocytochemical techniques could be used for the demonstration of homologous neurosecretory cells in the suboesophageal ganglion of the Colorado potato beetle and of the migratory locust. The antisera were anti-FMRFamide, anti-bovine pancreatic polypeptide, two antisera to vasopressin, which all had been used before in the work described in chapters 1 and 2, and an antiserum to α-MSH. It is shown that neurosecretory cells are present in the suboesophageal ganglion of both species and that they occur in similar locations and have the same immunoreactivity. This suggests that the two species contain homologous neurosecretory cells. It is speculated that they also have similar functions.

In chapter four, the concept of homologous neurons is further developed and ten different insect species were examined for the presence of neurons immunoreactive with a specific antiserum to gastrin-releasing peptide. Immunoreactive neurons were revealed in seven species, belonging to seven orders. The concentration of antiserum required to demonstrate immunoreactive cells was much higher in insects than in chicken proventriculus, known to contain gastrin-releasing peptide. Some immunoreactive neurons were found in the same numbers and approximately the same locations within the nervous system of different insect species. This suggests that the substances in these neurons have been relatively stable during evolution and that these neurons are homologous.

In chapter five, a large number of antisera to various regulatory vertebrate peptides was tested on the Colorado potato beetle, to reveal peptidergic neurons and neurosecretory cells and to differentiate between these cells. Immunoreactive cells were revealed by antisera to ACTH, gastrin, cholecystokinin, α-endorphin, β-endorphin, γ1-MSH, insulin, human calcitonin, motilin, growth hormone, somatostatin, corticotropin-releasing factor, ovine prolactin and rat prolactin. Some of these immunoreactive cells seem to function as neurons, whereas others function as neurosecretory cells. It is postulated that if the part of the substance recognized by the antiserum is of physiological importance to the insect, that part is retained in several species. The antiserum should then reveal homologous neurons in different insect species. Given the fact that insect species differ widely in their immunocytochemical responses, the criterion of staining of probably homologous neurons offers some help in separating relevant from irrelevant immunoreactions. The immunocytochemical data are evaluated according to this concept.

In chapter six, neurons in the central and peripheral nervous system of the Colorado potato beetle were described which contain substances immunoreactive with antiserum to the insect neuropeptide proctolin. This peptide was originally isolated from cockroaches, in which it stimulates contractions of the gut musculature. Immunoreactive axon terminals were also found on the muscles of the fore- and hindgut, and abdominal segments, as well as in the vas deferens of the testis. Furthermore, the nervous system was extracted to investigate whether proctolin-like bioactivity could be demonstrated. A proctolin-like bioactive peptide was demonstrated, that behaved chromatographically like proctolin. This suggests that at least some of the immunocytochemically demonstrated proctolin is identical with or at least similar to proctolin.

SAMENVATTING

Dit proefschrift beschrijft het voorkomen van peptiderge neuronen en neurosecretorische cellen in de Coloradokever, zoals die immunocytochemisch konden worden aangetoond met behulp van antisera tegen verschillende biologisch actieve peptiden van vertebraten, tegen het tetrapeptide FMRFamide, dat in weekdieren de hartslag versnelt, en tegen het neuropeptide proctoline dat bij insekten de darmperistaltiek versnelt.

In hoofdstuk één worden peptiderge neuronen in de Coloradokever beschreven, die reageren met antisera tegen FMRFamide en pancreatisch polypeptide. Uit adsorptieproeven bleek dat beide antisera zeer waarschijnlijk kruisreageren en dezelfde substantie(s) herkennen in de Coloradokever. Sommige neuronen die met de antisera reageren hebben axonen en dendrieten die zich binnen het neuropileem sterk vertakken, hetgeen erop kan wijzen dat zij het peptide als neurotransmitter of neuromodulator gebruiken. In het corpus cardiacum reageren beide antisera met neurosecretorische axoneindigingen die afkomstig zijn van neurosecretorische cellen in het suboesophageale ganglion. Deze cellen gebruiken het peptide waarschijnlijk als hormoon.

In hoofdstuk twee zijn tien verschillende antisera gebruikt om immunocytochemisch cellen in de Coloradokever van elkaar te onderscheiden. Er werden vier antisera tegen vasopressine, drie tegen oxytocine, en één tegen vasotocine, neurophysine-1 en neurophysine-2 elk, gebruikt. Alle antisera waren immunoreactief, behalve die tegen de neurophysinen. De verschillende antisera tegen vasopressine reageerden alle verschillend. Eén antiserum tegen vasopressine herkende slechts twee neuronen, terwijl de andere antisera ook met andere cellen reageerden. Ook de drie oxytocine-antisera reageerden verschillend. Verder bleek de samenstelling van het fixatiemengsel van grote invloed op de immunocytochemische resultaten. Uit de gevonden resultaten werd geconcludeerd dat er in de Coloradokever verschillende vasopressine/oxytocine-achtige peptiden aanwezig zijn.

In hoofdstuk drie werd onderzocht in hoeverre immunocytochemische technieken bruikbaar zijn om homologe neurosecretorische cellen in het suboesophageale ganglion van de Coloradokever en de treksprinkhaan aan te tonen. Hiervoor werden antisera gebruikt tegen FMRFamide en pancreatisch polypeptide, ook gebruikt in hoofdstuk één, twee antisera tegen vasopressine, die ook in hoofdstuk twee zijn gebruikt, en een antiserum tegen α-MSH. In beide insektensoorten werden op vergelijkbare plaatsen in het suboesophageale ganglion neurosecretorische cellen gevonden, die met dezelfde antisera reageerden. Dit suggereert dat deze cellen homoloog zijn

en mogelijk vergelijkbare functies hebben.

In hoofdstuk vier werd het concept van homologe neuronen verder uitgewerkt met behulp van een specifiek antiserum tegen gastrine releasing peptide. Tien insektensoorten werden onderzocht op het voorkomen van neuronen die met dat antiserum reageren. Zulke neuronen werden gevonden in zeven soorten, die tot zeven insektenordes behoren. De concentratie waarin het antiserum gebruikt diende te worden, was voor insekten aanzienlijk hoger dan voor kippekliermaag, waarvan bekend is dat er gastrine releasing peptide in voorkomt. Sommige met het antiserum reagerende neuronen werden bij verschillende insektensoorten in vergelijkbare aantallen en op vergelijkbare plaatsen binnen de hersenen gevonden. Dit duidt erop dat de substanties die met het antiserum reageren in de loop van de evolutie weinig zijn veranderd en dat de neuronen die deze substantie bevatten homoloog zijn.

In hoofdstuk vijf werden een groot aantal antisera tegen verschillende biologisch actieve vertebratenpeptiden onderzocht op hun bruikbaarheid om immunocytochemisch cellen in het zenuwstelsel van de Coloradokever aan te tonen. Positief reagerende cellen werden gevonden met antisera tegen ACTH, gastrine, cholecystokinine, α-endorfine, β-endorfine, γ1-MSH, insuline, menselijk calcitonine, motiline, groeihormoon, somatostatine, corticotropine-releasing factor, schapeprolactine en rattenprolactine. Sommige van deze cellen zijn neurosecretorisch, anderen zijn waarschijnlijk gewone neuronen. De hypothese wordt naar voren gebracht dat indien een stukje van een peptide belangrijk is voor het goed functioneren van het hormoon of de neurotransmitter dat stukje dan waarschijnlijk ook in meer dan één soort insekt aanwezig zal zijn. Indien dat stukje tevens immunoreactief is, dan valt te verwachten dat met het betreffende antiserum in verschillende insektensoorten homologe neuronen kunnen worden aangetoond. Aangezien de uitkomsten van immunocytochemische reacties met een bepaald antiserum bij verschillende soorten zo sterk kunnen verschillen, is het criterium van het aantonen van mogelijk homologe neuronen bruikbaar om de irrelevante en de relevante immunocytochemische reacties van elkaar te onderscheiden. De gevonden resultaten werden vervolgens met dit criterium geëvalueerd.

In hoofdstuk zes worden neuronen in het centrale en perifere zenuwstelsel van de Coloradokever beschreven die met een antiserum tegen proctoline reageren. Dit peptide is oorspronkelijk uit kakkerlakken geïsoleerd, waar het contracties van spieren van de einddarm stimuleert. Op spieren van de slokdarm, van de einddarm, van abdominale segmenten en in de vas deferens van de testis werden axoneindigingen gevonden die met het antiserum tegen proctoline reageerden. Verder werd er in een extract van zenuwstelsels na toepassing van enige chemische scheidingsmethoden

een substantie gevonden die dezelfde biologische activiteit heeft als proctoline en daarvan met een HPLC systeem niet kan worden onderscheiden. Dit duidt erop dat op zijn minst sommige van de neuronen die met het antiserum reageerden proctoline bevatten, dan wel een peptide dat sterk op proctoline lijkt.

DANKWOORD/ACKNOWLEDGEMENTS

Bij het verschijnen van dit proefschrift wil ik graag een aantal mensen met name bedanken voor hun bijdrage aan deze dissertatie.

Wijlen Prof.Dr. J. de Wilde ben ik dankbaar voor het vertrouwen dat hij in mij stelde en voor de vrijheid die hij me gegeven heeft bij de uitvoering van het onderzoek.

Prof.Dr. L.M. Schoonhoven was na het overlijden van Prof.Dr. J. de Wilde bereid als mijn promotor op te treden, hiervoor ben ik hem zeer erkentelijk.

De dagelijkse begeleiding van het onderzoek was in handen van Dr. H. Schooneveld. Beste Huug, met genoegen denk ik terug aan de afgelopen jaren. Voor het geduld en de zorgvuldigheid waarmee je de manuscripten corrigeerde, maar vooral ook voor de vrijheid die je me gegeven hebt, ben ik je dankbaar.

De meeste manuscripten zijn ook door Prof.Dr. H.H. Boer kritisch doorgelezen, behalve hiervoor ben ik hem en Dr. L.P.C. Schot erg erkentelijk voor de gastvrijheid die ik als student van hen aan de Vrije Universiteit heb ontvangen.

Een belangrijk deel van de experimenten is op nauwkeurige wijze door mw H.M. Romberg-Privee uitgevoerd. Beste Helga, voor de vlotte en plezierige manier waarop je heel wat werk voor me hebt opgeknapt ben ik je dankbaar.

C.A. Bishop, H.H. Boer, F.T. Bosman, R.E. Chance, K. Dierickx, G.J. Dockray, H. Duve, H.J.T. Goos, C.J.P. Grimmelikhuijzen, L. Heding, J.A.M. Mattheij, W.J. Nooyen, J.M. Polak, L.P.C. Schot, D.F. Swaab, A. Thorpe, F. Vandesande and N. Yanaihara are gratefully acknowledged for their generous gifts of antisera and peptides, without which this work would not have been possible.

Furthermore, I am grateful to Drs J.M. Polak and S. van Noorden for the opportunity they offered me to work in their laboratory for a month, and to Professor M. O'Shea, who kindly performed the bioassay for proctolin and HPLC separation of nervous extracts of the Colorado potato beetle.

Tenslotte dank ik F.J.J. von Planta voor het verzorgen van het omslag, mw M. Koopman voor het typewerk en W.J. Brangert voor het maken van de foto's.

CURRICULUM VITAE

Jan Adrianus Veenstra werd geboren op 11 april 1956 te Groningen. Hij behaalde in 1974 het eindexamen Gymnasium-B aan het Willem-Lodewijk Gymnasium te Groningen. In hetzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen. Het doctoraal examen Planteziektenkunde (met entomologie als verzwaard hoofdvak en dierfysiologie en informatica als bijvakken) legde hij in juni 1981 met lof af. Van 1 juli 1981 tot 1 juli 1984 was hij als wetenschappelijk assistent aangesteld bij de vakgroep Entomologie van de Landbouwhogeschool te Wageningen, waar het in dit proefschrift beschreven onderzoek werd verricht.