LOCATION AND FUNCTION OF SEROTONIN IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM OF THE COLORADO POTATO BEETLE

Theo van Haeften

Promotor: dr. L.M. Schoonhoven
Emeritus-hoogleraar in de Entomologie
(fysiologie der insekten)

Co-promotor: dr. H. Schooneveld
Universitair hoofddocent
Vakgroep Entomologie

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Theo van Haeften

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Voor mijn ouders
Voor mijn broers en zussen
Voor Olga

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Stellingen

1. Serotoninerge functionele compartimenten in het ventrale zenuwstelsel van de Coloradokever zijn ontstaan als gevolg van ganglionfusie.

   Dit proefschrift.

2. Fysiologische effecten, die optreden na toediening van serotonine aan "in vitro" darmpreparaten, dienen met voorzichtigheid te worden geinterpreteerd, omdat het optreden van deze effecten sterk afhankelijk is van de gekozen bioassay en de fysiologische uitgangsconditie van deze preparaten.

   Dit proefschrift.
   Banner SE, Osborne RH, Cattell KJ. Comp Biochem Physiol [C] (1987) 88: 131-138.
   Cook BJ, Eraker J, Anderson GR. J Insect Physiol (1969) 15: 445-455.

3. De aanwezigheid van omvangrijke serotoninerge neurohemale netwerken in de kop van de Coloradokever duidt op een snelle afbraak van serotonine in de hemolymph.

   Dit proefschrift.
   Trimmer BA. J Exp Biol (1985) 114: 307-328.

4. Immunocytochemische detectie van serotonine bevattende neuronen in het zenuwstelsel van insekten is afhankelijk van de fysiologische condities waarin de insekten zich bevinden.

   Dit proefschrift.

5. De diversiteit binnen het insektenrijk lijkt te worden weerspiegeld in het grote aantal benamingen voor één en hetzelfde type neuronen.

40951

6. Het model van Jones, dat de relatie weergeeft tussen de mate van informatietransfer van de entorhinale cortex naar de hippocampus en de frequentie van de neocorticale input in de entorhinale cortex, beschrijft niet de projecties van laag III cellen in de entorhinale cortex naar het CA1-gebied van de hippocampus en is derhalve incompleet.

   Jones RSG. TINS (1993) 16(2): 58-64.

7. De bijzondere effecten van temperatuurverhoging door microgolven op histologische processen, worden vaak ten onrechte toegeschreven aan niet nader omschreven fysische eigenschappen van deze golven, omdat de juiste controles ontbreken. Deze effecten zouden vaker vergeleken moeten worden met de effecten die optreden na een vergelijkbare conventionele temperatuurverhoging.

   Smid HM. In: Microwave Cookbook for Microscopists: Art and Science of Visualization. (1992), pp: 379-389.

8. De desinteresse van de overheid in de bijenteelt, als een voor de landbouw belangrijke vorm van vrije-tijdsbesteding, komt duidelijk tot uiting in de weigering van subsidiëring van bijensuiker.

9. Het lijdt geen twijfel, dat beperking van het gebruik van wasverzachters een grotere bijdrage levert aan een schoner milieu, dan het verpakken van deze milieubelastende middelen in zogenaamde navulverpakkingen.

10. Het gezegde: "het hemd is nader dan de rok", is vrouw-onvriendelijk.

Stellingen behorend bij het proefschrift:

Location and function of serotonin in the central and peripheral nervous system of the Colorado potato beetle.

Wageningen, 23 juni 1993 — Theo van Haeften

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CHAPTER 1

General introduction

The Colorado potato beetle

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), belongs to the family Chrysomelidae (leaf beetles), which is the third largest family of Coleoptera with 35,000 recorded species worldwide and estimates of an 25,000 species yet to be discovered (Jacques 1988; Jolivet 1988). This beetle feeds on the leaves of plants of several different genera within the family of the Solanaceae. Eggs are deposited on the leaves of the same host plant. The larvae hatch after 7 days and feed during 4 larval instars. The prepupal and pupal stages occur in the soil and transformation takes 10 days under laboratory conditions. Depending on their geographic location, there are one or two generations per year. There are two strategies of seasonal adaptation (Jacques 1988): (1) in the tropics, as the dry season approaches, the beetles enter diapause and wait for the onset of the summer rains; (2) in the temperate zone, the beetles respond to the shortening of the photoperiod in autumn; they dig themselves into the soil and enter diapause, to emerge in spring.

The Colorado potato beetle is native to Mexico, where its primary host plants are *Solanum augustifolium* and *S. rostratum* (buffalo spur) (Casagrande 1987). It moved to the western part of the United States of America were it got adapted to the cultivated potato (*S. tuberosum*) which was brought to the West by settlers. About 1859, it spread to the east, even crossed the Atlantic Ocean and moved into Europe. In the year 1920, it was reported in the Bordeaux region of France. During the following decades the beetle spread to the north-east and reached North Europe in 1945 (Roessingh 1971). The beetle proved to be a severe pest in potato on the european continent. Its control depended mainly on the use of pesticides, which resulted in the development of resistance to most of the pesticides used. Research on alternative management strategies for this insect was neglected and recent intensive research has not resulted in a simple solution of this pest problem (Casagrande 1987).

Research on the Colorado potato beetle at the Wageningen Agricultural University

In the Department of Entomology at Wageningen physiological research on the Colorado potato beetle has since 25 years been focused on two major topics. Most work has been concentrated on the endocrine regulation of reproduction and

diapause behaviour. Depending on e.g. the availability of food, temperature, and photoperiod, the beetle will either reproduce or enter diapause. Reproduction requires a high juvenile hormone titre in the hemolymph (de Wilde et al. 1968). Diapause is initiated when the juvenile hormone titre in the hemolymph becomes low due to a slow rate of juvenile hormone production by the corpora allata, and an accelerated breakdown in the blood by an specific juvenile hormone esterase (de Wilde et al. 1968; Kramer et al. 1977; de Kort 1981). The activity of the corpora allata appears to be partly regulated by peptide containing (peptidergic) neurons in the brain (Schooneveld 1970; Khan 1984). In the past many of such peptidergic neurons in the central and peripheral nervous system of the beetle have been identified, first histochemically (Schooneveld 1970), and later immunohistochemically, with polyclonal antisera to vertebrate and invertebrate peptides (Veenstra and Schooneveld 1984; Veenstra et al. 1985) and with monoclonal antibodies (Schooneveld et al. 1989; Schooneveld and Smid 1990; Smid and Schooneveld 1992). Some of these peptides are currently being isolated and identified, and their physiological significance will be established in the future.

Another major line of research dealt with the analysis of host plant selection by the Colorado potato beetle. Guided by the odour of the plant and the taste of the leaves, the beetle recognizes the host plant and feeds until satiation. The complex molecular composition of odour eliciting a locomotory response has been studied by Visser (1976), whereas the mechanisms of host odour recognition have been analyzed by de Jong (1988).

In spite of much effort spent on understanding the function of the nervous system, the brain is still a 'black box' regarding our knowledge of the routes along which afferent information from sensory neurons is processed to efferent information controlling target organs. The nature of the neurochemicals present in these neurons along the route is largely unknown. Earlier histochemical studies in the Colorado potato beetle have shown that certain sensory neurons in the antennae may contain a corticotropic releasing factor-like (CRF) peptide (Smid, personal communication). Studies with a monoclonal anti-body (MAC-2), raised against brain homogenates (Schooneveld et al. 1989), revealed the presence of a hitherto unknown peptide in sensory neurons, in afferent and efferent nerve tracts, and in the central nervous system. The peptides proctolin and FMRFamide have been demonstrated in efferent neurons innervating the gut (Veenstra and Schooneveld 1984; Veenstra et al. 1985;

Schooneveld et al. 1993). In most of these cases, peptides are supposed to function as co-transmitters. Co-localization studies in other insects, revealed that neuropeptides are often co-localized with biogenic amines (Homberg and Hildebrand 1989).

Biogenic amines have been demonstrated in the nervous system of several insect species, in the early days with the histochemical Falck-Hillarp fluorescence technique (Falck and Owman 1965), and more recently with immunohistochemistry e.g. in: *Calliphora* (Nässel 1988; Nässel et al. 1988); *Locusta migratoria* (Konings et al. 1988); and *Apis mellifera* (Schäfer et al. 1988). Circumstantial evidence suggest that biogenic amines play an important role in several aspects of feeding physiology, e.g. control of gut function, salivary glands, and processing of olfactory information (for a review see Evans 1980; Nässel 1986a, 1986b, 1988). No information is available, however, on the occurrence and function of these important neuroactive substances in the nervous system of the Colorado potato beetle.

Aim of the study described in this thesis

The aim of this study is to localize aminergic neurons in the central and peripheral nervous system of the Colorado potato beetle by means of immunohistochemistry and to assess the possible role of aminergic neurons in feeding physiology. Emphasis was laid on the position of immunoreactive neurons involved in: (1) the channelling of sensory information from antennal and gustatory detector systems to the central nervous system; (2) the central organization of the serotoninergic neuron system providing information on possible central processing of this information; and (3) the routes of innervation of target organs. The selection of suitable anti-amine antisera was of prime importance.

Biogenic amines present in the brain

We collected antisera to different biogenic amines from various suppliers (listed below) and tested them primarily for their staining of mono-aminergic neurons in the nervous system of the beetle (Table 1.). It appeared that the antisera to serotonin were among the few staining neurons in the brain. The anti-serotonin antiserum was therefore selected for further delineating the aminergic networks. It should be stated that a negative immunohistochemical staining does not necessarily imply that the

biogenic amine is altogether absent. Rather, that the presence of the antigen could simply not be demonstrated, possibly due to fixation artefacts or to antiserum specificity failures.

Table 1. Comparison of antisera to biogenic amines with repect to staining specificity and staining intensity of aminergic neurons in serial sections of beetle brains.

antiserum	supplier	antigen	fixation	type of sections	dilution 1:	results[1]
rabbit-anti serotonin	Immunonuclear	serotonin-FA-BSA	formaldehyde	paraffin	1500	+++
rabbit-anti serotonin # 7-7	Dr. Steinbusch Amsterdam	serotonin-FA-BSA	formaldehyde	paraffin	1000	++
rabbit-anti-dopamine # 9-5	Dr. Steinbusch Amsterdam	dopamine-GA-THY	glutaraldehyde	paraffin cryostat	500 500	-- +
rabbit-anti-dopamine	Immunonuclear	dopamine-GA-BSA	glutaraldehyde	paraffin cryostat	500 500	-- --
rabbit-anti-dopamine	Serotec	dopamine-GA-BSA	glutaraldehyde	paraffin cryostat	500 500	-- --
rabbit-anti-taurine	Serotec	taurine-GA-BSA	glutaraldehyde	paraffin cryostat	500 500	-- --
rabbit-anti-octopamine[2]	Dr. Vullings Utrecht	octopamine -GA-THY	glutaraldehyde	paraffin cryostat	500 500	-- --
rabbit-anti-octopamine # 2-7	Dr. Steinbusch Amsterdam	octopamine -GA/FA-BSA	glutaraldehyde /formaldehyde	paraffin cryostat	500 500	-- --
rabbit-anti-histamine	Immunonuclear	histamine-CDI-KLH	carbodiimide	paraffin cryostat	500 500	-- --

[1])

+++ reproducible staining with high intensity
++ reproducible staining with low intensity
+ variable staining results with low intensity
-- no staining
[2]) this antiserum stains Locust metathoracic DUM-neurons

FA: formaldehyde
BSA: bovine serum albumin
GA: glutaraldehyde
THY: thryroglobuline
CDI: carbodiimide
KLH: succinylated keyhole limpet hemocyanin

The commercial antiserum to serotonin (Immunonuclear) was the only one to give a reproducible staining of neurons with high intensity. We used this Immunonuclear

antiserum for the remainder of the studies mentioned in this thesis.

For practical reasons, the work is presented in subsequent chapters. The location of serotoninergic neurons in the cerebral ganglion complex and the pathways along which these neurons process antennal, visual, and intracerebral information are described in **Chapter 2.** The location of serotoninergic neuron cell bodies in the ventral nerve cord and their specialized role in inter- and intraganglionic communication are described in **Chapter 3.** The innervation of fore- and hindgut by efferent serotoninergic neurons in the stomatogastric and central nervous system and a bioassay for studying the effects of serotonin on contractions of isolated hindguts in vivo are described in **Chapter 4.** We report on the anatomy of two diffuse neurohormonal release systems for serotonin in the head and the possible target organs associated with these systems in **Chapter 5.** The results of an immunohistochemical inventory on the possible presence of serotonin in sensory neurons associated with sensilla on maxillary and labial palps, galea, the preoral cavity, antennae, eyes, and legs is described in **Chapter 6.**

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CHAPTER 2

Serotonin-like immunoreactive neurons in the cerebral ganglion complex of the Colorado potato beetle, *Leptinotarsa decemlineata.*

Submitted in a slightly revised form to Cell and Tissue Research
With: *H. Schooneveld*

SUMMARY

An immunohistochemical study was undertaken to assess the role of serotoninergic neurons in the cerebral ganglion complex of *Leptinotarsa decemlineata.* Paraffin and whole-mount preparations were made to locate immunoreactive neurons and to follow their processes within the brain. About 100 neurons were present in each brain hemisphere, representing interneurons serving both short- and long-range communication. Every major brain compartment has its complement of neurons. These neurons can be grouped according to their place, number, and distribution of their processes. Their processes have specific destinations. One category of protocerebral neurons and one in the deutocerebrum are the sole source of immunoreactivity in the central complex and the mushroom bodies. Neurons in the optic lobes send processes to one or more neuropils of the optic ganglia, whereas those in the deutocerebrum project to all of the antennal glomeruli in the deutocerebrum. It seems that the innervation may span several adjacent neuropil areas, and there is an extensive left-right communication by certain protocerebral neurons. With regard to their position, serotoninergic neurons are probably important mediators in the processing of visual, olfactory, and intracerebral information. No neurons with a secretory function were found, nor were there structural indications that serotoninergic neurons could be involved with the control of known neurosecretory neurons in the protocerebrum.

INTRODUCTION

Neural and neuroendocrine communication in the insect nervous system occurs through the transmission of chemical messengers. Several types of messengers have been identified in the nervous systems of representatives of several insect orders (Evans 1980; Nässel 1986; Rémy and Vieillemaringe 1988; Raabe 1989; Holman et al. 1990). They can be grouped into different categories, i.e.: classical neurotransmitters (e.g. biogenic amines) and peptides. We are interested in the occurrence, distribution and function of both peptides and amines in the Colorado potato

beetle, *Leptinotarsa decemlineata*, because of their probable involvement in the hormonal and neural regulation of complex behaviour.

Several putative peptidergic centres have been identified, first histochemically (Schooneveld 1970) and later immunohistochemically, with polyclonal antisera to vertebrate and invertebrate peptides (Veenstra and Schooneveld 1984; Veenstra et al. 1985) and with monoclonal antibodies (Schooneveld et al. 1989; Schooneveld and Smid 1990). The function of some of these peptides is currently under investigation.

Until now, no information has been available on the occurrence of biogenic amines in the central and peripheral nervous system of the Colorado potato beetle. In other insects, several biogenic amines have been demonstrated, either by means of the histochemical Falck-Hillarp fluorescence technique (Falck and Owman 1965; Klemm 1980), or by chemical identification methods such as the reversed-phase HPLC (Nagao and Tanimura 1988), autoradiography (Lafon-Cazal and Arluison 1976), and immunohistochemistry (Konings et al. 1988; Schäfer et al. 1988; Nässel 1986, 1988a, 1988b; Nässel et al. 1988). One of the biogenic amines, serotonin, has been particularly well studied. This is due to the availability of specific antisera and the relative abundance of serotonin in the nervous system of insects.

In our studies on the control of feeding physiology in the Colorado potato beetle, *Leptinotarsa decemlineata*, we are investigating the topography of serotonin-like immunoreactive (SLI) neurons in the central and peripheral nervous system. This paper describes the serotoninergic neural network in the brain. Preliminary studies indicated that such neurons are situated in strategic positions relative to the channels through which intracerebral information and sensory stimuli are processed (van Haeften and Schooneveld 1990). Information about the possible role of brain SLI neurons is the processing of olfactory, visual, and intracerebral information has been obtained for *Manduca sexta* (Kent et al. 1987; Homberg and Hildebrand 1989a, 1989b), *Periplaneta americana* (Salecker and Distler 1990), *Schistocerca* (Homberg 1991), and several other insect species (Nässel 1988a, 1991).

We used paraffin and whole-mount preparations to obtain an accurate and detailed map of SLI perikarya and the course of their processes. Our aim is to relate neuron position and brain areas covered by their processes for the assessment of the probable function of these neurons in intracerebral communication. Extra attention was paid to the possible occurrence of a brain-located serotoninergic communication

system controlling nervous centres lower in hierarchy. We also made a inventory of neurons with a secretory function, or those involved in the control of other neurosecretory neurons in the protocerebrum.

MATERIALS AND METHODS

Sexually mature, continuously fed Colorado potato beetles (16 h photophase, 8 h scotophase) and diapause beetles (5 months diapause) both from a laboratory culture were used in all experiments. Brains were dissected under ice-cold physiological saline (Khan et al. 1982), and fixed either for 4-5 h in a 4% formaldehyde solution, freshly prepared from paraformaldehyde and 0.1 M phosphate buffer (NaH_2PO_4) pH 7.3, or for 1-2 h in a BHS (Bouin-Hollande sublimé) solution. Fixation quality of formaldehyde and BHS fixed tissues for serial sections was improved by an additional fixation of tissues in a microwave oven according to the method of Smid et al. (1990). Next, tissues were rinsed in 0.1 M phosphate buffer pH 7.3 for 15 h at 4 °C, dehydrated, embedded in Paraplast Plus (Lancer, Oxford), sectioned at 5 μm, and mounted on poly-L-lysine (Sigma) coated slides.

Immunohistochemistry

Serial sections were deparaffinized and rinsed in phosphate-buffered-saline (PBS, Dulbecco 'A', Oxoid Corp.), pH 7.3. Next, sections were preincubated for 30 minutes in a 10% normal swine serum solution in PBS (NSS), followed by an overnight incubation at 4 °C in rabbit anti-serotonin antiserum (Immunonuclear Corp., Stillwater, Minnesota, USA), diluted 1:1500 in PBS. Immunostaining was developed according to the PAP method (Sternberger 1979) and the slides were counterstained with Mayer's haematoxylin.

Tissues for whole-mounts were, after fixation in formaldehyde, rinsed in phosphate buffer pH 7.3, and prepared according to the method of Breidbach (1990). In essence, after fixation in paraformaldehyde and several rinses in phosphate buffer pH 7.3, tissues were dehydrated to 90% ethanol and treated with n-heptane for 20 seconds to further increase membrane permeability for antibody. Next, tissues were

rehydrated to PBS + 0.25 Triton-X-100 (Merck) (PBS-T) buffer and after preincubation for 4 h in 10% NSS in PBS-T, tissues were incubated in anti-serotonin antiserum diluted 1:1000 in PBS-T + 0.1% sodium azide, for 2 days at 4 °C. The staining was developed with peroxidase conjugated swine-anti-rabbit (Dakopatts) for two days at 4 °C. The peroxidase reaction was carried out with 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2. After staining, tissues were cleared in xylol and embedded in Depex on glass slides.

Specificity controls

Antiserum specificity was tested by preadsorption of one ml diluted antiserum with 100 µg serotonin-BSA conjugate (Immunonuclear, Stillwater Minn., USA), or 100 µg BSA. The adsorped antiserum was tested on whole-mount and serially sectioned preparations. These conditions resulted in a total absence of immunostaining. The absence of endogenous peroxidase was confirmed by incubating sections and whole-mount preparations in the absence of the primary antiserum.

RESULTS

The fixation protocols used greatly influenced the selectivity of staining of serotonin-like immunoreactive (SLI) neurons in paraffin sections. The use of formaldehyde fixation allows the staining of perikarya and cell processes with equal intensity. The use of Bouin-Hollande sublimé gives a selective and strong staining of cell processes which stand out clearly against the surrounding tissue. The formaldehyde fixation gave better results for the delineation of neurons in whole-mounts.

Earlier studies on the localization of peptidergic secretory neurons in the nervous system showed that neuron visualization could be greatly improved by manipulating the physiological condition of the beetle (Schooneveld et al. 1989). Adult animals in various phases of long- and short day development were therefore used here to see if accumulation of SLI material in brain neurons could be induced in a similar way. That was not the case. The label intensity, though variable to some extend, showed

no relation with age and reproductive or diapause behaviour. Therefore, only long-day beetle of both sexes were used in all experiments.

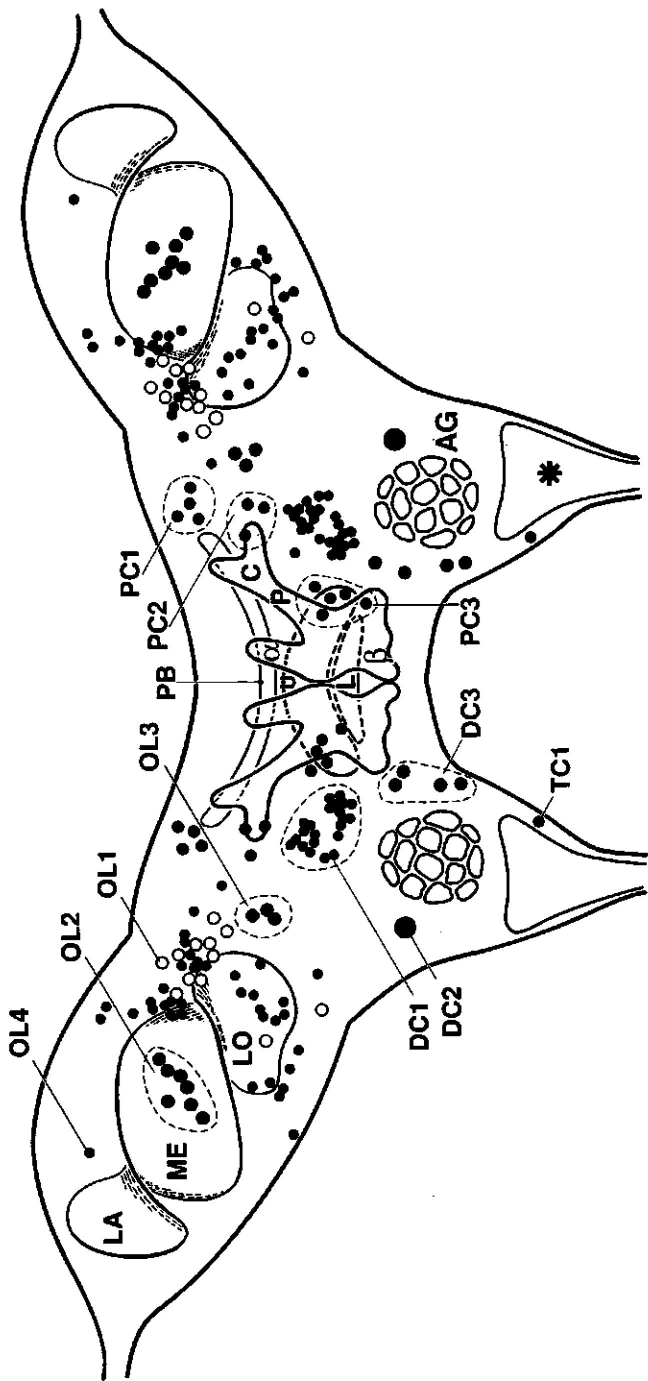
General distribution of serotonin-like immunoreactive material in the brain

The general organization of the brain and the positions of the SLI neurons and neuropils innervated by them is shown in Fig. 1. About 200 neurons were stained in the whole brain and we grouped them according to their position and the course of their processes into 11 segmentally arranged bilateral groups. Although the distribution of processes of individual neurons in each group may differ slightly, they all share a great number of characteristics. The morphology of the most conspicuous neurons in those groups is described below.

Protocerebrum.

Three distinct paired sets of neurons can be distinguished in the front-medial area (Fig. 1). (1) Eight dorsal neurons (PC1, size 10 μm) with processes that split: one branch arborizing in the ipsilateral protocerebrum, the other running into the posterior commissure, to merge into the contralateral hemisphere where it arborizes profusely (Figs. 2a, 3). (2) Six neurons (PC2, size 10 μm) with processes surrounding the ipsi-and contralateral pedunculi of the mushroom bodies with a dense network of fibres, without actually penetrating the pedunculi (Figs. 2b, 4). Other branches do innervate the calyces. Branches also run to the upper division of the central body (Figs. 2b, 5). (3) Ten neurons (PC3, size 10 μm) are present in the ventral protocerebrum, their projections could not be traced.

Fig. 1. Highly diagrammatic representation of brain of the Colorado potato beetle showing general organization and position of serotonin-like immunoreactive neurons and neuropils innervated by them, frontal view. *PC1*, *PC2*, *PC3* protocerebral neuron clusters; *OL1*, *OL2*, *OL3*, *OL4* optic lobe neuron clusters; *DC1*, *DC2*, *DC3* deutocerebral neuron clusters; *TC1* tritocerebral neuron; *AG* antennal glomeruli; *LA* lamina; *ME* medulla; *LO* lobula; *PB* protocerebral bridge; *C* calyx; *P* pedunculus; α alpha-lobe, *β* beta-lobe of mushroom body; *U* upper, *L* lower division of central body; *asterisk* tritocerebral neuropil.



AG
PC1
PC2
PC3
C
P
α
β
L
PB
OL3
DC3
TC1
OL1
OL2
OL4
LO
ME
LA
DC1
DC2

Optic lobes.

Immunoreactivity is strong in this part of the brain. Intense staining could be observed in the three optic neuropils, i.e. lamina, medulla, and lobula. We found neurons that were specifically associated with the lobula, medulla, or lamina (Figs. 1, 6a-b). (1) One group of 10-12 neurons (OL1, size 10-15 µm) lies close to the dorsal medulla. These neurons have two processes, one projecting distally to the medulla and lamina, the other projecting centrally to terminate near the central complex in the median protocerebrum (Figs. 6a-b, 7). (2) A group of 7 neurons (OL2, size 10-15 µm) near the caudal medulla innervate the medulla and the protocerebral neuropil (Figs. 6a-b, 8). Due to this massive staining, it was impossible to establish whether both neurons innervate the same or different areas of the medulla. (3) A group of three neurons (OL3, size 10-15 µm) in the constriction between protocerebral and optic lobe have major projections in the lobula and the frontal protocerebrum (Figs. 6a-b). (4) Numerous small neurons (OL4, size 10 µm) with untraceable processes are scattered over all parts of the optic lobe (Figs. 6a-b, 8).

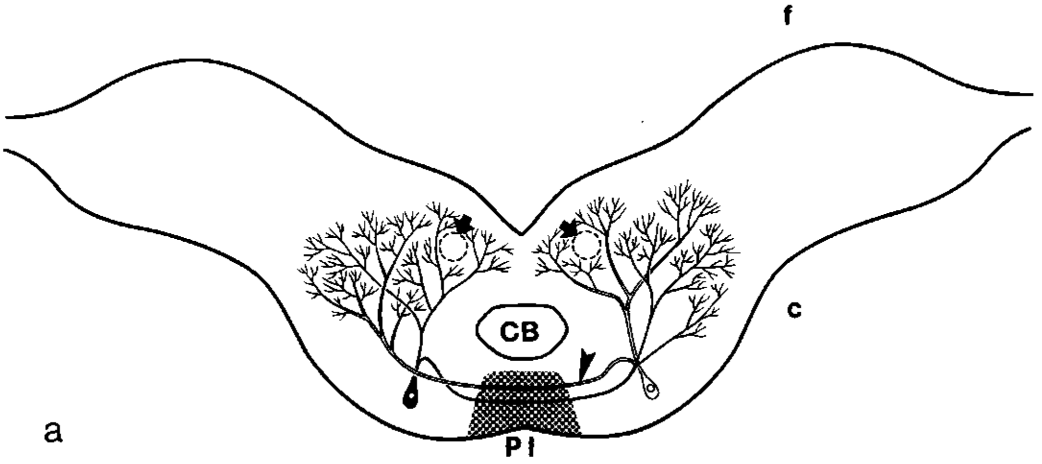
Deutocerebrum

There are three bilateral neuron groups which have in common that they innervate the antennal glomeruli. (1) One group, comprising 20-25 neurons (DC1, size 10 µm) lies close to the protocerebrum (Figs. 1, 9). Their other processes form an intensily stained bundle that runs via the antennal-glomerular tract, first to the lower and then to the upper division of the central body (Figs. 9, 10).

Figs. 2a-b. Diagrammatic representations showing location of protocerebral neurons and distribution pattern of their processes in protocerebrum. *f, c, d, v* indicates frontal, caudal, dorsal, and ventral side of brain.

2a. Processes of bilateral PC1-neurons in protocerebrum, run into posterior commissure (*arrowhead*) to terminate in contralateral hemisphere of brain. Other processes arborize extensively in ipsilateral hemisphere. *arrow*, pedunculus; *CB* central body; *PI* pars intercerebralis.

2b. Processes of bilateral PC2-neurons surround pedunculi of mushroom bodies without penetrating them (*double arrowhead*). Other branches project into contralateral hemisphere and innervate upper division of central body (*UCB*) and calyces (*C*). Note absence of processes on ß-lobes of mushroom bodies (*arrowhead*).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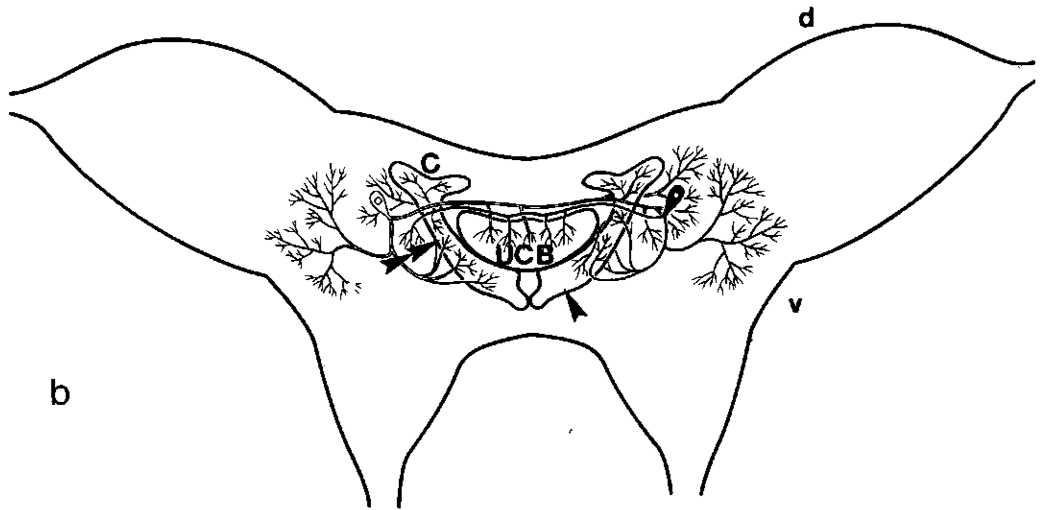
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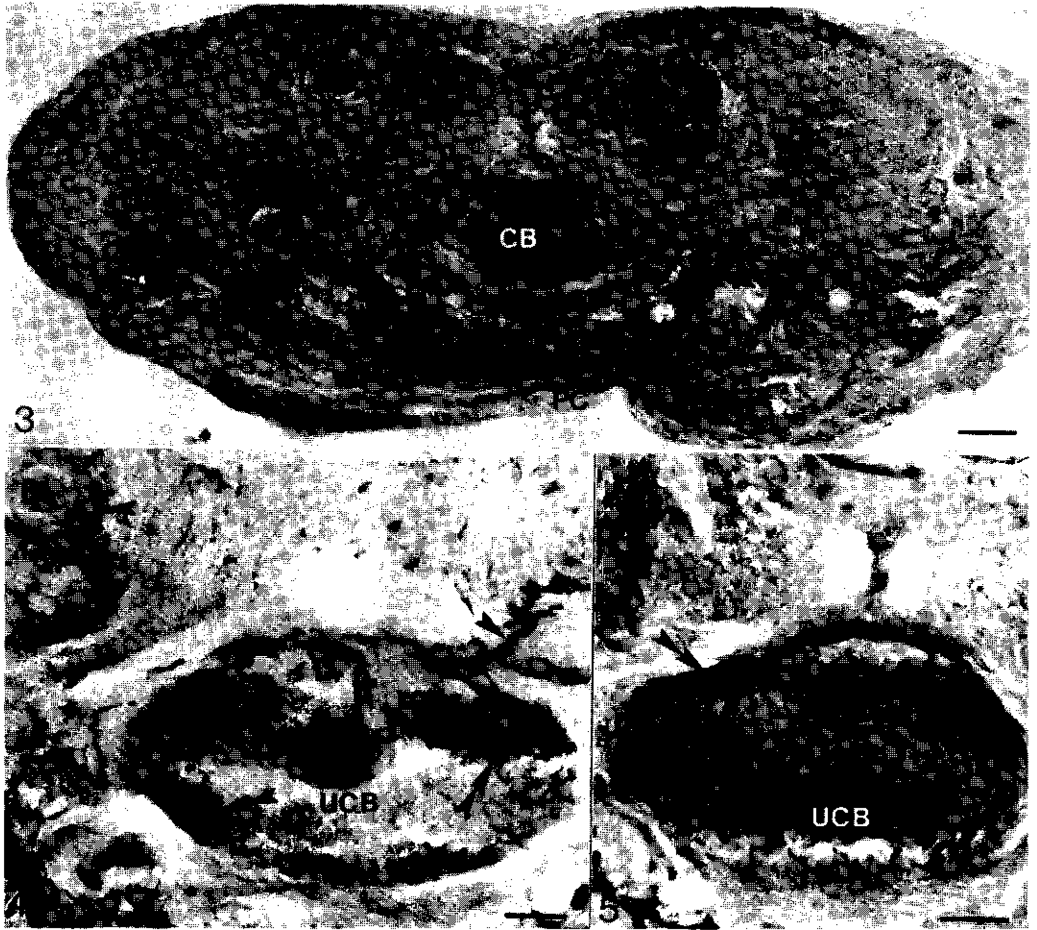


Fig. 3. Horizontal section of protocerebrum showing intense immunoreactivity in central body (*CB*) and posterior commissure (*PC*). Processes of PC1-neurons arborize profusely in contralateral hemisphere of protocerebrum (*double arrowheads*). *asterisks* pedunculi of mushroom bodies. **Bar:** 35 μm.

Fig. 4. Serial section of protocerebrum showing contralateral processes of PC2-neurons surrounding contralateral pedunculi (*P*) of mushroom bodies (*arrowheads*). Other branches innervate (*arrow*) upper division of central body (*UCB*). Note presence of dense network of varicosities in central body (*double arrowheads*). **Bar:** 20 μm.

Fig. 5. Serial section of protocerebrum showing innervation of upper division of central body (*UCB*) in detail. Branches of contralateral processes of PC2-neurons penetrate into central body (*double arrowheads*) and ramify extensively. **Bar:** 20 μm.

(2) A single large bilateral neuron (DC2, size 20-25 μm) (Fig. 9) invades the glomerular neuropil and arborizes extensively at the periphery of the glomeruli, without entering them, however. Its other process projects outside the deutocerebrum to the protocerebrum, but its final destination could not be determined. (3) A group of 5 neurons (DC3, size 10-15 μm) send processes to the glomeruli without communicating with other centres (Fig. 11).

Tritocerebrum.

The neuropil is densely packed with immunoreactive processes. Some of the processes originate from a single bilateral neuron (TC1, size 10 μm).

Communication between brain and other parts of the central nervous system

Several of the immunoreactive axon profiles in the tritocerebrum originate from perikarya in the ventral nerve cord. A few of these processes seem to continue their course, ascend into the protocerebrum, and terminate either close to the central complex or in the frontal ganglion. We found no cerebral neurons that send processes down into the ventral nerve cord.

The frontal ganglion is not innervated by immunoreactive processes originating from perikarya in the brain, whereas SLI neurons in the frontal ganglion do not project into neuropil areas of the brain.

Relation between protocerebral serotonin-like immunoreactive neurons and peptidergic neurosecretory neurons

The protocerebral SLI neurons lie close to the pars intercerebralis where several known peptidergic neurosecretory neurons are located (Schooneveld 1970). We investigated the presence of SLI material in the pars intercerebralis, but none of the secretory neurons appeared to be immunoreactive, nor did their cell processes seem to contact SLI processes in the neuropil bordering the pars intercerebralis.

DISCUSSION

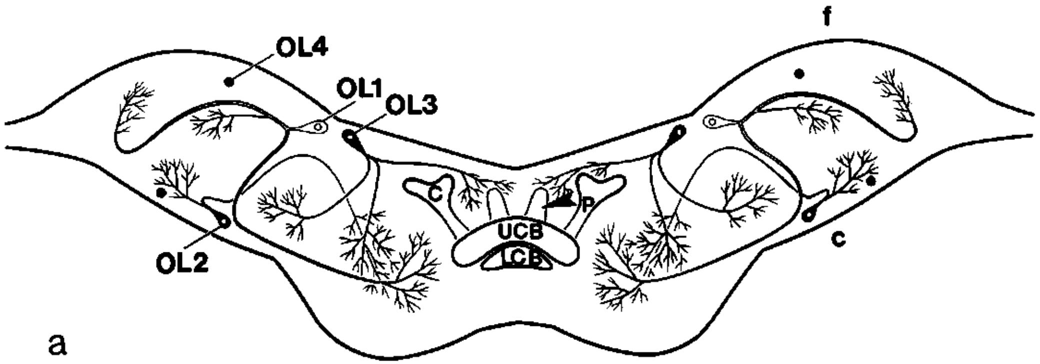
In the present study, we investigated the position and possible role of serotonin-like immunoreactive (SLI) neurons in the cerebral ganglion complex of *Leptinotarsa decemlineata*. We were able to reconstruct neuron morphologies in considerable detail by applying immunohistochemistry on paraffin sections and whole-mount preparations.

About 200 neurons are arranged in bilateral clusters in such a way that each major compartment of the brain has its complement of neurons. No neurons with a secretory function are present. All processes remain within the brain and we therefore assume that these neurons are interneurons serving both short- and long distance intracerebral communication. We grouped them according to their place, number, and the distribution of their processes. Most interneurons have processes spanning several adjacent neuropils within the same brain segment. Their processes have specific destinations. Neurons in the optic lobe send processes to one of more optic neuropils of the optic ganglia, whereas those in the deutocerebrum project to all of the antennal glomeruli. Protocerebral neurons project into the ipsi- and contralateral hemisphere of the protocerebrum. One group of protocerebral neurons and two groups in the deutocerebrum are special in that their processes run to other brain segments. These neurons appear to be the sole source of processes innervating the central complex and the mushroom bodies. We can only speculate as to the function of these brain interneurons.

Figs. 6a-b. Diagrammatic representations of brain and optic lobe showing location of representative of each optic lobe neuron cluster and distribution of its processes. *f, c, d, v* indicates frontal, caudal, dorsal, and ventral side of brain.

6a. Processes of OL1-neurons innervate both lamina and medulla, and protocerebral neuropil. Projections of OL2-neurons near caudal medulla innervate both medulla and protocerebral neuropil. Major projections of OL3-neurons in lateral protocerebrum innervate both lobula and frontal protocerebrum. OL4-neurons are found in all parts of optic lobe, their processes could not be traced. *C* calyx; *P* pedunculus of mushroom body; *arrowhead* α-lobe; *UCB* upper division of central body; *LCB* lower division of central body.

6b. Diagrammatic representation showing spatial distribution of processes of optic lobe neurons in optic lobe and lateral protocerebrum. *LA* lamina; *ME* medulla; *LO* lobula.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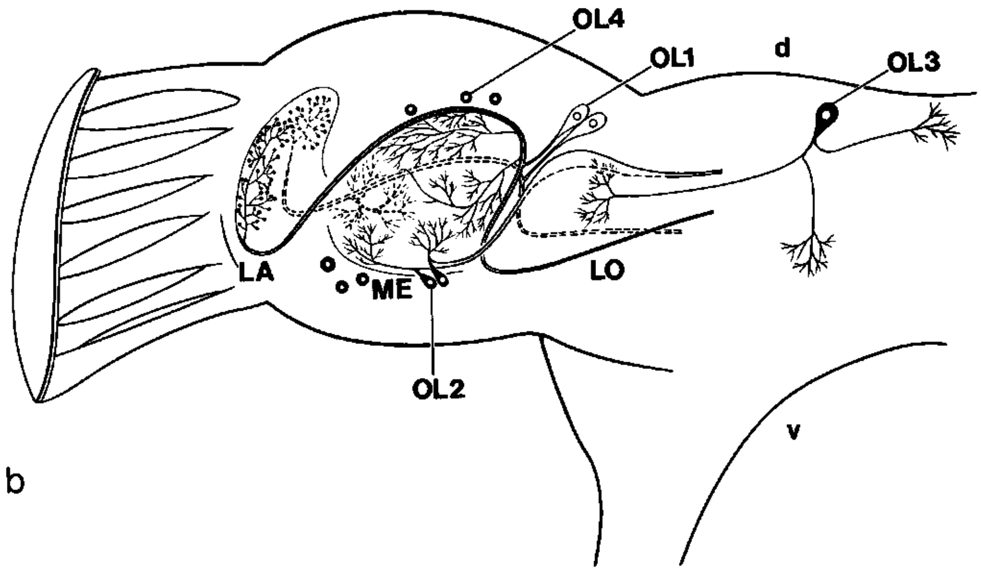
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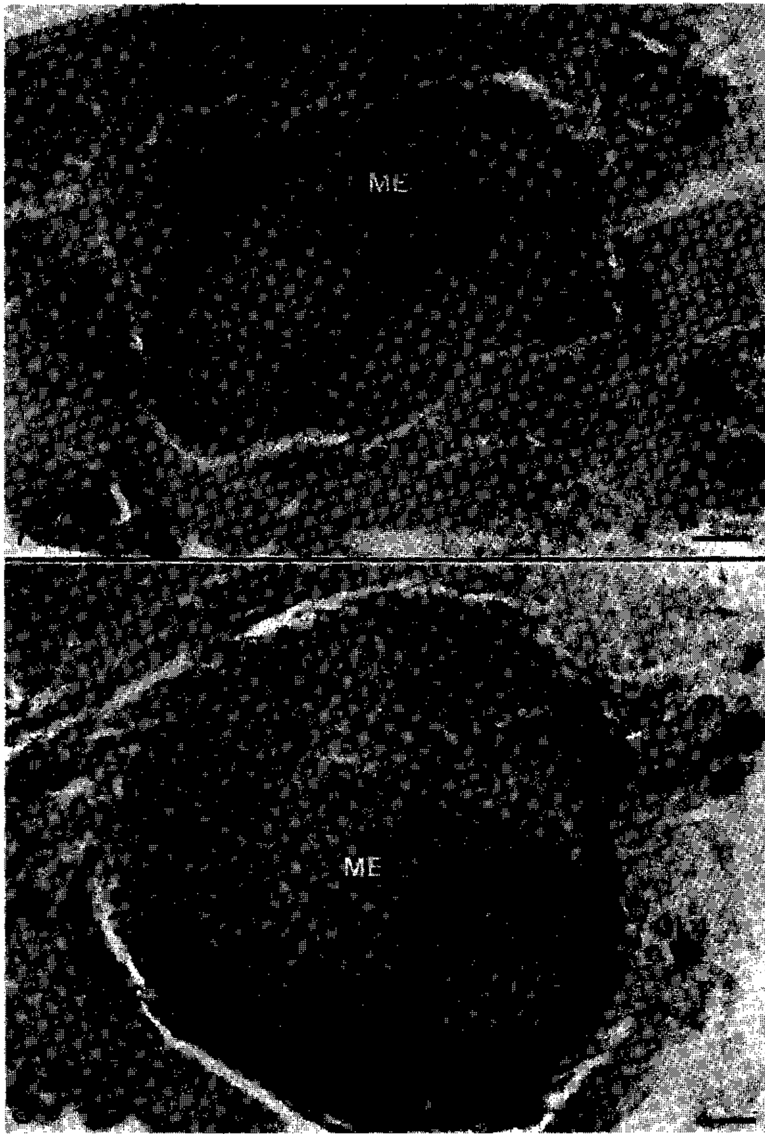

Fig. 7. Serotonin-like immunoreactivity in optic lobe. Processes of OL1-neuron in optic lobe innervate medulla (*ME*) or run along outer surface of medulla (*double arrowhead*) to terminate in lamina (*LA*). Its other process (*arrowhead*) projects to protocerebrum. Note presence of two distinct layers of immunoreactive varicosities in medulla. *asterisks* chiasmata; *LO* lobula. **Bar:** 20 µm.

Fig. 8. Serotonin-like immunoreactivity in medulla (*ME*). Medulla is innervated by processes originating from OL2-neurons. Smaller OL4-neurons are found in all parts of optic lobes. **Bar:** 20 µm.

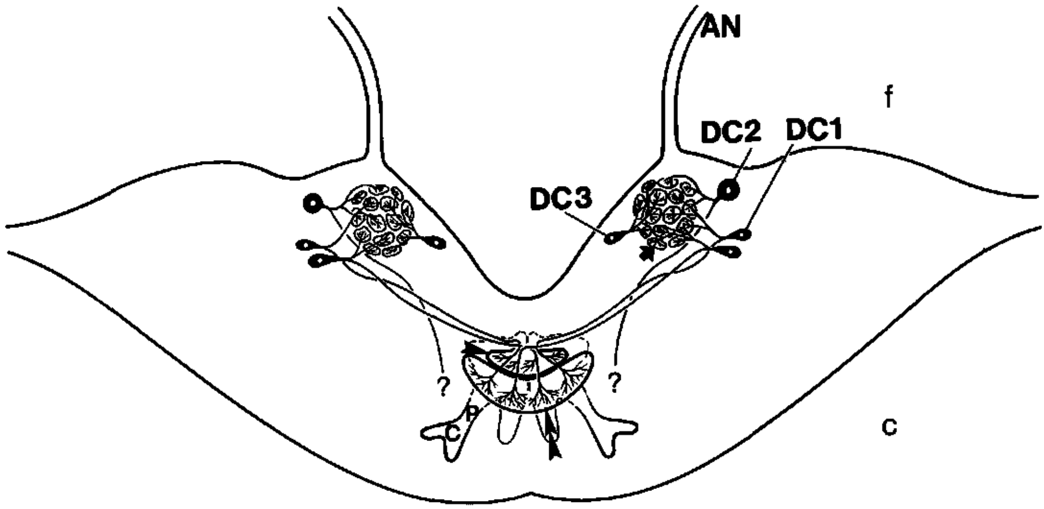

Fig. 9. Diagrammatic representation showing location of deutocerebral (*DC*) neurons in deutocerebrum and distribution pattern of their processes. DC1-neurons innervate antennal glomeruli (*arrow*). Their processes run as a bundle to protocerebrum, to terminate in lower (*arrowhead*) and upper (*double arrowhead*) division of central body. Processes of large DC2-neurons ascend to protocerebrum and presumably terminate near mushroom bodies. Processes of DC3-neurons innervate glomeruli without communicating with other centres. *AN* antennal nerves; *f*, *c* frontal and caudal side of brain; *C* calyx; *P* pedunculus.

Their position relative to the channels along which sensory and intracerebral information is relayed suggests a function in the processing of this information. Interneurons in the optic lobe may thus play a role in the processing of visual information, whereas those in the deutocerebrum are probably involved in the processing of antennal information. Protocerebral neurons with arborizations in both the ipsi- and contralateral hemisphere also have processes innervating the central complex and/or the mushroom bodies. These neurons thus appear to mediate information from both hemispheres to the central complex and the mushroom bodies. The function of these structured neuropil structures is not fully understood, but

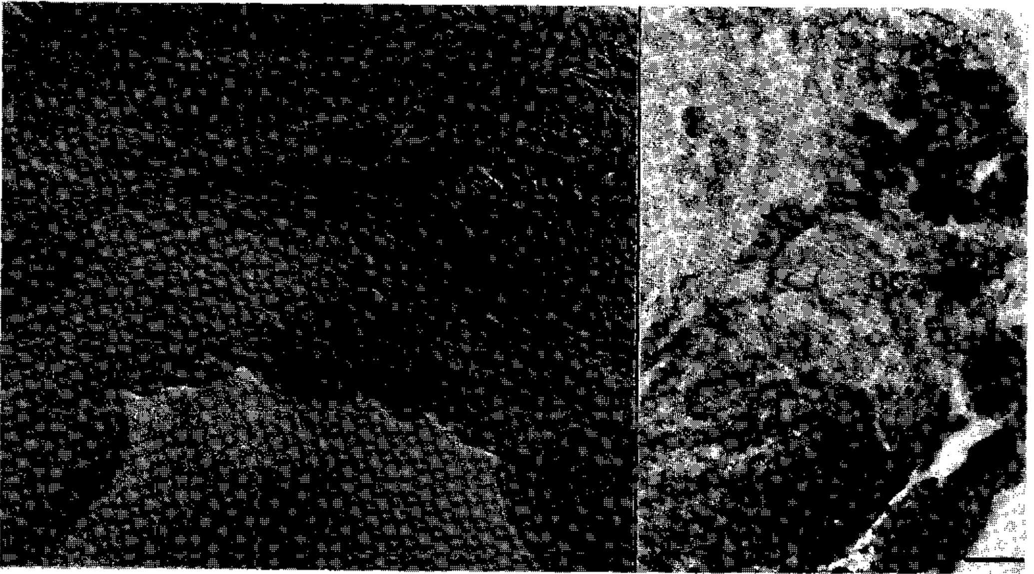

Fig. 10. Transverse serial section of brain showing serotonin-like immunoreactive processes of DC1-neurons running as a bundle (*arrowhead*) to protocerebrum to terminate in lower (*LCB*) and upper (*UCB*) division of central body. **Bar:** 30 µm.

Fig. 11. Distribution of serotonin-like immunoreactivity in deutocerebrum. Weakly stained processes of DC1-neurons (*arrowhead*) innervate antennal glomeruli (*AG*). Larger DC3-neurons lie close to DC1-neurons and glomeruli. **Bar:** 20 µm.

evidence indicates a role in central processing of intracerebral information from both hemispheres of the brain (Erber et al. 1987; Homberg 1987; Schürmann 1987). Several neurons in the brain of *L. decemlineata* are also consistently found in other insect species. This holds true for the optic lobe and the deutocerebral neurons. Although there are slight differences in the number of these neurons and in the distribution of their processes, similar neurons have been described in various insect species (Schürmann and Klemm 1984; Nässel et al. 1985; Ohlsson and Nässel 1987; Homberg and Hildebrand 1989a, 1989b; Tyrer et al. 1984; Klemm et al. 1984; Salecker and Distler 1990). Other neurons, i.e. those in the protocerebrum, show large variability between species in their number and morphology (Tyrer et al. 1984; Schürmann and Klemm 1984; Homberg and Hildebrand 1989a; Homberg

1991).

SLI processes connecting the brain to the ventral nerve cord or to the frontal ganglion are absent. Thus, the serotoninergic neural network in the brain has the appearance of an individual serotoninergic neural unit. This morphological arrangement indicates that a brain-centred serotoninergic system controlling other serotoninergic neural units lower in hierarchy is absent. It may well be, however, that individual neural units communicate through larger integrative neural systems employing other neurochemical messengers, not detected in our present methodologies.

There are no structural indications that protocerebral interneurons are involved with the control of known neurosecretory neurons in the pars intercerebralis of *L. decemlineata*, since SLI is absent in this part of the brain. There are, however, indications in other insects that serotonin-containing neurons in the pars intercerebralis may control neurosecretory neurons at a peripheral level. Studies in *L. migratoria* showed that certain neurons send processes into the storage lobe of the corpora cardiaca, where they are supposed to modulate the release of neurohormones from these glands (Konings et al. 1988). Although we did not find connections between the SLI neural network and peptidergic neurosecretory neurons in the protocerebrum, other relations might exist. There is ample evidence that serotonin co-localizes with peptides in certain cerebral peptidergic neurons (Homberg and Hildebrand 1989b; Raabe 1989). The next challenge will be to map neurons elsewhere in the nervous system of *L. decemlineata* that employ both serotonin and peptides as neurochemical messengers.

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CHAPTER 3

Serotonin-like immunoreactivity in the ventral nerve cord of the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of five different neuron classes.

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With : *H. Schooneveld*

SUMMARY

In an immunohistochemical study of the ventral nerve cord of *L. decemlineata*, five distinct neuron categories were distinguished: 1) Two paired segmental twin interneurons occur in each ganglion or neuromere; their axons distribute processes over almost the entire nerve cord and run to the cerebral ganglion complex, in contrast, other axons are distributed locally. 2) Four large frontal neurosecretory neurons occur in the suboesophageal ganglion (SOG), two of which have axons that run into the mandibular nerves to form a neurohemal plexus on the surface of cerebral nerves. 3) A pair of large caudal neurons occur in the terminal ganglion and innervate the hindgut. 4) Small local miniature interneurons occur in the SOG. 5) Terminal neurons are present in the terminal ganglion. Segmental twin interneurons appear to be grouped into 3 'functional units' spanning several ganglia. Their axons run to specific projection areas, which separate the functional units, and which mark the externally visible separation of condensed ganglion complexes. A possible role of the most caudal functional unit might be the synaptic control of caudal neurons innervating the hindgut.

INTRODUCTION

In our studies on the control of feeding physiology in the Colorado potato beetle, *Leptinotarsa decemlineata*, we are investigating the topography of serotonin-like immunoreactive (SLI) neurons in the central and peripheral nervous system. Immunohistochemical studies so far revealed the presence of 11 segmentally arranged bilateral neuron clusters in the cerebral ganglion complex, representing interneurons serving both short- and long range communication (van Haeften and Schooneveld, submitted)(Chapter 2). Every major brain compartment has its complement of neurons and their processes span several adjacent neuropil areas. The tritocerebrum is special in that it is densely packed with immunoreactive processes originating from perikarya in the ventral nerve cord (VNC).

In this paper we present a study on the topography of these SLI neurons in the

VNC of *L. decemlineata*. The ventral nervous system presents an interesting model for studying the internal organization of the insect nervous system because the adult VNC of this species shows a considerable degree of ganglion condensation (Fig. 1). The metathoracic ganglion is fused with abdominal ganglia 1 and 2 (Fig. 2d), and abdominal ganglia 3-8 are fused, or only separated by rudimentary connectives (Fig. 2e). It is conceivable that such a condensation may lead to, or be the result of, a specialization of neurons of importance to intra- or interganglionic communication. We collected immunohistochemical data on neuron morphology from nerve cords in paraffin sections and whole-mounts. We describe here the location of neuron cell bodies and the distribution of axons and dendrites to provide evidence of a specialized role of SLI neurons in intra- and interganglionic communication.

MATERIALS AND METHODS

Sexually mature, continuously fed Colorado potato beetles (16 h photophase, 8 h scotophase) and diapause beetles (5 months diapause), both from a laboratory culture, were used. Ventral nerve cords were dissected under physiological saline (Khan et al. 1982). All tissues were fixed for 4-5 h in a 4% formaldehyde solution, freshly prepared from paraformaldehyde and dissolved in 0.1 M phosphate (NaH_2PO_4) buffer pH 7.3. For serially sectioned material, an additional microwave-accelerated formaldehyde fixation was carried out according to the method of Smid et al. (1990). It is our experience that this improves the structural preservation. Tissues were rinsed in 0.1 M phosphate buffer pH 7.3 for 15 h at 4 °C, dehydrated, and embedded in Paraplast Plus (Lancer, Oxford). Serial sections of 5 µm were mounted on poly-l-lysine (Sigma) coated slides.

Immunostaining

Deparaffinized sections were rinsed in phosphate-buffered saline (PBS, Dulbecco 'A', Oxoid Corp.) pH 7.3 and preincubated for 30 minutes in 10% normal swine serum (NSS) in PBS. Rabbit-anti-serotonin antiserum (Immunonuclear Corp., Stillwater, USA) was diluted 1:1500 in PBS and tissues were incubated for one day

at 4 °C. Immunostaining was developed with the PAP method (Sternberger 1979). The slides were counterstained with Mayer's haematoxylin.

Whole-mount preparations were prepared according to the method of Breidbach (1990). After a heptane treatment, tissues were rehydrated to PBS + 0,25% Triton-X-100 (Merck) (PBS-T) buffer. After preincubation for 4 h in 10% NSS in PBS-T, tissues were incubated in the antiserum, diluted 1:1000 in PBS-T, for two days at 4 °C. Washing steps were prolonged and the staining was developed with peroxidase conjugated swine-anti-rabbit (Dakopatts) for two days at 4 °C. The peroxidase reaction was carried out with 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2. After staining, tissues were cleared in xylol, trimmed to the desired size, and embedded in Depex on glass slides.

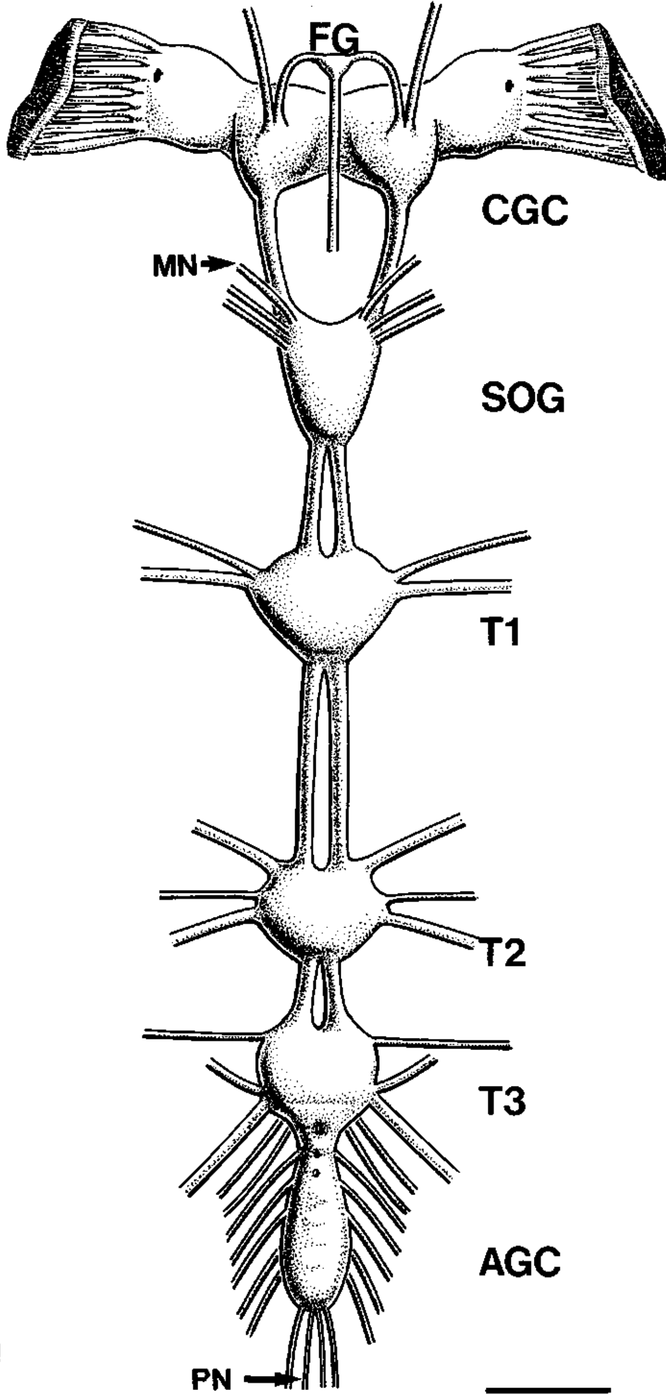
Specificity controls

Antiserum specificity was tested by preadsorption of one ml diluted antiserum with 100 μg serotonin-BSA conjugate (Immunonuclear Corp., Stillwater, USA), or 100 μg BSA (Sigma). The absence of endogenous peroxidase was confirmed by incubating sections in the absence of the primary antiserum.

RESULTS

The preadsorption of antiserum with serotonin-BSA conjugate and the omission of the primary antiserum resulted in a total absence of specific staining, whereas preadsorption of the antiserum with BSA did not influence the staining results. This indicates that our staining is specific for serotonin. The gross morphology of the central nervous system of *L. decemlineata* is shown in Fig. 1.

Fig. 1. Schematic representation of central nervous system of adult Colorado potato beetle showing condensed thoracico-abdominal ganglia, viewed from below. *CGC* cerebral ganglion complex; *SOG* suboesophageal ganglion; *T1* prothoracic ganglion; *T2* mesothoracic ganglion; *T3* metathoracic ganglion; *AGC* abdominal ganglion complex; *FG* frontal ganglion; *MN* mandibular nerve; *PN* proctodaeal nerve. **Bar:** 400 μm.


FG
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Whole-mounts of the VNC of both long-day and diapause beetles were used to study the distribution of entire SLI neurons, together with their axonic and dendritic arborizations. Paraffin sections were used to verify the location of perikarya and to trace the finest arborizations of processes in the neuropil. A complete map of SLI neurons, based on both methods of analysis is presented in Figs. 2 and 3. We distinguish five classes of SLI neurons on basis of their morphology and position in the nerve cord.

Segmental twin neurons

The most conspicuous neurons in the VNC are those arranged symmetrically in each ganglion. Fifty-six neurons (size 10-15 μm) are distributed over 14 segmentally arranged caudal sets of 4 neurons, each set consisting of a bilateral pair of twin neurons (Figs. 2, 4, and 7). They have a fixed location in the latero-caudal part of the ganglia and have cell processes running in different directions.

Figs. 2a-e. Schematic representation of location and distribution of SLI neurons in ventral nerve cord, based on camera lucida drawings.

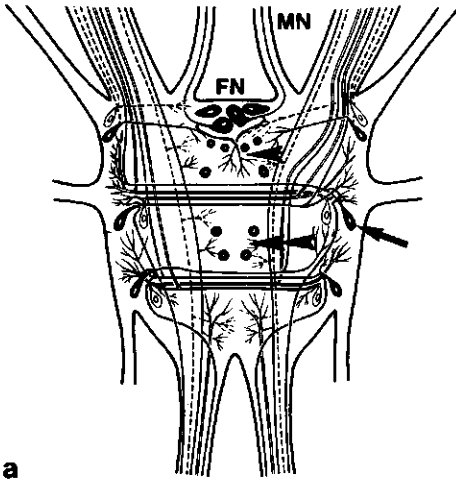
2a. Suboesophageal ganglion showing frontal secretory neurons (*FN*), dorsal miniature neurons (*arrowhead*) and ventral miniature neurons (*double arrowhead*), and 12 segmental twin interneurons (*arrow*). Neurons indicated in white project only anteriorly. Neurons indicated in black project posteriorly or anteriorly, or both. Note mandibular nerves (*MN*), comprising axons from frontal neurons.

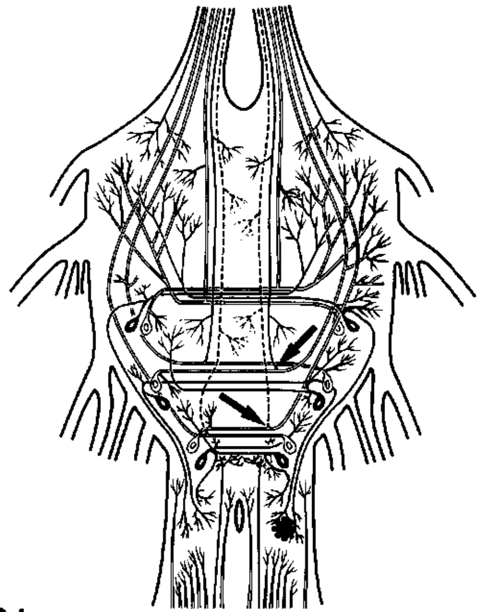
2b. Prothoracic ganglion with paired segmental twin interneurons. Neurons indicated in white project anteriorly, those in black posteriorly. Contralateral dendrites are present in lateral part of ganglion (*arrow*).

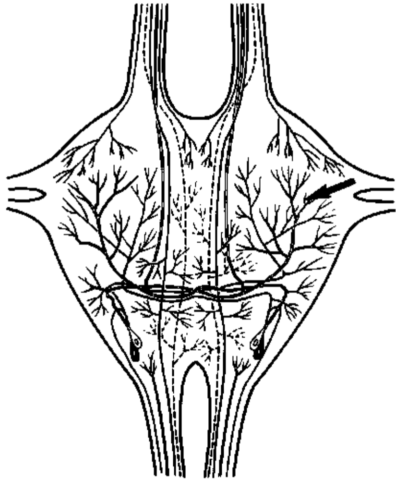
2c. Mesothoracic ganglion, showing contralateral axons projecting to prothoracic ganglion (*arrow*), and ipsilateral axons projecting to metathoracic ganglion (*arrowhead*). Rostral and caudal synaptic areas are drawn (*asterisks*).

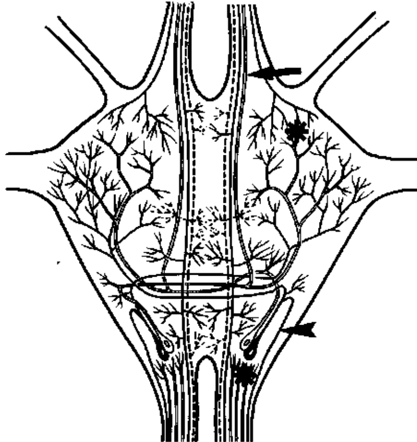
2d. Metathoracic ganglion, fused with abdominal ganglion 1 and 2, showing the origin of long-distance axons in abdominal ganglion 1 and 2 (*arrows*). Projection area of caudal functional unit is indicated with *asterisk*.

2e. Abdominal ganglion complex, consisting of abdominal ganglion 3-8. Large caudal neurons (*CN*) are present with contralateral axons leaving the complex via proctodaeal nerves (*PN*). Terminal neurons (*TN*) are present in last abdominal ganglion and project to caudal projection area (*asterisk*). Neurons indicated in white project to projection area between abdominal ganglion 2 and 3, whereas neurons indicated in black project to projection area in last abdominal ganglion. **Bar:** 100 μm.


MN
FN
2a


2d


2b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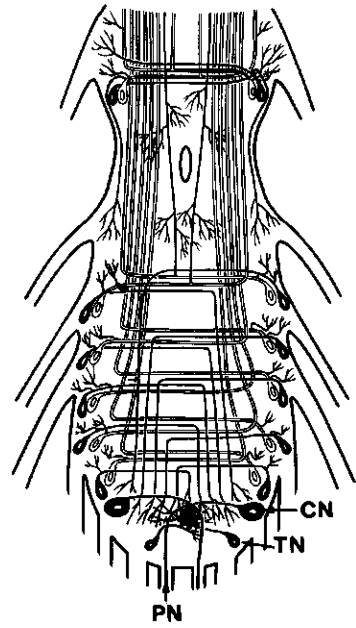
CN
TN
PN
2e

It is not possible to differentiate between cell processes conveying afferent and efferent messages, i.e. processes with dendritic and axonal functions, because they are morphologically similar. For descriptive reasons, we therefore use the term 'axon' for the longest of the processes that communicate with other ganglia, and the term 'dendrites' for those branched processes that terminate in the same ganglion.

We distinguish three principal morphologies:

A. Neurons with ipsilateral axons that run posteriorly to the rostral neuropil of the more caudal ganglion in the VNC (Figs. 2, 3, and 8); **B.** Neurons with contralateral axons that cross the ganglion via a ventral commissure and run to the caudal neuropil of the more frontal ganglia (Fig. 10); **C.** Neurons with contralateral axons that run through several of the more frontal and caudal ganglia and connect those ganglia via their axonal arborizations at different levels of the VNC (Fig. 8). One group of the latter neurons is special in that their cell bodies lie in abdominal ganglia 1 and 2 (Fig. 8) whereas their axons run through the whole VNC to terminate in the brain. These long-range axons, too, have arborizations in each ganglion that is passed. Neurons in the abdominal ganglia 3-8 run either posteriorly (Fig. 9) or anteriorly; none of their axons ascends to ganglia beyond abdominal ganglion 3. Several neuropil areas are richly innervated by immunoreactive processes from long-and short-range neurons (Figs. 2, 3, and 9). These neuropil areas with densely arborizing SLI processes are located precisely at those sites at which ganglion condensation has not occurred, i.e. between thoracic ganglia 2 and

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Fig. 3. Highly diagrammatic projection pattern of SLI neurons in one half of ventral nerve cord. *SOG1-3* mandibular, maxillary, and labial neuromere of suboesophageal ganglion, respectively; *T1-3* pro, meso, and metathoracic ganglia, respectively; *A1-8* abdominal ganglia 1 to 8; ⅄ axon projection; ⋔ dendrite projection; *FN* frontal secretory neurons; *CN* caudal neurons; ●○ segmental twin interneurons; *asterisk* projection areas.

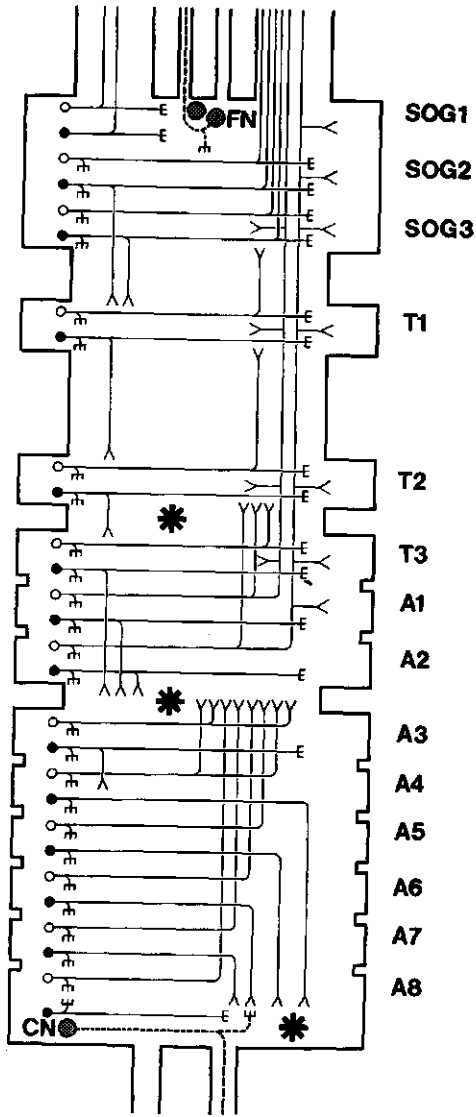
Figs. 4a-d. Serial sections of suboesophageal ganglion, showing serotonin-immunoreactive neurons in three neuromeres in transversal sections.

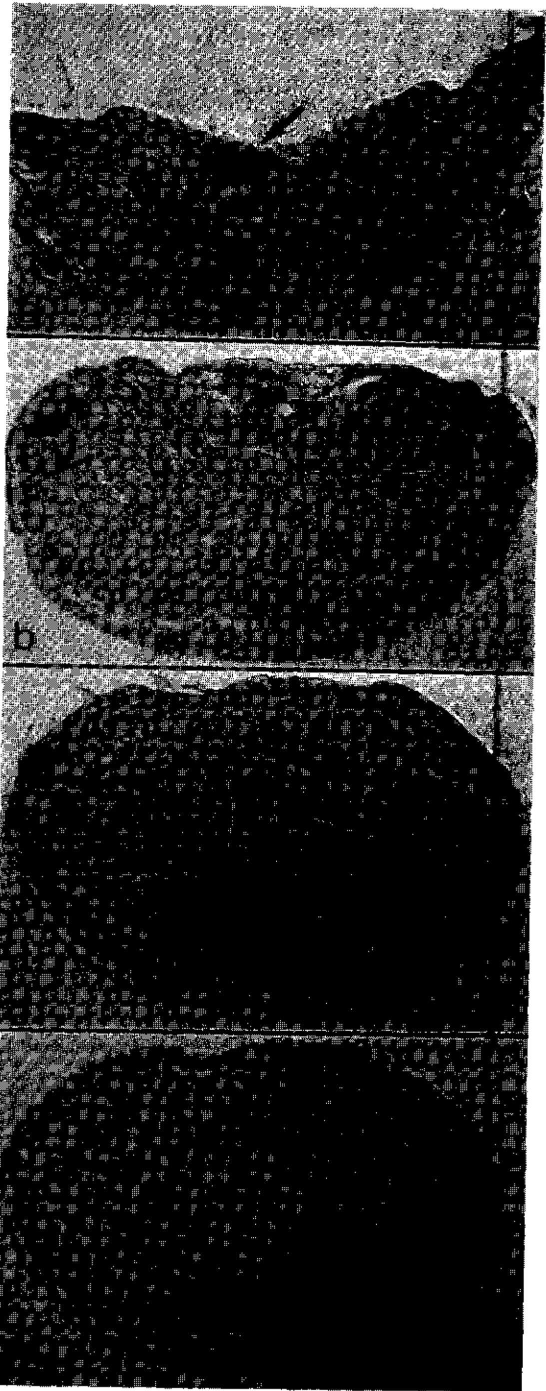
4a. Section through mandibular neuromere, showing large frontal neurons (*arrow*).

4b. Section through mandibular neuromere, showing first set of segmental twin interneurons (*arrowhead*) and dorsal miniature neurons (*arrow*).

4c. Section through maxillary neuromere, showing second set of segmental twin interne2urons with contralateral projection (*arrow*).

4d. Section through labial neuromere, showing third set of segmental twin interneurons and ventral miniature neurons (*arrow*). **Bar:** 50 µm.


FN
SOG1
SOG2
SOG3
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
CN
3


b

3, between abdominal ganglia 2 and 3, and in the caudal part of the last abdominal ganglion (Fig. 9).

Dendritic processes in the SOG and thoracic ganglia either lie in the close proximity of the cell bodies, or form dendritic fields in the contralateral hemisphere (Figs. 8 and 10). Dendrites in the abdominal ganglia appear to occur only close to the cell body.

Frontal neurosecretory neurons

Four large neurons, 30-35 μm in diameter, have variable positions on the fronto-dorsal side of the SOG (Fig. 4a). Their dendrites remain on the ipsilateral side of the ganglion. Axons of two of the neurons run into the mandibular nerve. They emerge from the nerve at some distance from the SOG and then branches to form an immunoreactive plexus around the mandibular nerve. A detailed description of this neurohemal plexus will be given elsewhere (van Haeften and Schooneveld 1993) (Chapter 5). Projections of the other two SLI neurons could not be traced.

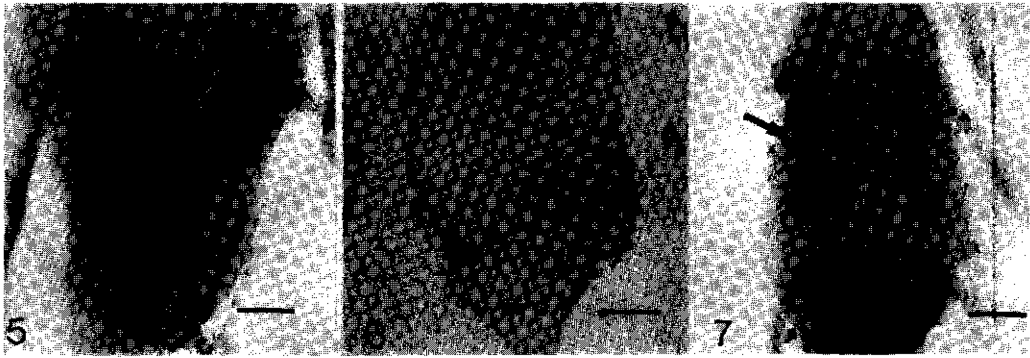
Fig. 5. Abdominal ganglion complex, showing two terminal neurons in caudal part. **Bar:** 40 μm.

Fig. 6. Serial section through abdominal ganglion complex of 5-month diapause beetle showing large caudal neuron (*thick arrow*) and small terminal neuron (*thin arrow*). **Bar:** 45 μm.

Fig. 7. Abdominal ganglion complex, showing several sets of segmental twin interneurons (*arrow*). **Bar:** 60 μm.

Fig. 8. Mesothoracic (top) and fused metathoracico-abdominal (bottom) ganglion with serotonin-like immunoreactive structures. Three pronounced commissures are present (*double arrowhead*) in thoracico-abdominal ganglion, each representing one fused ganglion. Contralateral dendrites are present in each hemisphere of mesothoracic and metathoracic ganglion (*arrowhead*). Thin ipsilateral axons (*long arrow*) project posteriorly to metathoracic ganglion and contralateral axons (*short arrow*) project anteriorly to the mesothoracic ganglion. Long-distance axons originate from abdominal ganglion 2 (*thin arrow*). **Bar:** 60 μm.

Fig. 9. Abdominal ganglion complex showing axons originating from abdominal twin interneurons projecting posteriorly (*arrow*) to caudal projection area (*asterisk*). **Bar:** 40 μm.


5
7

Caudal neurons

The last abdominal ganglion contains two large paired ventral neurons (diameter 30-35 μm) (Fig. 6). The caudal neurons are stained only in diapause beetles. These neurons are, however, also present in beetles which are kept under long-day conditions, but exhibit under these conditions no SLI. They each send a large axon into the contralateral proctodaeal nerve and innervate the hindgut. The axons then divide, one branch innervating the hindgut musculature, the other innervating the cryptonephridial system. A detailed innervation study of the entire alimentary canal will be reported elsewhere (van Haeften et al. 1993) (Chapter 4).

Miniature neurons

Two clusters of neurons (8 μm diameter) are present in the SOG. A dorsal cluster of six neurons is present in the first neuromere (Fig. 4b), a ventral cluster of four neurons is present in the third neuromere (Fig. 4d). No axons or dendrites were discernible.

Terminal neurons

Two neurons (size 10 μm) are present in the last abdominal ganglion (Figs. 5 and 6). Each of the neurons project contralaterally to a neuropil area with densely arborizing axon processes in the caudal part of the last abdominal ganglion. With exception of the SLI axons in the mandibular and proctodaeal nerves, immunoreactive processes remain within the neuropil of the VNC. All other nerves are devoid of immunoreactivity.

DISCUSSION

This paper describes the location and morphology of serotonin-like immunoreactive (SLI) neurons in the ventral nerve cord (VNC) of *L. decemlineata*. We were able to study the serotoninergic system in considerable detail by applying improved technical protocols on beetles of different physiological conditions.

Tissues were satisfactorily fixed with the assistance of a microwave oven (Smid et al. 1990), and the permeability of tissues for antibody was enhanced by a heptane treatment (Breidbach 1990). The use of beetles of different physiological conditions resulted in differential staining. Certain neurons stain only in diapause beetles. The state of diapause seems to induce the accumulation of serotonin in these neurons. This effect has also been observed in certain peptidergic neurons of this beetle (Schooneveld 1970; Schooneveld et al. 1989). For that reason, we used more than one physiological condition to construct an accurate and comprehensive map of SLI distribution.

Altogether 74 neurons and their processes were revealed and we grouped them into five neuron categories, differing in organization pattern. Most numerous are the segmental twin neurons. A set of two bilateral pairs is present in each of the ganglia or SOG neuromeres. These neurons have extensive dendritic arborizations in the contralateral hemisphere of the ganglion, and small arborizations in the close proximity of the perikaryon.

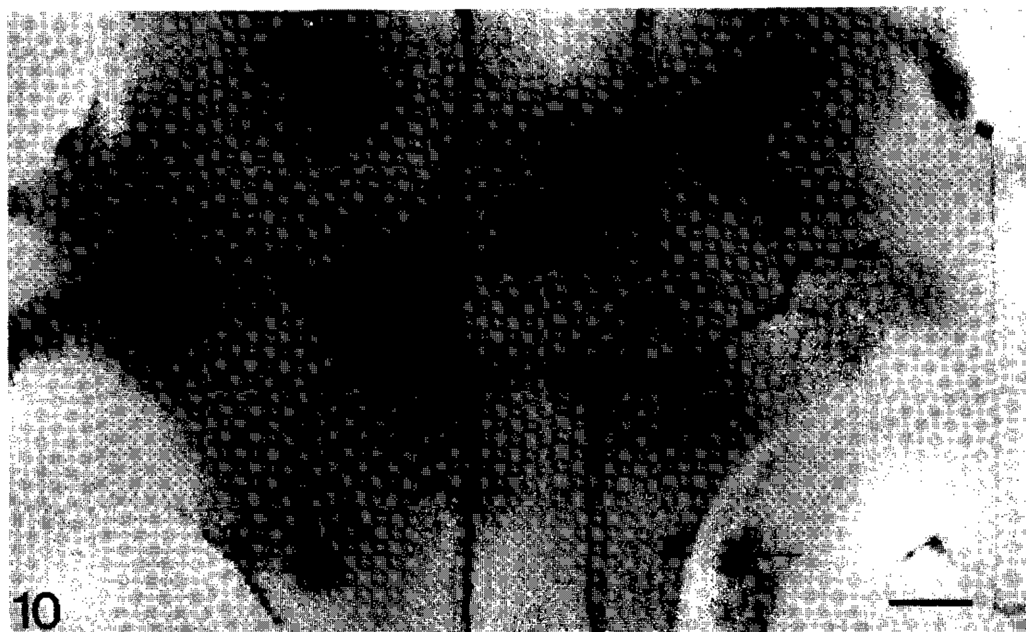


Fig. 10. Mesothoracic ganglion with segmental twin interneurons (*thin arrow*) whose contralateral axons (*thick arrow*) join long-distance axons (*double arrowhead*) on their way to the brain. Large dendritic fields are present in each hemisphere (*arrowhead*). **Bar:** 40 μm.

Their axons take a contra- and ipsilateral course to the more frontally and/or caudally located ganglia. All processes remain within the neuropil of the VNC and we therefore assume that these neurons are interneurons.

The distribution of axons is indicative of a division of labour between these interneurons. Half of the interneurons (those indicated in black, Figs. 2 and 3) communicate via their axons with the posterior ganglia, whereas the other interneurons (indicated in white, Figs. 2 and 3) communicate with the anterior ganglia. In addition, all thoracic ganglia are connected to the brain by means of long-distance axons from interneurons in abdominal ganglia 1 and 2 (Figs. 2 and 3). The intersegmental axon projections of the twin interneurons suggest a function of these neurons in interganglionic communication. Rehder et al. (1987) assumed that the intersegmental projections in *Apis mellifera* have a function in the coordination of neural activity among the various segments. The contralateral dendritic projections may have a function in the coordination of left/right neural activity within the ganglia.

Neurons with similar characteristics have been reported in several other insect species, be it under different names, i.e. 'segmentally repeated interneurons' in *Locusta migratoria* (Tyrer et al. 1984); 'large bilateral paired neurons' in *Periplaneta americana* (Bishop and O'Shea 1983); 'bilateral paired neurons' in larvae of *Calliphora erythrocephala* (Nässel and Cantera 1985; Nässel 1988); and intrasegmental interneurons in *Drosophila melanogaster* (Válles and White 1989). Although there is variability between insect species in the number of these neurons and in the distribution of their processes, these similarities show that various insects have a highly conserved common basic SLI organization pattern (Bishop and O'Shea 1983; Tyrer et al. 1984; Nässel and Cantera 1985; Rehder et al. 1987; Válles and White 1988).

Four large frontal neurosecretory neurons are present in the mandibular neuromere of the SOG. They form one dorsal cluster close to the mandibular nerve roots. Two of the neurons establish a plexus of fine arborizations on the surface of the mandibular nerves, as will be reported in detail elsewhere (van Haeften and Schooneveld 1993) (Chapter 5). Similar frontal neurons have been described in several other insect species under a variety of names, and are generally supposed to have a neurosecretory function (Griss 1989; Davis 1985, 1987; Nässel and Elekes 1985; Bräunig 1987, 1988; Homberg and Hildebrand 1989).

The caudal neurons in the last abdominal ganglion send their axons into the contralateral proctodaeal nerves, which innervate the posterior part of the alimentary canal. We observed that the proctodaeal nerves divide, one branch innervating the muscles of the hindgut, the other branch reaching for the cryptonephridial system (van Haeften et al. 1993) (Chapter 4). Similar neurons have been reported in *Acheta domesticus* (Hustert and Topel 1986) and their axons were reported to innervate the hindgut as well.

The terminal neurons in the last abdominal ganglia are described here for the first time. Their function is unknown. In the SOG, two clusters of miniature neurons are weakly stained and resemble those described by Griss (1989) in the SOG of larvae of *Manduca sexta*. Neuronal processes could not be traced. Griss (1989) suggested that these neurons function as local interneurons.

The distribution pattern of SLI neurons in the VNC enabled us to distinguish three putative functional units. These units are represented by VNC segments between the posterior neuropils of thoracic ganglion 2, abdominal ganglion 2, and abdominal ganglion 8 (Fig. 3). These neuropils are projection areas of the twin interneurons located in the functional units between these neuropils. The twin interneurons in each unit project either to the posterior or anterior projection area and seem to provide the functional unit with a communication channel to the adjacent functional units. There is a striking correspondence between the presence of functional units (segments) and ganglion condensation: those ganglia showing condensation are precisely those containing such a functional unit. The role of these functional units in neural control is not clear. We speculate, that one of the functions of the caudal functional unit might be the control of the caudal neurons innervating the hindgut muscles. All ganglia of this caudal unit have an identical internal SLI organization and have interneurons projecting to the caudal projection area. Processes of the caudal neurons also terminate in this area (Figs. 2 and 3).

SLI processes connecting the functional units are scarce or absent (Fig. 3). The long-range axons of abdominal ganglia 1 and 2 connect the more frontal segments. It therefore appears that the most caudal functional unit is more or less isolated from the other units. This morphological arrangement raises the question whether these functional units represent individual integrative centres, or form part of some larger integrative system. It may well be that they are part of a larger system which integrates all individual units. A comparison of this serotoninergic system with other

aminergic or peptidergic systems could provide more detailed information on neural interactions in this complex neuronal circuitry of the ventral nervous system.

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CHAPTER 4

Serotoninergic innervation of the alimentary canal of the Colorado potato beetle, *Leptinotarsa decemlineata*: Structural and functional aspects.

Accepted for publication in Cell and Tissue Research
With: *H.M. Smid and H. Schooneveld*

SUMMARY

Immunohistochemical studies showed that the alimentary canal of *Leptinotarsa decemlineata* receives serotoninergic innervation from different neurons in the central and stomatogastric nervous system. The foregut is innervated by the frontal ganglion. Four of the 6-8 large neurons present in this ganglion have axons which run to the musculature of the oesophagus, crop, sphincter, and frontal area of the midgut. They are accompanied by axons from neurons in the suboesophageal ganglion, and by axons from as yet unidentified non-immunoreactive neurons in the brain and/or the ventral nerve cord. The posterior part of the midgut is essentially devoid of serotoninergic innervation. The hindgut is innervated by two large neurons in the caudal tip of the last abdominal ganglion. The axons always run to the circular and longitudinal muscles of the crop, the circular muscles of the sphincter, and the longitudinal muscles of the hindgut. Immunohistochemical electron microscopy suggests that exocytosis of the immuno-labelled vesicles may occur at some distance from the muscle fibres, implying a neurohormonal release of this neurochemical. A bioassay used to demonstrate the type of effect of serotonin on isolated hindguts in vitro, indicated a clear inhibitory effect on spontaneous contractions at concentrations of 10^{-8}-10^{-5} M. This effect was dose-dependent. Axons found in association with the cryptonephridial system on the hindgut might be involved in the control of diuresis although we have not tested this possibility experimentally.

INTRODUCTION

In our studies on the role of serotonin in the feeding physiology of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), we came across two indications that this neurochemical messenger is involved in the control of gut functions. (1) An extensive study on the distribution of neurons containing a serotonin-like immunoreactive product (SLI neurons) in the brain indicated the presence of axonal tracts between the ventral nerve cord/brain and the frontal ganglion (van Haeften and Schooneveld, submitted; van Haeften and Schooneveld 1992) (Chapters 2 and 3).

The frontal ganglion is commonly held responsible for the control of foregut functions (Kirby et al. 1984; Chapman 1985b; Luffy and Dorn 1991). (2) A similar study on the distribution of SLI neurons in the ventral nerve cord showed that two neurons in the last abdominal ganglion have axons projecting to the hindgut via the proctodaeal nerves (van Haeften and Schooneveld 1992) (Chapter 3). The procto-daeal nerves control the caudal part of the alimentary canal (Nagy 1977; Chapman 1985a, 1985b). These data prompted us to analyze the control of gut functions by the SLI system by immunohistochemistry and physiological methods.

Earlier studies on the Colorado potato beetle have revealed the morphology of the alimentary canal (Patay 1939) and the histology of the stomatogastric nervous system (Bounhiol 1927; Schooneveld 1970). Serotonin is not the only neuro-chemical mediator present in the nervous circuits controlling gut functions: both FMRF-amide and proctolin-like substances have also been found (Veenstra and Schooneveld 1984; Veenstra et al. 1985).

The present study focuses on the serotoninergic innervation of the fore- and hindgut. We analyzed the axonal distribution over the diverse gut segments and determined the origin of the axons in both stomatogastric and central nervous system. Electron microscopy was used to investigate if serotonin is released either to function synaptically or neurohormonally. Bioassays on isolated hindguts were used to determine the effect of this biogenic amine on gut motility, and its physiological relevance as a neurotransmitter, modulator, or hormone.

MATERIALS AND METHODS

Sexually mature, continuously fed Colorado potato beetles from a laboratory culture (18 h photophase, 8 h scotophase) were used in all experiments. For light microscopy, entire nervous systems, together with adhering stomatogastric ganglia and alimentary canals were dissected under physiological saline (Khan et al. 1982). Care was taken not to damage the fragile nerve connections. All tissues were fixed in a freshly prepared 4% paraformaldehyde solution in 0.1 M phosphate buffer (NaH_2PO_4) pH 7.3. Nervous systems were incubated for 4-5 h, whereas entire alimentary canals were incubated for 18 h in the fixative at 4 °C. The quality of

tissues used for serial sectioning was improved by fixation of tissues in a microwave oven according to Smid et al. (1990). Next, tissues were rinsed in 0,1 M phosphate buffer pH 7.3 for 18 h at 4 °C, dehydrated, and embedded in Paraplast Plus (Lancer, Oxford), sectioned at 5 µm, and mounted on poly-l-lysine (Sigma) coated slides.

For electron microscopy, alimentary canals were dissected in ice-cold physiological saline, fixed for 4-5 h in an ice-cold solution of 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate (NaH_2PO_4) buffer, pH 7.3, and washed overnight in ice-cold phosphate buffer.

Immunohistochemistry

Serial sections were deparaffinized and rinsed in phosphate-buffered-saline (Dulbecco 'A', Oxoid) (PBS) pH 7.3. Next, the sections were preincubated for 30 min in 10% normal swine serum (NSS) in PBS, followed by an overnight incubation at 4 °C in rabbit-anti-serotonin antiserum (Immunonuclear, Stillwater, USA), diluted 1:1500 in PBS. The immunostaining was developed with the PAP-method (Sternberger 1979). The peroxidase reaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2. The sections were counterstained with Mayer's haemotoxyline.

Whole-mount preparations for light microscopy were fixed in 4% formaldehyde, rinsed in phosphate buffer pH 7.3, dehydrated to 90% alcohol and treated with normal-heptane for 20 sec to further increase membrane permeability for antibodies (Breidbach 1990). Next, the tissues were rehydrated to PBS + 0,25% Triton-X-100 (Merck) (PBS-T) buffer and after preincubation for 4 h in 10% NSS in PBS-T, tissues were incubated in anti-serotonin antiserum diluted 1:1000 in PBS-T + 0,1% sodium azide, for 2 days at 4 °C. The labelling was detected with peroxidase conjugated swine-anti-rabbit (Dakopatts), for 2 days at 4 °C. The peroxidase reaction was visualized with 0.05% DAB and 0.01% H_2O_2. After staining, tissues were trimmed to the desired size and embedded in Depex (Fluka) on glass slides.

Whole-mount preparations for electron-microscopy were immunostained as described above, but with omission of the heptane treatment and Triton-X-100. After the immunostaining, tissues were rinsed in 0.1 M sodium cacodylate buffer pH 7.3, postfixed for 1 h in 1% OsO_4 (TAAB) in 0.1 M sodium cacodylate buffer

pH 7.3, and embedded in epon. Ultrathin sections were cut on an LKB Ultrotome, and collected on formvar coated 100-mesh copper grids.

Specificity controls

The antiserum specificity was tested by preadsorption controls. One ml of diluted antiserum was incubated with 100 μg of serotonin-BSA conjugate (Immunonuclear Corp., Stillwater, USA) for 8 h at 20 °C. After centrifugation, the adsorped antiserum was tested on whole-mount preparations of the entire alimentary canal and nervous system. Additionally, incubations in the absence of the primary antiserum were carried out. These conditions resulted in a total absence of immunostaining.

Cobalt filling of nerves

After low temperature anaesthesia of beetles, entire central nervous systems with adhering stomatogastric ganglia were dissected under physiological saline (Khan et al. 1982). The nerve to be filled was sucked into a glass micropipette, pulled from Clark GC-150F-15 capillaries with a DKI micropipette puller (DKI, model 700C, Tujunga, USA). The micropipette was filled with a 5% cobalt chloride solution in distilled H_2O and filling took place for 18 h at 4°C. The cobalt ions were precipitated by immersing the tissues in a $(NH_4)_2S$ solution. Next, tissues were fixed in a 4% paraformaldehyde solution in 0.1 M phosphate buffer pH 7.3 for 6-8 h at 20 °C, dehydrated, and embedded in Depex on glass slides.

Bioassay of gut contractions

Equipment.

Our bioassay equipment is based on an impedance converter (model 2991, Biocom Inc., Culver City, California), which generates a 50 kHz alternating voltage and modulates the impedance changes (measured between two electrodes) into a proportional DC voltage, which is visualized with a pen-recorder. Since movement of tissues between electrodes, e.g. peristaltic movements of the gut, is accompanied by changes in impedance of the field between electrodes, it is possible to register

movements of tissues by simply recording the changes in impedance (Tublitz and Truman 1985).

The two electrodes were made of 25 mm long stainless steel wire, thickness 0.5 mm, and were insulated with nail polish, except for 1 mm from the tip. The electrodes were mounted on a micro-manipulator, and connected to the impedance converter. A Brush recorder (Gould, type 220) was used for the recording of data. All experiments were also visually monitored with a stereo microscope. A more detailed description of the bioassay equipment has been given by Schooneveld et al. (1993).

Dissection.

The abdomen was removed from the thorax, and the hindgut with the distal part of the midgut was rapidly dissected. The anus and the anterior part of the ileum were removed with micro-scissors, and the remaining part of the hindgut was stored in saline (Khan et al. 1982) at room temperature for later use. The physiological saline was refreshed frequently, and all hindguts were used between 30 min and 4 h after dissection.

Assay conditions.

All assays were performed in 1 ml of physiological saline solution at room temperature. The gut was loosely pinned on the sylgard-coated (Dow Corning Co., Seneffe, Belgium) bottom of the assay-chamber with two minute needles, to ensure a fixed position combined with maximum freedom of movement. The uncoated tips of the two electrodes were positioned close to and about half-way the hindgut.

Serotonin-hydrochloride (Janssen, Tilburg, The Netherlands) was diluted in a ten-fold range from 10^{-12} to 10^{-4} M in saline. The activity of a serotonin solution was tested by replacing the saline solution of the assay chamber with the serotonin solution, during which the recording was shortly interrupted. All incubations were preceded by a control incubation with saline. Spontaneously active, as well as inactive guts, were used to measure possibly inhibitory or stimulatory effects of serotonin. Series of ten specimens were tested per serotonin concentration, beginning with the lowest concentration.

We defined the term "effect" as a distinct change either in amplitude or frequency, or both, which starts within 1 min after the replacement of saline with test medium (saline with serotonin), and lasts for at least 1 min.

RESULTS

Gross anatomy of the alimentary canal

The muscularis of the foregut consists of a smaller inner longitudinal muscle layer and an outer circular muscle layer. Additionally, several longitudinal muscle strands are arranged around the circular muscle layer. Circular muscles in the distal part of the foregut are well developed and form a sphincter which separates the fore- and midgut (Fig. 1). The mid- and hindgut muscularis consists of an inner layer of circular muscles and an outer layer of longitudinal muscles. The longitudinal muscles are arranged as six muscle strands spiralling around ileum and rectum (Fig. 2).

There is a pronounced cryptonephridial system (Figs. 2a, b). A highly convoluted mat of Malpighian tubules lies close to the rectal epithelium. This complex is covered by a perinephric membrane which consists of an outer sheath of epithelial cells and underlying tracheal cells and an inner sheath composed of a few undefined cell layers. Two Malpighian tubules emerge from the cryptonephridial system, bifurcate twice to give rise to 3 tubules which run to the anterior hindgut (Fig. 2a).

Innervation of the foregut

The musculature of the foregut is supplied by SLI axons in three oesophageal nerves originating from the stomatogastric nervous system (Figs. 1, 3).

Two of the oesophageal nerves, originating from the hypocerebral ganglion, follow a lateral course to the crop and branch a few times (Fig. 4). The most dorsal branch runs laterally along the crop and the anterior midgut and ramifies extensively. The ventral branch follows the ventral side of the anterior midgut and ramifies likewise. The third oesophageal nerve follows a medial course and ramifies on the dorsal side of crop and anterior midgut (Fig. 5). At the position of the sphincter, all branches

ramify, follow a circular course and penetrate into the muscle layers of the sphincter (Fig. 6).

Axons may innervate different sets of muscles. The nerves on the crop run to both circular and longitudinal muscles, whereas those on the midgut run only to the longitudinal muscles. Axons follow the direction of the longitudinal muscles on the midgut, ramify and form a network of axon swellings. These axon swellings have the appearance of neurohemal release sites (see below).

Where do these axons come from ? The frontal and hypocerebral ganglia are commonly held responsible for the innervation of the foregut and we have analyzed the distribution of SLI axons in these ganglia in more detail using immunohistochemistry (Fig. 3). In the frontal ganglion six to eight large neurons (size 30-35 µm) are present (Fig. 7). Two of the neurons send their axon into the frontal connectives. These axons emerge from the connective and enter a small-diameter nerve, which forms a neurohemal plexus over several of the cerebral nerves (van Haeften and Schooneveld 1993)(Chapter 5).

These neurons are not involved in gut regulation. Axons of the other neurons run through the recurrent nerve, split after passing the hypocerebral ganglion and enter the oesophageal nerves (Figs. 3, 8). Several other immunoreactive axons are present in the frontal and hypocerebral ganglion, but these appear to originate from pericarya in the central nervous system (Fig. 3). Some of the latter axons run straight through the frontal ganglion to the contralateral connective, or take a turn and enter the recurrent nerve. Other axons terminate in the neuropil of the frontal ganglion.

To determine the origin of these axons and the distribution of cell processes, and to understand the role of the frontal ganglion in foregut innervation, cobalt fillings have been performed on selected nerves. The results can be summarized as follows

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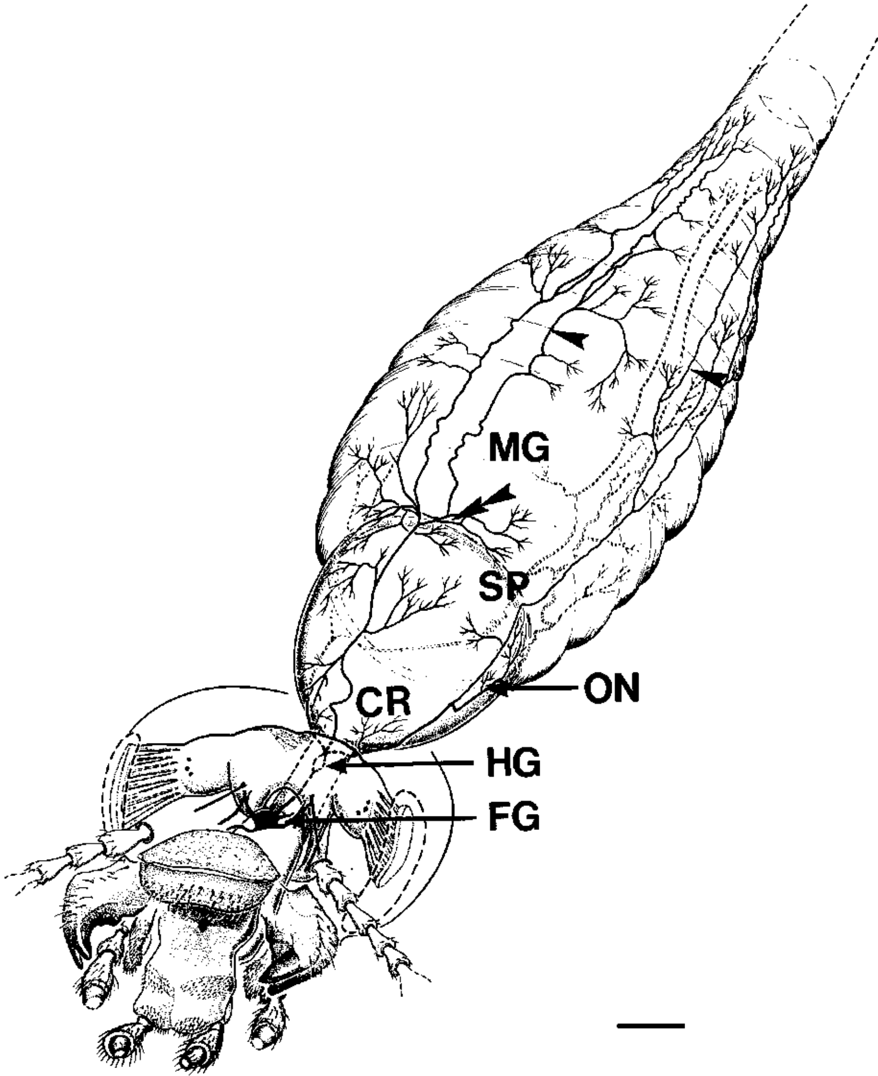
Fig. 1. Diagram of anterior alimentary canal of adult Colorado potato beetle showing origin and distribution of serotonin-immunoreactive nerves on foregut and anterior midgut, frontal view. Three serotonin-like immunoreactive oesophageal nerves (*ON*) originate from hypocerebral ganglion (*HG*), run laterally and dorsally along oesophagus and crop (*CR*), and terminate on muscles of anterior midgut (*MG*) (*arrowheads*). Nerves ramify extensively on sphincter (*SP*) (*double arrowhead*). *FG* frontal ganglion. **Bar:** 450 µm.


MG
SP
CR
ON
HG
FG

(Fig. 3): (1) Filling of the recurrent nerve in the direction of the frontal ganglion reveals 5-8 large neurons in the frontal ganglion (Fig. 9), four of which are identical with the large immunoreactive neurons projecting into the recurrent nerve. Additionally, some small neurons are stained, which thus far have never shown any immunoreactivity. (2) Filling of the frontal connectives towards the brain reveals neurons in the suboesophageal ganglion that are probably identical with those previously found to be immunoreactive (van Haeften and Schooneveld 1992) (Chapter 3). Several small neurons are revealed in the brain which are not immunoreactive. (3) Filling of one of the circumoesophageal connectives (COC) towards the brain reveals one large neuron in the ipsilateral part of the frontal ganglion, that is identical with the immunoreactive neuron giving rise to the neurohemal plexus on the cerebral nerves described above (Fig. 10). Several small non-immunoreactive neurons are revealed as well (Fig. 10).

Innervation of the hindgut

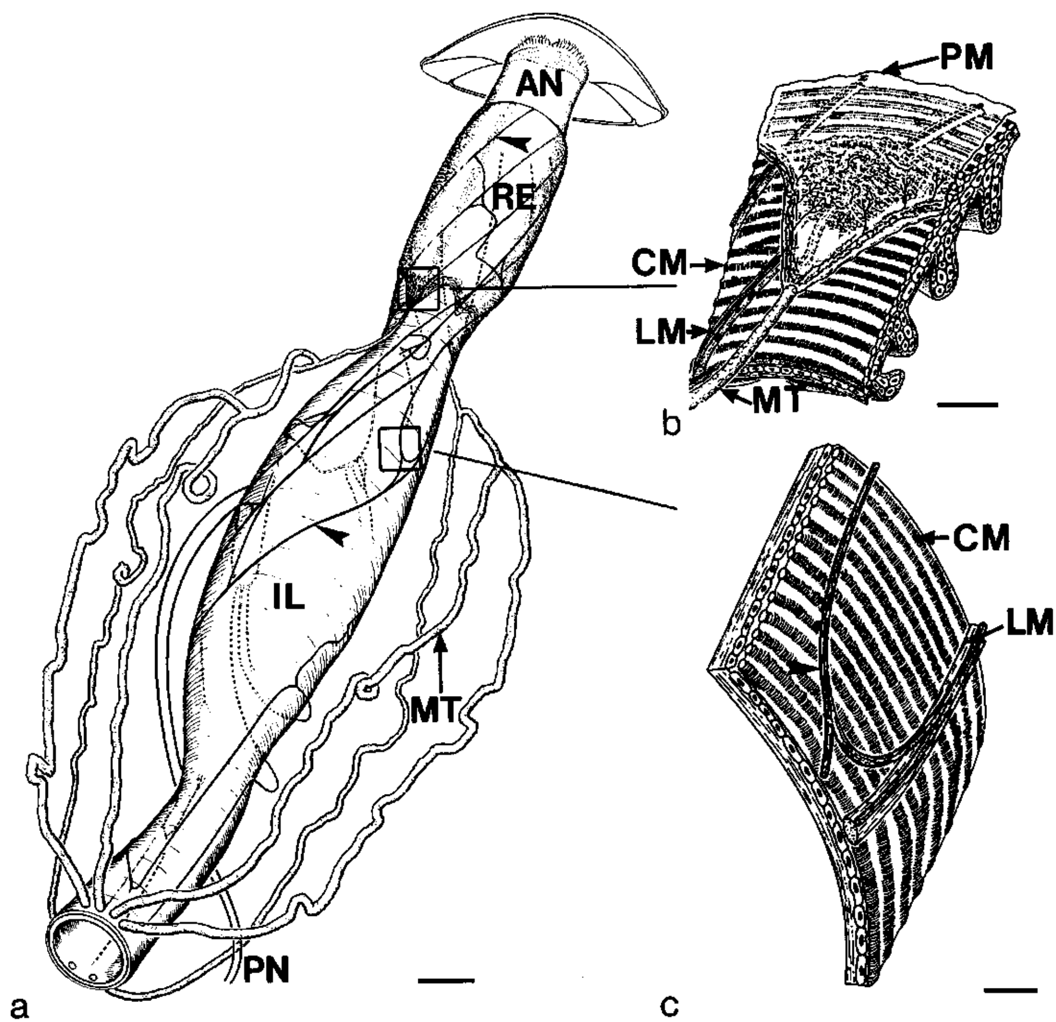
The proctodaeal nerves arrive at the hindgut half-way at the ileum. One immunoreactive branch innervates the anterior ileum, the other the posterior ileum and the rectum with its cryptonephridial system (Fig. 2a). The branches are closely associated with the six longitudinal muscle strands described above (Fig. 2) and several anastomoses between the branches are present (Fig 2c). The immunoreactive axons in these nerves have swellings (Figs. 11-13), identical with those seen on the foregut muscles. We have never observed such immunoreactive swellings on the circular muscles of ileum and rectum.

Figs. 2a-c. Diagram of hindgut of adult Colorado potato beetle showing distribution of serotonin-immunoreactive proctodaeal nerves on hindgut, viewed from above.

2a. Distribution of proctodaeal nerves (*PN*) on ileum (*IL*) and rectum (*RE*). Proctodaeal nerves are associated with longitudinal muscle strands and spiralize around ileum and rectum (*arrowheads*). Immunoreactivity is absent on anus (*AN*). *MT* Malpighian tubule. **Bar:** 500 μm.

2b. Cryptonephridial system on rectum. Malpighian tubules (*MT*) within a double layered perinephric membrane (*PM*) that envelops rectum. *CM* circular muscles; *LM* longitudinal muscles. **Bar:** 80 μm.

2c. Branching (*arrowhead*) of immunoreactive proctodaeal nerve on longitudinal muscle (*LM*) of ileum. *CM* circular muscles. **Bar:** 80 μm.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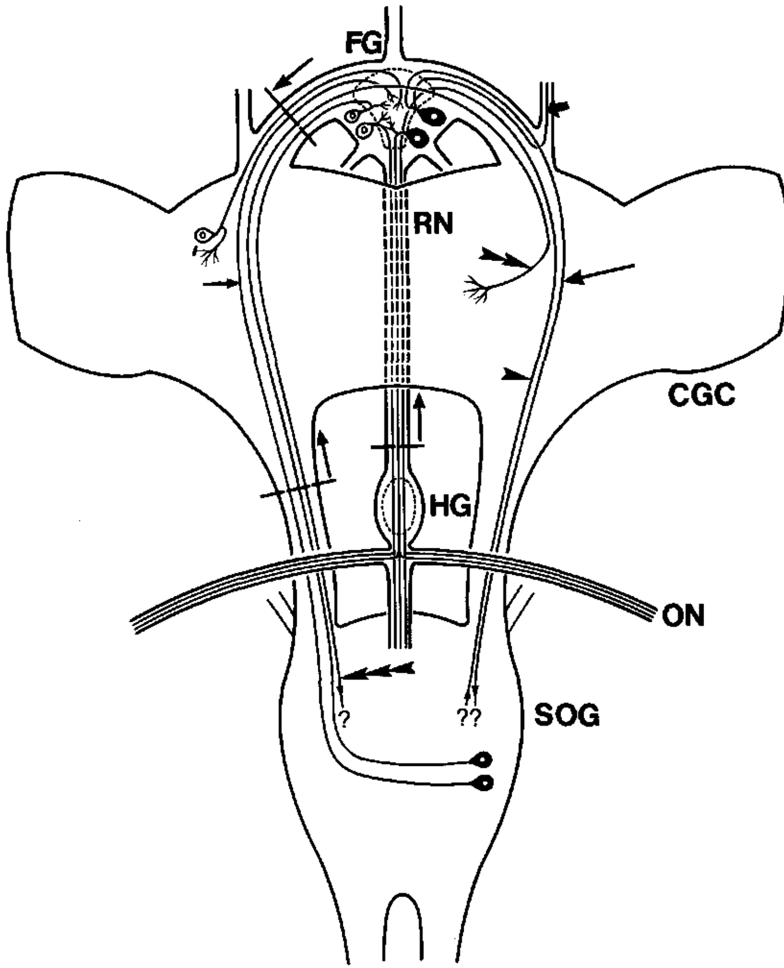


Fig. 3. Highly diagrammatic projection pattern of neurons in cerebral ganglion complex (*CGC*), suboesophageal ganglion (*SOG*), frontal ganglion (*FG*), and hypocerebral ganglion (*HG*), involved in regulation of foregut, as revealed by immunohistochemistry and cobaltfilling of nerves. Axons of immunoreactive neurons (indicated in black) in frontal ganglion run through recurrent nerve (*RN*), split after passing hypocerebral ganglion and enter three oesophageal nerves (*ON*). Axons from immunoreactive neurons in SOG either terminate in frontal ganglion (*thin arrow*), or run into recurrent nerve (*arrowhead*) to innervate musculature, or run straight (*double arrowhead*) through frontal ganglion and terminate in CGC. Axons of small non-immunoreactive neurons in CGC (indicated in white) terminate in frontal ganglion. Similar neurons in frontal ganglion project to SOG (*triple arrowhead*) and into oesophageal nerves. Axon of secretory neuron in frontal ganglion leaves frontal connective and forms neurohemal plexus on cerebral nerves (*thick arrow*), its other process terminate in SOG (*long arrow*). Dashed line and arrow indicates cobalt-filled nerve and direction of filling. Question mark indicates unresolved origin or projection of axon in SOG.

Axon swellings of the proctodaeal nerves are also observed on the perinephric membrane over the meandering Malpighian tubules of the cryptonephridial system (Fig. 14). They originate from two large neurons in the caudal tip of the abdominal ganglion complex (van Haeften and Schooneveld 1992) (Chapter 3).

Neurohemal release of serotonin-like immunoreactive substance

The axon swellings on the muscles have the appearance of neurohemal structures and the possible release of serotonin from these structures has been further studied by electron microscopy. The immunoreactive axon profiles are in close proximity to the longitudinal muscles of the fore- and hindgut (Fig. 15) and of the circular sphincter muscles of the foregut (Fig. 6), and contain small clear vesicles with a diameter of 40-50 nm, large granular vesicles of 80-100 nm, and large dense-core vesicles of 100-120 nm (Fig 16). They occur both on the outside of these muscles and between the muscle fibres. In all cases, a glia layer is present between the immunoreactive profiles and the sarcolemma, and no synapses have been observed. Many axon profiles on the outside of the muscles are in direct contact with the extracellular stroma and face the hemolymph (Fig. 16). This suggests that axon swellings represent neurohemal release sites for serotonin, although we have not observed exocytotic axon profiles in the present material.

On the other hand, there is evidence for the occurrence of synaptic innervation of muscles, but only by non-immunoreactive axon profiles. These profiles are found in the deeper muscle layers of both fore- and hindgut. They make close contact with the myofibrils and contain large granular, presumably peptidergic vesicles of 200-250 nm (Fig. 17).

Serotoninergic modulation of hindgut contractions

About 50 percent of the dissected hindguts show rhythmic low-frequency (5 contractions /min.) coiling and contraction movements in vitro, resulting from an alternating contraction of the circular and longitudinal muscles. We have investigated whether serotonin has a stimulatory or inhibitory effect on hindguts by administering graded dosages of serotonin to the incubation bath.

The possibly inhibitory effects have been studied with guts that are spontaneously

active after dissection. Concentrations of serotonin of 10^{-8} or 10^{-7} M reduce contraction frequencies (Fig. 18). The amplitude of the resulting peaks is not reduced. A full recovery to the basal contraction rate occurs after rinsing. At higher concentrations (10^{-6} to 10^{-5} M), a complete relaxation of both muscle types occurs. Here too, the autonomous contraction rhythm is reestablished after washing. The possibly stimulatory effects have been studied with guts that are either inactive or have lost activity after dissection. Such guts can never be activated. We conclude that serotonin acts as an inhibitory compound in this in vitro assay.

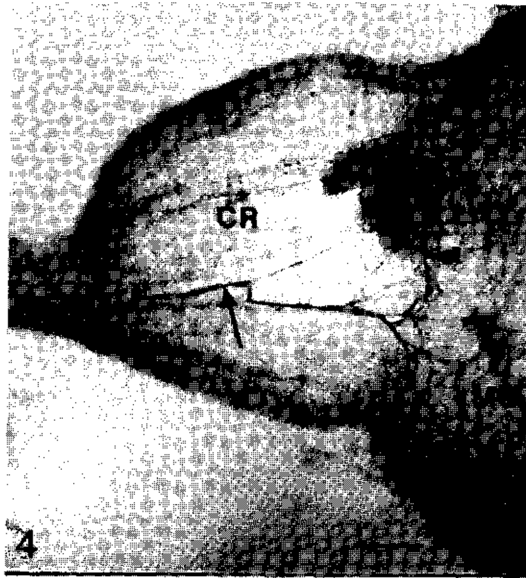
DISCUSSION

This paper describes the distribution of serotonin-immunoreactive axons over the diverse gut segments. We have analyzed the origin and course of these axons in both the central and stomatogastric nervous system and we have investigated the possible effects of serotonin on hindgut contractions. It appeared that axons present on the foregut and crop are associated with the circular and longitudinal muscles, whereas those present on the hindgut are associated only with the longitudinal muscles. The functional significance of this selective innervation is not yet clear, but we speculate here that serotonin is involved with specific regulation of coordinated peristaltic movements.

Fig. 4. Distribution of a lateral immunoreactive nerve on foregut and anterior midgut (*MG*), lateral view. Oesophageal nerve (*arrow*) follows a lateral course on oesophagus (*OE*), runs along crop (*CR*), and ramifies extensively on sphincter (*SP*) (*arrowhead*). **Bar:** 250 μm.

Fig. 5. Distribution of medial oesophageal nerve (*arrow*) on crop (*CR*) and anterior midgut (*MG*), dorsal view. On sphincter (*SP*), the nerve ramifies (*arrowhead*) and follows a circular course around sphincter. Two branches continue and innervate midgut (*double arrowhead*). **Bar:** 250 μm.

Fig. 6. Serial section through sphincter, showing immunoreactive axon terminals (*arrows*) on circular sphincter muscles (*CM*). **Bar:** 30 μm.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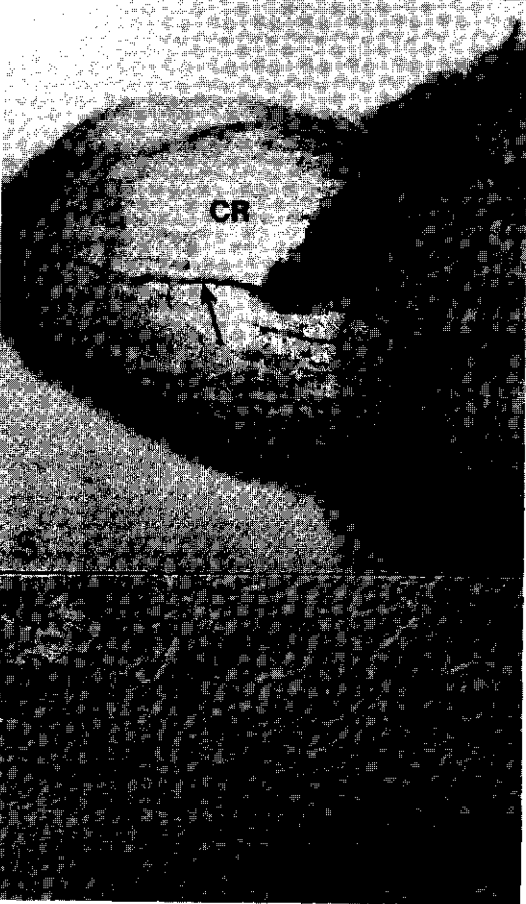
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The muscles of the fore- and anterior midgut are innervated by three sources: (1) four large immunoreactive neurons in the frontal ganglion, (2) immunoreactive neurons in the SOG (van Haeften and Schooneveld 1992), and (3) as yet unidentified non-immunoreactive neurons in the brain and/or ventral nerve cord. The foregut thus receives innervation from both the stomatogastric and the central nervous system. Whether the neurons in the central and stomatogastric nervous system innervate different sets of muscles is unclear.

The longitudinal muscles of the hindgut and the cryptonephridial system are innervated by axons originating from two large immunoreactive neurons in the caudal part of the central nervous system (van Haeften and Schooneveld 1992). These neurons are assumed to be located in the caudal visceral nervous system, which is part of the abdominal ganglion complex (Penzlin 1985). Similar neurons innervating the hindgut have been reported in the terminal ganglion of *Acheta domesticus* (Elekes et al. 1987). In some other insect species the hindgut receives serotoninergic innervation from the stomatogastric nervous system as well (Klemm et al. 1986).

How is the gut musculature innervated by the serotoninergic nervous system?

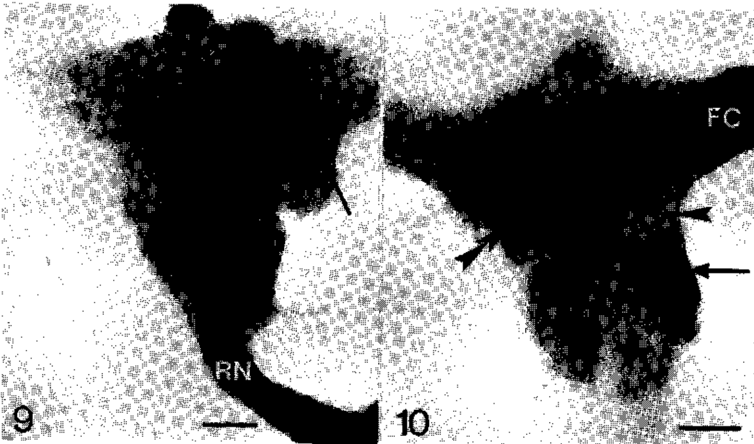
Fig. 7. Frontal ganglion with immunoreactive neurons (*thick arrow*). Some axons in frontal connective (*FC*) run through the ganglion (*arrow*). Other axons (*arrowhead*) enter the recurrent nerve (*RN*). Note neurohemal release sites on cerebral nerves (*double arrowhead*). **Bar:** 30 μm.

Fig. 8. Hypocerebral ganglion (*HG*) with oesophageal nerves (*ON*). Serotonin-immunoreactive axons (*arrowhead*) in recurrent nerve (*RN*) run through hypocerebral ganglion, split, and enter oesophageal nerves (*arrow*) to innervate foregut muscles. Note absence of immunoreactive neurons. **Bar:** 30 μm.

Fig. 9. Frontal ganglion with cobalt-stained neurons (*arrow*) after filling of recurrent nerve (*RN*). **Bar:** 40 μm.

Fig. 10. Frontal ganglion with cobalt-stained neurons after filling of circumoesophageal connective. Large lateral serotoninergic neuron (*arrow*) supplies neurohemal plexus on cerebral nerves. Additionally, several small non-immunoreactive neurons stained (*double arrowhead*). Note large unstained neurons (*arrowhead*). *FC* frontal connective. **Bar:** 40 μm.

FC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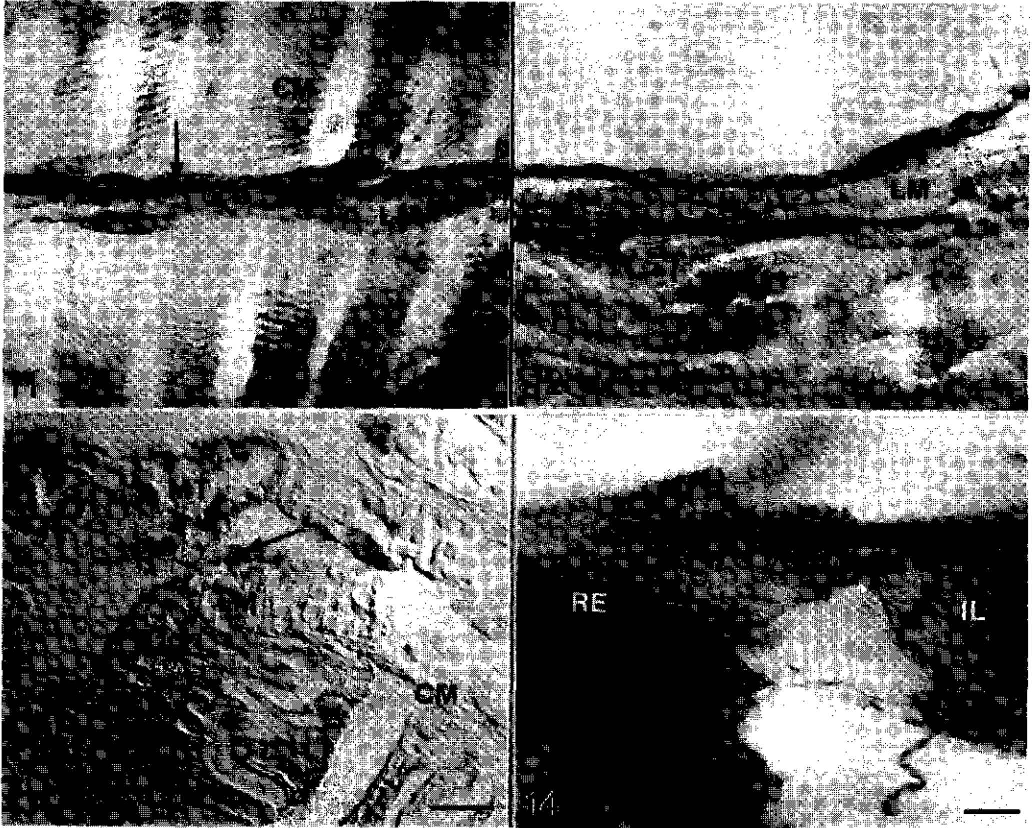


Fig. 11. Ileum with outer longitudinal muscle strand (*LM*) and circular muscles (*CM*). Serotonin-immunoreactive axons (*arrow*) follow longitudinal muscles. **Bar:** 30 μm.

Fig. 12. Neurohemal structures (*arrow*) on longitudinal muscle (*LM*) strand of ileum. **Bar:** 20 μm.

Fig. 13. Transverse serial section through rectum and associated cryptonephridial system. Serotonin-immunoreactive neurohemal structure (*arrow*) on longitudinal muscle strand (*LM*). *CM* circular muscles; *MT* Malpighian tubule; *asterisk* rectum lumen. **Bar:** 40 μm.

Fig. 14. Cryptonephridial system on rectum (*RE*). Nerves (*arrowhead*) on ileum (*IL*) form serotonin-immunoreactive neurohemal structures on perinephric membrane (*arrow*). **Bar:** 50 μm.

The immunoreactive axons establish dense plexuses of axon swellings on the surface of both longitudinal and circular muscles. Electron microscopy has revealed that although axon profiles are in contact with the muscle sarcolemma, no serotoninergic synaptic contacts are available. Instead, the profiles are in direct contact with the extracellular stroma and hence in direct contact with the hemolymph, suggesting a neurohormonal, rather than a neurotransmitter-type relationship. Similar neurohemal release structures have been reported to occur on visceral muscles of other insect species, e.g.: of *Schistocerca gregaria* and *A. domesticus* (Klemm et al. 1986; Elekes and Hustert 1988). Synaptic serotoninergic contacts exist, but seem to occur only in the neuropil of ganglia (Salecker and Distler 1990).

What kind of message is delivered to the gut musculature? Using a bioassay to demonstrate the nature of the activity of serotonin on isolated hindguts in vitro, we have determined that serotonin has an inhibitory effect on spontaneous contractions of longitudinal and circular muscles. Effective concentrations are in the range of 10^{-8}-10^{-5} M and this effect is dose-dependent. A total arrest of contractions is usually obtained at 10^{-5} M. Both circular and longitudinal muscles are brought into a state of total relaxation. Serotoninergic release sites are only present on the longitudinal muscles, but we speculate that the circular muscles may also respond to serotonin after it is released as a neurohormone from the neurohemal release sites on the longitudinal muscles given the small dimensions of the gut.

There is no general consensus as to an inhibitory function of serotonin on insect visceral muscles in several species. Banner et al. (1987) have shown that serotonin caused a dose-dependent relaxation of muscles of *S. gregaria* in vitro. Other reports show serotonin to have an excitatory effect on visceral muscles in vitro, e.g. in *Blaberus giganteus* (Cook et al. 1969), and in *Locusta migratoria* (Huddart and Oldfield 1982).

The seemingly conflicting results may be the result of the fact that the alimentary canal is a complex organ, and its normal functioning is the result of perhaps more than one regulatory substance and many exogenous factors. In our experience, the use of an impedance converter has proved to be valuable for recording gut contractions (Schooneveld et al. 1993). A major advantage of this bioassay over other bioassay methods is that guts are used now in the unrestrained state.

Some branches of the proctodaeal nerves supply the cryptonephridial system with

a neurohemal plexus. We suggest that serotonin, released from this plexus, might be involved in the regulation of diuresis in the cryptonephridial system since serotonin has been shown to stimulate the fluid secretion in isolated Malpighian tubules in a number of species, e.g. in *L. migratoria* (Morgan and Mordue 1984) and in *Aedes aegypti* (Veenstra 1988).

It is becoming probable that the gut of the Colorado potato beetle is regulated by more than one neuroactive substance. Apart from serotonin, the myogenic peptides FMRF-amide and proctolin have been demonstrated immunohistochemically in the gut of the beetle (Veenstra and Schooneveld 1984; Veenstra et al. 1985) and electron microscopy has revealed the presence of putative peptidergic synaptic contacts on visceral muscles. Schooneveld et al. (1993) have shown that proctolin stimulates the contractions of the longitudinal muscles in a dose-dependent way. It thus becomes obvious that the hindgut is under both stimulatory and inhibitory neurohormonal control, and the next challenge will be to disentangle the specific effects of these substances on specific sets of muscles.

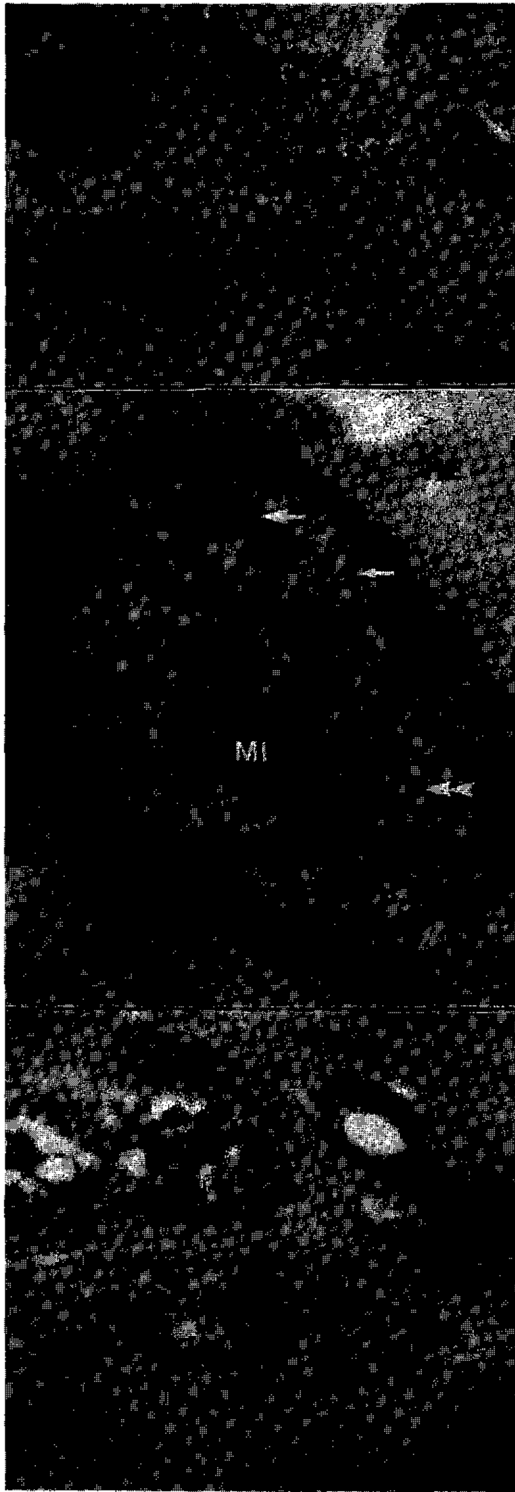
Figs. 15-17. Electron-micrographs of axons innervating hindgut musculature.

Fig. 15. Serotonin-immunoreactive axon profile (*arrow*) on longitudinal muscle strand on ileum. *GL* glia; *SL* sarcolemma; *LM* longitudinal muscles. **Bar:** 1.5 μm.

Fig. 16. Serotonin-immunoreactive axon terminal making contact with longitudinal muscles of ileum. Terminal faces hemolymph (*HE*) and is filled with clear vesicles (*thin arrow*), dense-core vesicles (*thick arrow*), and large granular vesicles (*double arrowhead*). Note well-developed glia-layer (*GL*) between sarcolemma (*SL*) of muscles and terminal. *MI* mitochondria. **Bar:** 0.5 μm.

Fig. 17. Non-immunoreactive axon terminal on longitudinal muscle (*LM*) of ileum. Terminal is in close contact with muscle (*arrowhead*) and contains several large granular, presumably peptidergic vesicles (*arrow*). **Bar:** 0.5 μm.

Fig. 18. Effect of administration of graded dosages of serotonin on contractions of spontaneously active hindguts in a impedance converter bioassay. *Arrowhead*, time of serotonin administration, *arrow*, time of washing. **Bar:** 1 min.
Administration of **a.**10^{-8} M, **b.** 10^{-7} M, **c.** 10^{-6} M, **d.** 10^{-5} M, of serotonin-hydrochloride respectively.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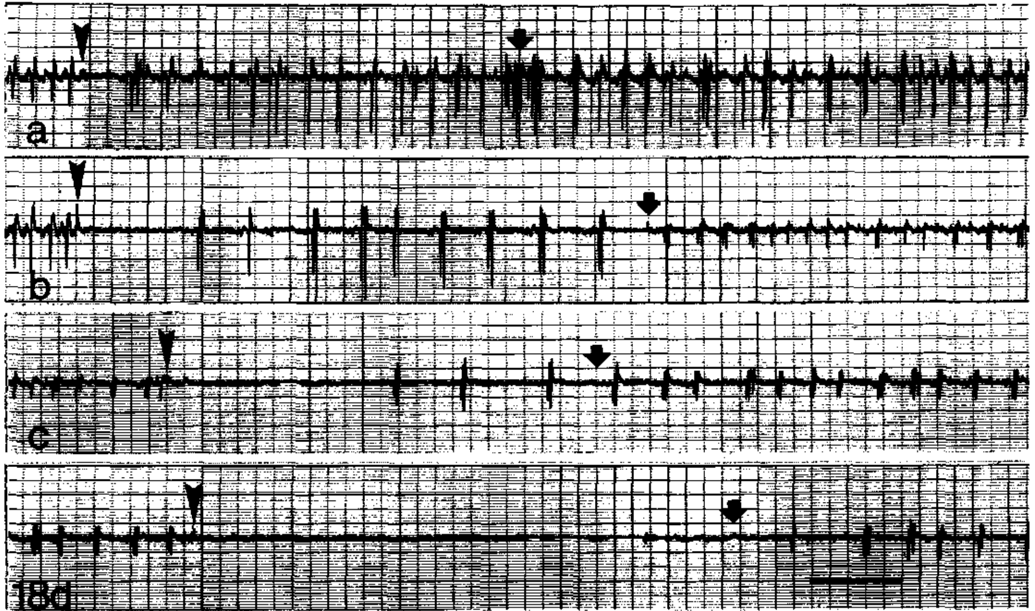

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CHAPTER 5

Diffuse serotoninergic neurohemal systems associated with cerebral and suboesophageal nerves in the head of the Colorado potato beetle, *Leptinotarsa decemlineata*.

Accepted in a slightly revised form for publication in Cell and Tissue Research
With: *H. Schooneveld*

SUMMARY

We analyzed the anatomy of two distinct diffuse neurohemal systems for serotonin in the head of *Leptinotarsa decemlineata* by means of immunohistochemistry. One such system is formed by axons from two bilateral pairs of neurons in the frontal margin of the SOG that enter the ipsilateral mandibular nerve, emerge from this nerve at some distance from the SOG and cover all branches of the mandibular nerve with a dense plexus of immunoreactive axon swellings. The other system is formed by axons from two large neurons in the frontal ganglion that enter the ipsilateral frontal connectives, emerge from these connectives and form a network of axon swellings on the labro-frontal, pharyngeal, and antennal nerves, and on the surface of the frontal ganglion. Immunohistochemical electron microscopy demonstrated that the axon swellings are located outside the neural sheath of the nerves and hence in close contact with the hemolymph. We therefore suggest that these plexuses represent extensive neurohemal systems for serotonin. Most immunoreactive terminals are in direct contact with the hemolymph, other terminals are closely associated with the muscles of the mandibles, labrum, and anterior pharynx, as well as with the salivary glands, indicating that these organs are under serotoninergic control.

INTRODUCTION

Serotonin (5-hydroxytryptamine) has been demonstrated biochemically and immunohistochemically in the nervous systems of many insect species (Nässel 1988). This neurochemical is usually assumed to have a neurotransmitter or neuromodulator function in the central nervous system, but it also has a peripheral function, for instance on visceral muscles (Cook et al. 1969; Banner et al. 1987; van Haeften et al. 1993) (Chapter 4), salivary glands (Trimmer 1985), Malpighian tubules (Morgan and Mordue 1984; Veenstra 1988), and heart (Tublitz and Truman 1985). Some of these physiological effects can be explained only by assuming that serotonin is released into the hemolymph as a neurohormone. In the context of our

studies on the control of feeding physiology of the Colorado potato beetle, *Leptinotarsa decemlineata*, we are investigating the central and peripheral distribution of serotonin. We showed that it is rather abundant in the ventral nerve cord (van Haeften and Schooneveld 1992) (Chapter 3) and in the brain (van Haeften and Schooneveld, submitted) (Chapter 2) and that it is carried to the fore- and hindgut and associated areas (van Haeften et al. 1993) (Chapter 4).

Release of serotonin as a neurohormone into the hemolymph of insects usually occurs through specialized neurohemal organs, usually the corpora cardiaca and in many species also through the perisympathetic organs (Raabe 1989). This is not the case in *L. decemlineata*, however. The corpora cardiaca could never be shown by immunohistochemical methods to contain serotonin, and no perisympathetic organs appear to be present (van Haeften, unpublished observation). Instead, a pilot study indicated that another release system for serotonin is in operation: a diffuse system of serotoninergic axons carried by several nerves in the head. We here report on the immunohistochemical studies to describe the sources of serotonin-immunoreactive (SLI) material in the major ganglia of the head, and the spatial organization of peripheral axon branches. Structural evidence for neurohemal axon specializations was sought by electron microscopy.

MATERIALS AND METHODS

Sexually mature male and female Colorado potato beetles from a laboratory culture (16 h photophase, 8 h scotophase), fed on fresh potato foliage, were used in all experiments. Cerebral ganglion complexes together with frontal and suboesophageal ganglia and adhering nerves, here referred to as cephalic nervous systems, and entire heads were dissected under ice-cold physiological saline (Khan et al. 1982).

Fixation

For whole-mount light microscopy, cephalic nervous systems and entire heads were fixed in a freshly prepared 4% paraformaldehyde solution in 0.1 M phosphate

buffer (NaH_2PO_4) pH 7.3 for 4-6 h at room temperature and washed overnight in ice-cold phosphate buffer. For entire head preparations, the head capsule was carefully removed with a pair of tweezers.

For electron microscopy, cephalic nervous systems were fixed for 4-6 h in an ice-cold solution of 4% paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 and washed overnight in ice-cold phosphate buffer.

The preservation of the delicate peripheral nerves for routine paraffin serial sections was achieved by injecting chill-anaesthetized beetles with 0.5 ml of a 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.3. After 30 minutes the beetles were decapitated and the heads were subjected to an additional microwave-accelerated formaldehyde fixation according to the method of Smid et al. (1990). After removal of the jaws, the heads were infiltrated with Heidenhain's susa for 16 h under vacuum. Next, the heads were rinsed, dehydrated, and embedded in Paraplast Plus (Lancer, Oxford), sectioned at 7 µm, and mounted on poly-l-lysine (Sigma) coated slides.

Immunohistochemistry

Tissues for whole-mount preparations for light microscopy were dehydrated to 90% alcohol and treated with n-heptane for 20 seconds to further increase membrane permeability for antibodies (van Haeften and Schooneveld 1992). Next, tissues were rehydrated to phosphate-buffered-saline (Dulbecco 'A', Oxoid) (PBS) pH 7.3. After rinsing in PBS + 0.25% Triton-X-100 (Merck) (PBS-T) and preincubation for 4 h in 10% normal swine serum (NSS) in PBS-T, tissues were incubated in rabbit anti-serotonin antiserum (Immunonuclear, Stillwater, USA), diluted 1:1000 in PBS-T + 0.1% sodium azide, for two days at 4 °C. The immunostaining was developed with peroxidase conjugated swine-anti-rabbit (Dakopatts), for two days at 4 °C. The peroxidase reaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2. After staining, tissues were trimmed to the desired size and embedded in Depex (Fluka) on glass slides.

Whole-mount preparations for electron microscopy were immunostained as described above, but with omission of the heptane treatment and Triton-X-100. After the immunostaining, tissues were rinsed in 0.1 M sodium cacodylate buffer (Merck) pH 7.3, post-fixed for 1 hour in 1% osmium tetroxide (TAAB) in 0.1 M

sodium cacodylate buffer pH 7.3, and embedded in Epon. Ultrathin sections were cut on an LKB Ultrotome, collected on formvar coated 100-mesh copper grids, and counterstained with a 1% lead citrate and a 2% uranyl acetate solution in destilled water.

Serial paraffin sections were deparaffinized, rinsed in PBS, and the sections were preincubated in 10% NSS in PBS-T, followed by an overnight incubation at 4 °C in anti-serotonin antiserum diluted 1:1500 in PBS-T. The immunostaining was developed with the PAP-method (Sternberger 1979) and the peroxidase reaction was carried out with 0.05% DAB and 0.01% H_2O_2. The sections were counterstained with Mayer's haematoxylin.

Specificity controls

One ml of diluted antiserum was incubated with 100 μg of serotonin-BSA conjugate (Immunonuclear, Stillwater, USA) for 8 h at 20 °C. After removal of the precipitate, the preadsorped antiserum was tested on whole-mounts and serial sections. In addition, sections were incubated in absence of the primary antiserum. Under these conditions no immunostaining was observed.

RESULTS

Serotonin-like immunoreactive neurons with a possible neurohemal function

Whole-mount immunohistochemistry is a powerful method for revealing SLI neurons in the ventral nervous system in the Colorado potato beetle (van Haeften and Schooneveld 1992). We made a detailed examination of SLI neurons in the suboesophageal ganglion (SOG) and frontal ganglion (FG) (Fig. 1) and noted that axons from altogether 6 of the SLI neurons in these ganglia left the central nervous system along unexpected routes (Fig. 2). Of two bilateral pairs of large neurons in the anterio-medial-part of the SOG (diameter 30-35 μm) (Figs. 2, 3) with dendritic arborizations elsewhere in the ganglion, each pair send their axons into the ipsilateral mandibular nerve. This nerve branches several times and runs to the mandibular musculature. Their SLI axons, however, emerge from a proximal part of

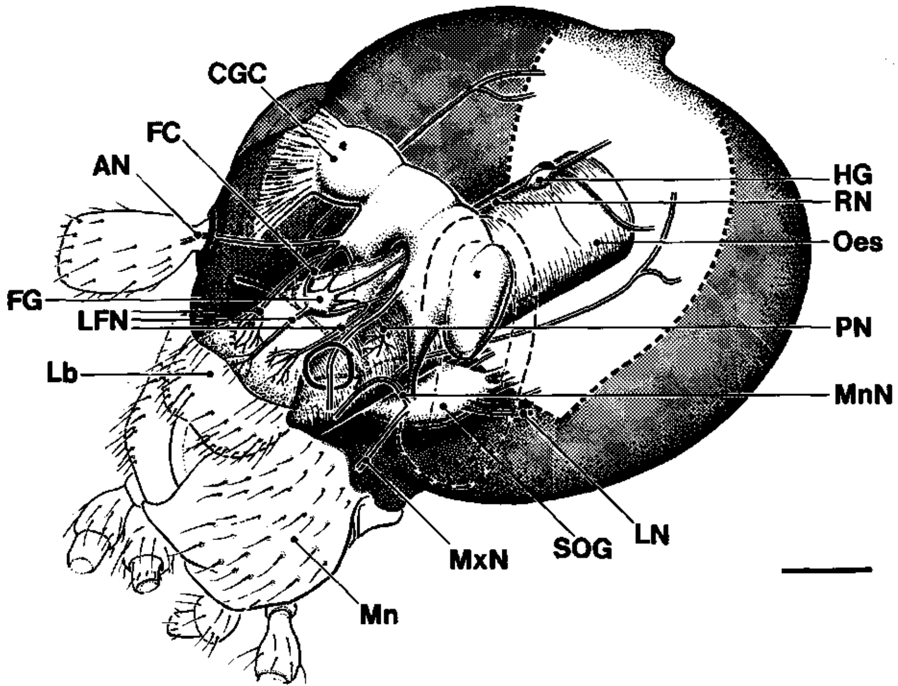


Fig. 1. Schematic representation of head of adult Colorado potato beetle showing location of cephalic ganglia and distribution of cephalic nerves in head, frontal view. Three labro-frontal nerves (*LFN*) originate from cerebral ganglion complex (*CGC*) and frontal ganglion (*FG*) and run to labrum (*Lb*) to innervate labral muscles. Mandibular nerves (*MnN*) originate from suboesophageal ganglion (*SOG*), split and run to muscles of mandibles (*Mn*). Pharyngeal nerves (*PN*) from frontal ganglion run to visceral muscles of anterior pharynx. Retrocerebral glands are not drawn. *AN* antennal nerve; *FC* frontal connective; *HG* hypocerebral ganglion; *LN* labial nerve; *MxN* maxillary nerve; *Oes* oesophagus; *Rn* recurrent nerve. **Bar:** 340 µm.

the mandibular nerve (Figs. 2, 4) and continue their complex course over the mandibular nerve, frequently branching to form a network of immunoreactive fibres (Figs. 2, 4), referred to here as the lateral neurohemal plexus. The axons show a beaded appearance characteristic for axon swellings with a neurohemal function (Fig. 4). Some of the branches run over the circumoesophageal connectives but other nerves originating from the SOG are not approached. We have not been able to delineate axon tracts of each of the SOG neurons separately; it is therefore not possible to say if there is a division of function between the neurons.

The two large, paired, neurons in the FG (diameter 30-35 μm) have their dendrites in the deutocerebrum (Figs. 2, 5). The axons run into the ipsilateral connectives but do not reach the brain: they emerge from the connectives at a point close to the brain to give rise to an even more developed neurohemal system, the fronto-medial plexus. Each axon branches several times, some branches covering nearby structures such as the labro-frontal nerves (Figs. 2, 6), the frontal connectives, and the FG (Figs. 2, 7), other branches running free through the hemocoel to associate with remote nerves such as the antennal and pharyngeal nerves, the recurrent nerve and the tritocerebral connective (Fig. 2).

There seems to be no overlap between these two neurohemal plexuses. On the other hand, such plexuses have not been observed in other parts of the body.

Structural evidence suggesting a neurohemal release of serotonin

The beaded axon swellings have the appearance of neurohemal structures and the possible release of serotonin from the plexuses has been studied. The pre-embedding whole-mount immunostaining procedure produces a clearly visible DAB precipitate inside axon swellings, which facilitates the selection, orientation, and trimming of tissue for electron microscope inspection. Electron micrographs show that such swellings stand out clearly against the surrounding tissues. The electron opacity of the swelling content is caused by the black vesicles, probably containing serotonin, and the flocculent opaque precipitate between the vesicles (Figs. 8, 9). The preservation of axoplasm integrity is poor, inescapably due to ruptures by the oxygen generated by the peroxidase enzyme.

Such specifically stained axon profiles are found in the interior of the mandibular nerves as well as just underneath the neural lamella (Fig. 8). Axon swellings on the

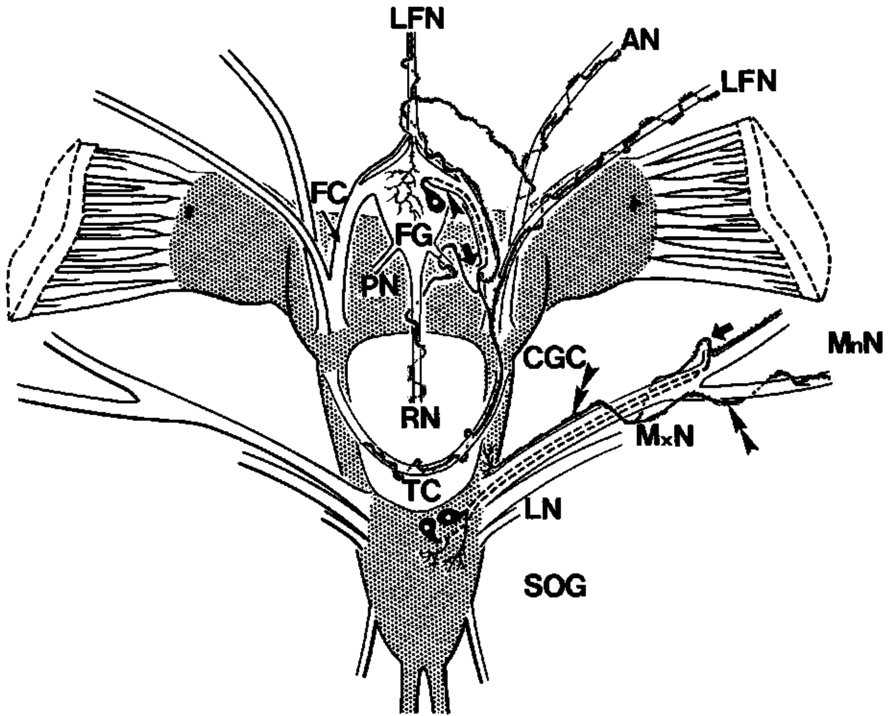


Fig. 2. Highly diagrammatic representation of cephalic nervous system of Colorado potato beetle showing origin and distribution of lateral and fronto-medial serotoninergic neurohemal plexuses on lateral half of cephalic nervous system, view from below. Axons of two large neurons in suboesophageal ganglion (*SOG*) run into ipsilateral mandibular nerve (*MnN*), emerge (*thick arrow*) and form a network of immunoreactive fibres (*double arrowheads*) on all branches of nerve. Large lateral neuron in frontal ganglion (*FG*) has dendrites (*arrowhead*) in cerebral ganglion complex (*CGC*). Its axon runs into frontal connective (*FC*), emerges (*thick arrow*) and forms a network of immunoreactive fibres on surface of labro-frontal nerves (*LFN*), antennal (*AN*), pharyngeal (*PN*) and recurrent nerve (*RN*), tritocerebral connective (*TC*), as well as on surface of frontal ganglion. *MxN* maxillary nerve; *LN* labial nerve.

same nerve as well as those of the fronto-medial plexus facing the hemolymph often contain large granular vesicles of 100 nm and numerous vesicles of 50-60 nm (Fig. 9) that are associated with the axolemma. Although no exocytotic profiles have been encountered we think that these axon swellings represent sites for the release of serotonin as a neurohormone into the hemolymph. The stroma layer covering such swellings is often rather thin (Fig. 9), thus allowing the easy liberation of secretion into the bloodstream of the head.

Targeted release of serotonin

Besides emptying its content in the bloodstream, an axon may also release its content rather directly to a potential target organ as follows from following axon profiles in serial paraffin sections. Axon swellings on peripheral branches of the mandibular nerves occur close to the mandibular muscles (Fig. 10) and the strongly convoluted tubules of the salivary glands (Fig. 11), but no evidence of participation in synaptic control has been obtained. We refer to this situation as a targeted release.

A similar targeted release close to the labral muscles was observed, but in this case the axons run to the labral musculature with the assistance of the labro-frontal nerves (Fig. 12). Branches of the pharyngeal nerves carry a neurohemal plexus which extends to the visceral muscles of the anterior pharynx.

DISCUSSION

The present observations reveal that the Colorado potato beetle has a special way of releasing serotonin in the head: through a neurohemal system comprising a fronto-medial and a lateral diffuse neurohemal plexus. Both plexuses are acting independently and are fed by neurons in the FG and SOG, respectively. No similar systems occur elsewhere in the body, and only certain cephalic nerves carry the immunoreactive axons, never the trachea or other nearby structures. The spatial arrangement is similar among individuals and no influence of age was found, the plexuses therefore appear to be pre-programmed in the ontogeny of our species. The

arrangement is reminiscent of that of a few other species that have been examined, i.e. *Periplaneta americana* (Davis 1985, 1987), *Locusta migratoria* (Bräunig 1987, 1988), and *Calliphora erythrocephala* (Nässel and Elekes 1985). In these species, too, neurohemal axons originate from the SOG, but the route along which different nerves are supplied by the free-running axons is characteristic for each species. Certain abdominal nerves in *C. erythrocephala* may carry immunoreactive axons as well (Nässel and Elekes 1985; Duve et al. 1988). The contribution of the frontal ganglion to a fronto-medial neurohemal plexus in *L. decemlineata* has not been reported before for other species.

The morphological differences among species are probably not fundamental to the question as to the physiological significance of the elaborate release apparatus. Veenstra (1987) already pointed out that the branching pattern of secretory neurons has hardly been under evolutionary pressure and hence strong variations between species may be anticipated.

What then is the function of the serotoninergic plexuses? Although our evidence for a probable release function of these 'open' swellings needs to be verified with physiological experiments, our observations, combined with similar observations by others (Davis 1985, 1987; Nässel and Elekes 1984, 1985), make it likely that this is

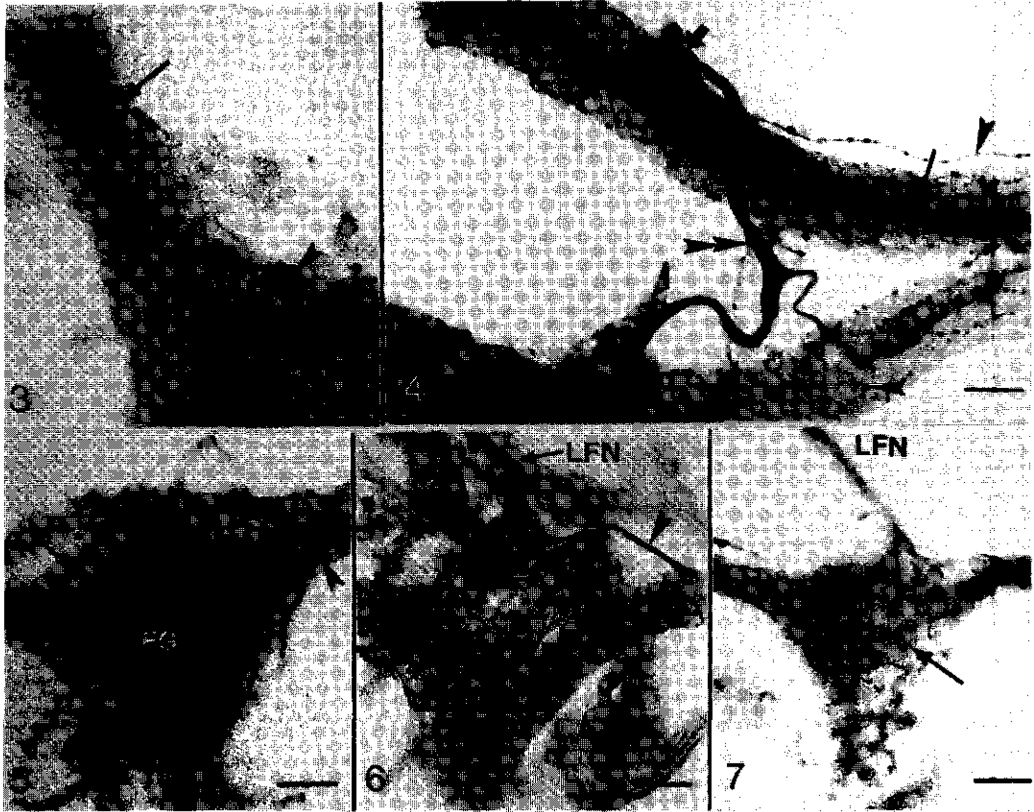
Fig. 3. Suboesophageal ganglion (*SOG*) with large serotonin-immunoreactive neurons (*arrowhead*), whose axons (*arrows*) run into mandibular nerve (*MnN*) to form lateral neurohemal plexus. **Bar:** 50 μm.

Fig. 4. Branched mandibular nerve with two serotonin-immunoreactive core axons (*long arrow*) originating from two anterio-medial neurons in suboesophageal ganglion. These axons emerge (*thick arrow*) and ramify extensively on surface of this nerve. Some ramifications (*double arrowhead*) run free through hemocoel and cover surface of other mandibular nerve branches with plexus of neurohemal axon swellings (*arrowheads*). **Bar:** 30 μm.

Fig. 5. Frontal ganglion (*FG*) with serotonin-immunoreactive neurons (*arrow*). Two neurons supply fronto-medial neurohemal plexus (*arrowhead*). **Bar:** 50 μm.

Fig. 6. Frontal ganglion (*FG*) with labro-frontal nerves (*LFN*). Large serotonin-immunoreactive axon (*arrowhead*) from frontal connective (*FC*) covers surface of these nerves with dense neurohemal network of axon swellings. **Bar:** 25 μm.

Fig. 7. Frontal ganglion with superficial network of serotonin-immunoreactive axon swellings (*arrow*). *LFN* labro-frontal nerve. **Bar:** 75 μm.



a huge system with a high release-capacity for serotonin. A likely explanation for the fact that the corpora cardiaca in our species does not function as a neurohemal organ for serotonin (in contrast to a number of other species, see: Davis 1985; Klemm et al. 1986; Konings et al. 1988; Raabe 1989; Strambi et al. 1989), is that it is too tiny for this release function. Serotonin is known to be degraded in the blood with high efficiency (Trimmer 1985). A considerable amount must therefore be released in the bloodstream to achieve sufficient concentrations, even perhaps in remote places and far from the head.

We can only speculate as to the physiological function of serotonin release through the plexuses. The mandibular and labro-frontal nerves are among the nerves consistently carrying part of the plexus and the corresponding muscles are directly

addressed by nearby axon terminals. It seems plausible, therefore, that these nerve-muscle units are under a serotoninergic control. This would fit in with suggestions of Baines and Tyrer (1989) and Baines et al. 1990) who worked on this problem from an electrophysiological angle. Also the salivary glands may be under a direct serotoninergic control, as proposed by Trimmer (1985), working on the in vitro serotonin-modified saliva production in *C. erythrocephala*

Apart from the diffuse release of serotonin, a targeted release seems to occur in *L. decemlineata*. This holds true for several muscles involved with the movements of the mouthparts and for the salivary glands. This provision might ensure that these organs are addressed in an early phase of serotonin release from the diffuse system.

The present observations contribute to our appreciation of the complexity of the serotoninergic system. Earlier studies described the elegant region-specific organization of serotoninergic networks in the ventral nerve cord (van Haeften and Schooneveld 1992) (Chapter 3) and recent studies showed the likely involvement of serotonin in the channelling of optic stimuli in the protocerebrum (van Haeften and Schooneveld, submitted) (Chapter 2). On the other hand, a much smaller number of large neurons in the ventral nerve cord, supplying the hindgut, have a secretory

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Figs. 8 and 9. Electron-micrographs of serotonin-imunoreactive axon swellings on peripheral nerves.

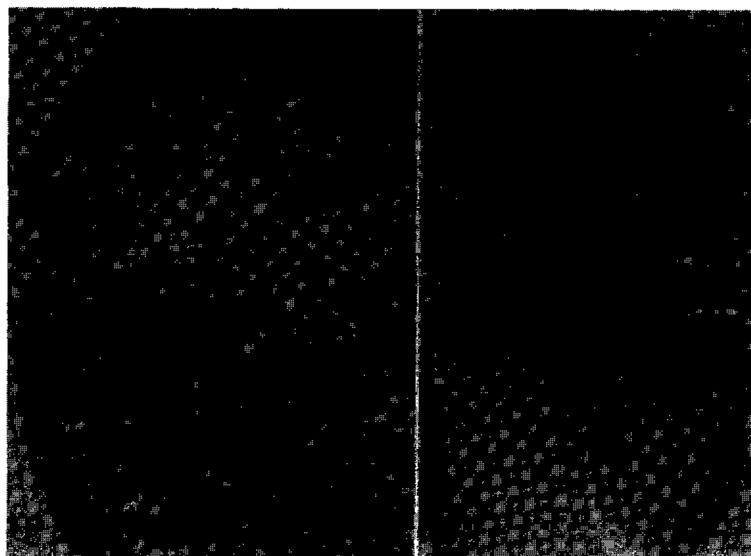
Fig. 8. Transverse section through mandibular nerve with two immunoreactive axon profiles (*double arrowheads*) underneath extra-cellular stroma layer (*SL*), which are in close contact with hemolymph (*HE*). Note presence of presumable peptidergic axon profile (*asterisk*). **Bar:** 0.5 µm.

Fig. 9. Immunoreactive axon swelling on neural sheath (*NS*) of mandibular nerve. The swelling contains large granular vesicles of 100 nm (*thick arrow*) and numerous small vesicles of 50-60 nm (*thin arrow*). *HE* hemolymph; *SL* stroma layer. **Bar:** 0.3 µm.

Fig. 10. Serotonin-immunoreactive axon terminals (*arrows*) making contact with mandibular muscles (*MM*). **Bar:** 20 µm.

Fig. 11. Serotonin-immunoreactive terminals (*arrows*) on salivary gland tubule (*SG*). *asterisk* fatbody; *arrowhead* tubule lumen. **Bar:** 20 µm.

Fig. 12. Labro-frontal nerve (*LFN*) running between labral muscles (*LM*). Serotonin-immunoreactive axon terminals (*arrowheads*) on labro-frontal nerve are in close contact with labral muscles. **Bar:** 20 µm.



LM

function.

The present extension of the number of secretory neurons seems to complete the inventory of serotoninergic neurons in this species.

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CHAPTER 6

Immunohistochemical studies on the occurrence of serotonin in peripheral sensory neurons in the Colorado potato beetle, *Leptinotarsa decemlineata*.

SUMMARY

In the present study we investigated the possible presence of a serotonin-like immunoreactive substance in sensory neuronal cell bodies in the peripheral nervous system of *L. decemlineata*, with an antiserum to serotonin. Sensory neurons from sensilla on labial and maxillary palps, galea, ventral labrum, tarsi, and compound eyes were inspected but no immunoreactive neurons were found in this part of the peripheral nervous system.

INTRODUCTION

Our studies on the localization and role of the neurochemical messenger serotonin in *Leptinotarsa decemlineata*, indicated that serotonin-containing neurons have a function in the regulation of feeding behaviour at both the central and peripheral level. Serotonin-like immunoreactive (SLI) neurons are rather abundant in the brain (van Haeften and Schooneveld, submitted) (Chapter 2) and in the ventral nerve cord (van Haeften and Schooneveld 1992) (Chapter 3). Most of these neurons appear to function as interneurons, specialized either in the processing of optic and antennal information within the brain, or in the inter- and intraganglionic communication between the various segments of the central nervous system. Other SLI neurons in the ventral and stomatogastric ganglia are efferent neurons. Their processes leave the central nervous system and innervate peripheral targets organs either directly, e.g. with terminals on the muscles of mouthparts, fore- and hindgut, salivary glands, or indirectly via the hemolymph (van Haeften et al. 1993 (Chapter 4); van Haeften and Schooneveld 1993) (Chapter 5).

Until now no information was available on the possible occurrence of afferent SLI neurons in the sensory nervous system of *L. decemlineata* and there is still little information with regard to afferent SLI neurons in other insect species. Tyrer et al. (1984) suggested that afferent fibres in some of the peripheral nerves of *Locusta migratoria* might originate from serotoninergic sensory neurons. Lutz and Tyrer (1988) demonstrated the presence of serotoninergic sensory neuronal cell bodies

associated with internal proprioreceptors in the leg of *Schistocerca gregaria.*

The present study focuses on the possible presence of serotonin-containing sensory neurons in the peripheral nervous system of *L. decemlineata* that play a role in feeding behaviour. We paid attention to sensory neurons from the sensilla on galea, maxillary and labial palps, antennae, from the preoral cavity, legs, and eyes in paraffin sections and whole-mount preparations, and investigated whether such neurons use serotonin as a neurochemical.

MATERIALS AND METHODS

One-day-old beetles (16 h photophase, 8 h scotophase) from a laboratory culture were used in all experiments. At this stadium, the cuticle of the beetle is soft and transparent and can easily be sectioned with a microtome. For entire beetle preparations, mandibles, legs, and elytra were removed and the beetle was perfused with 1 ml of Heidenhain's Susa. The beetles were next immersed in a 4% formaldehyde solution, freshly prepared from paraformaldehyde and 0.1M NaH_2PO_4 pH 7.3, and an additional microwave-accelerated fixation was carried out according to the method of Smid et al. (1990). Next, the tissues were incubated in the same fixative for 1 night under vacuum. Tissues were rinsed in 0.1 M NaH_2PO_4 buffer pH 7.3 for 1 day, dehydrated, and embedded in Paraplast Plus (Lancer, Oxford). Serial sections of 5 μm were mounted on poly-L-lysine coated slides.

For whole-mount preparations, legs and antennae were removed with micro-scissors and fixed in a formaldehyde solution for 6-8 h under vacuum.

Immunostaining

Immunostaining of whole-mounts and serial sections was carried out with a rabbit-anti-serotonin antiserum (Immunonuclear, Stillwater, USA) according to the method of van Haeften and Schooneveld (1992) and van Haeften et al. (1993), with some minor changes. All washing steps were prolonged and to ensure a sufficient penetration of rinsing buffers and antisera, all incubations of whole-mounts took place under vacuum.

Staining controls

Antiserum specificity was tested by preadsorption of one ml diluted antiserum with 100 μg serotonin-BSA conjugate (Immunonuclear, Stillwater, USA), or 100 μg BSA (Sigma). The preadsorpted antiserum was tested on serially sectioned brain preparations.

To ensure that an absence of immunostaining is not due to failures in the staining protocols, in all cases we simultaneously performed anti-serotonin immunostainings on sectioned or whole-mount preparations of brains as a positive control. These tissues always stained under the conditions used.

RESULTS

The preadsorption of the antiserum with serotonin-BSA conjugate resulted in a total absence of immunostaining, whereas preadsorption of the antiserum with BSA did not influence the staining results. This indicates that our staining is specific for serotonin.

Earlier studies on the Colorado potato beetle have described the morphology, the ultrastructure, and physiology of the galeal sensilla (Mitchell and Harrison 1984), the sensory complexus on the maxillary and labial palps (Schanz 1953; Sen 1988), and the sensilla of the antennae (Schanz 1953).

Mouthparts

The galea of adult beetles are well supplied with both mechanosensory hairs and chemosensitive apical pegs (Mitchell and Harrison 1984). The perikarya of the sensory neurons are located near the tip under the cuticle of the galea and were devoid serotonin-like immunoreactivity.

The apical tips of both the maxillary and labial palps carry 370 and 130 sensilla respectively of four morphologically different types. Type A sensilla, which are unique to the maxillary palps, and type C sensilla both have characteristics of olfactory sensilla. Type B and D sensilla are considered to be contact chemoreceptors. All sensilla are innervated by several bipolar neurons located at the

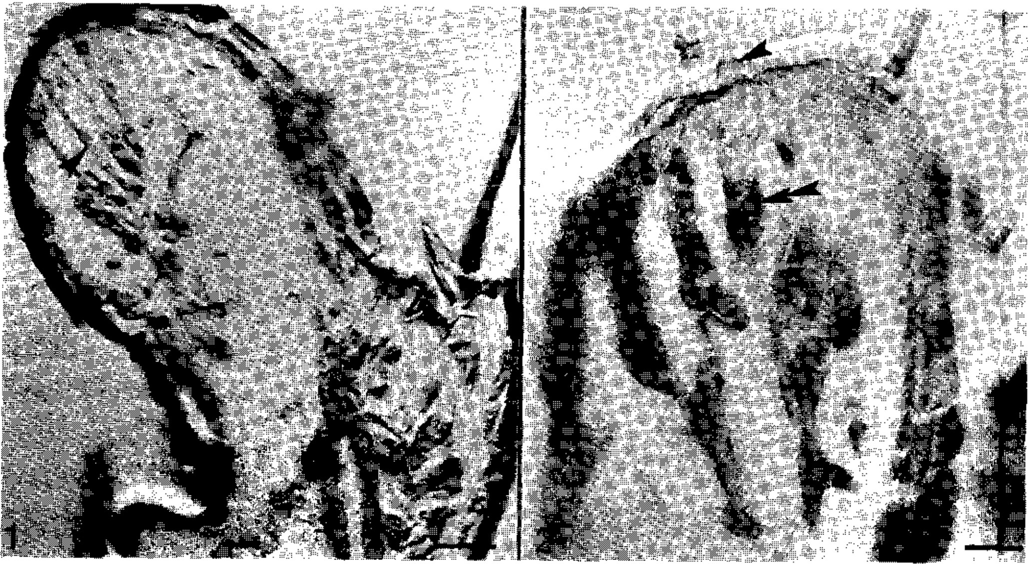

Fig. 1. Serial section of labial palp showing elongate non-immunoreactive sensory neurons (*arrowhead*). **Bar:** 20 μm.

Fig. 2. Serial section of maxillary palp showing sensillum (*arrowhead*) and associated non-immunoreactive sensory neurons (*double arrowhead*). **Bar:** 20 μm.

basis of the sensillum (Sen 1988). None of these sensory perikarya appeared to contain a serotonin-like neurochemical (Figs. 1, 2).

Serial sections of entire heads showed the presence of sofar unknown sensilla on the medio-ventral surface of the labrum with the appearance of chemoreceptors. Their sensory perikarya are located under the cuticle of the labrum and were likewise devoid of immunoreactivity.

Antennae

Most of the sensilla in the antennae are situated in the five distal segments. Schanz (1953) distinguished three morphological types of sensory hairs in the Colorado

potato beetle: (1) straight and (2) bent hairs, which are supposed to function as mechanoreceptors; and (3) small hairs, which may function as chemoreceptors. The sensory perikarya associated with these sensilla showed no immunoreactivity in both serially sectioned and whole-mount preparations of antennae.

Photoreceptors of the compound eyes

No serotonin-like immunoreactivity was found in the photoreceptor cells and no immunoreactive axons were present in the optic nerves.

Receptors in legs

Mechanoreceptor hairs are present only on the tarsi of the legs (Schanz 1953). We were unable to demonstrate serotonin-immunoreactive sensory perikarya associated with these hairs. In some leg preparations internal proprioreceptors are present. Their sensory perikarya were non-immunoreactive as well.

DISCUSSION

In this study we have investigated the presence of serotonin-immunoreactive sensory neurons associated with chemo-and mechanoreceptors of the mouthparts, antennae, legs, and the photoreceptors of the eyes of *L. decemlineata* to complete our assessment of the role of serotonin in the central and peripheral nervous system. No serotonin could be demonstrated. This negative result cannot be attributed to technical failures in our staining protocols because the proper control experiments have been carried out. In view of the fact that immunohistochemical studies on serially sectioned preparations of entire beetle never showed the presence of serotoninergic sensory cells (van Haeften, unpublished results), we thus conclude that serotonin does not belong to the neurochemicals used by sensory neurons in the peripheral nervous system of Colorado potato beetle. In contrast, we have shown that serotoninergic interneurons play a role in the processing of sensory information at a more central level. Serotoninergic interneurons in the brain have the morpho-

logical specializations that enable them to connect primary sensory input areas, such as the antennal glomeruli and the optic lobes, to the higher integration centres in the protocerebrum (van Haeften and Schooneveld, submitted) (Chapter 2).

It must be emphasized that very little is known about the type of transmitters generally used by afferent systems in insects. There is circumstantial evidence that most afferent neurons are cholinergic (Sanes and Hildebrand 1976; Lutz and Tyrer 1988; Kral and Schneider 1981). There good evidence now that also the biogenic amines histamine and taurine are important neurochemical messengers in afferent systems. Histamine has been demonstrated immunohistochemically in the photoreceptors of both ocelli and compound eyes of *Locusta migratoria* (Simmons and Hardie 1988; Schlemermeyer et al. 1989), *Musca domestica* (Hardie 1987; Nässel et al. 1988), and *Calliphora erythrocephala* (Nässel et al. 1988), and it is generally assumed that histamine functions as a neurotransmitter. There is also strong evidence that taurine is a neurotransmitter in the photoreceptors of the ocelli and compound eyes of *Apis mellifera* (Schäfer et al. 1988).

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CHAPTER 7

Summary and concluding remarks

In this thesis we have localized serotoninergic neurons in the central and peripheral nervous system of the Colorado potato beetle, *Leptinotarsa decemlineata* by means of immunohistochemistry with a specific antiserum to serotonin and assessed the possible role of these neurons in feeding physiology. Emphasis was laid on the location of serotoninergic neurons involved in: (1) channelling of sensory information from antennal and gustatory sensory systems to the central nervous system; (2) the central organization of the serotoninergic neuron system providing information on possible central processing of this information; and the routes of innervation of possible target organs.

We have shown that about 200 serotoninergic neurons are present in the cerebral ganglion complex of the beetle, representing interneurons serving short- and long range communication. These neurons were grouped according to their location, number, and distribution of their processes. Clusters of paired protocerebral neurons appeared to be responsible for left-right communication within the brain and are the sole source of immunoreactivity in the central complex and the corpora pedunculata. Other neurons in the optic lobes and the deutocerebrum are likely to play an important role in the processing of visual and olfactory information respectively. This serotoninergic network in the brain does not project to other parts of the central nervous system and has the appearance of an individual serotoninergic neural unit. No neurons with a secretory function are present in the cerebral ganglion complex, nor are there structural indications that serotoninergic neurons are involved with the control of peptidergic neurosecretory neurons in the protocerebrum **(Chapter 2)**

In the remainder of the central nervous system, the ventral nerve cord, altogether 74 serotoninergic neurons were found and their organization pattern enabled us to group them into five neuron classes. Two paired segmental twin interneurons are present in each ganglion or neuromere. These neurons have extensive dendritic arborizations in the contralateral hemisphere of the ganglion, and small arborization close to the perikaryon. Their axons take a contra- and ipsilateral course to more frontally and/ or caudally located ganglia. The distribution of processes is indicative of a division of labour among these neurons. The intersegmental projection are indicative of a function in interganglionic communication, whereas the dendritic projections suggest a function in the coordination of left-right neural activity within the ganglia. Four large frontal secretory neurons are present in the suboesophageal

ganglion with axons projecting to a diffuse neurohemal system on oesophageal nerves. A pair of large caudal efferent neurons in the terminal ganglion send their processes in the proctodaeal nerves and innervate the proximal part of the hindgut. The function of miniature and terminal neurons is unknown. The distribution pattern of serotonin-like immunoreactivity enabled us to distinguish three separate putative functional units. The function of the caudal functional unit might be the synaptic control of caudal neurons innervating the alimentary canal, the function of the other two units is unknown (**Chapter 3**).

The presence of serotoninergic axons in the proctodaeal nerves indicated that the gut might be under serotoninergic control. By means of immunohistochemistry, it was shown that the alimentary canal of the beetle receives innnervation from two separate sources. Large efferent neurons in both the stomatogastric and central nervous system innervate the gut. Four neurons in the frontal ganglion have axons which run via the recurrent nerve to the circular and longitudinal muscles of the fore- and anterior midgut and supply the surface of these muscles with neurohemal axon swellings. The posterior midgut is devoid of immunoreactivity. The longitudinal muscles of the hindgut are supplied by the two caudal neurons described in **Chapter 3**. Electron-microscopical inspections of the axon swellings showed that exocytosis of immunolabelled vesicles occurs at some distance from the muscles fibres, indicating that the gut muscles might be under neurohormonal control. No serotoninergic synapses are observed on muscle fibres. A possible serotoninergic neurohormonal control of gut muscles, was confirmed in a bioassay. It appeared that administration of graded dosages of serotonin to the incubation medium has a clear inhibitory effect on spontaneous contractions of hindguts in vitro at concentrations of 10^{-8}-10^{-5} M. This effect was dose-dependent (**Chapter 4**).

Other organs might be under serotoninergic control as well. Two diffuse neurohemal systems for serotonin are present in the head of the beetle. Axons of four secretory neurons in the suboesophageal ganglion, described in **Chapter 3**, enter the ipsilateral mandibular nerve and cover the surface with a dense network of immunoreactive swellings. Two efferent neurons in the frontal ganglion have processes that run into the frontal connectives. Here, the axons emerge and form a similar network of axon swellings on the surface of the labro-frontal, pharyngeal, and antennal nerves, and on the frontal ganglion. Electron microscopy showed that these axon swellings are located outside the impermeable neural sheath, surrounding the nerves,

and hence in close contact with the hemolymph. Next to this neurohemal release, a targeted release of serotonin occurs near the muscles of labrum, mandibles, pharynx, and salivary glands, indicating that these organs are under serotoninergic control (**Chapter 5**).

We have investigated the presence of serotoninergic sensory neuronal cell bodies in sensilla on labial and maxillary palps, galea, ventral labrum, tarsi, and compound eyes. It appeared that no afferent serotoninergic neurons are present in the peripheral nervous system of the beetle (**Chapter 6**).

The studies presented in this thesis show that the biogenic amine serotonin (5-hydroxytryptamine) is a ubiquitous and versatile neuroactive substance in both the central and peripheral nervous system of the Colorado potato beetle. In the central nervous system it is present in interneurons serving intra- and interganglionic communication. Here, it probably functions as a neurotransmitter and/or neuromodulator. A small number of serotoninergic neurons release serotonin, via elaborate ways, as a neurohormone into the hemolymph and/or close to their target organs.

In this thesis we have provided evidence that serotoninergic neurons are involved in the regulation of some aspects of feeding physiology at both the central and peripheral level. In the central nervous system, several serotoninergic interneurons, i.e. those in the cerebral ganglion complex, participate in the channelling and the central processing of antennal and optic information, whereas other interneurons, i.e. those in the ventral nerve cord, are part of functional neural units which are proposed to control efferent neurons, e.g. the caudal neurons innervating the hindgut. Serotoninergic neurosecretory neurons are involved in control of gut functioning, as shown in immunohistochemical and bioassay studies. Another class of efferent neurosecretory neurons were shown to innervate salivary glands and muscles of labrum, mandibles, and anterior pharynx, indicating that these organs might also be under serotoninergic control.

Algemene inleiding en samenvatting

INLEIDING

De Coloradokever

De Coloradokever, *Leptinotarsa decemlineata* (Say), is een lid van de op twee na grootste keverfamilie, de Chrysomelidae (Haantjes). Deze familie omvat wereldwijd 35.000 beschreven en ongeveer 25.000 nog te beschrijven soorten (Jacques 1988; Jolivet 1988). De Coloradokever voedt zich met bladeren van planten behorend tot verschillende geslachten van de familie Solanaceae (Nachtschadeachtigen). Eieren worden afgezet op bladeren van dezelfde waardplant. De jonge larven komen na 7 dagen uit de eieren en voeden zich gedurende 4 larvale stadia. De prepupale en pupale stadia voltrekken zich in de grond en de verpopping duurt ongeveer 10 dagen onder laboratorium condities. Afhankelijk van de geografische locatie zijn er 1 of 2 generaties per jaar. De kevers kennen twee vormen van seizoensadaptatie (Jacques 1988): (1) in de tropen gaan de dieren voor het begin van de droge periode in diapauze en wachten op het begin van de zomerregens; (2) in de gematigde gebieden reageren de dieren op de verandering van de lichtperiode in de herfst; ze graven zich in de grond en gaan in diapauze om in de lente weer te voorschijn te komen.

De Coloradokever is inheems in Mexico, waar het nog steeds leeft op zijn oorspronkelijke waardplanten, *Solanum augustifolium* en *S. rostratum* (Casagrande 1987). Begin vorige eeuw verspreidde de kever zich naar het westelijk deel van de Verenigde Staten, wisselde daar van waardplant en voedde zich vervolgens met bladeren van de gecultiveerde nachtschade, de consumptieaardappel (*S. tuberosum*), die door kolonisten meegenomen was naar het westen. Rond 1859 begon de verspreiding naar het oosten, over de Atlantische Oceaan, naar Europa. In 1920 werd de kever in de buurt van Bordeaux, Frankrijk, waargenomen en gedurende de volgende decennia verspreidde het zich naar het noordoosten en bereikte Noord-Europa in 1945 (Roessingh 1971). De kever bleek een ernstige belager van aardappeloogsten op het europeese continent te zijn. Bestrijding geschiedde voornamelijk met chemische bestrijdingsmiddelen hetgeen resulteerde in resistentie tegen de meest gebruikte middelen. De ontwikkeling van alternatieve bestrijdingsmethoden werd verwaarloosd en recent intensief onderzoek heeft nog niet geleid tot een oplossing van dit probleem (Casagrande 1987).

Coloradokever onderzoek aan de Landbouwuniversiteit Wageningen

Fysiologisch onderzoek aan de Coloradokever bij de vakgroep Entomologie heeft zich de afgelopen 25 jaar geconcentreerd op twee belangrijke onderzoekslijnen. De eerste onderzoekslijn omvat de hormonale regulatie van voortplantings- en diapauzegedrag. Afhankelijk van bijvoorbeeld de aanwezigheid van voedsel, de temperatuur, en de lichtperiode planten de dieren zich voort of gaan in diapauze. Voortplanting gebeurt onder de aanwezigheid van een hoge juveniel hormoonspiegel in het bloed (de Wilde et al. 1968). Diapauze treedt op wanneer de juveniel hormoonspiegel in het bloed daalt door een lagere juveniel hormoon productie door de corpora allata en door een versnelde afbraak door een juveniel hormoon specifieke esterase (de Wilde et al. 1968; Kramer et al. 1977; de Kort 1981). De aktiviteit van de corpora allata blijkt gedeeltelijk te worden gereguleerd door peptiden (kleine eiwitten) bevattende (peptiderge) zenuwcellen in de hersenen (Schooneveld 1970; Khan 1984). In het verleden zijn een groot aantal van die peptiderge zenuwcellen (neuronen) in het centrale en perifere zenuwstelsel geidentificeerd, in eerste plaats met behulp van histochemische kleuringstechnieken (Schooneveld 1970) en later immunohistochemisch met behulp van polyklonale (Veenstra and Schooneveld 1984; Veenstra et al. 1985) en monoklonale antisera (Schooneveld et al. 1989; Schooneveld en Smid 1990; Smid en Schooneveld 1992) opgewekt tegen peptiden afkomstig uit vertebraten en evertebraten. Een aantal van deze peptiden wordt momenteel geisoleerd en geidentificeerd, en hun fysiologische betekenis zal worden onderzocht.

De tweede lijn omvat de analyse van waardplant selectie door de Coloradokever. Aangetrokken door de geur van de waardpant en de smaak van de bladeren herkent de kever de waardplant en zal het dier zich voeden tot het verzadigd is. De complexe moleculaire samenstelling van de plantgeur die verantwoordelijk is voor de oriëntatie op de waardplant is bestudeerd door Visser (1976), terwijl het mechanisme van waardplantgeur herkenning is geanalyseerd door de Jong (1988).

Ondanks de grote onderzoeksinspanningen om de functies van het zenuwstelsel van insekten te doorgronden, zijn de hersenen nog steeds een zogenaamde "zwarte doos" wat betreft onze kennis over de routes waarlangs zintuiginformatie wordt verwerkt tot informatie die het functioneren van organen reguleert. De aard van de neuroactieve stoffen aanwezig in neuronen langs deze routes is bijna geheel onbekend. Eerdere histochemische studies in de Coloradokever hebben aangetoond

dat bepaalde zintuigneuronen in de antennen een "corticotropic releasing factor" - achtig (CRF) peptide bevatten (Smid, persoonlijke mededeling). Studies met een monoklonaal antilichaam (MAC-2), opgewekt tegen hersenhomogenaten (Schooneveld et al. 1989), toonden de aanwezigheid van een tot nu toe onbekend peptide in sensorische neuronen, in aan- en afvoerende zenuwbanen, en in het centrale zenuwstelsel aan. De peptiden proctoline en FMRFamide zijn aangetoond in neuronen die darmfuncties reguleren (Veenstra and Schooneveld 1984; Veenstra et al. 1985; Schooneveld et al. 1993). In de meeste gevallen worden de peptiden verondersteld te functioneren als co-transmiters. Co-localisatie studies in andere insekten hebben aangetoond dat neuropeptiden vaak samen worden aangetroffen met biogene aminen (Homberg and Hildebrand 1989).

Biogene aminen zijn aangetoond in het zenuwstelsel van verschillende insektesoorten. Vroeger gebeurde dat met behulp van de Falck-Hillarp fluorescentie techniek (Falck and Owman 1965) en meer recent met behulp van immunohistochemische technieken b.v. in: *Calliphora* (Nässel 1988; Nässel et al. 1988); *Locusta migratoria* (Konings et al. 1988); en *Apis mellifera* (Schäfer et al. 1988). Deze onderzoeksresultaten maken duidelijk dat biogene aminen een belangrijke rol spelen in verschillende aspecten van de voedingsfysiologie van insekten, b.v. de regulatie van de darmfunctie, de aktiviteit van de speekselklieren en de verwerking van geurinformatie (voor een overzicht zie Evans 1980; Nässel 1986a, 1986b, 1988). Echter, niets is echter bekend over de aanwezigheid en functie van biogene aminen in het zenuwstelsel van de Coloradokever.

Doel van het onderzoek beschreven in dit proefschrift

Het doel van deze studie is met behulp van immunohistochemische technieken de plaats en morfologie van biogene aminen bevattende neuronen in het centrale en perifere zenuwstelsel van de Coloradokever te beschrijven om vervolgens de mogelijke functie van deze neuronen in de voedingsfysiologie te analyseren. Nadruk in het onderzoek werd gelegd op die neuronen die mogelijk betrokken zijn bij: (1) de kanalisatie van zintuiginformatie afkomstig van antennale- en smaakzintuigsystemen naar het centrale zenuwstelsel; (2) de centrale organisatie van het aminerge neuronsysteem betrokken bij de centrale verwerking van deze informatie; en (3) de routes waarlangs de doelorganen worden geinnerveerd. De selectie van een geschikt anti-

amine antiserum was van het grootste belang.

Biogene aminen aanwezig in de hersenen

Antisera, opgewekt tegen verschillende biogene aminen, afkomstig van verschillende leveranciers (zie beneden), zijn getest op vermogen om biogene aminen bevattende neuronen in het zenuwstelsel van de kever te detecteren (Tabel 1.)

Tabel 1. Vergelijking van antisera tegen biogene aminen met betrekking tot specificiteit en intensiteit van kleuring van aminerge neuronen in seriële coupes van keverhersenen.

antiserum	leverancier	antigeen	fixatief	type coupes	verdunning 1:	resultaat[1]
konijn-anti serotonine	Immunonuclear	serotonine-FA-BSA	formaldehyde	paraffine	1500	+++
konijn-anti serotonine # 7-7	Dr. Steinbusch Amsterdam	serotonine-FA-BSA	formaldehyde	paraffine	1000	++
konijn-anti-dopamine # 9-5	Dr. Steinbusch Amsterdam	dopamine-GA-THY	glutaar-aldehyde	paraffine cryostaat	500 500	-- +
konijn-anti-dopamine	Immunonuclear	dopamine-GA-BSA	glutaar-aldehyde	paraffine cryostaat	500 500	-- --
konijn-anti-dopamine	Serotec	dopamine-GA-BSA	glutaar-aldehyde	paraffine cryostaat	500 500	-- --
konijn-anti-taurine	Serotec	taurine-GA-BSA	glutaar-aldehyde	paraffine cryostaat	500 500	-- --
konijn-anti-octopamine[2]	Dr. Vullings Utrecht	octopamine-GA-THY	glutaar-aldehyde	paraffine cryostaat	500 500	-- --
konijn-anti-octopamine # 2-7	Dr. Steinbusch Amsterdam	octopamine-GA/FA-BSA	glutaar-aldehyde/ formaldehyde	paraffine cryostaat	500 500	-- --
konijn-anti-histamine	Immunonuclear	histamine-CDI-KLH	carbodiimide	paraffine cryostaat	500 500	-- --

[1])
+++ reproduceerbare kleuring met hoge intensiteit
++ reproduceerbare kleuring met lage intensiteit
+ variabele kleuring met lage intensiteit
-- geen kleuring
[2]) dit antiserum kleurt DUM-neuronen in *Locusta migratoria*

FA: formaldehyde
BSA: bovine serum albumine
GA: glutaaraldehyde
THY: thryroglobuline
CDI: carbodiimide
KLH: succinylated keyhole limpet hemocyanin

Van alle geteste antisera bleken alleen de antisera tegen serotonine, serotonine bevattende (serotoninerge) neuronen in de hersenen reproduceerbaar aan te kleuren. Om die reden werd het anti-serotonine antiserum gekozen voor de bestudering van aminerge neuronale netwerken. Het moet duidelijk zijn dat een negatief kleuringsresultaat niet vanzelfsprekend betekent dat het betreffende aminerge antigeen niet aanwezig is. Waarschijnlijk was het niet detecteerbaar door mogelijke fixatie-artefacten of antiserum-specificiteits problemen. Het commerciële antiserum opgewekt tegen serotonine (Immunonuclear) bleek het enige antiserum dat serotoninerge neuronen met hoge betrouwbaarheid herkende. We hebben dit Immunonuclear antiserum verder gebruikt in alle studies beschreven in dit proefschrift.

Om praktische redenen worden de resultaten gepresenteerd in opeenvolgende hoofdstukken. De plaats van serotoninerge neuronen in het cerebrale ganglion complex (hersenen) en de routes waarlangs deze neuronen antennale, visuele en intracerebrale informatie verwerken worden beschreven in **Hoofdstuk 2**. De plaats van serotoninerge neuronen in het ventrale touwladderzenuwstelsel en hun rol in inter- en intraganglionaire communicatie wordt beschreven in **Hoofdstuk 3**. De innervatie van de voor- en einddarm door serotoninerge neuronen in het stomatogastrische en centrale zenuwstelsel en een bioassay voor de bestudering van de effecten van serotonine op contracties van geisoleerde eindarmen worden beschreven in **Hoofdstuk 4**. De anatomie van twee diffuse neurohormonale afgiftesystemen voor serotonine in de kop en de mogelijke doelorganen geassocieerd met deze systemen worden beschreven in **Hoofdstuk 5**. De resultaten van een immunohistochemische inventarisatie van de mogelijke aanwezigheid van serotonine in zintuigneuronen in sensilla op maxillaire en labiale palpen, galea, de preorale mondholte, antennen, ogen en poten wordt beschreven in **Hoofstuk 6**.

SAMENVATTING

In dit proefschrift is met behulp van immunohistochemische kleuringstechnieken de aanwezigheid van serotoninerge neuronen in het centrale en perifere zenuwstelsel van de Coloradokever onderzocht. Daarnaast is de mogelijke rol van een aantal van deze neuronen in de voedingsfysiologie geanalyseerd. Nadruk werd gelegd op de

locatie van neuronen betrokken bij: (1) de kanalisatie van sensorische informatie van antennale en smaakzintuigen naar het centrale zenuwstelsel; (2) de centrale organisatie van het serotoninerge neuronsysteem in relatie tot de mogelijke centrale verwerking van deze informatie; en de routes waarlangs mogelijke doelorganen worden geinnerveerd.

Ongeveer 200 serotonine bevattende neuronen zijn aanwezig in het cerebrale ganglion complex van de kever, die waarschijnlijk fungeren als interneuronen betrokken bij communicatie binnen de ganglien over korte en lange afstand. Deze neuronen werden onderverdeeld op grond van locatie, aantal, en het verspreidingspatroon van hun uitlopers. Protocerebrale neuronen zijn verantwoordelijk voor links-rechts communicatie in de hersenen en blijken de bron van immunoreactiviteit in het centrale complex en de corpora pedunculata te zijn. Andere neuronen in de optische lobben en het deutocerebrum blijken een belangrijke rol te spelen in de verwerking van respectievelijk visuele en geurinformatie. Dit serotoninerge systeem in de hersenen projecteert niet naar andere delen van het centrale zenuwstelsel en lijkt te fungeren als een zelfstandig serotoninerg neuronaal systeem. Neuronen met een secretoire functie zijn afwezig in de hersenen, noch zijn er aanwijzingen dat serotoninerge neuronen betrokken zijn bij de regulatie van peptiderge secretoire neuronen in het protocerebrum (**Hoofdstuk 2**).

In totaal zijn 74 serotoninerge neuronen aanwezig in het ventrale touwladder-zenuwstelsel, die op grond van hun vorm en plaats werden ingedeeld in vijf neurontypes. Twee gepaarde segmentale "tweeling" interneuronen zijn aanwezig in ieder ganglion of neuromeer. Deze neuronen hebben uitgebreide dendritische vertakkingen in de contralaterale helft van het ganglion en kleine dendritische vertakkingen dicht bij het cellichaam. Hun axonen lopen ipsi- en contralateraal naar meer frontaal en/of caudaal gelegen ganglien. De verspreiding van deze uitlopers duidt op een taakverdeling tussen deze neuronen. De intersegmentale uitlopers duiden op een functie in communicatie tussen de ganglien, terwijl de dendritische uitlopers een functie in de coördinatie van de neurale activiteit in linker en rechterhelft van het ganglion lijken te hebben. Vier grote frontale secretoire neuronen zijn aanwezig in het frontale ganglion. Een paar grote caudale efferente neuronen in het terminale ganglion sturen hun uitlopers in de proctodale zenuwen. De functie van de miniatuur en terminale neuronen is onbekend. Het verspreidingspatroon van serotonine-immunoreactiviteit in het ventrale zenuwstelsel maakt het mogelijk om drie

afzonderlijke functionele eenheden te onderscheiden. De functie van de meest caudale unit is de synaptische controle van darm-innerverende caudale neuronen (**Hoofdstuk 3**).

Grote efferente neuronen in zowel het stomatogastrische als het centrale zenuwstelsel innerveren de darm. Vier neuronen in het frontale ganglion hebben axonen die naar de circulaire en longitudinale spieren van de voordarm projecteren. Het voorste deel van de middendarm wordt niet geinnerveerd door serotoninerge axonen. De longitudinale spieren van de einddarm worden geinnerveerd door de caudale neuronen beschreven in **Hoofdstuk 3**. Studies in het electronenmicroscoop tonen aan dat exocytose van serotonine bevattende blaasjes optreedt op enige afstand van de spiervezels, hetgeen op een neurohormonale afgifte van serotonine aan de spieren duidt. Geen serotonine bevattende synapsen zijn waargenomen op de spiervezels. In een bio-assay werd aangetoond dat toediening van 10^{-8}-10^{-5} M. serotonine een duidelijk inhiberend effect heeft op de spontane contracties van einddarmen in vitro. Dit effect was dosis-afhankelijk (**Hoofdstuk 4**).

Twee sterk vertakte neurohemale systemen voor de afgifte van serotonine aan de hemolymf zijn aanwezig in de kop van de kever. Axonen van vier secretoire neuronen in het suboesophageale ganglion, beschreven in hoofdstuk 3, lopen in de ipsilaterale mandibulaire zenuw en bedekken het oppervlak van deze zenuw met een dicht netwerk van immunoreactieve blaasjes. Twee efferente neuronen in het frontale ganglion hebben axonen die in de frontale connectieven lopen. In de connectieven treden deze axonen naar buiten en bedekken het oppervlak van de labro-frontale, de pharyngeale en de antennale zenuwen en het frontale ganglion met een vergelijkbaar netwerk van immunoreactieve blaasjes. In het electronen-microscoop blijkt dat deze axonblaasjes buiten het ondoordringbare vlies, dat de zenuwen omgeeft, liggen en dus in nauw contact staan met de hemolymf. Naast deze neurohormale afgifte van serotonine, vindt een meer gerichte afgifte van serotonine plaats in de buurt van de spieren van het labrum, de mandibels en de pharynx en de speekselklieren. Dit duidt erop dat deze organen onder controle staan van serotoninerge neuronen (**Hoofdstuk 5**).

De aanwezigheid van serotoninerge zintuigneuronen in sensilla op de labiale en de maxillaire palpen, de galea, het ventrale labrum, de tarsen en de facetogen is onderzocht. Geen afferente serotoninerge neuronen konden worden aangetoond in het perifere zenuwstelsel van de kever (**Hoofdstuk 6**).

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NAWOORD

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Theo

LIJST VAN PUBLICATIES

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CURRICULUM VITAE

Op 28 december 1960 werd ik, Theodorus van Haeften, geboren in Arnhem, als jongste in een gezin van elf kinderen. Na het behalen van mijn MAVO-diploma in 1978 op de Theresia MAVO te Arnhem en vervolgens het HAVO (1980) en VWO (1982) op het Thomas à Kempis College te Arnhem, ben ik in september 1982 begonnen aan de studie Biologie (orientatie organisme) aan de toenmalige Landbouwhogeschool te Wageningen. Doctoraalonderzoeken heb ik verricht bij de vakgroepen Entomologie (bij Dr. H. Schooneveld) en Fytopathologie (bij Dr.ir. L.C. Davidse). Van januari 1987 t/m september 1987 werkte ik in het kader van mijn praktijktijd in het agrobiologisch laboratorium van Duphar B.V. te 's Graveland (bij Dr.ir. A.C. Grosscurt). Na het behalen van mijn doctoraal examen Biologie in 1988 ben ik van april 1988 t/m april 1992 als Assistent in Opleiding werkzaam geweest bij de vakgroep Entomologie, sectie endocrinologie, van de Landbouwuniversiteit, onder begeleiding van Dr. H. Schooneveld. Het onderzoek tijdens deze periode, beschreven in dit proefschrift, omvatte de analyse van transmitter-specifieke communicatiebanen in het zenuwstelsel van de Coloradokever: Non-peptiderge neuronen.

Vanaf 1 januari 1993 ben ik als post-doctoraal onderzoeker, werkzaam bij de vakgroep Anatomie en Embryologie van de Vrije Universiteit te Amsterdam, waar ik onderzoek verricht aan de intrinsieke organisatie van de entorhinale cortex van de rat, een project gefinanciëerd door Human Frontier Science Program.

Correspondentie adres: Tullekenshof 9, 6666 EC Heteren