Dissertation

Neurobiological Study of the Reproductive Organs in Crickets (*Gryllus bimaculatus*)

Kouji Yasuyama

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Neurobiological Study of the Reproductive Organs in Crickets (*Gryllus bimaculatus*)

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CONTENTS

1 General Introduction

In many insects including crickets, the sperm transfer from males to females during copulation is performed by spermatophores, which consist of sperm and secretions of the male accessory gland (Khalifa, 1949; Davey, 1960). The basic process of spermatophore formation in male insects requires the release at a certain period in the mating cycle of both various spermatophore-forming materials from the accessory gland and sperm from the epididymis to the ejaculatory duct according to a temporal motor program (Loher, 1974).

In the cricket Gryllus bimaculatus, a new spermatophore protrudes from the phallic cavity to be exposed to the air several minutes after the termination of each mating, and the secretions and sperm for the formation of such a spermatophore are released within about one minute prior to the occurrence of spermatophore protrusion (Kimura et al. unpublished data). This implies that spermatophore formation occurs so rapidly that it may be controlled directly by motor commands. It is most probable that at the time of release of the accessory gland materials, the contraction of muscles of the accessory gland triggered by neural signals causes the extrusion of materials from the lumen of each gland tubule. In fact, in the male desert locust, thin single muscle layers surround individual tubules of the accessory gland and the axons originating from the terminal abdominal ganglion make synapses onto the muscles (Odhiambo, 1970). Furthermore, distribution of the axons among the muscle layers of the accessory gland has been described in

other insects (e.g., in Leptinotarsa, De Loof and Lagasse, 1972; in Periplaneta, Adiyodi and Adiyodi, 1974). However, there is still little information available about the musculature and innervation of the male accessory gland with regard to the neural control of spermatophore formation.

Recently, Yamaguchi et al. (1985) reported that, in the male cricket, the dorsal unpaired median neurons (DUMR7 neurons) of the terminal abdominal ganglion extend their axons bilaterally through the seventh nerve roots of this ganglion toward the accessory gland. Dorsal unpaired median neurons (DUM neurons) exhibiting this characteristic bilateral morphology have been described in several insect orders (Plotnikova, 1969; Crossman et al. 1971a, b; Bentley, 1973; Hoyle et al. 1974; Clark, 1976; Casaday and Camhi, 1976; Davis, 1977; Davis and Alanis, 1979; Christensen and Carlson, 1981; Lange and Orchard, 1984a; Watson, 1984). Among these DUM neurons, the DUMETi innervating the extensor tibiae muscle of the locust is thought to release a transmitter adjacent to the muscle fibers without forming an anatomically specific neuromuscular junction, although its preterminal fine branches contain numerous large dense-cored vesicles (Hoyle, 1978; Hoyle et al. 1980). This DUM neuron contains octopamine, a biogenic amine, which modulates neuromuscular transmission (Evans and O'Shea, 1977, 1978; Evans and Siegler, 1982). The DUM neurons in the terminal abdominal ganglion of the firefly, on the other hand, have the primary function of direct initiation and regulation of an effector response, the bioluminescent response of the lantern through the release of octopamine, long presumed to

be the lantern neurotransmitter (Christensen and Carlson, 1981, 1982; Christensen et al. 1983). The functional role of DUMR7 neurons in the spermatophore formation of the male cricket, however, has not yet been established.

The first aim of this thesis is to provide a detailed account of the neurons related to the neural control of spermatophore formation in the male cricket. In Chapter 3 of this thesis, the author will describe the gross innervation of the reproductive organs of the male cricket and the fine structures of the morphologically identified neurons, especially of the DUMR7 neurons, and their association with the neural control of these organs. In Chapter 4, evidence obtained using electrophysiological, biochemical, and immunocytochemical techniques will show that the DUMR7 neurons are proctolinergic excitatory motoneurons which induce the neurogenic contraction of the accessory gland by releasing proctolin from their nerve endings.

The sperm discharged into the female by way of a spermatophore is transferred through the spermathecal duct to the spermathecal bulb which is a sperm reservoir. The mechanism of sperm transfer in Gryllidae has been described by Khalifa (1949): the sperm transfer for the spermathecal bulb is attributed to the pressure forcing the sperm out of the spermatophore and the sperm's own motility. In many other insects, in contrast, the migration of sperm takes place as a result of contractions of the female's genital duct (e.g. Davey, 1958). In Rhodnius prolixus, the secretions of the male accessory glands stimulate the rhythmic contractions of the female genital ducts (Davey, 1958). Furthermore, Kimura

and Yamaguchi (1987) have reported that in the female cricket the peristaltic movements of the spermathecal duct occur in response to the injection of the spermatophore contents through the copulatory papilla into the lumen of the spermathecal duct to facilitate sperm migration. This fact implies that the physiologically active substances contained in the spermatophore may penetrate from the lumen of the spermathecal duct to the haemocoel or the vicinity of the muscle fibers winding around the spermathecal duct, which induce the peristaltic movement of the duct.

The fine structures of the spermatheca and spermathecal duct have been described in several orders of insects (Clements and Potter, 1967; Guputa and Smith, 1969; Happ and Happ, 1970; Jones and Fischman, 1970; Lawson, 1970; Conti et al. 1972; Dallai, 1975; Filosi and Perotti, 1975; Happ and Happ, 1975; Huebner, 1980; Ahmed and Gillott, 1982 a,b), but there is still little information available regarding the fine structure of the cricket spermatheca and spermathecal duct.

The second aim of this thesis is to describe the detailed morphology of the spermathecal duct of the female cricket and clarify the passage which permits the physiologically active substances delivered into the lumen of the spermathecal duct to pass from there to the haemocoel. In Chapter 5, the fine structure of the spermathecal duct is described, and the presence of a transverse passage from the haemocoel to the lumen of the spermathecal duct is shown by using ionic lanthanum as a tracer.

2 Materials and Methods

Adult male and female crickets (Gryllus bimaculatus) used in this study were reared under 12 hr light- 12 hr dark at 27°C.

Experimental Procedures for the Study on Male Reproductive Organs

Anatomical and histological techniques

Back-filling: for nickel back-filling the proximal cut end of one of the nerve branches (Br3s) emerging from the right and left seventh nerve roots of the terminal abdominal ganglion was plunged into a thin capillary filled with 1 M $NiCl_2$ for 12-14 hr at 4°C. Then, nickel was precipitated within the neurons by addition of rubeanic acid to the saline (Levine and Murphey, 1980) in which the preparation was immersed. After fixation with 10% formalin, the preparation was dehydrated and cleared for whole-mount viewing. Usually, the preparation stained with nickel was intensified following principally the method of Bacon and Altman (1977).

For horseradish peroxidase (HRP) back-filling the proximal cut end of Br3 was plunged into a thin capillary filled with 20% HRP for 24 hr at 10°C, and then the terminal abdominal ganglion with the reproductive organs was fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 2 hr, followed by washing in the buffer. The enzyme reaction was performed with three drops of 3% H_2O_2 in 1% 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution for 15 min (Nässel,

1983). After washing in the buffer, the preparation was fixed with 2% OsO_4 in the buffer for 2 hr, then dehydrated through an ethanol series and embedded in epoxy resin. Sections, 10 μm thick, were photographed, then reembedded and cut for electron microscopy.

Electron microscopy: for the scanning electron microscopic study the abdomen of which the reproductive organs were exposed, was detached from the body and immersed in an ice-cold solution of 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 12 hr. It was washed and trimmed in the same buffer to uncover the nerve branches running from the terminal abdominal ganglion to the reproductive organs. This preparation was post-fixed with 1% OsO_4 buffered in the same buffer for 3 hr, and dehydrated through an ethanol series, then dried in a CO_2 critical-point dryer. After coating with platinum palladium, the preparation was examined in an Hitachi S 570 electron microscope.

For the transmission electron microscopic study the reproductive organs were isolated carefully with the terminal abdominal ganglion from the body. This preparation was fixed with an ice-cold 2.5% solution of glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 6 hr without cutting into small pieces. The preparation was washed with the same buffer and postfixed in buffered 1% OsO_4 for 2 hr. After dehydration in an ethanol series, the preparation was embedded in epoxy resin. Semiserial thick (10 μm) sections were cut and mounted on glass slides. After examination with light microscope to reconstruct the nerves innervating the reproductive organs, appropriate sections were reembedded

and thin sections were cut for electron microscopy. In some cases, the preparation was cut into small pieces after fixation with buffered same fixative for 30 min, and fixed in renewed fixative for 2 hr. These pieces were post-fixed and embedded as described above. All thin sections were stained with uranyl acetate and lead citrate and examined in an Hitachi H 500 electron microscope.

Physiological techniques

The isolated accessory gland preparation was obtained from the male cricket: after the dorsal cuticle and gut were removed without damaging the terminal abdominal ganglion, from which emerge the nerves innervating the reproductive organs; the accessory gland was isolated with or without the terminal abdominal ganglion in a perfusing chamber. This preparation remained viable for several hours as long as it was perfused or frequently flushed over with saline (in mM/l: NaCl, 150; KCl, 9; $CaCl_2$, 5; dextrose, 40; buffered with 10 mM/l Tris buffer to pH 7.2).

Mechanical movements of an intact or isolated accessory gland preparation were recorded with a strain gauge (Toyo-balbwin T7-8-240) which was set to press the preparation gently with a thin plastic rod, and these were displayed on a pen-recorder (Riken Denshi, PH-S5V). For electrical stimulation of the nerve branch (Br3) through which DUMR7 neurons pass, a pair of stimulating electrodes made of thin tungsten wires (50 μm in diameter) was used. The part which the Br3 made contact with the electrodes was covered locally with a small amount of vaseline for preventing desiccation and improving insulation.

Drug application: effects of the following drugs on the accessory gland were examined at several concentrations; dopamine, norepinephrine, epinephrine, octopamine, serotonin, acetylcholine, GABA, glutamate, proctolin, FMRFamide, cyclic AMP and cyclic GMP. Bath-applied drugs were perfused through the experimental chamber at a rate of 1 ml/min. Drugs were prepared daily as 10^{-2} M stock solutions from which serial dilutions were made as needed. Bath temperature was maintained between 24 and 27°C. High Mg^{2+} saline was used in order to block chemical synaptic transmission within the terminal abdominal ganglion: final concentration of Mg^{2+}/0 Ca^{2+} saline was 15 mM, and the concentration of Na^{+} was reduced to 123 mM to compensate for the increased osmolarity due to high Mg^{2+}. In this case, only the ganglion was in high Mg^{2+} saline and the accessory gland was in normal saline. When using high K^{+} saline to induce a sustained contraction of the accessory gland, the concentration of K^{+} was increased to 90 mM and that of Na^{+} was reduced to 70 mM.

Transplantation procedures

In some experiments, the anlage of the accessory gland in 6th or 7th instar was transplanted into a last instar. Prior to the transplantation, the anlage to be transplanted was dissected out in saline from a donor, picked up with a sterilized pincette and inserted into the body cavity of low temperature (4°C)- anesthetized host through an incision (1.2 mm in length) made in the 5th-7th intersegmental membranes on the dorsal side of the abdomen. The wound was closed with vaseline after inserting a few crystals of penicillin-G. The operated animal was kept in a Petri-dish

at room temperature and fed as usual. The transplanted accessory gland, that developed fully in the host after eclosion, was used as a preparation.

High-performance liquid chromatography (HPLC) and bioassay

The isolated accessory glands were homogenized in methanol-water-acetic acid (90:9:1, by vol). After centrifugation (12,500 g, 30 min) at 4°C, the supernatant was dried in a rotary vacuum evaporator (30°C) and resuspended in 0.1% TFA (trifluoroacetic acid). The solution was applied to a C18 Sep-Pak cartridge that had been washed with 10 ml of 50% acetonitrile, 0.1% TFA solution and 10 ml of 0.1% TFA solution, then with 10 ml of 0.1% TFA solution and 10 ml of 25% acetonitrile. The bioactive substance inducing the contraction of the accessory gland was eluted only in the 25% acetonitrile solution. Then the solution containing the bioactive substance was evaporated to resuspended the substance in 1 ml of 5% acetonitrile + 0.1% TFA solution for HPLC analysis.

HPLC separations were performed on a C18 reverse phase column (ODS-bondazol). The column was equilibrated with 15% acetonitrile, and the samples were injected in 200 μl of this solvent. The flow rate was 1 ml/min and fractioned every minute over 30 min. Each of the collected fractions was evaporated and resuspended in 1 ml of saline and then, applied to an accessory gland to determine its bioactivity. The retention time of standard proctolin was determined by detecting the absorbance of synthesized proctolin (10 μg /ml) at 228 nm. In each experiment it was about 21 min in this system.

To examine the release of the bioactive substance from the accessory gland, the bioactivity of the surrounding medium of accessory glands was detected by the following procedure. First, 80 accessory glands isolated from adult males were washed with 100 ml of saline for 15 min, followed by immersing in 60 ml of normal or high K^+ saline for 15 min. After filtering the surrounding medium through Toyo No. 2 filter, the filtrate was collected in a beaker chilled with ice cubes. Then, the filtrate was concentrated using a C18 Sep-Pak cartridge, and the bioactive substance containing in the filtrate was examined by HPLC and bioassay.

Immunocytochemical techniques

Antiserum preparation and testing: rabbit anti-proctolin antisera were obtained from Seikagaku Kogyo Co.(Osaka, Japan). The rabbits were immunized with conjugates of synthetic proctolin and bovine serum albumin (BSA) or bovine thyroglobulin, linked together with glutaraldehyde (Bishop et al. 1981; O'Shea and Bishop, 1982; Eckert and Ude, 1983). Proctolin antiserum was purified by affinity chromatography according to Eckert and Ude (1983). The purified anti-proctolin-BSA serum was mainly used in the light microscopic immunocytochemistry, and in electron microscopic study, the purified anti-proctolin-thyroglobulin serum was used. Controls were performed as follows: the purified proctolin antiserum was preincubated with synthetic proctolin at a concentration of 0.5 mg/ diluted antiserum for 16 hr, and processed for light and electron microscopy. Tests were also performed in which the primary antiserum was excluded. All immunoreactivity was eliminated by such treatment.

Light-microscopic immunocytochemistry: each of paired Br3s was tied by a single knot using a hair. The ganglia were isolated together with ligated nerve branches after 6 hr of ligation, and fixed. Ligation and dissections were undertaken in buffered cricket saline described above. The tissues were fixed in a glutaraldehyde-picric acid mixture containing 2.5% glutaraldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.3) for 15 hr at 4°C (Newman et al. 1983). After washing for 4 hr in the buffer, the tissues were dehydrated, embedded in paraffin, sectioned at 10 μm, and mounted on gelatin-coated slides. Immunoreactivity was visualized with the Vectastain avidin-biotin-peroxidase complex (ABC) method (Vector Lab.) After deparaffinization and rehydration, the sections were rinsed in a 0.01M phosphate-buffered saline (PBS, pH 7.4) for 10 min, incubated in methanol (100%) with 0.3% H_2O_2 for 30 min to block endogeneous peroxidase activity, and rinsed again in PBS for 30 min. The sections were then incubated with normal goat serum diluted 1:50 in PBS for 30 min, and were incubated with primary antiserum for 48 hr at 4°C. Proctolin antiserum used was diluted 1:200 - 1:800 with PBS containing 0.5% BSA and 0.01% Triton X-100. The sections were washed three times in PBS for 5 min each, incubated with biotinylated goat anti-rabbit IgG diluted with PBS (1:200) at room temperature for 40 min, and washed three times in PBS for 5 min each. Thereafter, the sections were incubated with PBS-diluted ABC reagent (1:100) for 1-2 hr, washed three times in PBS for 5 min each, and were incubated for 5-30 min with a solution containing 0.05% DAB and 0.01% H_2O_2 in 0.05 M Tris buffer

(pH 7.2). Afterward, the sections were washed in distilled water for 15 min, dehydrated, and were mounted.

Electron-microscopic immunocytochemistry combined with HRP back-filling: one of paired Br3 was tied by a single knot using a hair, and the proximal cut end of the other Br3 was plunged in to a thin capillary filled with 5% HRP for 24 hr at 4°C, and then the terminal abdominal ganglion with Br3s was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 3 hr, followed by washing overnight in the buffer. The procedures for enzyme reaction using DAB were performed according Nässel (1983). After the enzymatic reaction, the tissues were washed in the buffer for 5 hr, dehydrated and embedded in epoxy resin. No osmium postfixation was carried out. For electron-microscopic immunocytochemistry without HRP back-filling, the accessory glands, or the terminal abdominal ganglia with ligated Br3s for 6 hr, were fixed with the same fixative for 3 hr after dissection, washed in the buffer, dehydrated, and embedded in the same resin. The postembedding protein A-colloidal gold method (Roth, 1983) was employed as follows: ultrathin sections were collected on formvar-carbon coated nickel grids and soaked for 30 min on drops of PBS with 1% BSA. The proctolin antiserum was then applied at 1:200 - 1:1000 (in PBS with 1% BSA) for 24 hr at 4°C. Afterward, the grids were washed in PBS furiously agitated by a magnetic stirrer for 30 min. The protein A-gold complex (Janssen Pharmaceutica; particle size 10 or 15 nm) was applied at a concentration of 1:40 (in PBS with 1% BSA) for 1 hr at room temperature. After washing for 40 min, the sections were stained in uranyl acetate and lead

citrate. For the sections of HRP labeled tissues, lead citrate staining was eliminated. The sections were examined with a Hitachi H 500 electron microscope.

Experimental Procedures for the Study on Female Reproductive Organs

Virgin and mated female crickets were used for this experiment.

Histological techniques

Light microscopy: the spermathecal ducts with spermathecae were fixed in Bouin's solution, dehydrate through an ethanol series and embedded in paraffin. Serial sections were made and stained by the Azan staining methods.

Electron microscopy: the spermathecal ducts were fixed with ice-cold 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 2-4 hr, washed in the same buffer for 2 hr, and post-fixed with ice-cold 1% osmium tetroxide in the same buffer for 2 hr. These specimens were dehydrated through an ethanol series and embedded in epoxy resin. The sections were doubly stained with uranyl acetate and lead citrate solution and examined with an Hitachi H-500 electron microscope.

For scanning electron microscopy, the specimens were dried in a CO_2 critical-point dryer after fixation and dehydration as for transmission electron microscopy, coated with gold-palladium, and examined with an Hitachi S-570 electron microscope.

Ultrastructural tracer experiment

The spermathecal duct was isolated together with the copulatory papilla and spermatheca. The tissue was incubated in solutions of 5, 10, 20, 50 mM ionic lanthanum (made from $LaCl_3$) dissolved in physiological saline at room temperature for 2 hr. The tip of the copulatory papilla was kept in the air and its opening was closed with vaseline during the course of the incubation period, so that only the external muscular wall of the spermathecal duct was exposed to the tracer. Following this, the preparation was fixed in phosphate-buffered (0.1 M phosphate buffer, pH 7.2) 2.5% glutaraldehyde to precipitate the lanthanum. The preparation was then washed in several changes of the buffer, and treated with 1% osmium tetroxide in the same buffer for 1 hr. After dehydration in an ethanol series, the preparation was embedded in epoxy resin. Unstained thin section were examined by in a Hitachi H-500 electron microscope, and the appropriate sections were analyzed in a Hitachi HU-12 electron microscope equipped with an X-ray analytical unit (EDAX 711A) to verify the presence of lanthanum. An accelerating voltage of 25 kV was used with a specimen current of 1.2×10^{-12}A. The electron beam was focused at a 10 nm spot on the selected area of the section, and the spectrum was collected for 100 sec. Counts were collected on 800 channels by 20 eV/channel with a full scale height of 100 counts.

3 Musculature and Innervation of the Reproductive Organs in the Male Cricket

It is well known that the accessory gland of male cricket provides the ingredients necessary for the formation of the spermatophore (e.g. Khalifa, 1949; Davey, 1960). However, there is rather as yet very little information about not only the musculature and innervation of the accessory gland relating to the neural control of spermatophore formation, but also those of other internal reproductive organs. In this section, the author will describe the gross innervation of the male reproductive organs, and the morphology of the neural components associated with it, with special reference to the projection of the DUMR7 neurons.

3-1 Gross Innervation of the Reproductive Organs

The accessory gland of male cricket comprises more than 600 slender tubules, and each gland tubule opens into the lobed anterior part of ejaculatory duct. A pair of epididymides, which are the compactly coiled parts of vasa deferentia, also open into there. These reproductive organs are located in the mass just over the terminal abdominal ganglion (Fig. 1A). Each tubule of the accessory gland is surrounded by a thin muscle layer which consists of striated muscle fibers arranged in a circular pattern around the longer axis of tubule (Fig. 2A). This muscular layer is only one myofibril thick for most of the length of the tubule, but it becomes multifibrillar toward the junction with the

anterior end of ejaculatory duct (Fig. 2B). Epididymides also are wound around by multilayered muscle fibers. The anterior part of ejaculatory duct is enveloped by a thick muscular layer, but the remaining parts by an irregular network of muscle fibers.

Accessory gland, epididymides and the anterior part of ejaculatory duct are innervated by paired nerve branches (Br3s) emerging from the left and right seventh nerve roots of the terminal abdominal ganglion (Fig. 1B). Each of the seventh nerve roots arises closely to the eighth nerve root (cercal nerve) from the postero-lateral margin of the terminal abdominal ganglion, and soon after running under the eighth nerve root toward the posterior, it bifurcates: one branch (Br1) innervates the cercal muscles, and the other (Br2) divides further into two branches (Br3 and Br4). The Br3 runs into the mass of the reproductive organs lying over the terminal abdominal ganglion, and is joined with the contralateral Br3, so that both Br3s form a loop close to where paired epididymides open to the anterior end of ejaculatory duct (Fig. 1C). Two thick nerve twigs spreading many thin twiglets arise from the region corresponding to the junction of both Br3s in the loop, and they intrude into the muscle layers of the reproductive organs (Fig. 1D). The Br4 reaches the muscular wall of spermatophore sac.

Nickel back-filling through unilateral Br3 revealed one lateral and three (first-third) median clusters of somata in the posterior half of the terminal abdominal ganglion (Fig. 3). The lateral cluster lies ipsilateral to the back-filled nerve branch around the site where the seventh nerve root

leaves the terminal abdominal ganglion, and consists of 13 somata with the diameters ranging from 6 to 35 μm. The axons of the neurons having their somata in this cluster reach the mass of reproductive organs. This type of neurons is called "LC neurons". The first and second median clusters are located near the dorsal midline of the terminal abdominal ganglion and the third median cluster occupies rather a large area near the caudal midline (Fig. 3B). The position of these median clusters correspond to the embryonic segment 8-11, respectively (Panov, 1964). The features of soma position and profile of neurites show that the neurons having their somata in the median clusters are identical with DUMR7 neurons as described by Yamaguchi et al. (1985). The closer the median cluster is to the posterior end of the terminal abdominal ganglion, the larger the total number of somata is in the median cluster. This number, however, varies in different preparations: 4 to 7 in the first median cluster, 26 to 43 in the second median cluster and 69 to 89 in the third median cluster. The diameters of somata range from to 17 to 42 μm in the first median cluster, from 8 to 46 μm in the second median cluster and from 4 to 46 μm in the third median cluster.

On the other hand, nickel back-filling through unilateral Br4 showed the presence of a neuron having its soma (45 μm in diameter) which is located in seclusion near the third median cluster (Fig. 3B). The pathway of this axon is rather complex: after leaving the terminal abdominal ganglion the axon runs through the Br3 and loop, passes the contralateral Br3 and Br4, and then, terminates at one of

the retractor muscles of the spermatophore sac. This neuron is called "SR neuron". It is probable that the retractor muscle is homologous to the muscle 117 identified by DuPorte (1920). That is, it originates from the anterior end of the ventral valve at its junction with the ductus, runs obliquely round the sac and is inserted into the lateral side of the dorsal valve at the posterior end where the chitinous plates are given off.

3-2 Projection of the Dorsal Unpaired Median Neurons into the Reproductive Organs

HRP applied to a severed Br3 was transported to the contralateral axons of DUMR7 neurons beyond their somata within the terminal abdominal ganglion. Figure 4 shows a HRP-filled soma with its primary neurite in the second cluster and the labeled axons running through the contralateral Br3 and projecting via the site, where both Br3s are joined by their appearances, into the accessory gland.

In the somata of labeled DUMR7 neurons there were many mitochondria, ribosomes and Golgi bodies. Large granular vesicles (100-150 nm in diameter) were present associated with Golgi body (Fig. 5A). Similar vesicles could be found even in the primary and secondary neurites within the terminal abdominal ganglion (Fig. 5B,C), and also in the peripheral axons projecting into the mass of reproductive organs (Fig. 5D). These large granular vesicles seem to be produced in the somata and transported from there to the periphery for local storage and release. To confirm this presumption,

short-term ligature experiments of Br3 was performed.

One of paired Br3s was tied by double knots using a silk thread in the neighborhood of the loop of Br3s. The other Br3 was not tied as a control. The preparation was fixed after 6 hr of ligation, and then it was prepared for electron microscopy. In the control Br3, the distribution of large granular vesicles in the axons remained unchanged (Fig. 6A), whereas in the ligated Br3 numerous large granular vesicles accumulated in the cytoplasm of axons proximal to the ligature (Fig. 6B). A cross section of Br3 which was made from an intact animal showed about 260 transverse profiles of axons with the diameters ranging from 0.2 to 3.2 μm. Accumulation of the large granular vesicles in ligated axons of Br3 was seen in more than 20 axons with relatively large diameters. It is, therefore, probable that DUMR7 neurons produce the large granular vesicles in their somata and transport them bilaterally to the periphery.

3-3 Fine Structure of Axon Terminals on the Muscle Fibers of the Reproductive Organs

The axons containing large granular vesicles are widely distributed along the muscle fibers associated with accessory gland (Figs. 8,9), epididymides (Fig. 11) and anterior part of ejaculate duct (Fig. 10), and make neuromuscular junctions with them. Some axons which have large granular vesicles are ensheathed by the glial cells and sent from Br3 to the muscular layer (Fig. 7A). Small clear vesicles (40-60 nm) besides large granular vesicles appear in the cytoplasm

of axons near the neuromuscular junctions (Fig. 7B). At the axon terminal, the glial cells are absent partially, and the axon makes synaptic contact with the muscle fiber (Fig. 8). The neuromuscular junction is distinguished both by dense postsynaptic membrane and by a cluster of small clear vesicles at the presynaptic membrane. The aggregation of large granular vesicles is never found in the axon terminals. This type of neuromuscular junction is observed in every muscle layers of accessory gland, epididymides (Fig. 11) and anterior part of ejaculatory duct (Fig. 10).

The region of the muscle fiber making synaptic junction with the axon is in the form of extension from the main body of muscle fiber. The extension often contains granular sarcoplasm and scare contractile filaments (Fig. 8). These are prominent feature at the multifibrillar layers enveloping the proximal part of accessory gland tubules where they open to the anterior end of ejaculatory duct (Fig. 9). In this region, the extension of muscle fiber forms a complex series of folds about the axon terminal.

Unfortunately I could not succeed in transporting HRP as far as the axon terminals under my experimental procedure. These axon terminals, however, may be identical with those of DUMR7 neurons, because large granular vesicles were present in the axons of HRP labeled DUMR7 neurons (Fig. 5D) as well as in their somata (Fig. 5A). The axons which do not bear large granular vesicles are also distributed to the musculature of the massed reproductive organs. The identified LC neurons may contribute to these axons.

3-4 Discussion

Nickel back-filling of Br3 revealed the presence of 13 bilaterally paired LC neurons and 100 or more dorsal unpaired median neurons (DUMR7 neurons)(Fig. 3). All these neurons project into the muscle fibers of the internal reproductive organs, accessory gland, epididymides and ejaculatory duct (Fig. 8-11). In addition, the nickel backfilling of Br4 showed the presence of SR neuron innervating the contralateral retractor muscle of spermatophore sac.

The ultrastructural investigation revealed that there are two types of muscles in the accessory gland as illustrated in Fig. 12B, i.e. single-fibrillar thin muscle (MF-1) winds around most of each glandular tubule's length, and multifibrillar thick muscle (MF-2) surrounds the opening of each tubule in the anterior region of the ejaculatory duct. These muscles are innervated by LC neurons and DUMR7 neurons whose axons run down through the nerve branches (Br3s) emerging from the seventh nerve roots of the terminal abdominal ganglion. (Fig. 12A). There is, therefore, the possibility that the antagonistic excitation of MF-1 and MF-2 mediated by both excitatory and inhibitory motoneurons results in squeezing the spermatophore-forming materials from the glandular tubules (Fig. 12B), though the question which of two types of neurons are excitatory or inhibitory, remains unsolved.

Odhiambo (1970) described that the individual tubule of accessory gland of the male desert locust has highly developed muscular layer with innervation, and also that

innervating axons have synaptic vesicles at their junctional regions. In his observations, however, no large secretory vesicles were found in any of the axons attached to the locust accessory gland. In the present experiment it was found that the axons containing large granular vesicles widely distributed along the muscle fibers of the reproductive organs (Fig. 7), and they make neuromuscular junctions with the muscle fibers, where large granular vesicles coexist with small clear vesicles clustering especially at the presynaptic membranes (Fig. 8). Probably the terminal of these axons seems to be identical with those of the axons of DUMR7 neurons, since vesicles similar to the large granular vesicles in appearance are present in the peripheral axons of HRP-labeled DUMR7 neurons as well as in their somata (Fig. 5A,D) and the ligation of a Br3 carrying the axons of DUMR7 neurons reveals remarkable accumulation of the same type of vesicles as the large granular vesicles in the cytoplasm of axons proximal to the ligature (Fig. 6B). Further, these facts suggest that DUMR7 neurons produce large granular vesicles in their somata and transport them bilaterally to the periphery.

On the other hand, the electron microscopic observation showed also that there exist the axons containing only small clear vesicles on the muscle fibers of reproductive organs. These axons may be derived from LC neurons. It is probable that the muscle fibers of reproductive organs of male cricket are innervated by at least two types of neurons, i.e., DUMR7 and LC neurons originating from the terminal abdominal ganglion.

Dorsal unpaired median neurons (DUM neurons) exhibiting the characteristic bilateral morphology have been described in several insect species (e.g. Crossman et al. 1971a, b; Bentley, 1973; Hoyle et al. 1974; Clark, 1976; Casaday and Camhi, 1976; Davis, 1977; Christensen and Carlson, 1981; Lange and Orchard, 1984a; Watson, 1984), since first description of them in locust by Plotnikova (1969). Among these DUM neurons, the DUMETi innervating to the extensor tibiae muscle of locust is thought to release transmitter adjacent to the muscle fibers without forming an anatomically specific neuromuscular junction, although its preterminal fine branches contain numerous large dense-cored vesicles (Hoyle, 1978; Hoyle et al. 1980) and it contains the biogenic amine, octopamine to modulate the neuromuscular transmission (Evans and O'Shea, 1977,1978; Evans and Siegler, 1982). In contrast, the DUM neurons in the terminal abdominal ganglion of firefly have the primary function of direct initiation and regulation of an effector response, bioluminescent response of the lantern through the release of octopamine which serves a more direct role as a neurotransmitter than that postulated for its modulatory and hormonal functions in other arthropod system (Christensen and Carlson, 1981,1982; Christensen et al. 1983).

In this experiment, it was evident that there coexist two types of vesicles, large granular vesicles and small clear vesicles in the axons which are presumed to be those of DUMR7 neurons: at the axon terminals forming the direct synaptic junctions on the muscle fibers, small clear vesicles are distributed throughout the profile and aggregate at

the presynaptic membrane, but large granular vesicles do not aggregate near the presynaptic membrane. This fact suggests the possibility of dual functions of DUMR7 neurons serving the control of movements of reproductive organs which are involved in the spermatophore formation. Agricola et al. (1985) reported that the terminal abdominal ganglion of cockroach contains numerous proctolinergic fibers whose terminals contain large dense granules (140 to 150 nm) and numerous clear vesicles (40 to 50 nm), and proctolin immunoreactivity is present in the large dense granules. This fact suggests the possibility that the large granular vesicles in the DUMR7 neurons may contain proctolin.

3-5 Abstract

1) In the male cricket, nickel back-filling of the nerve branches (Br3s) emerging from the seventh nerve root of the terminal abdominal ganglion reveals the presence of two types of neurons running down to the internal reproductive organs (accessory gland, epididymis and ejaculatory duct).

2) The first type is the neurons (LC neurons) which have the somata forming cluster in the postero-lateral region of the terminal abdominal ganglion, and 13 somata are present in the cluster.

3) The second types is the dorsal unpaired median neurons (DUMR7 neurons), of which the somata form three clusters along the midline of the posterior half of the terminal abdominal ganglion and the bifurcating axons extend symmetrically to the left and right seventh nerve roots. Total

number of somata in these clusters is 100 or more.

4) The DUMR7 neurons probably terminate on the muscle fibers of the reproductive organs. This is based on the following evidence. First, the axon terminals, which contain both small clear vesicles (40-60 nm) and large granular vesicles (100-150 nm), are found in the musculature of the reproductive organs. Second, HRP back-filled DUMR7 neurons exhibit that large granular vesicles are present in their somata as well as in their axons running to the reproductive organs. Third, the ligation of either Br3 results in the remarkable accumulation of large granular vesicles in the cytoplasm of axon proximal to the ligature.

4 Proctolinergic Innervation of the Accessory Gland in the Male Cricket

In the previous chapter, the author described the morphology of two types of neurons (DUMR7 and LC neurons) which innervate the accessory gland, epididymis, and ejaculatory duct, and suggested the possibility that DUMR7 neurons are motoneurons directly terminating on the muscle fibers of the reproductive organs on the basis of morphological characteristics. In this section, the author will demonstrate (1) the control of mechanical activity of the male accessory gland by DUMR7 neurons, (2) the effects of various transmitters, and neuromodulators on the mechanical activity of the accessory gland, (3) the release of proctolin by the electrical stimulation of DUMR7 neurons, and (4) the presence of proctolin-like immunoreactivity in the DUMR7 neurons.

4-1 Myogenically- and Neurally-evoked Contractions of the Accessory Gland

Myogenically-evoked contractions of the accessory gland

Mechanical movements of the isolated accessory gland preparation were recorded with a strain gauge which was set to pass through the cluster of glandular tubules, pressing gently on the anterior part of the ejaculatory duct. The cluster of tubules, however, were allowed to move freely in the bath. This arrangement permitted us to record mainly the movements evoked by the muscles, MF-2s which are embedded in the anterior part of the ejaculatory duct (Fig.12B). The

movements of individual glandular tubules evoked by the muscle, MF-1, winding around the tubule were observed directly under a binocular microscope.

Usually, rhythmic contractions of small amplitude occurred spontaneously in an isolated accessory gland preparation with the terminal abdominal ganglion once it was suspended in saline (Fig. 13). The frequency of rhythmic contractions varied from 5 to 10/min according to the level of activity of the preparation. Even when the neural connections between the accessory gland and terminal abdominal ganglion were severed, the basic pattern of contractions and the wide variation in rhythmicity of contractions remained unchanged, except for a decrease in the basal tonus of the preparation.

Individual intact gland tubules also showed spontaneously rhythmic contractions with a frequency ranging from 4 to 7/min, and the principal pattern of contractions was a slow undulatory motion which presumably reflected shortening of the obliquely winding muscle, MF-1. When a glandular tubule was severed from its base, an annular constriction occurred in a short time (less than 1 s) just distal to the severed surface (Fig. 22). This constriction served as a stopper for preventing the leak of secretion from the lumen of the glandular tubule. Spontaneous contractions of the glandular tubule were, however, seldom evident after the tubule was completely isolated from the main body of the accessory gland.

In order to determine whether the origin of the spontaneously rhythmic contractions was neurogenic or myogenic,

the anlage of an accessory gland of a 6th or 7th instar was transplanted to the abdomen of a last instar (8th instar). As already described in Plebeiogryllus guttiventris by Ranganathan (1977), the transplanted anlage developed tubules in synchrony with that of the host, irrespective of the sex of the host. Although the tubules of the transplanted accessory gland were short, stumpy and few in most cases, close observation of their structure revealed that the gland cells possessed endoplasmic reticula and Golgi bodies, and the muscular layer surrounding each tubule developed as well as those of a normal accessory gland. No axons were seen near the muscular layer in any transplanted accessory gland (Yasuyama et al. 1984). However, in the absence of any evidence of intrinsic neural control the transplanted accessory gland showed spontaneously rhythmic contractions (Fig. 21), and their frequency was much the same as the those measured in the normal accessory gland. It therefore may be said that the spontaneous contractions recorded form the accessory gland with or without innervation are myogenic.

Neurally-evoked contractions of the accessory gland

As shown in the schematic illustration of Fig. 13A, after severing one of the nerve branches, Br3s of the seventh nerve roots at its distal region, the electrical stimulation applied antidromically to the proximal region of the severed Br3 elicited a strong sustained contraction of the accessory gland. The amplitude of contraction increased in proportion to the intensity and frequency of stimulation. Electrical stimulation applied orthodromically to the intact

Br3 induced a strong contraction which was followed by a lowering of basal tonus and suppression of myogenically evoked contractions for a short time: this time was roughly proportional to the intensity, frequency and duration of stimulation (Fig. 13B). The severance of both Br3s resulted in the decrease of basal tonus (not shown in Fig. 13). Electrical stimulation of one of the Br3s which were disconnected from the terminal abdominal ganglion elicited a strong sustained contraction showing the same type of characteristic as shown in Fig. 13B (Fig. 13C). It was often observed that when the electrical stimuli were antidromically applied to the Br3, secretory granules were released from the accessory gland through the wounds which were inflicted accidentally. These facts imply a direct link between nervous excitation and mechanical activity of the accessory gland through the DUMR7 neurons, and further, these suggest the presence of the unilateral motoneurons, which suppress the mechanical activity, in either Br3.

On the other hand, even when the accessory gland with the terminal abdominal ganglion was exposed to Ca^{2+} free, high Mg^{2+} saline for more than 60 min, the myogenically evoked contractions of the accessory gland apparently remained unchanged and the accessory gland was responsive to electrical stimulation through either Br3, though the amplitude of such neurally evoked contractions became slightly smaller within 5 min after the exposure (Fig. 14). This result suggests that the DUMR7 neurons having the characteristic bilateral morphology may provide a direct pathway for antidromic conduction through the terminal abdominal gan-

glion.

4-2 Pharmacological Properties of the Accessory Gland

Application of proctolin at threshold concentrations of 10^{-11} to 10^{-10} M produced an increase in basal tonus of the accessory gland as revealed by an elevation of the baseline. At 10^{-8} M and above this increase of basal tonus was accompanied by a slight reduction in the amplitude of neurally evoked contractions (Fig. 15). This amount of proctolin generally reduced the frequency of myogenic contractions of the accessory gland during the increase in its basal tonus, and in some preparations inhibited completely the myogenic contractions. In the absence of electrical stimulation the application of proctolin produced a strong contraction (Fig. 16). All of these proctolin-induced effects were dose-dependent, with a threshold concentration between 10^{-11} M to 10^{-10} M and maximal effects at 10^{-6} M (Fig. 17), and these were completely reversible following washing in saline.

Glutamate-induced effects were basically similar to proctolin-induced ones. That is, at concentrations of 10^{-6} M and higher glutamate reversibly reduced the amplitude of neurally evoked contractions of the accessory gland and also resulted in an increase of basal tonus (Fig. 15). Most of the neurally evoked contractions were completely inhibited at the high concentration of 10^{-2} M. In the absence of neural stimulation the application of glutamate induced a contraction of the accessory gland (Fig. 16). During glutamate-induced contractions the frequency of myogenic contrac-

tions was strongly reduced. All of these glutamate-induced effects were dose-dependent (Fig. 17), and reversed by washing with saline.

Octopamine produced little or no effect on the neurally evoked contractions at concentrations up to 10^{-4} M (Fig. 18). Application of octopamine at 10^{-7} M and above increased the frequency of myogenic contractions in a dose-dependent manner (Figs. 19 and 20). At concentrations of 10^{-7} M and above serotonin reversibly reduced the amplitude of neurally evoked contractions and also resulted in a relaxation of the accessory gland as revealed by a lowering of baseline (Fig. 18). Both of these effects were reversed by washing with saline. However, it took 10 min or more after washing the preparation to recover the initial amplitude of neurally evoked contractions and the initial basal tonus. In the absence of electrical stimulation the application of serotonin resulted not only in the suppression of myogenic contractions, but also in the reduction of basal tonus (Fig. 19). These effects were dose-dependent, with the threshold serotonin concentration at 10^{-7} M and maximal effect at 10^{-4} M (Fig. 20). Furthermore, proctolin- and glutamate-produced contractions were suppressed by the application of serotonin: the suppressive action of serotonin was competitive with proctolin and glutamate, and the presence of serotonin shifted the dose-response curve for proctolin or glutamate to the right without significant change in slope (not shown).

Dopamine, at 10^{-5} M, produced a small increase in the frequency of myogenic contractions of the accessory gland,

but there was no effect upon the neurally evoked contractions. However, epinephrine, norepinephrine, acetylcholine, GABA, FMRFamide, cyclic AMP and cyclic GMP produced no observable effects upon the mechanical activity of the accessory gland up to 10^{-4} M.

The effects of drugs on the transplanted accessory gland, which developed muscles lacking any innervation by host neurons, were examined. Both proctolin at 10^{-8} M and glutamate at 10^{-4} M induced contraction of the transplanted accessory gland (Fig. 21). However, the proctolin- and glutamate-produced contractions differed in that the former accompanied a suppression of myogenic contractions which was followed by a transient increase in the frequency of myogenic contractions during relaxation, where the latter accompanied the opposite change in myogenic contractions. Application of serotonin at 10^{-5} M always resulted both in a lowering of the basal tonus and in a suppression of myogenic contractions (not shown), but octopamine at concentrations up to 10^{-4} M had no effect on the transplanted accessory gland in most cases.

Figure 22 shows that an isolated glandular tubule became long and slender when it was treated with proctolin at 10^{-7} M, and such change in the configuration was accompanied by squeezing the secretory granules from the lumen through the cut end. Similar changes in configuration were observable also when the tubule was treated with glutamate at 10^{-4} M. However, as far as I have observed the isolated glandular tubules under the binocular microscope, they were relatively insensitive to octopamine and serotonin, even when the

concentration of these drugs was 10^{-4} M or more. These results suggests that the muscle, MF-1, winding around the tubule is sensitive, at least, to both proctolin and glutamate, and its contraction is responsible for squeezing the secretory granules from the lumen.

4-3 Chromatographic Evidence of Proctolin in the Accessory Gland

Homogenized extracts of isolated accessory glands with reverse-phase HPLC were analyzed to compare the tissue-derived proctolin with authentic proctolin. The extracts were purified with C18 Sep-Pak cartridges and injected into a reverse-phase column, and the solvent elutes were collected every minute. Fractions from HPLC were bioassayed for proctolin-like bioactivity on the accessory gland, with a threshold detectability of 0.002 pmol/accessory gland. The lower histogram in Fig. 23 obtained from the bioassays shows an elution pattern of a substance which is able to induce the contraction of accessory gland. In these experiments, the elution patterns always had only a single peak relating to that of authentic proctolin, as shown in the upper graph of Fig. 23. These facts are strong evidence for the presence of proctolin in the accessory gland. It was calculated by reading directly form the dose-response curve that the proctolin activity of an accessory gland is equivalent to about 3 pmol/animal (n=4 HPLC runs). The terminal abdominal ganglion averaged 7 pmol/animal (n=4).

In order to examine the possibility of release of proc-

tolin from the axon terminals onto the accessory gland, the nerve branch, Br3 of an isolated accessory gland preparation was electrically stimulated at 10 Hz for 5 min. Then, the filtrates of the surrounding medium (normal saline), in which the preparation was immersed for 15 min, was concentrated using a C18 Sep-Pak cartridge to detect the presence of proctolin by HPLC and bioassay. No proctolin was, however, detectable in the surrounding medium. Next, 80 isolated accessory glands were immersed in high K^+ saline for another 15 min (see Chapter 2, Materials and Methods), using a K^+ concentration which was high enough to produce sustained contraction of the accessory glands, and then the presence of proctolin in the surrounding medium was examined. Figure 24 shows that proctolin was detectable in the surrounding medium of the accessory glands while they contracted tonically in response to high concentration of K^+, and the bioactivity of concentrated filtrate was high enough to produce the contraction of an accessory gland. The amount of proctolin which was detected in the high K^+ saline was calculated to be about 1% of the total amount of proctolin contained in one accessory gland.

The homogenized extracts of the transplanted accessory glands, whose muscles had developed fully without any innervation from the host ganglia, were examined to decide whether or not they contained proctolin. However, no proctolin or biologically active substance was detectable in any of the extracts. The crude extracts of a normal accessory gland (wet weight, 36 mg) and of six transplanted accessory glands (wet weight in total, 68 mg) through a C18 Sep-Pak cartridge

were bioassayed for bioactivity on an accessory gland. As shown in Fig. 25, the extract obtained from normal accessory gland produced the contraction of accessory gland, while the extract from the transplanted accessory glands did not have any influence upon the mechanical activity of the accessory gland. These results strongly indicate that the axons innervating the accessory gland contain proctolin which is released from the axon terminals on the muscles in response to high K^+ in the surrounding medium, and a small amount of released proctolin diffuses through the synaptic clefts into the medium.

4-4 Proctolin-like Immunoreactivity in the Dorsal Unpaired Median Neurons innervating the Accessory Gland

Fig. 26A shows the dorsal, medial neurons and the axons with proctolin-like immunoreactivity (PLI) locating in the caudal region corresponding to segment 10 of the terminal abdominal ganglion, where the DUMR7 neurons form their most caudal cluster. This section was made from the animal of which Br3s were ligated for 6 hr before processing for immunocytochemistry. The medial neurons usually stained rather weakly, but the ventral commissure and dorso-medial neuropile constantly revealed strong PLI. This ventral commissure carries the bilaterally bifurcating axons of DUMR7 neurons located in the caudal region of the ganglion (Fig. 26B). Ligation of Br3s through which the DUMR7 neurons send their axons to the accessory gland, led the intensified immunoreactivity of the medial neurons. These fact

suggest that these medial neurons with PLI may be the DUMR7 neurons innervating the accessory gland.

The protein A-gold method revealed the wide distribution of the axons with PLI in the musculature associated with the accessory gland, that is, in the single-fibrillar muscles (MF-1) winding around most of each glandular tubule's length, and in the multi-fibrillar thick muscles (MF-2) surrounding the opening of each tubule in the anterior region of the ejaculatory duct. PLI was restricted to large electron-dense granular vesicles with a diameter of 100-115 nm in the axons (Fig. 27A). Proctolin-like immunoreactive vesicles were also detected in the ligated Br3s (Fig. 27B). The ligation of Br3s causes the remarkable accumulation of large electron-dense granular vesicles in the axoplasm proximal to the ligature (Fig. 6B). Fig. 27B shows the axon profile containing proctolin-like immunoreactive vesicles (ca. 100 nm in diameter) accumulated by the ligation. These results suggest that the proctolin-like immunoreactive vesicles are produced in the somata lying in the terminal abdominal ganglion, and transported to the accessory gland through Br3s. It can be also seen in Fig. 27B, that there is a non-immunoreactive axon profile: it has smaller granular vesicles (ca. 80 nm in diameter) than the immunoreactive ones.

In order to localize PLI in DUMR7 neurons, electron-microscopic immunocytochemistry combined with HRP labeling and ligation was performed. When one of Br3s was ligated and HRP was applied to the other Br3, HRP labeling occurred not only to the somata of DUMR7 neurons, but also to the axons

in the ligated site, where numerous large granular vesicles accumulated by ligation. Fig. 26B shows the HRP labeled somata of DUMR7 neurons and their secondary neurites running through the lateral commissure locating in the caudal region of the terminal abdominal ganglion. Protein A-gold method was worked in the region of the terminal ganglion shown in Fig. 26B. A few proctolin-like immunoreactive, electron-dense granular vesicles were found dispersively in HRP labeled somata (Fig. 28A). The size of vesicles with PLI ranged from 50 nm to 200 nm and mean diameter was 108 nm. The vesicles with PLI were seen associated Golgi bodies, but not seen in the cisternae of these structures. In close to lysosome-like structure, proctolin-like immunoreactive vesicles were also seen (Fig. 28B), as described for PLI neurons in cockroach hypocerebral ganglion (Ude and Eckert, 1988). Application of protein A-gold method in the ligated Br3, revealed PLI of the large electron-dense granular vesicles (ca. 100 nm in diameter) accumulated in the HRP labeled axons (Fig. 28C).

Proctolin-like immunoreactivity could not be localized in the somata and axons strongly labeled with HRP, as the dense HRP reaction product prevented the identification of the vesicles as well as the cell organelles. However, as shown in Fig. 28A-C, in the moderately HRP labeled somata and axons, PLI could be detectable within the electron-dense granular vesicles. Although PLI within the vesicles contained in HRP labeled axons, was weakened in comparison with PLI of the vesicles in the axons applied no HRP labeling (Fig. 28A,B), however, the gold label was apparently present

over the accumulated vesicles in the HRP labeled axons (Fig. 28C).

4-5 Discussion

Innervation and physiological properties of the muscles of accessory gland

In the present experiments it was found that the selective antidromic stimulation of the DUMR7 neurons evoked a contraction of the accessory gland, while orthodromic stimulation to both DUMR7 and LC neurons evoked not only a contraction, but also a transient reduction of basal tonus after relaxation (Fig. 13). This fact suggests that the DUMR7 and LC neurons are excitatory and inhibitory motoneurons respectively, though the question where each muscle is innervated dually by these motoneurons or not, remains unsolved. There is, therefore, the possibility that both excitatory and inhibitory motoneurons mediate the antagonistic excitation of MF-1 and MF-2 to squeeze the spermatophore-forming materials form the glandular tubules (Fig. 12B). According to Cook and Meola (1987), a fine network of nerve fibers covers the entire surface of the accessory gland tubules of the cockroach Leucophaea maderae, and the innervation of the muscles shows multiterminal-type innervation.

As has been described for many other visceral muscles (e.g. Miller and James, 1976; Lange et al. 1984), the accessory gland of the cricket is capable of spontaneous contrac-

tions. The observation of these contractions in the transplanted accessory gland which developed without innervation in a host offers strong evidence for the myogenic origin of the spontaneous contractions. In the cockroach, Cook and Meola (1987) reported that the spontaneous contractions of the accessory gland tubule may be myogenic on the basis of both the occurrence of spontaneous action potentials in the accessory gland isolated form the terminal abdominal ganglion and the arrangement of muscle fibers to form a structural syncytium on the tubule.

In the present experiments, it was examined that the effects of various pharmacologically active drugs on neurally and myogenically evoked contractions of the accessory gland. These contractions were practically unaffected by epinephrine, norepinephrine, acetylcholine, GABA, FMRFamide, cyclic AMP and cyclic GMP. The pentapeptide, proctolin, at 10^{-9} M, and the putative neurotransmitter at insect skeletal muscle, glutamate, at 10^{-6} M, induced both the reduction of amplitude of neurally evoked contractions (Fig. 15) and the production of contraction which usually was followed by the reduction in amplitude of myogenically evoked contractions (Fig. 16). The effects of these drugs on the accessory gland were fundamentally similar to those described already in other insect visceral muscle preparations (e.g. Brown, 1967; Cook and Holman, 1975; Lange and Orchard, 1984b). However, octopamine, at 10^{-7} M, increased only the frequency of myogenic contractions, and serotonin, at 10^{-7} M, decreased both the amplitude of neurally evoked contractions and the frequency of myogenic contractions, and also resulted in the

reduction of basal tonus (Figs. 18-20). In the oviducal muscles of locust, it was shown by Lange and Orchard (1984b) that octopamine inhibited neurally evoked contractions, and in addition inhibited the actions of both proctolin and glutamate. In the accessory gland of the cricket, dopamine had a small effect of increasing the frequency of myogenic contractions. Probably dopamine had its effects via the octopamine receptor, since it has already been reported that octopamine receptors have a weak affinity for dopamine and several biogenic amines (Evans, 1980; Lange and Orchard, 1984b). On the other hand, the evidence drawn from the study of the pharmacological properties of transplanted accessory gland indicated that properties of proctolin and glutamate receptors of transplanted accessory glands may be different from those of normal accessory glands (Fig. 21). The innervation from the terminal abdominal ganglion to the accessory gland may, therefore, be necessary for the normal differentiation of their receptors during postembryonic development.

The role of the DUMR7 neurons

The neurons which were found to initiate directly the contraction of the accessory gland may be positively identified as DUMR7 neurons on the basis of the following morphological and physiological criteria. First, the accessory gland is bilaterally innervated by 100 or more DUMR7 neurons the somata of which are found in the terminal abdominal ganglion, and the axon terminals lying on the muscles of the accessory gland contain large electron-dense vesicles and

somata of DUMR7 neurons which are identical with those found also in the neurites (see Chapter 3). Second, as mentioned before, due to the intrinsic bilateral morphology, DUMR7 neurons can be antidromically activated through either Br3, and this results in the initiation of contraction of the accessory gland (Fig. 13). Third the antidromically initiated-contraction of the accessory gland is basically unaffected by high Mg^{2+} saline, indicating that the DUMR7 neurons may provide a direct pathway for conduction through the terminal abdominal ganglion (Fig. 14). Of course, the presence of electrical junctions between DUMR7 neurons or between a DUMR7 neuron and any other neuron such as LC neuron cannot be ruled out. However, so far we have intracellularly stained about 40 DUMR7 neurons with Lucifer yellow CH and no evidence for the existence of dye-coupling between these neurons was found (Kimura et al. unpublished data). As shown in Fig. 14, the amplitude of antidromically induced contraction decreased slightly under the high Mg^{2+} condition. This decease occurred within five minutes after the local application of high Mg^{2+} saline only on the terminal abdominal ganglion and lasted as long as the ganglion was immersed in the high Mg^{2+} saline. Thus, the decrease may reflect the presence of other neurons providing an indirect pathway for conduction through the terminal abdominal ganglion. However, this is only one of the possibilities and further experimentation is necessary to reveal what mechanism is involved in this phenomenon.

The functional role of the dorsal unpaired median neuron which innervates the extensor tibiae of the locust metatho-

racic leg (DUMETi) has been extensively studied (e.g. Hoyle and Dagan, 1978; Evans,1980). The DUMETi neurons suppresses an intrinsic rhythm of slow contraction and relaxation in a small group of muscle fibers but the primary function of this neuron appears to be the modulation of the responses of the extensor tibiae muscle to spikes in the fast and slow extensor motoneurons (e.g. Evans and O'Shea, 1977, 1978; O'Shea and Evans, 1979; Evans, 1981; Evans and Siegler, 1982). Two neurons (DUMOV1 and DUMOV2) lying in the penultimate abdominal ganglion of the locust appear to be responsible for modulating contraction of the oviducal muscles (Orchard and Lange, 1985; Lange and Orchard, 1986). In contrast, the primary function of larval firefly DUM neurons running from the terminal abdominal ganglion is the direct initiation and regulation of light organs (Christensen and Carlson, 1982). As mentioned before the evidence presented here may demonstrate the direct role for the DUMR7 neurons of cricket in inducing the contraction of muscles of the accessory gland. On the other hand, perhaps, the paired LC neurons running down to the accessory gland may contain serotonin which has an inhibitory effect on all neurally and myogenically evoked contractions and basal tonus.

Presence of proctolin in the DUMR7 neurons

The pentapeptide proctolin was initially purified from the hindgut of the cockroach (Brown and Starratt, 1975). In recent years it has become evident that proctolin exists in nervous systems of insects (Brown, 1975; Bishop et al. 1981;

Eckert et al. 1981; O'Shea and Adams, 1981; Bishop and O'Shea, 1982; O'Shea and Bishop, 1982; Agricola et al. 1985; Lange et al. 1986; Sobek et al. 1986; Orchard and Lange, 1987; Anderson et al. 1988; Lange et al. 1988; Orchard et al. 1989; Lange, 1990) and crustaceans (Sullivan, 1979; Schwarz et al. 1980; Schwarz et al. 1984; Marder et al. 1986; Schiwicki and Bishop, 1986; Schiwicki et al. 1987). The most thoroughly documented action of proctolin is as a neuromuscular transmitter or neruomodulator. In the horsefly Tabanus (Cook and Meola, 1978; Cook, 1981) and in the cockroach Leucophaea maderae (Holman and Cook, 1985) proctolin, at low concentrations (10^{-10} M), results in an increase in the amplitude and frequency of spontaneous phasic contractions of the oviducts and an increase in the muscle tonus. In locust oviduct, exogeneously applied proctolin (10^{-11} M to 10^{-10} M) increases the amplitude of neurally evoked contractions, results in a sustained tonic contraction, and increases the frequency and amplitude of myogenic contractions (Lange and Orchard, 1984b). However, the present study showed that in the cricket male accessory gland, proctolin at threshold concentrations of 10^{-11} M to 10^{-10} M increased the basal tonus, and at concentrations of 10^{-7} M and above decreased the amplitude of neurally evoked contractions. The use of proctolin at neuromuscular junctions may be related to the special requirements of accessory gland muscles to develop and maintain sustained contractions over-extended periods during which the glandular tubules release the secretions from their lumens to form a spermatophore in the ejaculatory duct.

In the present experiment, it was shown by using the methods of HPLC and bioassay, that a large amount of proctolin is present in the innervated accessory gland, but not in the accessory gland without innervation (transplanted accessory gland), and also that the high K^+ saline induces the release of proctolin only from the accessory gland with innervation. These facts are strong evidence for the existence of proctolinergic innervation in normally developed accessory gland.

The evidence presented here, by electron microscopic immunocytochemistry combined with HRP back-filling, indicates that the vesicles with PLI are probably produced in the somata of DUMR7 neurons and transported to the accessory gland through their bilaterally paired axons. This was demonstrated to be based on the characteristic morphology of DUMR7 neurons that project their axons bilaterally to the left and right Br3s, and to be based on the fact that ligature of Br3 makes the granular vesicles accumulate in the axoplasm proximal to the tied site. One of Br3s was ligated and the other Br3 was applied HRP antidromically. This manipulation enabled the HRP labeled axons with accumulated vesicles to be identified as the projection originating from DUMR7 neurons. The proctolin-like immunoreactive vesicles were localized in HRP labeled DUMR7 somata and axons by the post-embedding protein A-gold method (Fig. 28A-C). It is probable, therefore, that the DUMR7 neurons are proctolinergic excitatory motoneurons and they induce the neurogenic contractions of the accessory gland by releasing proctolin from their nerve endings.

The ultrastructure of the proctolin-like immunoreactive vesicles are characterized in the ganglion and peripheral of a few insects with pre-embedding PAP technique (Eckert et al. 1981; Agricola et al. 1985; Nässel and O'Shea, 1987, Nässel et al. 1989), and with the post-embedding immunogold technique (Ude and Eckert, 1988). The immunoreactive terminals in the dorso-caudal neuropile within the terminal abdominal ganglion of cockroach, were demonstrate to contain large dense vesicles with a diameter of 140-150 nm and numerous small clear vesicles (Agricola et al. 1985). In blowfly, the immunoreactive granular vesicles with a diameter of about 100 nm were found in the immunoreactive somata of the protocerebrum and in the terminal of the pars intercerebralis (Nässel and O'Shea, 1987). The immunoreactive terminals in the lateral abdominal nerves contained clear vesicles (diameter about 75 nm) and granular vesicles with diameters of ca. 115 nm (Nässel et al. 1989). With the post-embedding immunogold technique, in cockroach, three types of proctolin-like immunoreactive vesicles were found: the electron-dense, small vesicles having a mean diameter of 80 nm in the frontal and hypocerebral ganglion and in the musculature of oviduct and hindgut, the electron-dense, large vesicles of 150 nm in the frontal ganglion, and the vesicles of 150 nm with an electron-lucent, flocculent content in the hypocerebral ganglion (Ude and Eckert, 1988). In the DUMR7 neurons of cricket, the immunoreactive vesicles in the HRP labeled somata ranged from 50 nm to 200 nm in diameter (mean diameter 108 nm) (Figs. 27A, 28A). In the HRP labeled axons within the ligated Br3, and in the axons

distributed in the accessory gland muscle, immunoreactive vesicles with a diameter of 100-110 nm occurred (Fig. 28C). Ude and Eckert (1988) suggest the different size of the vesicles with PLI might be due to the occurrence of proctolin with different neuroactive substances in the vesicle. The ligation of Br3 also revealed the accumulation of non-immunoreactive vesicles (ca. 80 nm in diameter) (Fig. 27B). The neurons producing these vesicles were not demonstrated in the present study.

Light-microscopic observations showed the weak stainability of the medial neurons, which are presumed to be the DUMR7 neurons, in the cricket terminal abdominal ganglion (Fig. 26). This is in accordance with electron-microscopic observation: the vesicles with PLI were dispersively found in the identified DUMR7 somata (Fig. 28A,B). Probably, the immunoreactive vesicles produced in the somata are rapidly transported to the peripheral organs. This is supported by the fact that the accumulation of vesicles occurs in a rather short period, 3 hr or less, after ligation (Yasuyama, unpublished data).

The DUMETi of the locust metathoracic ganglion has been shown to contain the biogenic amine, octopamine (Evans and O'Shea, 1977, 1978) and the effect of stimulating it is mimicked by the application of octopamine directly on to the muscle at low concentration of 10^{-9} M (Evans and O'Shea, 1978). Four dorsal unpaired median neurons of the larval firefly terminal abdominal ganglion contain octopamine which serves a direct role as a neurotransmitter, regulating directly the larval luminescence intensity (Christensen and

Carlson, 1982; Christensen et al. 1983). As mentioned above, it was evident that in the cricket male accessory gland, octopamine was effective in increasing the frequency of myogenically evoked contractions in concentrations as low as 10^{-7} M. The DUMR7 neurons innervating the accessory gland stain selectively with neutral red which is the vital dye for the localization of biogenic amine containing cells (Stuart et al. 1974; Yamaguchi et al. 1985). There is, therefore, the possibility that octopamine coexists with proctolin (and probably glutamate) in the DUMR7 neurons. Furthermore, another possibility may be proposed that, as suggested by Pflüger and Watson (1988), there are the neurons which are morphologically similar to DUM neurons but have a different function from them, concerning the innervation of visceral muscle. I will further discuss this assumption in Chapter 6.

4-6 Abstract

1) Spontaneous contractions were observed in the cricket male accessory gland which is innervated by dorsal unpaired median neurons (DUMR7 neurons) and paired neurons (LC neurons) arising from the terminal abdominal ganglion.

2) These contractions were myogenic: identical mechanical activity was observed in a transplanted accessory gland which lacked innervation.

3) Selective antidromic electrical stimulation of the DUMR7 neurons evoked a contraction of the accessory gland, which was graded and dependent upon both frequency and

duration of stimulation.

4) Glutamate at 10^{-4} M, and proctolin, at low concentrations of 10^{-9} M, produced contractions of the accessory gland. These drugs at higher concentrations (glutamate, $>10^{-4}$ M; proctolin, $>10^{-8}$ M) reduced the amplitude of neurogenically evoked contractions and increased the basal tonus.

5) Octopamine, at 10^{-7} M, increased the frequency of myogenically evoked contractions. Application of serotonin, at 10^{-5} M, reduced the amplitude of neurally evoked contractions and also resulted in a lowering of the basal tonus.

6) By use of high-performance liquid chromatography and bioassay it was shown that proctolin was present in the accessory gland, and it was released from the accessory gland in response to the application of high K^+ saline. No proctolin was detected in the transplanted accessory gland.

7) Proctolin-like immunoreactivity (PLI) was shown in the dorsal median neurons (DUMR7 neurons) by means of post-embedding immunogold method combined with horseradish peroxidase (HRP) back-filling and ligation of nerve branch.

8) The ligation of one of Br3s and HRP back-filling of the opposite Br3 caused labeling not only the somata of DUMR7 neurons, but also, in the ligated site, the axons containing numerous granular vesicles (ca. 100 nm in diameter) accumulated by ligature. PLI was found within the granular vesicles (50-200 nm, mean diameter 108 nm) in the HRP labeled somata of DUMR7 neurons, as well as within the granular vesicles accumulated in the HRP labeled axons.

9) The axons containing granular vesicles (100-115 nm) with PLI occurred widely in the visceral muscle associated

with the accessory gland. It seems to be obvious that the HRP labeled axons with accumulation of granular vesicles originate from the DUMR7 neurons from the characteristic bilateral morphology of these neurons.

5 Functional Morphology of the Reproductive Organs of the Female Cricket

Recently, Kimura and Yamaguchi (1987) have reported the existence of physiologically active substances in the cricket spermatophore, which mediate the peristaltic movements of the female spermathecal duct to facilitate sperm migration. In this chapter, on the basis of this physiological observation, the author will describe the fine structure of the spermathecal duct, and show the presence of transverse passage, through which physiologically active substances injected into the lumen of spermathecal duct during copulation may reach from there to the haemocoel or the vicinity of the muscle winding around the duct, using ionic lanthanum as a tracer.

5-1 Histology of the Spermathecal Duct

The spermathecal bulb of the female cricket is an oval pouch about 1 mm long and 0.8 mm wide. It is connected to the genital chamber by the highly convoluted spermathecal duct (Fig. 29). The elongated spermathecal duct is about 20 mm in length. The spermatheca and spermathecal duct is surrounded by circularly arranged muscle fibers. Thin branches of nerves and tracheoles are attached to the muscle layer.

The spermathecal duct can be subdivided into three regions: the proximal region near the junction of the duct with the genital chamber, the median region, and the distal

region near the junction with the spermathecal bulb. The proximal region is composed of a single layer of slender epithelial cells with basally located elliptic nuclei. The duct in this region is about 80 to 90 μm in diameter with an inner lumen of about 6 to 8 μm in diameter. The cuticular intima is about 4 μm in thickness. The median region is highly convoluted, and constitutes most of the duct's length. The diameter of the duct is about 60 to 70 μm. The lumen of the median region is narrow (about 2 μm in diameter), and the cuticular intima is thin (about 2 μm in thickness). This region is composed of three cell types: (a) slender epithelial cells with basally located oval nuclei, (b) columnar cells with basally located large round nuclei, and (c) cells with small nuclei. The columnar cells has a large vacuole containing a stainable substance (Fig. 30A). In addition to these cells, apically recurved tubular structures, which extend from the cuticular intima into the wall of the duct, are present in the median region of the duct (Fig. 30B). These tubular structures and the cells containing large vacuoles are peculiar to the median region. Near the junction to the spermathecal bulb, the spermathecal duct becomes thicker (ca. 100 μm in diameter) and passes straight along the surface of the spermatheca (Fig. 29). The lumen of the duct is about 4 μm in diameter, and the cuticular intima is about 8 μm in thickness. In this region, the wall of duct consists of a single layer of slender epithelial cells with medially located elliptic nuclei.

5-2 Fine Structure of the Spermathecal Duct

Glandular Cells: each glandular cell has a large central cavity (described above as a vacuole). The central cavity is formed by an invagination of the cytoplasmic membrane and is lined with many microvilli (Fig. 31A). The cavity of each glandular cell contains the end apparatus which consists of a loosely packed, finely filamentous material (Fig. 31B), the so-called 'felt-work' (Mercer and Brunet, 1959).

In the glandular cells of newly emerged virgin females (2 or 3 days old), the microvilli fill the narrow cavity and are in close contact with the felt-work. A small number of electron-dense, membrane-bound granules are scattered in the cytoplasm surrounding the cavity, but no secretory substance is visible within the cavity (Fig. 32A). In older, mated females, the secretory granules within the cytoplasm increase in size and number, and a small amount of secretory substance is present between the microvilli within the cavity (Fig. 31A,B). In addition to these granules, large spherical, electron-dense vesicles and irregularly shaped, moderately electron-dense vesicles are found throughout the cytoplasm (Fig. 32C). As the secretory cycle advances, the microvilli lining the cavity become more loosely arranged and separated by the secretory substance which is electron-dense and finely granular (Fig. 32B).

In newly emerged females, some flattened cisternae of rough endoplasmic reticulum are sparsely scattered in the peripheral region of the cytoplasm of the glandular cells, while in older mated females, the cisternae of rough endoplasmic reticulum are dilated and mostly occupy the cyto-

plasm (Fig. 32D). Mitochondria, randomly arranged microtubules, several Golgi bodies, and glycogen deposits are found in the glandular cells of both mated and newly emerged females.

The glandular cells were not found in the wall of spermathecal bulb.

Epithelial Cells: the basal cytoplasmic membranes of the epithelial cells are infolded. The intercellular spaces, which invade deeply between the adjacent epithelial cells (Fig. 33B), contain the same material that forms the basement membrane. The lateral membrane facing these spaces possesses a dense area associated with microtubules. The apical half of lateral cytoplasmic membranes of adjacent cells are extensively interdigitated, and adjacent cells are linked by a septate junction (Fig. 33D). The apical region of epithelial cells shows a highly irregular outline due to the numerous complicated invaginations of the cytoplasmic membranes (Fig. 33A). The invaginated membranes surround narrow projections of the innermost layer of the cuticular intima, and possess dense areas associated with tufts of microtubules (Fig. 33C,D).

A small amount of rough endoplasmic reticulum is sparsely scattered in the cytoplasm. Golgi bodies are present but not prominent. Mitochondria, although present throughout the cell, are distributed mostly in the apical cytoplasmic zone (Fig. 33A). The concentration of mitochondria in the apical zone is particularly prominent in the epithelial cells which compose the distal and proximal region of the spermathecal duct, but this phenomenon is not prominent in the epithelial

cells composing the median region of the duct. Glycogen deposits are present in the basal zone of the epithelial cells. In old, mated females, large glycogen areas are especially noticeable in the epithelial cells at the proximal region of the spermathecal duct (Fig. 33A), while, in newly emerged, virgin females, they are prominent in the cells composing the distal region of the duct. The apical cytoplasmic protuberances of the epithelial cells often contain glycogen granules (Fig. 33A).

Cuticular Ductules and Ductule Forming Cells: the secretory substance released into the central cavity of each glandular cell is conveyed to the lumen of the spermathecal duct through an cuticular ductule (described above as a tubular structure). The tip of the ductule within the cavity is surrounded by a loosely-knit felt-work (Fig. 31B). The cuticular ductule approaches the cuticular intima of the spermathecal duct passing tortuously through a ductule forming cell (described above as a cell with a small nucleus) (Fig. 34A). Although the ductule through the cuticular intima of the spermathecal duct is not accompanied by a ductule forming cell, it maintains its structural identity (Fig. 34B). The lumen of the cuticular ductule lined by the cuticular intima is about 0.2 to 0.3 μm in diameter.

The intima of the ductule consists of (1) a superficial electron-dense region, (2) an underlying homogeneous layer of moderate electron density, and (3) a fibrous region lying immediately beneath the cytoplasmic membrane of the ductule forming cell (Fig. 34C). The superficial region consists of a pair of dense layers: the one closest to the lumen is

about 10 nm thick, and the other is thinner, about 7 nm in thickness. The electron-transparent zone between the two electron-dense layers is about 7 nm. The homogeneous underlying layer is about 20-30 nm in thickness. The fibrous zone is not uniform in thickness. The outer surface of this zone is extremely irregular, and often extends into the cytoplasm of the ductule forming cell. Occasionally, the irregularly shaped deposits of electron-dense substance are present in this extension of the fibrous zone (Fig. 34A). The fibrous zone becomes very thin or disappears at the region where the ductule invades the glandular cell, and changes into a felt-work structure within the cavity of the glandular cell (Fig. 31B).

The infolded cytoplasmic membrane of the ductule forming cell encloses the wall of the cuticular efferent ductule (Fig. 34A). The adjoining cytoplasmic membrane of the glandular and ductule forming cell are linked by a septate junction (Fig. 31B). The cell organella of these cells are poorly developed. Microtubules, small mitochondria, scattered profiles of rough endoplasmic reticulum are dispersed throughout the cytoplasm. Occasionally, small Golgi bodies are observed.

Muscles and Axons: the muscle wall enveloping the spermathecal duct consists of two or three layers of insect visceral muscle. Axons containing electron-dense vesicles (ca. 100 nm in diameter) approach the muscle fibers and make neuromuscular junctions with them. In the axon terminals, both large dense and small clear vesicles (ca. 50 nm in diameter) are present (Fig. 35).

5-3 Transverse Ionic Passage from the Haemocoel to the Glandular Cavity and the Lumen of the spermathecal Duct

Bifurcation of a cuticular ductule was occasionally observed. In those cases, one branch ran into the secretory cavity of the glandular cell, and the other ran down towards and terminating in the region of the basement membrane, where the superficial electron-opaque layer of the intima is not apparent, and the lumen of the ductule is surrounded by a homogeneous substance. The outer surface of this substance was extremely irregular and abutted on the intercellular space (Fig. 37A).

When the spermathecal duct was incubated in the ionic lanthanum solution, electron-dense deposits were found in the lumina of the cuticular ductules (Figs. 36, 37A), the secretory cavities (Figs. 36, 37B), and the openings of the cuticular ductules leading to the lumen of the spermathecal duct (Fig. 37C), as well as in the surface of the muscle fibers and the intercellular space (Fig. 36). These deposits were analyzed with an X-ray dispersive technique to verify they were in fact lanthanum. The resultant histograms shown in Fig. 37 represent the counts attributable to the presence of lanthanum in the areas indicated as a-c in Figure 37. Lanthanum was identified by detecting the L_{α} peak at 4.65 keV and L_{β} peak at 5.04 keV. Spectra taken from adjacent areas of the spermathecal duct without those deposits did not show a lanthanum peak. These results appeared to indi-

cate that the lanthanum applied to the external muscular wall of the spermathecal duct entered the cuticular ductules and traveled not only to the secretory cavity, but also to the lumen of the spermathecal duct. Figure 37A shows the basal end of the branch of the ductule near the basement membrane. It seems possible that the ionic lanthanum diffusively entered the lumen of the ductule through the homogeneous layer surrounding the basal end of the cuticular ductule from the intercellular space.

5-4 Discussion

The spermathecal duct of the cricket, G. bimaculatus, is characterized by the presence of glandular cells. In the cockroach, Periplaneta americana (Guputa and Smith, 1969), and the fruit fly, Drosophila melanogaster (Filosi and Perotti, 1975), glandular cells are present only in the wall of spermatheca. In the mosquito, Aedes aegypti (Clements and Potter, 1967; Jones and Fischman, 1970), glandular cells are present in both the spermathecal bulb and spermathecal duct, but their distribution in the spermathecal duct is restricted to a particular region near the junction of the duct with the spermathecal bulb. In contrast, in G. bimaculatus, the glandular cells were found only in the median region where the spermathecal duct is highly convoluted (Figs. 30, 31), and not found either near the junction with the genital chamber or near the junction with the spermathecal bulb. They were also absent from the wall of the spermathecal bulb.

The general form of the glandular cells in G. bimaculatus is similar to that in other insects (Guputa and Smith, 1969; Lawson, 1970; Ahmed and Gillott, 1982 a; Clements and Potter, 1967; Jones and Fischman, 1970; Filosi and Perotti, 1975; Happ and Happ, 1970; Conti et al. 1972), i.e., the glandular cell has a central cavity formed by invagination of its apical cytoplasmic membrane, and releases its product there (Fig. 32A, B). The secretory substance is exported to the lumen of the organ through the cuticle-lined efferent ductule (Fig. 31B). Thus, the secretory structure in the spermathecal duct of G. bimaculatus, like that of other insects, falls into the Class 3 category in the scheme devised by Noriot and Quennedey (1974) for insect epidermal glands.

The tip of the cuticular ductule within the cavity of the glandular cell was surrounded by a loosely-knit felt-work (Fig. 31B). This structure appears to be of general occurrence within the cavities of the glandular cells in the spermathecal duct and spermatheca (Guputa and Smith, 1969; Ahmed and Gillott, 1982 a; Clements and Potter, 1967; Jones and Fischman, 1970; Filosi and Perotti, 1975; Conti et al. 1972). Conti et al. (1972) demonstrated in Dytiscus marginalis that the felt-work structure contains resilin and neutral polysaccharides. The fine structure of the intima of the cuticular ductule is different from that of the cuticular ductule of P. americana (Guputa and Smith, 1969). The intima of the efferent ductule of P. americana consists of two distinct regions: an inner dense region and an underlying region of lower density where both longitudinal and

radial periodicity are observed. In contrast, the cuticular ductule intima of in G. bimaculatus could be subdivided into three regions, and the periodicity was not observed in any region (Fig. 34C).

In P. americana, Guputa and Smith (1969) observed the aggregation of moderately electron-dense vesicles in the glandular cell cytoplasm surrounding the central cavity. They suggested that the secretory vesicles gradually loose their initial density and increase in size prior to their release by a 'blebbing off' of pieces of cytoplasm. In G. bimaculatus, however, the aggregation of small electron-dense granules around the cavity was observed, but that of the moderately electron-dense vesicles was not (Fig. 32). Probably, the contents of the small electron-dense granules are released directly into the cavity. The relationship of the electron-dense granules and moderately electron-dense vesicles was not clarified in the present study. Ahmed and Gillott (1982 b) demonstrated in Melanoplus sanguinipes that the secretory substance of the glandular cells in the spermatheca contains protein and acidic mucopolysaccharide.

The aggregation of mitochondria within the apical cytoplasm of epithelial cells has been observed in the spermathecae of several species of insects (Guputa and Smith, 1969; Ahmed and Gillott, 1982 a; Huebner, 1980), and it is presumed that the aggregation of mitochondria concerns the ionic regulation necessary to maintenance of viable sperm (Huebner, 1980). In G. bimaculatus, the polarized distribution of mitochondria was prominent in the epithelial cells of the proximal region near the junction of the duct with

the genital chamber and in those of the distal region near the junction with the spermathecal bulb (Fig. 33C), but not so in the median region of the spermathecal duct. This fact suggests that the epithelial cells of the proximal and distal region of the duct are functionally specialized for the regulation of the microenvironment within the lumen of the spermathecal duct.

Glycogen deposits have been noted in the spermathecal epithelial cells of Tenebrio molitor (Happ and Happ, 1975), P. americana (Guputa and Smith, 1969), and M. sanguinipes (Ahmed and Gillott, 1982 a). In G. bimaculatus, they were noticed in the epithelial cells at the proximal region of the spermathecal duct in old mated females (Fig. 33A), whereas in newly emerged females they were prominent in the cells composing the distal region of the duct near the spermathecal bulb. This phenomenon may result from the difference in the degree of cellular maturation. Glycogen deposits are frequently found in developing insect epithelia (Filshie and Waterhouse, 1968).

Axons containing both large electron-dense and small clear vesicles were widely distributed among the muscle fibers of the spermathecal duct (Fig. 35). Electron microscopic immunocytochemistry reveals that the wide distribution of the axons with proctolin-like immunoreactivity (PLI) throughout the musculature associated the spermathecal duct. PLI is restricted to large electron-dense vesicles in axon terminals (Yasuyama et al. 1988). The female reproductive organs (genital chamber, spermathecal duct and spermathecal bulb) are innervated by several dorsal unpaired median neu-

rons (DUMR4 neurons) and paired lateral neurons emerging through the fourth nerve roots from the terminal abdominal ganglion (Yamaguchi et al. 1985; Kimura et al. 1987). The paired neurons are serotonergic neurons which innervate the genital chamber (Hustert and Topel, 1986), and the spermathecal duct near the junction with the genital chamber (Kimura et al. 1987). It is implied, therefore, the DUMR4 neurons may involve the axon terminals containing large-dense vesicles with PLI. Further experiments to identify the neurons contributing the axon terminals in the musculature of the spermathecal duct, are needed.

In the female cricket, the peristaltic movements of the spermathecal duct occur in response to the injection of the spermatophore contents through the copulatory papilla into the lumen of the spermathecal duct in order to facilitate sperm migration to the spermathecal bulb, which is a sperm reservoir (Kimura and Yamaguchi 1987). Furthermore, the peristaltic movements of an isolated spermathecal duct can be induced by the application of the spermatophore contents to the proximal region of an isolated spermathecal duct near the genital papilla (Kimura and Yamaguchi, 1987). Ai et al. (1986) referred to the possibility that prostaglandins, which are produced in the spermatheca of the female cricket, may diffuse into the haemocoel through the genital chamber wall to elicit egg deposition. In contrast, Sugawara (1987) reported that the cuticular layer lining within the lumina of the spermathecal duct and genital chamber is an obstacle to the penetration of prostaglandins.

The present tracer experiment using ionic lanthanum

clearly demonstrated the presence of transverse ionic passage through the cuticular ductules from the haemocoel to the lumen of the spermathecal duct and glandular cavities (Figs. 36, 37). It is likely, at least in G. bimaculatus that these passages permit the physiologically active substances, such as prostaglandins and the spermatophore contents to pass from the lumen of the spermathecal duct to the haemocoel. The present experiment, however, did not provide any direct evidence showing that the cuticular ductules act as two-way passages for large molecules from the haemocoel to the lumen of the spermathecal duct and vice versa. Further investigations to examine the validity of the conveyance of physiologically active substances through the cuticle ductules between the lumen of the spermathecal duct and the haemocoel are needed.

5-5 Abstract

1) The spermathecal duct of female crickets is lined with a cuticular intima. Its wall consists of three cellular types: glandular cells, ductule forming cells and epithelial cells.

2) The glandular cells are absent from the proximal region near the junction of the duct with the genital chamber and from the distal region near the junction with the spermathecal bulb. They are restricted to the median region where the spermathecal duct is highly convoluted. Each glandular cell has a cavity formed by an invagination of the apical cytoplasmic membrane.

3) The cavity of the glandular cells and the lumen of the spermathecal duct are connected by a cuticular efferent ductule which is surrounded by a ductule forming cell.

4) The epithelial cells contain bundles of longitudinally arranged microtubules and large deposits of glycogen. In the epithelial cells, mitochondria are distributed mostly in the apical cytoplasmic zone. The concentration of mitochondria in the apical zone suggests their involvement in ion transport required for the regulation of the microenvironment within the lumen of the spermathecal duct.

5) The muscle fibers surrounding the spermathecal duct are innervated by axons which contain electron-dense vesicles.

6) When an isolated spermathecal duct was incubated in an ionic lanthanum solution, deposits of lanthanum were found in the lumina of the cuticular ductules, the secretory cavities of the glandular cell, and in the opening of the cuticular ductules to the lumen of the spermathecal duct. This finding suggests that each cuticular ductule is a tripartite passage between the haemocoel, the glandular cavity, and the lumen of the spermathecal duct.

6 General Discussion

Function of DUMR7 neurons in the reproductive organs of the male cricket

On the basis of the morphological and physiological evidence described in Chapters 3 and 4, it is quite conclusive that the DUMR7 neurons in the male cricket are at least proctolinergic excitatory motoneurons: i.e., they terminate on the muscle fibers of the accessory gland and induce neurogenic contractions of the accessory gland by releasing proctolin from their nerve endings.

DUM neurons have been described in several insects (see Chapter 1). Hoyle (1975) first suggested that the DUMETi neurons, which innervate the extensor tibiae muscle of the locust, are octopaminergic. The presence of octopamine in their somata was shown by Evans and O'Shea (1978). DUM neurons of the firefly terminal abdominal ganglion have also been shown to contain significant levels of octopamine (Christensen et al. 1983). It is now established that octopamine is the substance produced and released as a neuromodulator by DUMETi and, probably, by other DUM neurons as well. Octopamine and other biogenic amine containing cells in invertebrates stain specifically with the dye neutral red (Stuart et al. 1974; Wallace et al. 1974; Evans and O'Shea, 1977). The DUMR7 neurons innervating the accessory gland stain selectively with this dye (Yamaguchi et al. 1985). In addition, octopamine has been effective in increasing the frequency of myogenically evoked contractions of the acces-

sory gland in concentrations as low as 10^{-7}M (Chapter 4). Therefore, the possibility that octopamine coexists with proctolin in DUMR7 neurons cannot be ruled out, and further experiments on the localization of octopamine in the DUMR7 neurons should be performed.

However, it is also possible, as suggested by Pflüger and Watson (1988), that there are neurons which are morphologically similar to DUM neurons but which have a different function. In the female locust, Lange and Orchard (1984a) reported that back-filling of the branches of the oviducal nerve reveals a total of eight neurons (three pairs of bilaterally symmetrical neurons and two posterior DUMOV neurons) within the seventh abdominal ganglion. Subsequently, it was shown by a radioenzymatic assay that the somata of DUMOV neurons contain octopamine (Orchard and Lange, 1985). However, another anterior cluster of DUM neurons within this ganglion has been also disclosed by back-filling the same nerve branches (Kiss et al. 1984; Pflüger and Watson, 1988; Eckert et al. 1989). Kiss et al. (1984) reported two types of axon terminals in the locust oviduct muscle; one containing mainly small clear vesicles together with some larger granular vesicles, which makes conventional synapses onto the muscle, and a second containing predominantly large granular vesicles of a similar type to those in the terminals of the DUMETi, which is thought to release the transmitter adjacent to the muscle fibers without forming anatomically specific neuromuscular junctions (Hoyle et al. 1980). Based on these findings, Pflüger and Watson (1988) suggested that the DUM neurons forming the anterior cluster

in the seventh abdominal ganglion of the female locust may be motoneurons having a different function from the posterior octopaminergic DUMOV neurons. On the basis of their back-filling experiment, they also suggested that in male locust some motor neurons which are morphologically similar to DUM neurons may be associated with the innervation of the visceral muscle of the reproductive organs (Pflüger and Watson, 1988). In the case of the DUMR7 neurons of the cricket, their somata contained large electron-dense vesicles which were identical with the vesicles found in the axon terminals making neuromuscular junctions with the accessory gland muscles as well as those in their neurites (Chapter 3). In addition, the selective stimulation of the DUMR7 neurons induced a monophasic contraction of the accessory gland (Chapter 4). These findings also suggest that the DUMR7 neurons are functionally different from such DUM neurons as the DUMETi neurons and others which modulate the response of insect skeletal muscles. It is probable that the DUMR7 neurons have a direct role in inducing the contraction of the visceral muscles associated with the reproductive organs.

In the present experiment, it was shown that the electrical stimulation of DUMR7 neurons caused the release of proctolin and proctolin-like immunoreactivity was observed in the DUMR7 neurons (Chapter 4). As far as we know, this is the first report to indicate that DUM neurons contain the neuropeptide proctolin. In a few cases, however, it has been reported that the unpaired neurons located along the midline of the ganglia have proctolin-like immunoreactivity. In the

cockroach terminal abdominal ganglion, one of four ventral unpaired median neurons (VUM neurons) which innervate the oviduct muscle and the musculus sphincter, has been characterized as a proctolin-like immunoreactive neuron (Stoya et al. 1989). In lepidopteran, Davis et al. (1989) reported a proctolin-like immunoreactive, dorsal, median, unpaired neuron (K neuron). This neuron occurs in all of the abdominal ganglion and appears to project bilaterally into the perivisceral organs. The K neuron is proposed to have a neurohaemal function like that of the midline bilateral (MB) cells in the abdominal ganglia of Manduca sexta (Tublitz and Truman, 1985), which have bilateral projections to the perivisceral organs and produce cardioacceleratory peptides.

The DUMR7 neurons in the male cricket form three clusters in the posterior half of the terminal abdominal ganglion (Chapter 3), and some of the DUMR7 neurons in the most caudal cluster were found to have proctolin-like immunoreactivity (Chapter 4). In several insects, proctolin-like immunoreactive neurons have been found in the caudal region in the terminal abdominal ganglion or in the fused abdominal ganglion (e.g. in the grasshopper, Keshishian and O'Shea, 1985; in the cockroach, Eckert et al. 1981; Bishop and O'Shea, 1982, in the Colorado potato beetle, Veenstra et al. 1985; in the dipteran, Anderson et al. 1988; Nässel et al. 1989). It has been proposed that some of them innervate the hindgut (Eckert et al. 1981; Anderson et al. 1988; Nässel et al. 1989). However, it is also possible that some of those proctolin-like immunoreactive neurons may be associated with innervation of the reproductive organs, since it is predict-

able that proctolin has a significant effect on the regulation of not only the visceral muscle associated with the alimentary canal, but also that associated with the reproductive organs. In the male cricket, the use of proctolin at neuromuscular junctions may be related to the special requirements of accessory gland muscles to develop and maintain sustained contractions over an extended period, during which the glandular tubules release the secretions from their lumina to form a spermatophore in the ejaculatory duct.

Since I could not characterize the individual DUM neurons physiologically or immunocytochemically, the question of whether all DUMR7 neurons are proctolinergic or not still remains unanswered.

Functional morphology of the spermathecal duct of the female cricket

In Chapter 5, the author described the local differentiation of the morphology of the female spermathecal duct. The spermathecal duct can be subdivided into three regions: the proximal region near the junction of the duct with the copulatory papilla, the median region, which is highly convoluted and constitutes most of the duct's length, and the distal region near the junction with the spermathecal bulb. Glandular cells and cuticular ductules are restricted to the median region. The polarized distribution of mitochondria is prominent in the epithelial cells of the proximal and distal regions, but not so in the median region.

This observation suggests that the epithelial cells of these regions are functionally specialized for regulation of the microenvironment with the lumen of the spermathecal duct.

The spermathecal duct of the female cricket is surrounded by circularly arranged muscles. These muscles are presumably responsible not only for transferring the sperm to spermatheca after mating, but also for releasing it from spermatheca onto the eggs at the time of fertilization; that is, they probably mediate the sperm transfer bi-directionally at different times; i.e., during mating and fertilization. Khalifa (1949) suggested that the sperm transfer for spermatheca in the cricket should be attributed to the pressure which forces the sperm out of the spermatophore and to the sperm's own motility.

On the other hand, Kimura and Yamaguchi (1987) reported that, in the female cricket, the peristaltic movements of the spermathecal duct occur in response to the injection of the spermatophore contents through the copulatory papilla into the lumen of the spermathecal duct to facilitate sperm migration. Furthermore, Kimura et al. (1988) indicated that the response to the spermatophore contents and some neurotransmitter substances (proctolin, serotonin and octopamine), differs in the three regions of the spermathecal duct; i.e., the proximal, median and distal regions. Our previous immunocytochemical study disclosed a noticeable difference among the three regions of the spermathecal duct; i.e., serotonin-like immunoreactive fibers are distributed in the proximal regions, whereas proctolin-like immunoreactive ones are extensively distributed throughout the duct

(Yasuyama et al. 1988). These observations suggest that the cricket spermathecal duct also has local differentiation in both its morphology and physiology, and that sperm migration to the spermatheca can probably be attributed to the peristaltic movement of the spermathecal duct induced by physiologically active substances contained in the spermatophore. The copulatory papilla, spermathecal duct, and spermatheca of the cricket are innervated by the nerve branches arising from the fourth nerve roots of the terminal abdominal ganglion (Hustert and Topel, 1986; Kimura et al. 1987). Although, at present time, we do not have enough information on the function of each of the neurons innervating the spermathecal duct, it could be proposed that some of the neurons may have a modulatory function with regard to the amplitude and frequency of the peristaltic movements of the spermathecal duct induced by the spermatophore contents to facilitate sperm migration.

The spermatophore contents, which are injected into the lumen of the spermathecal duct during copulation, can induce the peristaltic movement of the duct (Kimura and Yamaguchi, 1987). This observation suggests that physiologically active substances in the spermatophore contents may move from the lumen of this duct to the haemocoel and act directly on the muscle to induce the peristaltic movement of the duct. A similar proposal; that is, the possibility of passage of the male accessory gland substances through the wall of female internal reproductive organs into the haemocoel, has been made concerning the oviposition stimulating factor (Friedel and Gillott, 1977; Yamaoka and Hirano, 1977; Lange and

Loughton, 1985; Ai et al. 1986). Ai et al. (1986) suggested that prostaglandins produced in the spermatheca of the female cricket may diffuse into the haemocoel through the genital chamber wall to elicit egg deposition. However, Sugawara (1987) showed that no part of the genital chamber and its vicinity lacks a cuticular lining and suggested that the cuticle lining is an obstacle to the diffusion of prostaglandins.

Taking into consideration the above-mentioned findings, I performed a tracer experiment using ionic lanthanum and clearly demonstrated the presence of a transverse passage through the cuticular ductule from the haemocoel to the lumen of the spermathecal duct and glandular cavities (Chapter 5). In the basal end of the cuticular ductule, it loses the innermost electron-opaque layer of the intima, and the lumen of the ductule is surrounded by a homogeneous substance. The outer surface of this substance is extremely irregular and abuts on the intercellular space (Chapter 5; Fig. 37A). The ionic lanthanum applied to the external muscular wall of the spermathecal duct probably entered the lumen of the ductule from the intercellular space through the homogeneous layer and moved through its lumen, reaching not only the glandular cavity, but also the lumen of the spermathecal duct. This experiment still leaves unanswered the question, however, of whether or not the physiologically active substances presumed to be large molecules, which are injected into the lumen of the spermathecal duct during copulation, can be conveyed from there to the haemocoel. To solve this question requires the injection of a tracer of a

molecular size larger than lanthanum directly into the lumen of the spermathecal duct.

7 Conclusion

The purpose of this study is to clarify the structure and control systems of the reproductive organs, which play important roles during mating behavior, in both sexes of the cricket, Gryllus bimaculatus.

1) The male reproductive organs (accessory gland, epididymides and ejaculatory duct) are innervated by paired nerve branches (Br3s) emerging from the right and left seventh nerve roots of the terminal abdominal ganglion. Nickel back-filling experiment revealed the presence of two types of neurons running down to the reproductive organs: 100 or more dorsal unpaired median neurons (DUMR7 neurons) extending their axons bilaterally through both Br3s, and 13 ordinary paired neurons (LC neurons) extending their axons only through a Br3.

2) The male accessory gland comprises more than 600 slender tubules. Single-fibrillar thin muscle winds around most of each glandular tubule's length, and multifibrillar thick muscle surrounds the opening of each tubules in the anterior region of the ejaculatory duct. Electron microscopic examination of the accessory gland showed that the presence of the axon terminals, which contain small clear vesicles together with large electron-dense vesicles, and make conventional synapses directly onto the muscle. Similar large electron-dense vesicles were present in the axons of horseradish peroxidase (HRP) labeled DUMR7 neurons as well as their somata. These facts suggest that the DUMR7 neurons probably terminate on the muscle of the accessory gland.

3) The selective stimulation to the DUMR7 neurons induced a monophasic mechanical response (contraction) of the accessory gland. Simultaneous stimulation to the DUMR7 and LC neurons induced biphasic response of that: the first phase corresponded to the contraction and the second to a decrease in basal tonus. Therefore, it is likely that the DUMR7 neurons are excitatory or modulatory neurons, while the LC neurons are inhibitory neurons.

4) Either of glutamate (10^{-5} M) and the neuropeptide proctolin (10^{-9} M) induced sustained contraction of the accessory gland. Serotonin (10^{-7} M) induced a relaxation of the accessory gland by a lowering of the basal tonus. Octopamine (10^{-7} M) increased the frequency of contractions. A certain amount of proctolin was detectable, by using high-performance liquid chromatography (HPLC) and bioassay, in the surrounding medium of the accessory gland while the muscles of accessory gland contracted tonically in response to high K^+ saline. No proctolin was detectable in the transplanted accessory gland which lacked intrinsic neural elements. These facts suggest that the DUMR7 neurons are proctolinergic excitatory motoneurons and they induce the contractions of the accessory gland by releasing proctolin from their endings. Perhaps, the LC neurons may contain serotonin which has an inhibitory effects on the contraction and basal tonus of the accessory gland.

5) Proctolin-like immunoreactivity (PLI) was shown in the DUMR7 neurons by electron microscopic immunocytochemistry combined with HRP back-filling. PLI was found within the large electron-dense vesicles in the HRP labeled somata of

DUMR7 neurons and their axons running down through Br3s to the accessory gland. Furthermore, the large electron-dense vesicles with PLI identical with those of the HRP-labeled DUMR7 neurons, were found in the axons widely distributed in the muscles of accessory gland. Thus, these results indicate the possibility that some of DUMR7 neurons produce proctolin-like substance in their somata and transport through their axons to the muscles of accessory gland, where this substance may be used as a neuromuscular transmitter or cotransmitter.

6) The female spermathecal duct is a long (about 20 mm at full extension), highly convoluted duct connecting the copulatory papilla with the spermathecal bulb. Its wall consists of epithelial cells, glandular cells, and ductule forming cells, and contains thin cuticular ductules extending from the cuticular intima of the spermathecal duct. The glandular cells and cuticular ductules are widely distributed along the duct's length, except in the proximal and distal region of the spermathecal duct. The cuticular ductules approach the glandular cavities along a tortuous course and enter them, or run down towards to terminate in the region of the basement membrane.

7) Electron microscopic tracer experiment in the female spermathecal duct suggests that some of the cuticular ductules are a tripartite passage between the haemocoel, the glandular cavity, and the lumen of the spermathecal duct on the basis of the following observation: when an isolated spermathecal duct was incubated in an ionic lanthanum solution, deposits of lanthanum were found in the lumina of the

cuticular ductules, the secretory cavities of the glandular cells, and in the opening of the cuticular ductules to the lumen of the spermathecal duct, but also in the intercellular space and the surface of muscle fibers surrounding the spermathecal duct. Therefore, it is likely that these passages permit the physiologically active substances in the male accessory gland secretions delivered into the lumen of the female spermathecal duct during copulation, to pass from the lumen of this duct to the haemocoel in order to induce the peristaltic movements of the spermathecal duct.

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9 References

Adiyodi RG, Adiyodi KG (1974) Ultrastructure of the utriculi majores in the mushroom-shaped male accessory gland of Periplaneta americana (L.). Z Zellforsch 147:433-440

Agricola H, Eckert M, Ude J, Birkenbeil H, Penzlin H (1985) The distribution of a proctolin-like immunoreactive material in the terminal ganglion of the cockroach, Periplaneta americana L. Cell Tissue Res 239:203-209

Ahmed I, Gillott C (1982a) The spermatheca of Melanoplus sanguinipes (Fabr). II. Ultrastructure. Int J Invert Reprod 4:297-309

Ahmed I, Gillott C (1982b) The spermatheca of Melanoplus sanguinipes (Fabr.). I. Morphology, histology, and histochemistry. Int J Invert Reprod 4:281-295

Ai N, Komatsu S, Kubo I, Loher W (1986) Manipulation of prostaglandin-mediated oviposition after mating in Teleogryllus commodus. Int J Invertebr Reprod Dev 10:33-42

Anderson MS, Halpern ME, Keshishian H (1988) Identification of the neruopeptide transmitter proctolin in Drosophila larvae: characterization of muscle fiber-specific neuromuscular endings. J Neurosci 8:242-255

Bacon JP, Altman JS (1977) A silver intensification method for cobalt-filled neurones in wholemount preparations. Brain Res 138:359-363

Bentley DR (1973) Postembryonic development of insect motor systems. In: Young D (ed) Developmental Neurobiology of Arthropods. Cambridge Univ Press, Cambridge, pp 147-177

Bishop CA, O'Shea M (1982) Neuropeptide proctolin (H-Arg-

Tyr-Leu-Pro-Thr-OH): Immunocytochemical mappings of neurons in the central nervous system of the cockroach. J Comp Neurol 207:223-238

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

Brown BE (1967) Neuromuscular transmitter substance in insect visceral muscle. Science 155:595-597

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

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

Casaday GB, Camhi JM (1976) Metamorphosis of flight motor neurons in the moth Manduca sexta. J Comp Physiol 112:143-158.

Christensen TA, Carlson AD (1981) Symmetrically organized dorsal unpaired median (DUM) neurons and flash control in the male firefly, Photuris versicolor. J Exp Biol 93:133-147

Christensen TA, Carlson AD (1982) The neurophysiology of larval firefly luminescence: direct activation through four bifurcating (DUM) neurons. J Comp Physiol 148A:503-514.

Christensen TA, Sherman TG, McCaman RE, Carlson AD (1983) Presence of octopamine in firefly photomotor neurons. Neuroscience 9:183-189

Clark R (1976) Structural and functional changes in an

identified cricket neurons after separation from the soma. I: Structural changes. J Comp Neurol 170:253-256

Clements AN, Potter SA (1967) The fine structure of the spermathecae and their ducts in the mosquito Aedes aegypti. J Insect Physiol 13:1825-1836

Conti L, Ciofi-Luzzatto A, Autuori F (1972) Ultrastructural and histochemical observations on spermathecal gland of Dytiscus marginalis L. (Coleoptera). Z Zellforsch 134:85-96

Cook BJ (1981) The action of proctolin and 5- hydroxytryptamine on the oviduct of the horsefly, Tabanus proximus. Int J Invert Reprod 3:209-212

Cook BJ, Holman GM (1975) The neural control of muscular activity in the hindgut of the cockroach Leucophaea maderae: prospects of its chemical mediation. Comp Biochem Physiol 50C:137-146

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

Cook BJ, Meola S (1987) Nerve-muscle networks in the accessory gland tubules of a male insect: structural and physiological properties. J Insect Physiol 33:791-801

Crossman AR, Kerkut GA, Pitman RM, Walker RJ (1971a) Electrically excitable nerve cell bodies in the central ganglia of two insect species Periplaneta americana and Shistocerca gregaria. Comp Biochem Physiol 40A:594-597

Crossman AR, Kerkut GA, Walker RJ (1971b) Axon pathways of electrically excitable cell bodies in the insect nervous

system. J Physiol 218:55-56

Dallai R (1975) Fine structure of the spermatheca of Apis mellifera. J Insect Physiol 21:89-109

Davey KG (1958) The migration of spermatozoa in the female of Rhodnius prolixus Stal. J Exp Biol 35:694-701

Davey KG (1960) The evolution of spermatophores in insects. Proc R Ent Soc London (A)35:107-113

Davis NT (1977) Motor neurons of the indirect flight muscles of Dysdercus fulvoniger. Ann Entomol Soc Am 70:377-386.

Davis NT, Alanis J (1979) Morphological and electrophysiological characteristics of a dorsal unpaired median neuron of the cricket, Acheta domesticus. Comp Biochem Physiol 62A:777-788

Davis NT, Velleman, SG, Kingan TG, Keshishian H (1989) Identification and distribution of a proctolin-like neuropeptide in the nervous system of the Gypsy moth, Lymantria dispar, and in other Lepidoptera. J Comp Neurol 283: 71-85

De Loof A, Lagasse A (1972) The structure of the male accessory reproductive glands of the Colorado beetle. Z Zellforsch 130:545-552

DuPorte M (1920) The muscle system of Gryllus assimilis Fabr. (=Pennsylvanicus Barum.). Ann Entomol Soc Am 3:16-52

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

Eckert M, Ude J (1983) Immunocytochemical techniques for the

identification of peptidergic neurons. In: Strausfeld NJI (ed) Functional Neuroanatomy. Springer Series in Experimental Entomology. Springer, Heidelberg Berlin New York, pp 267-301

Eckert M, Varanka I, Benedeczky I (1989) Proctolinergic innervation of the locust oviduct. Zool Jb Physiol 93: 471-479

Evans PD (1980) Biogenic amines in the insect nervous system. Adv Insect Physiol 15:317-473

Evans PD (1981) Octopaminergic modulation of neuromuscular transmission in the locust. In: Rozsa KS (ed) Advances in Physiological Science, Vol.22 Neurotransmitters in Invertebrates. Pregamon Press, Oxford; Akademia Kiado, Budapest

Evans PD, O'Shea M (1977) The identification of an octopaminergic neurone which modulates neuromuscular transmission in the locust. Nature 270:257-259

Evans PD, O'Shea M (1978) The identification of an octopaminergic neurone and modulation of a myogenic rhythm in the locust. J Exp Biol 73:235-260

Evans PD, Siegler MVS (1982) octopamine mediated relaxation of maintained and catch tension in locust skeletal muscle. J Physiol 324:93-112

Filosi M, Perotti ME (1975) Fine structure of the spermatheca of Drosophila melanogaster Meig. J Submicr Cytol 7:259-270

Filshie BK, Waterhouse DF (1968) The fine structure of the lateral scent glands of the green vegetable bug, Nezara viridula (Hemiptera, Pentatomidae). J Microscopie 7:231-

244

Friedel T, Gillott C (1977) Contribution of male-produced protein to vitellogenesis in Melanoplus sanguinipes. J Insect Physiol 23:145-151

Gupta BL, Smith DS (1969) Fine structural organization of the spermatheca in the cockroach, Periplaneta americana. Tissue and Cell 1:295-324

Happ GM, Happ CM (1970) Fine structure and histochemistry of the spermathecal gland in the mealworm beetle, Tenebrio molitor. Tissue and Cell 2:443-466

Happ GM, Happ CM (1975) Fine structure of the spermatheca of the mealworm beetle (Tenebrio molitor L.). Cell Tissue Res 162:253-269

Holman GM, Cook BJ (1985) Proctolin, its presence in and action on the oviduct of an insect. Comp Biochem Physiol 80C:61-64

Hoyle G (1975) Evidence that insect dorsal unpaired median (DUM) neurons are octopaminergic. J Exp Zool 193:425-431

Hoyle G (1978) The dorsal unpaired median neurons of the locust metathoracic ganglion. J Neurobiol 9:43-57

Hoyle G, Dagan D (1978) Physiological characteristics and reflex activation of DUM (octopaminergic) neurons of locust metathoracic ganglion. J Neurobiol 9:59-79

Hoyle G, Dagan D, Moberly B, Colquhoun W (1974) Dorsal unpaired median insect neurons make neurosecretory endings on skeletal muscle. J Exp Zool 187:159-165

Hoyle G, Colquhoun W, Williams M (1980) Fine structure of an octopaminergic neuron and its terminals. J Neurobiol 11:103-126

Huebner E (1980) Spermathecal ultrastructure of the insect Rhodnius prolixus Stal. J Morphol 166:1-25

Hustert R, Topel U (1986) Location and major postembryonic changes of identified 5-HT-immunoreactive neurones in the terminal ganglion of a cricket (Acheta domesticus). Cell Tissue Res 245: 615-621

Jones JC, Fischman DA (1970) An electron microscopic study of the spermathecal complex of virgin Aedes aegypti mosquitoes. J Morphol 132:293-312

Keshishian H, O'Shea M (1985) The distribution of a peptide neurotransmitter in the postembryonic grasshopper central nervous system. J Neurosci 5:992-1004

Khalifa A (1949) The mechanism of insemination and the mode of action of the spermatophore in Gryllus domesticus. Quart J Micr Sci 90:281-292

Kiss T, Varanka I, Benedeczky, I (1984) Neuromuscular transmission in the visceral muscle of locust oviduct. Neuroscience 12: 309-322

Kimura T, Kotoh Y, Yamaguchi T (1987) Morphological and functional features of neurons innervating the reproductive organs in the female cricket. Zool Sci 4:961

Kimura T, Yamaguchi T (1987) Initiation of rhythmic movement of the spermathecal duct by the contents of spermatophore in the female cricket. Dobutsu seiri 4:130

Kimura T, Yasuyama K, Yamaguchi T (1988) Effect of some drugs and bioactive substance derived from the spermatophore on the mechanical activity of the spermathecal duct in female cricket. Zool Sci 5:1197

Lange AB (1990) The presence of proctolin in the reproduc-

tive system of Rhodnius prolixus. J Insect Physiol 36:345-351

Lange AB, Loughton BG (1985) An oviposition-stimulating factor in the male accessory reproductive gland of the locust, Locusta migratoria. Gen Comp Endocrinol 57:208-215

Lange AB, Orchard I (1984a) Dorsal unpaired median neurons, and ventral bilaterally paired neurons, project to a visceral muscle in an insect. J Neurobiol 15:441-453

Lange AB, Orchard I(1984b) Some pharmacological properties of neuromuscular transmission in the oviduct of the locust, Locusta migratoria. Arch Insect Biochem Physiol 1:231-241

Lange AB, Orchard I (1986) Identified octopaminergic neurons modulate contractions of locust visceral muscle via adenosine 3', 5'-monophosphate (cyclic AMP). Brain Res 363:340-349

Lange AB, Orchard I, Adams ME (1986) Peptidergic innervation of insect reproductive tissue: the association of proctolin with oviduct visceral musculature. J Comp Neurol 254:279-286

Lange AB, Orchard I, Barrett, FM (1988) The presence and distribution of proctolin in the blood-feeding bug, Rhodnius prolixus. J Insect Physiol 34: 379-386

Lange AB, Orchard I, Loughton BG (1984) Spontaneous and neurally evoked contractions of visceral muscles in the oviduct of Locusta migratoria. Arch Insect Biochem Physiol 1:179-190

Lawson FA (1970) Ultrastructural comparison of the sper-

mathecae in Periplaneta americana (Blattaria: Blattidae). J Kans Entomol Soc 43:418-434

Levine RB, Murphey RK (1980) Pre- and postsynaptic inhibition of identified giant interneurons in the cricket (Acheta domesticus). J Comp Physiol 135:269-282

Loher W (1974) Circadian control of spermatophore formation in the cricket Teleogryllus commodus Walker. J Insect Physiol 20:1155-1172

Marder E, Hooper SL, Siwicki KK (1986) Modulatory action and distribution of the neuropeptide proctolin in the crustacean stomatogastric nervous system. J Comp Neurol 243:454-467

Mercer EH, Brunet PCJ (1959) The electron microscopy of the left colleterial gland of the cockroach. J Biophys Biochem Cytol 5:257-262

Miller T, James J (1976) Chemical sensitivity of the hyperneural nerve-muscle preparation of the American cockroach. J Insect Physiol 22:981-988

Nässel DR (1983) Horseradish peroxidase and other heme proteins as neuronal markers. In: Strausfeld NJI (ed) Functional Neuroanatomy. Springer Series in Experimental Entomology. Springer, Heidelberg Berlin New York pp 44-91

Nässel DR, Holmqvist BI, Movérus BJA (1989) Vasopresin- and proctolin-like immunoreactive efferent neurons in blowfly abdominal ganglia: development and ultrastructure. J Comp Neurol 283: 450-463

Nässel DR, O'Shea M (1987) Proctolin-like immunoreactive neurons in the blowfly central nervous system. J Comp

Neurol 265: 437-454

Newman GR, Jasani B, Williams ED (1983) A simple post-embedding system for rapid demonstration of tissue antigens under the electron microscope. Histochem J 15:543-555

Noirot C, Quennedy A (1974) Fine structure of insect epidermal glands. Ann Rev Entomol 19:61-80

Odhiambo TR (1970) The architecture of the accessory reproductive glands of the male desert locust. 3. Components of the muscular wall. Tissue and Cell 2:233-248

Orchard I, Belanger JH, Lange AB (1989) Proctolin: a review with emphasis of insects. J Neurobiol 20:470-496

Orchard I, Lange AB (1985) Evidence for octopaminergic modulation of an insect visceral muscle. J Neurobiol 16:171-181

Orchard I, Lange AB (1987) The release of octopamine and proctolin from an insect visceral muscle: effects of high-potassium saline and neural stimulation. Brain Res 413:251-258

O'Shea M, Evans PD (1979) Potentiation of neuromuscular transmission by an octopaminergic neurone in the locust. J Exp Biol 79:169-190

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

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

Panov A (1964) Correlations in the ontogenetic development of the central nervous system in the house cricket Gryl-

lus domesticus L. and the mole cricket Gryllotalpa gryllotalpa L. (Orthoptera, Grylloidea). Entomol Rev 45:179-185

Pflüger H-J, Watson AHD (1988) Structure and distribution of dorsal unpaired median (DUM) neurones in the abdominal nerve cord of male and female locusts. J Comp Neurol 268:329-345

Plotnikova SI (1969) Effector neurons with several axons in the ventral nerve cord of the Asian grasshopper Locusta migratoria. J Evol Biochem Physiol 5:276- 278.

Ranganathan LS (1977) Endocrine regulation of development and differentiation of the accessory reproductive gland of male Plebeiogryllus guttiventris. Adv Invert Reprod India 1:252-257

Roth J (1983) The colloid gold marker system for light- and electron microscopic cytochemistry. In: Bullock GR, Petrusz P (ed) Techniques in Immunocytochemistry, vol. 2. Academic Press, London New York pp 217-284

Schwarz TL, Harris-Warrick RM, Glusman S, Kravitz EA (1980) A peptide action in a lobster neuromuscular preparation. J Neurobiol 11:623-628

Schwarz TL, Lee GM-H, Siwicki KK, Standaert DG, Kravitz EA (1984) Proctolin in the lobster: the distribution, release, and chemical characterization of a likely neurohormone. J Neurosci 4:1300-1311

Schiwicki KK, Bishop CA (1986) Mapping of proctolin-like immunoreactivity in the nervous systems of lobster and crayfish. J Comp Neurol 243:435-453

Schiwicki KK, Belz BS, Kravitz EA (1987) Proctolin in iden-

tified serotonergic, dopaminergic and cholinergic neurons in the lobster, Homarus americanus. J Neurosci 7:522-532

Sobek L, Eckert M, Penzlin H, Reissmann S (1986) Evidence for proctolinergic innervation of the cockroach oviduct. Zool Jb Physiol 90: 461-466

Stoya G, Agricola H, Eckert M, Penzlin H (1989) Investigations on the innervation of the oviduct muscle of the cockroach, Periplaneta americana (L.). Zool Jb Physiol 93:75-86

Stuart AE, Hudspeth AJ, Hall ZW (1974) Vital staining of specific monoamine-containing cells in the leech nervous system. Cell Tissue Res 153:55-61

Sugawara T (1987) Cuticular lining in the genital chamber of the cricket - an obstacle to prostaglandin diffusing? Int J Invertebr Reprod Dev 12:213-216

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

Tublitz NJ, Truman JW (1985) Identification of neurones containing cardioacceleratory peptides (CAPs) in the ventral nerve cord of the tabacco hawkmoth, Manduca sexta. J Exp Biol 116:395-410

Ude J, Eckert M (1988) Submicroscopic characterization of proctolin-like immunoreactivity in the nervous system of the cockroach Periplaneta americana L. Cell Tissue Res 254:197-202

Veenstra JA, Romberg-Privee HM, Schooneveld H (1985) A proctolin-like peptide and its immunocytochemical localization in the Colorado potato beetle, Leptinotarsa decemlineata. Cell Tissue Res 240: 535-540

Wallace BG, Talamo BR, Evans PD, Kravitz, EA (1974) Octopamine: selective association with specific neurons in the lobster nervous system. Brain Res 74: 349-355

Watson AHD (1984) The dorsal unpaired median neurons of the locust metathoracic ganglion: neuronal structure and diversity, and synapse distribution. J Neruocytol 13:303-327

Yamaguchi T, Kushiro N, Waki T (1985) Sexual dimorphism of the terminal abdominal ganglion of the cricket. Naturwiss 72:153-154

Yamaoka K, Hirano T (1977) Stimulation of original oviposition by male factor and its effect on spontaneous neurons activity in Bombyx mori. J Insect Physiol 23:57- 63

Yasuyama K, Kimura T, Yamaguchi T (1988) Ultrastructural and immunohistochemical investigation of the spermathecal duct of the female cricket, Gryllus bimaculatus. Zool Sci 5:1196

Yasuyama K, Yamaguchi T, Kimura T (1984) Morphological differentiation of transplanted male accessory glands and their mechanical activity in the cricket. Dobutsu Seiri 1:164

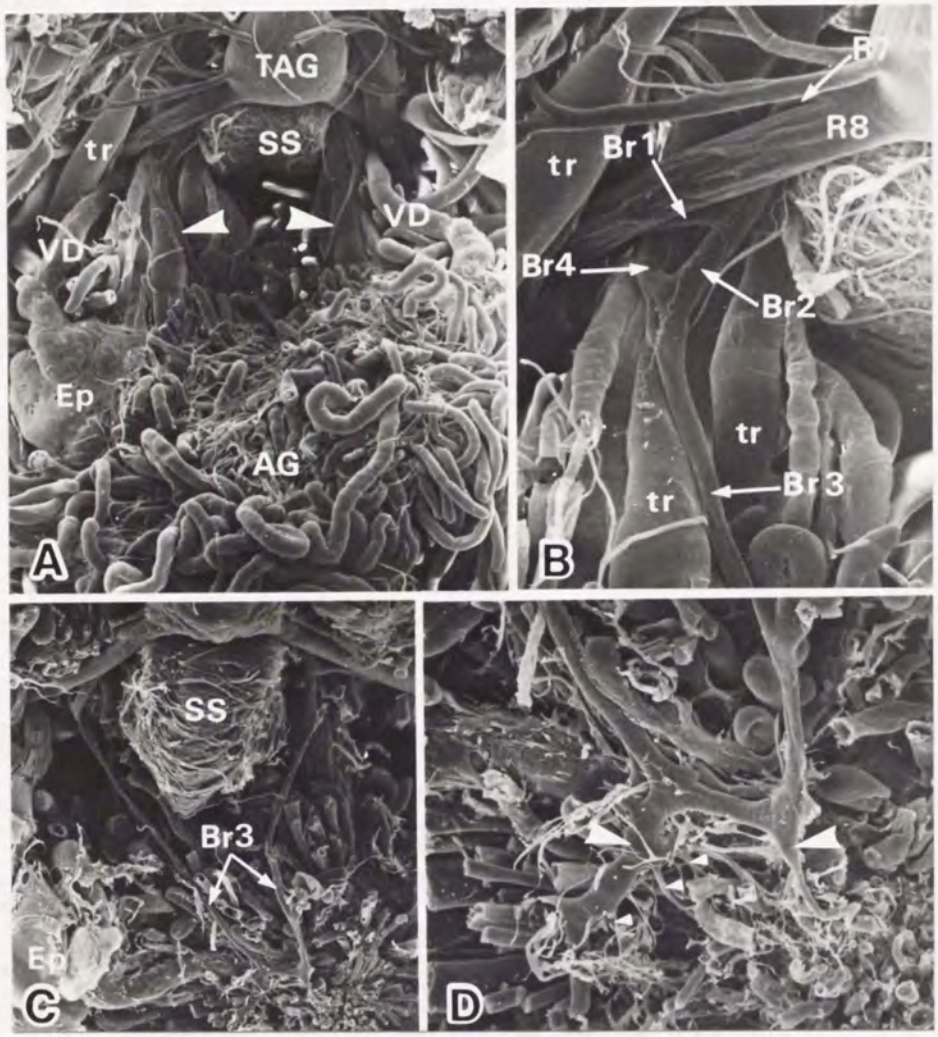

Fig. 1A-D. Scanning electron microphotographs of the male reproductive organs and their innervation. (A) Dorsal view of the terminal abdominal ganglion (TAG) and reproductive organs; X31. In this preparation, the accessory gland (AG) was drawn back to expose terminal abdominal ganglion. Arrow heads indicate the left and right nerve branches innervating the reproductive organs. (B) A higher magnification view of the left nerve branches shown in A; X82. Note that the seventh nerve root (R7) emerging from the terminal abdominal ganglion is divided into four nerve branches (Br1-Br4). (C) Dorsal view of Br3s innervating accessory gland; X33. In this preparation, the Br3s were exposed by removing the gland tubules just covering them and their neighborhood. Note that the left and right Br3s are joined to form a loop

(arrow head). (D) A higher magnification view of the loop in C; X100. Two nerve twigs (large arrow heads) with fine twiglets (small arrow heads) emerge from the loop. Ep, epididymis; R8, eighth nerve root; SS, spermatophore sac; tr, trachea; VD, vas deferens.

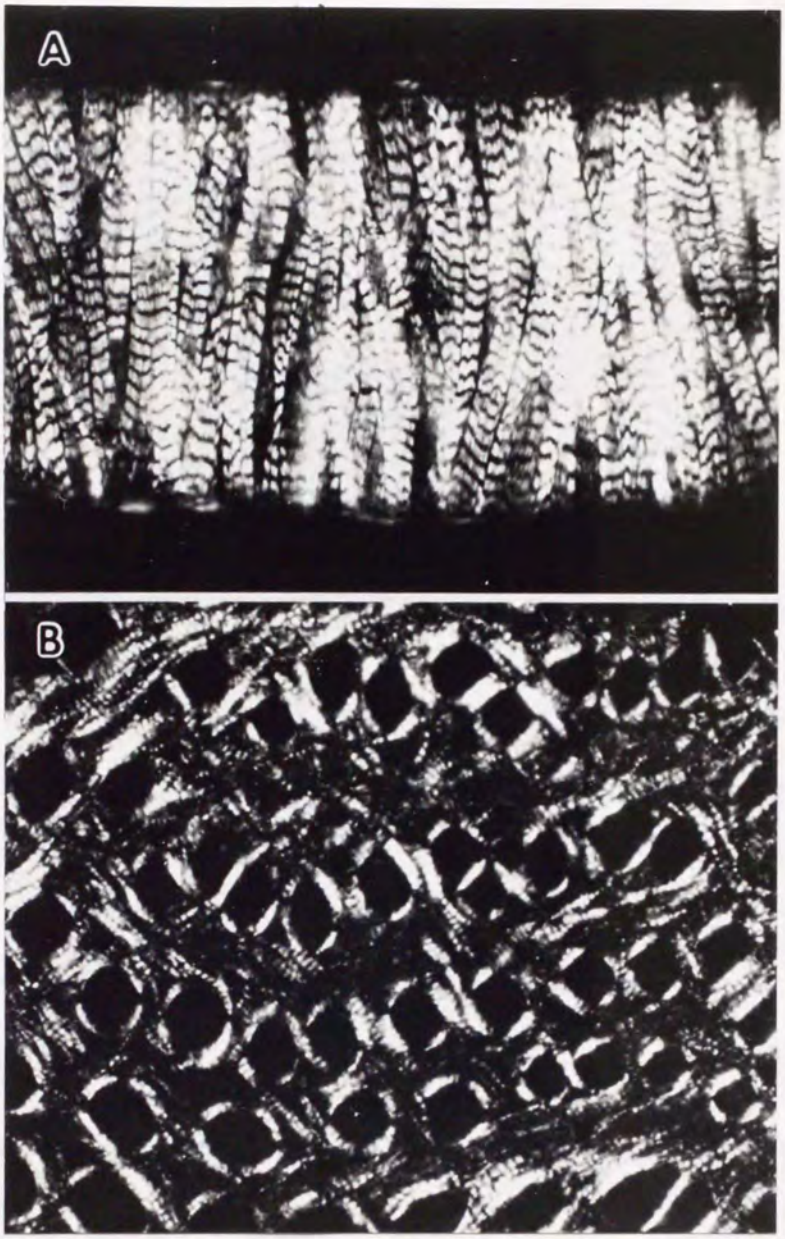

Fig. 2A,B. Polarized-light microphotographs of the muscle fibers. (A) The striated muscle fibers winding around an accessory gland tubule; X320. This microphotograph was taken after the secretions contained in the tubule were squeezed out by the contraction of tubule which was induced by application of high K^+ saline (in mM/l: NaCl, 70; KCl, 90; $CaCl_2$, 5; buffered with 10mM/l Tris to pH 7.2). (B) A cross section of the lobed anterior part of ejaculatory duct on which the orifices of the accessory gland are located; X160.

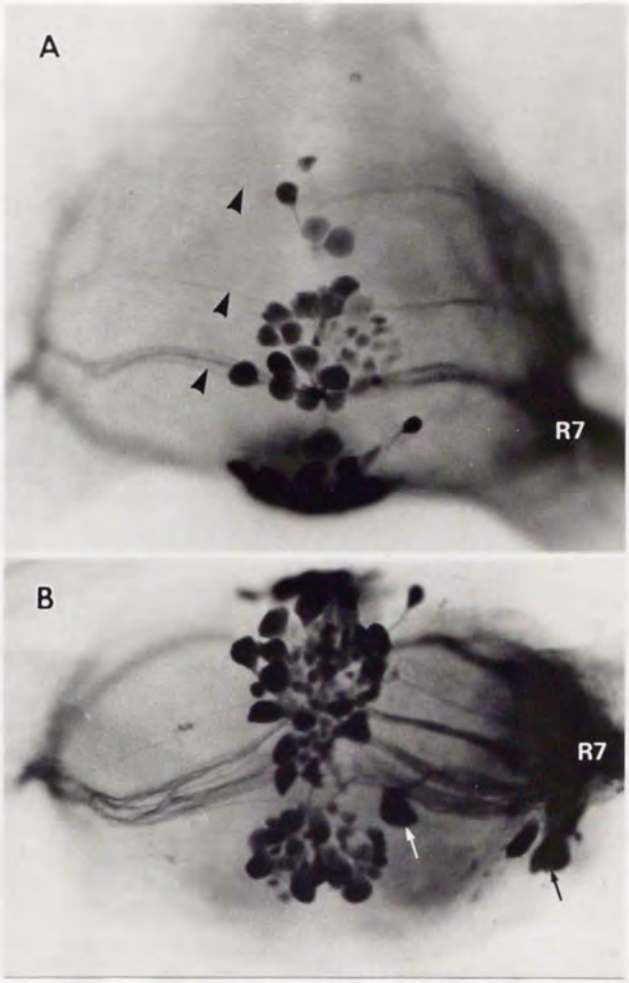

Fig. 3A,B. Light microphotographs of the terminal abdominal ganglion back-filled with nickel through the right Br3. (**A**) Dorsal view (montage); X90. (**B**) Caudal view; X90. Each arrow head indicates the secondary neurites bifurcating from the primary neurites of DUMR7 neurons and extending toward the left and right seventh nerve roots. Black arrows represent the lateral cluster of somata of LC neurons lying near the site where the right seventh nerve root (R7) emerges from the ganglion. A white arrow shows the somata of SR neuron which is located separately from the clusters.

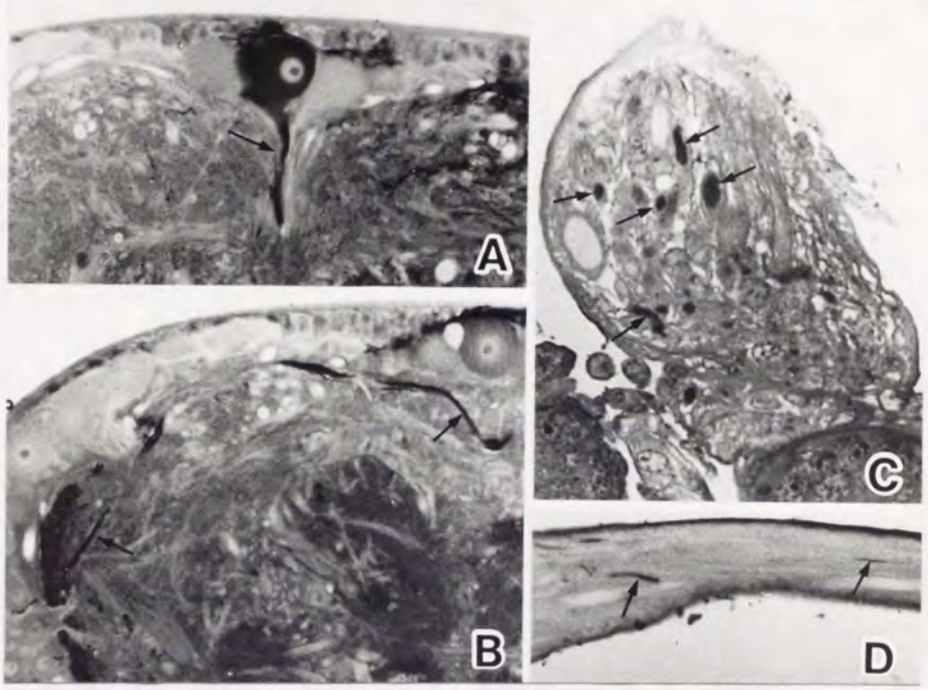

Fig. 4A-D. Light microphotographs of DUMR7 neurons labeled with HRP through the right Br3. **(A)** A transverse section through the terminal abdominal ganglion showing the soma and its primary neurite (arrow) of a labeled DUMR7 neuron; X200. **(B)** A transverse section through the terminal abdominal ganglion; X200. Arrows indicate the secondary neurites. **(C)** A transverse section of the left Br3 carrying labeled axons (arrows); X545. This section was made at the loop where the left and right Br3s were joined together. **(D)** A longitudinal section of a nerve twig arising from the loop; X310. Each arrow indicates the labeled axon running through the nerve twig.

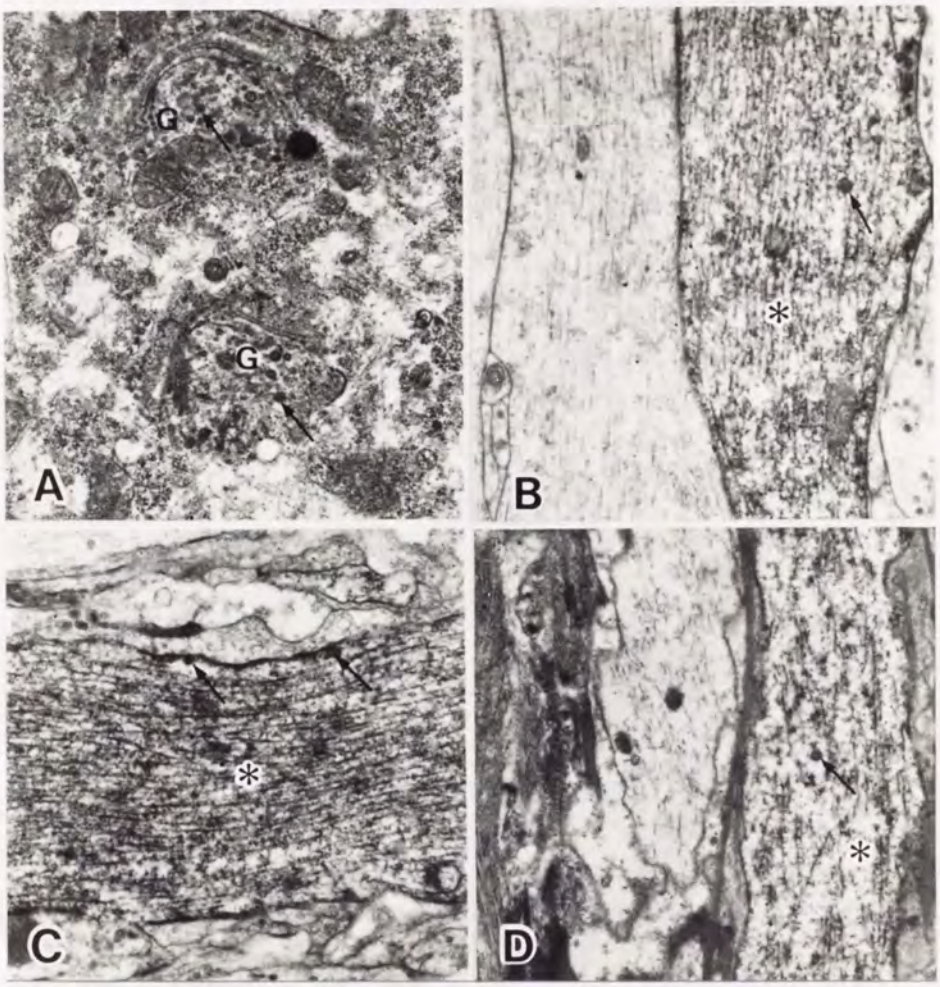

Fig. 5A-D. Electron microphotographs of HRP-labeled DUMR7. (**A**) The cytoplasm of labeled DUMR7 neuron soma; X12,500. Arrows indicate vesicles which appear to be associated with Golgi bodies (G). (**B**) A labeled primary neurite (asterisk) within the terminal abdominal ganglion; X12,500. The arrow shows a vesicle. (**C**) A labeled secondary neurite (asterisk) within the terminal abdominal ganglion; X12,500. Each arrow indicates a vesicle. (**D**) A labeled axon (asterisk) passing through the nerve twig shown in Fig. 4D; X12,500. This axon also has a vesicle (arrow).

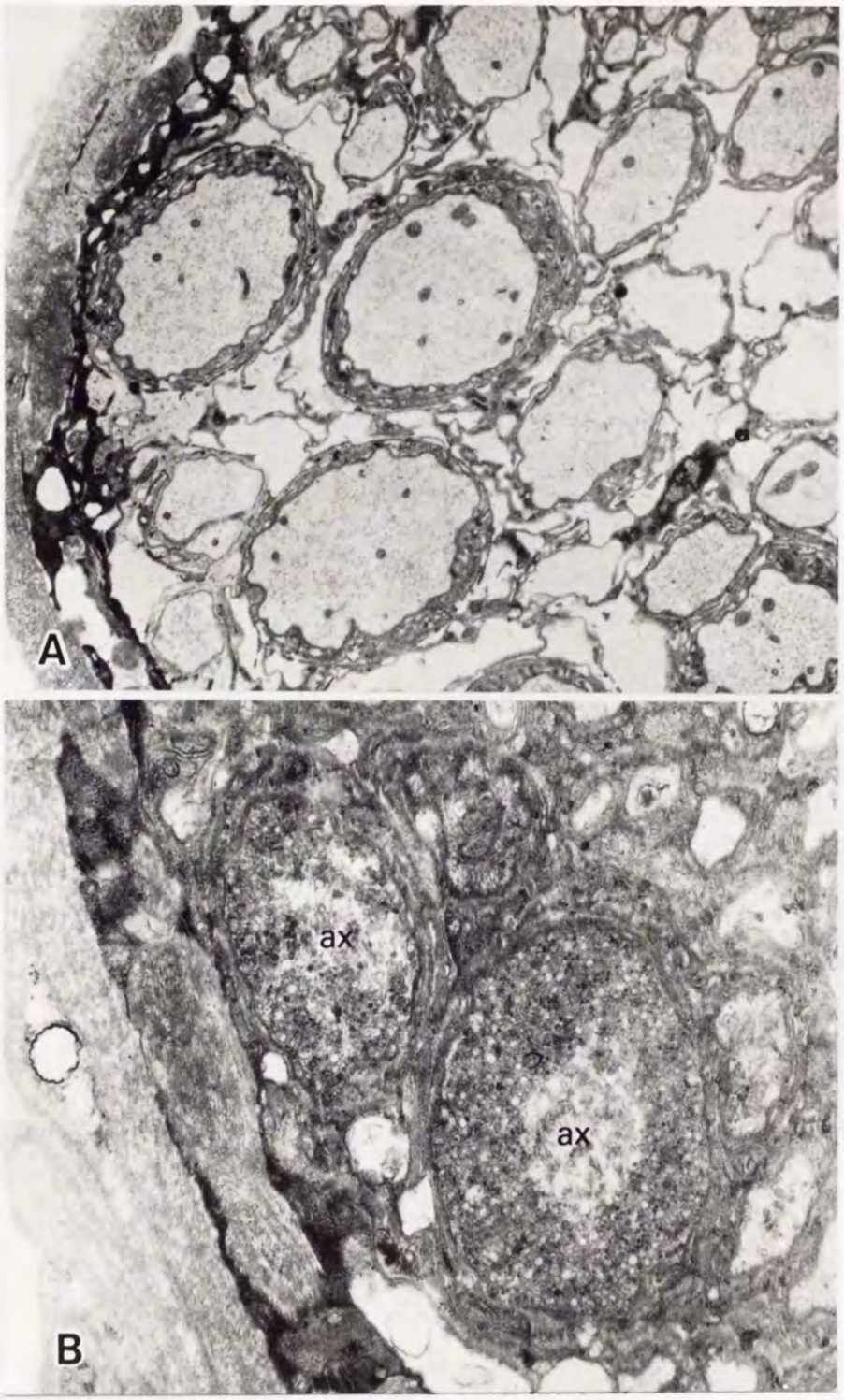

Fig. 6A,B. Transverse sections of the nerve branches, Br3. (A) The non-ligated (control) Br3 contralateral to the ligated Br3 shown in B; X7,000. (B) The ligated Br3; X14,000. Note that the accumulation of vesicles is seen in the axons (ax) with relatively large diameter.

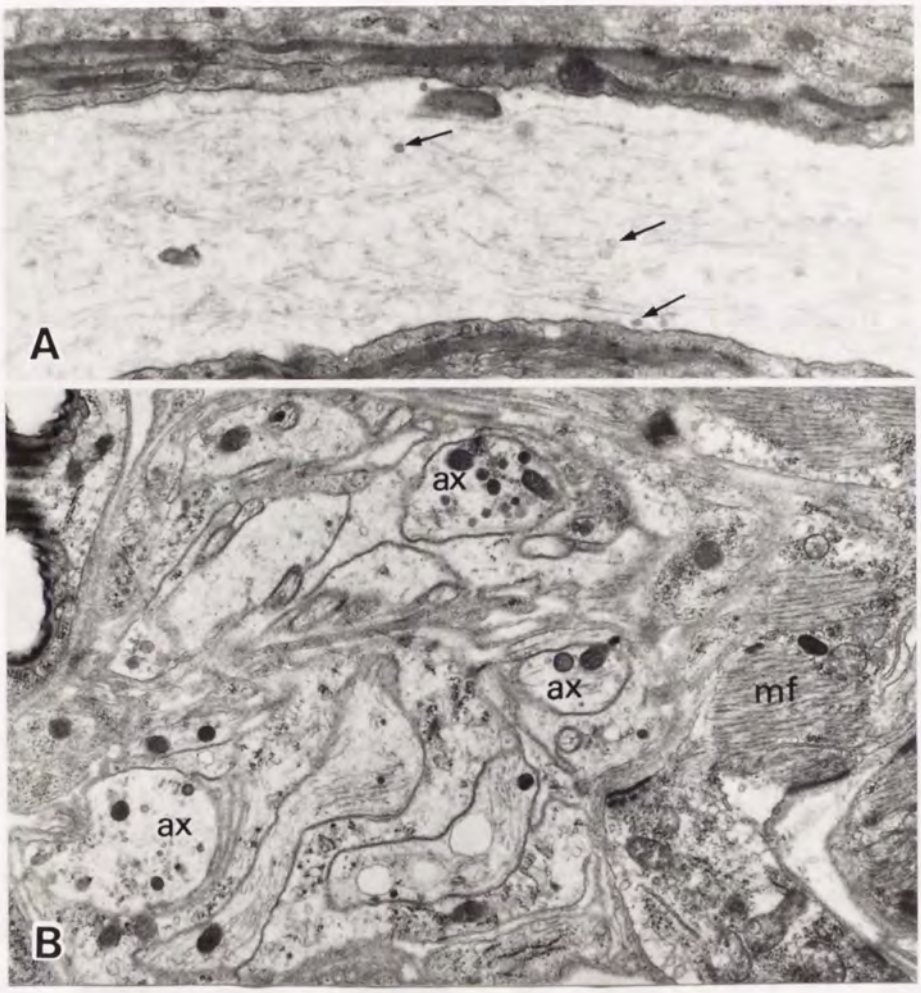

Fig. 7A,B. Axons which are distributed among the accessory gland tubules. (**A**) A longitudinal section of an axon passing through a thick nerve twig which emerges from the loop of Br3s; X15,000. Arrows indicate vesicles containing in the cytoplasm. (**B**) An oblique section of a thin nerve twiglet near the muscle fibers (mf) of accessory gland tubules; X16,000. Note that both small and large vesicles are visible in the cytoplasm of axons (ax).

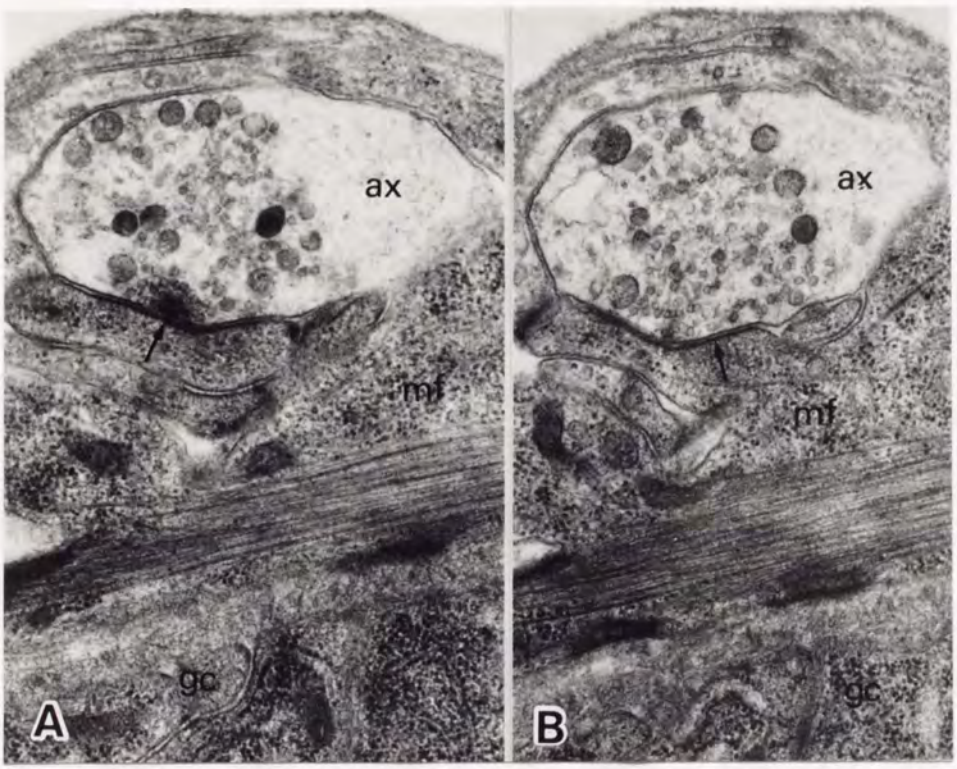

Fig. 8A,B. An axon terminal making the synaptic contact (arrows) with muscle fiber (mf) which envelops the accessory gland tubule; X27,000. Both **A** and **B** show the same axon terminal (ax) in different sections. Note that the axon terminal contains many large granular vesicles as well as small clear vesicles; the latter ones cluster along the presynaptic membrane. gc, epithelial cell of accessory gland tubule.

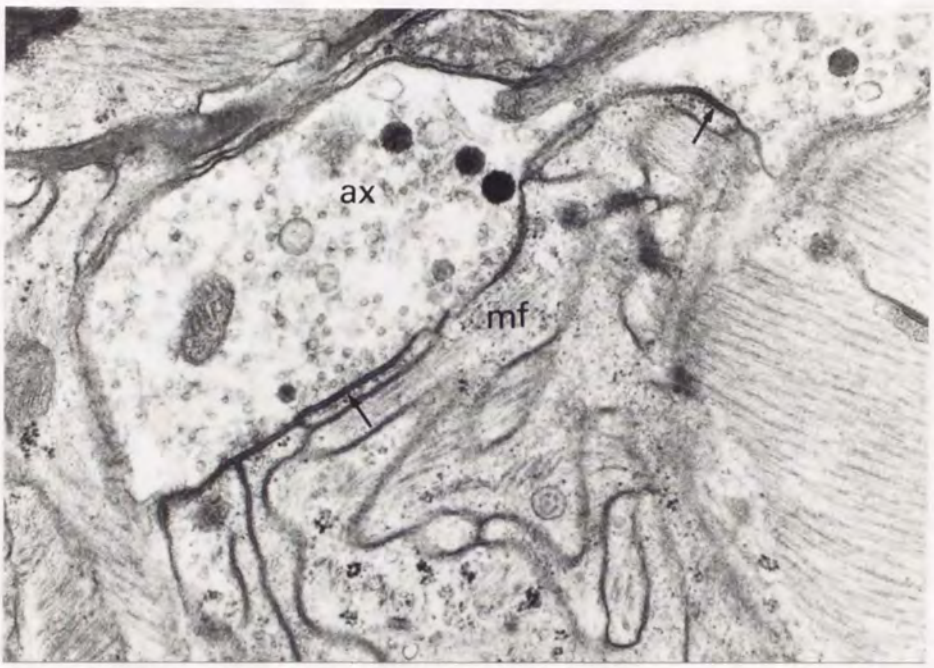

Fig. 9. An axon terminal within the proximal part of the accessory gland tubule, where multilayered muscle fibers are located; X29,000. Arrows indicate the dense postsynaptic membranes. Note that large and small vesicles are present within the axon terminal (ax), and the extension of muscle fiber (mf) forms a complex series of folds about the axon terminal.

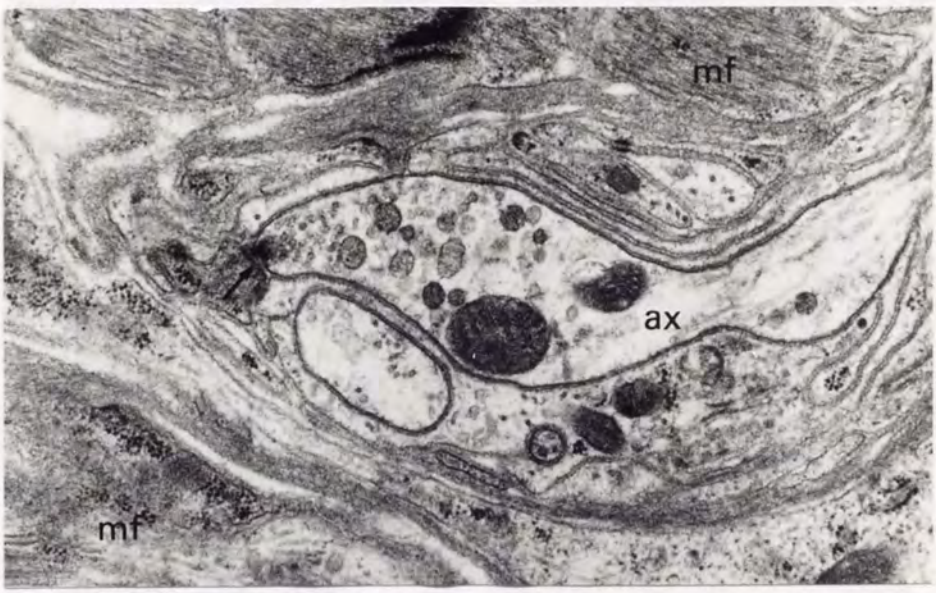

Fig. 10. An axon terminal within the anterior part of the ejaculatory duct where both the accessory gland tubules and epididymis open; X29,000. Large and small vesicles are also present in this axon terminal (ax). Numerous small vesicles cluster together at the presynaptic membrane (arrow). mf, muscle fiber.

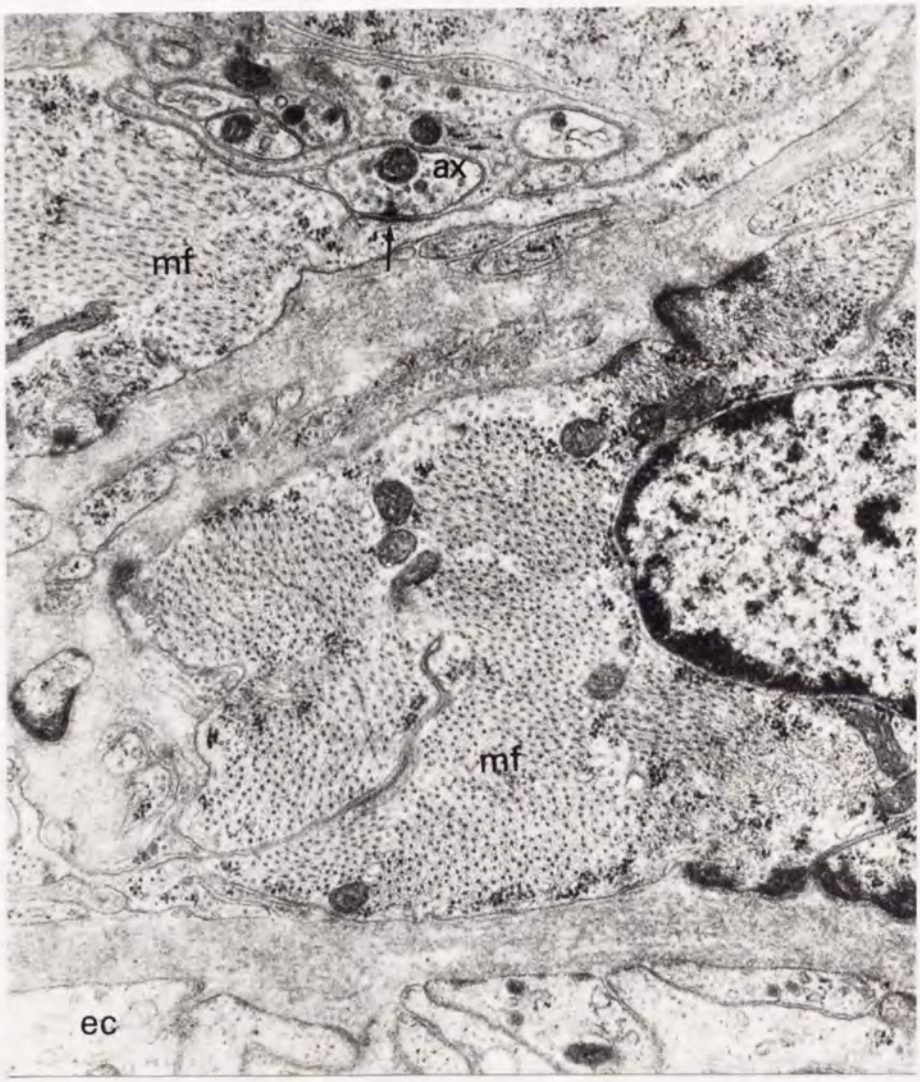

Fig. 11. A transverse section of the epididymis; X29,000. The epithelial cells of epididymis (ec) are enveloped by multilayered muscle fibers (mf). Note that an axon terminal (ax) containing both large and small vesicles makes synaptic contact (arrow) with the muscle fiber.

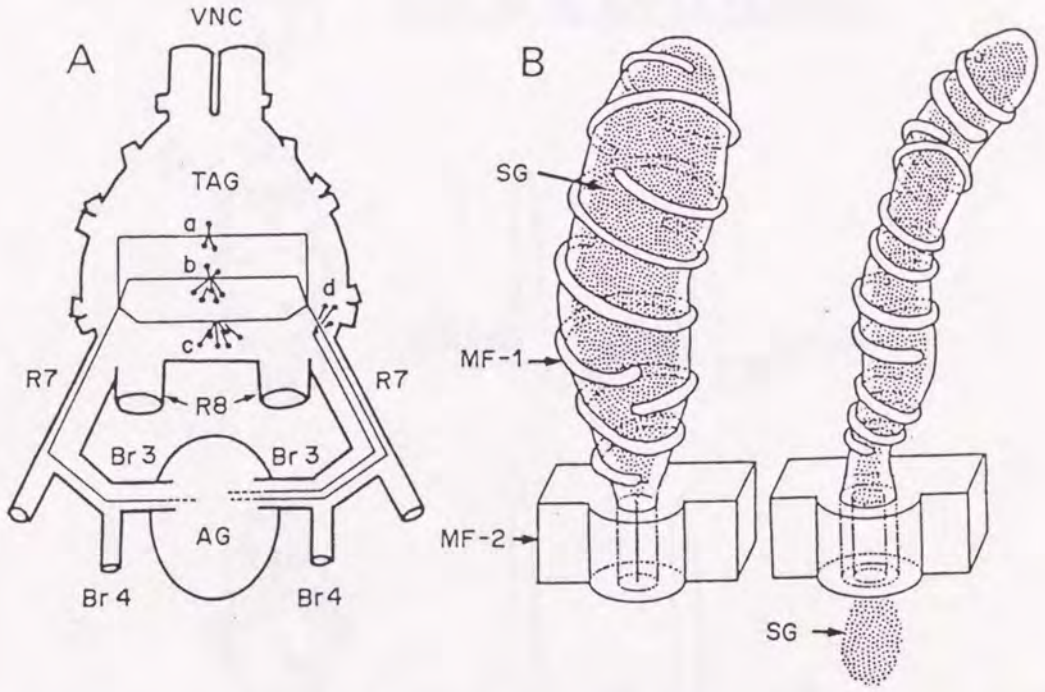

Fig. 12A,B. Schematic illustrations showing the innervation and musculature of the accessory gland. (**A**) Pathways of dorsal unpaired median neurons (DUMR7 neurons) and paired neurons (LC neurons) innervating the accessory gland. (**B**) Musculature of an accessory gland tubule. AG, accessory gland; a-c, soma clusters of DUMR7 neurons; d, soma cluster of LC neurons; R7 and R8, seventh and eighth nerve roots; Br3, Br4, nerve branches; SG, secretory granules; MF-1, muscle fiber winding around the tubule; MF-2, multilayered muscle fibers surrounding the exit of the accessory gland tubule; TAG, terminal abdominal ganglion; VNC, ventral nerve cord.

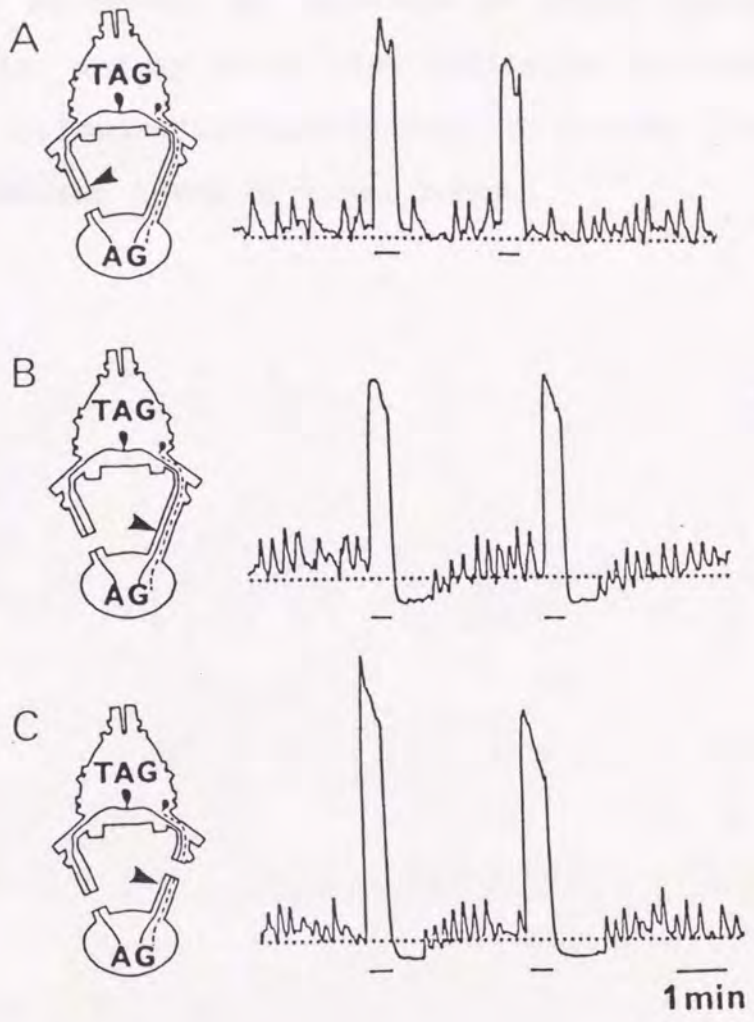

Fig. 13A-C. Spontaneous and neurally evoked contractions of the accessory gland. In **A** and **B**, electrical stimuli (2 Hz) were given antidromically to the left Br3 near its cut end or orthodromically to the intact right Br3. In **C**, after cutting the proximal region of the right Br3, the stimuli were given orthodromically to the same region as in **B**. Note the occurrence of spontaneous contractions, whether the accessory gland was connected to the terminal abdominal ganglion by the Br3s or not. Each horizontal line under the record indicates the duration (30s) of electrical stimulation. In each schematic illustration of the preparation, the solid and broken lines drawn within the contour of the prepa-

ration are represent the pathways of DUMR7 and LC neurons respectively, and an arrow head indicates the position of a paired of stimulating electrodes. A dotted line in each record shows the level of basal tonus.

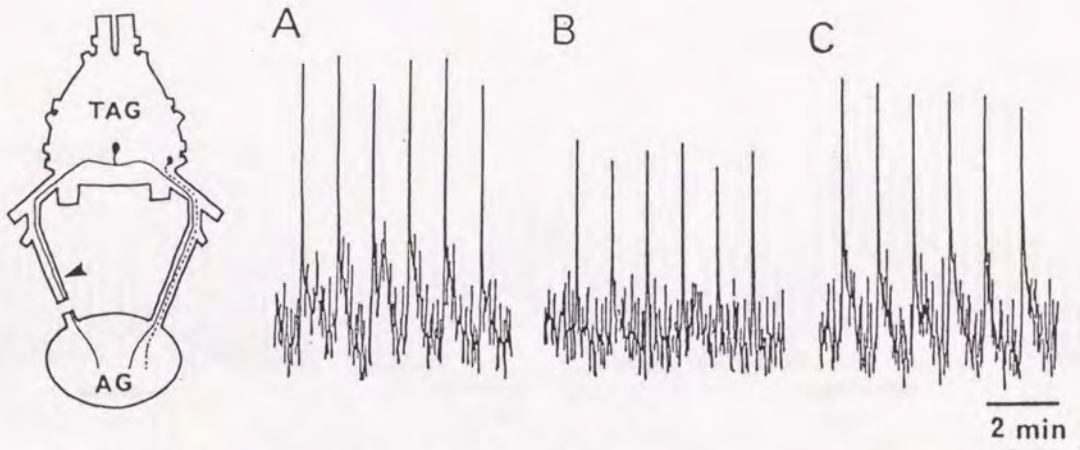

Fig. 14A-C. Effect of high Mg^{2+} saline on neurally evoked contractions of the accessory gland. **(A)** Neurally evoked contractions in normal saline. **(B)** Neurally evoked contractions in high Mg^{+2} saline. In an isolated accessory gland in preparation only the terminal abdominal ganglion was immersed in high Mg^{2+} saline, but the remainder in normal saline. The record was obtained 23 min after the ganglion was immersed in high Mg^{2+} saline. **(C)** Neurally evoked contractions 18 min after the high Mg^{2+} saline was replaced by normal saline. Electrical stimuli (frequency, 5 Hz; duration of each pulse, 0.1 ms; duration of stimulation, 4 s) were given antidromically to one of Br3s, as shown in the inset. See Fig. 13 for further explanations.

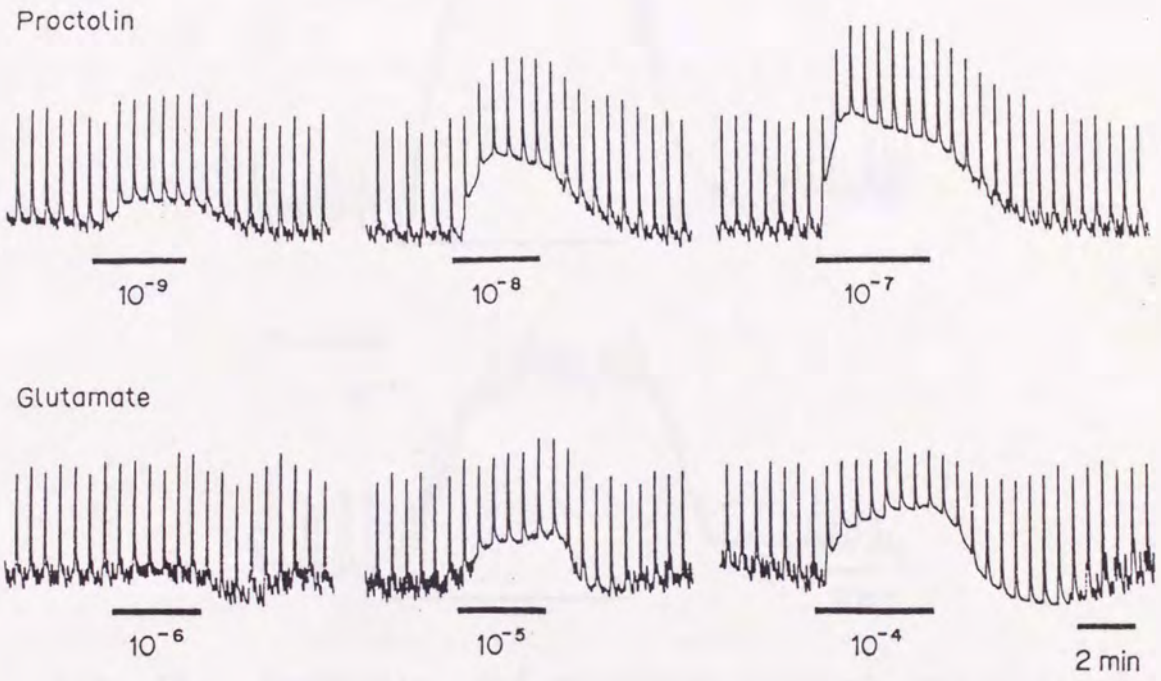

Fig. 15. Effects of proctolin and glutamate on neurally evoked contractions of the accessory gland. One of the Br3s innervating the accessory gland was antidromically stimulated at 10 Hz for 2 s every 30 s. These drugs were applied to the same preparation during the periods indicated by the horizontal lines under the records.

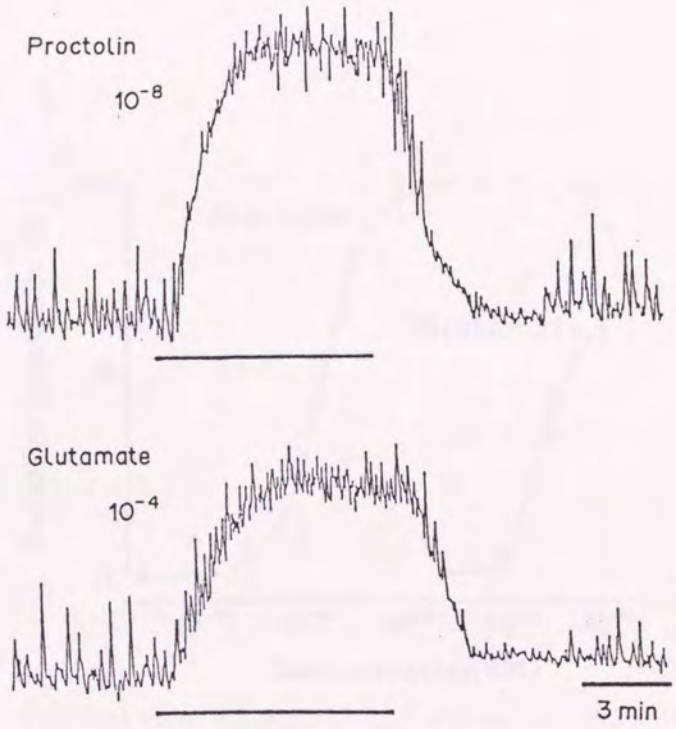

Fig. 16. Proctolin- and glutamate-induced contractions of the accessory gland. The horizontal lines under the records indicate the duration of application of drugs. The preparation was washed with saline following each drug. Note that the washing with saline resulted in an initial decrease in myogenic activity.

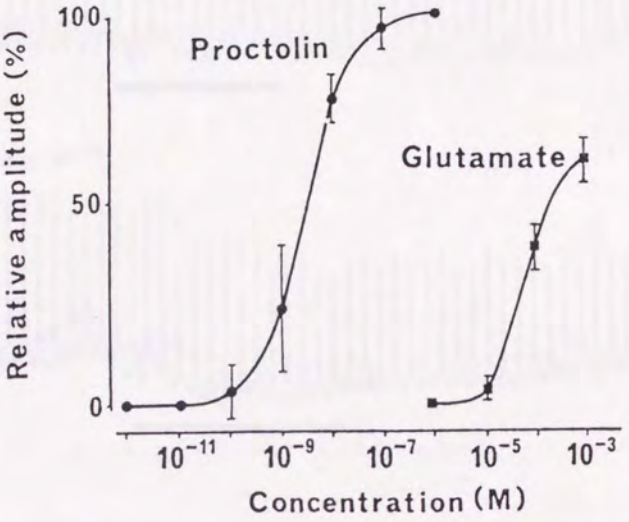

Fig. 17. Dose-response curves of proctolin and glutamate. The ordinate represents the relative amplitude of proctolin- or glutamate-induced contraction of the accessory gland: 100% corresponds to the amplitude of contraction induced by the application of 10^{-6} M proctolin. The preparation was washed in saline between each dose of drug.

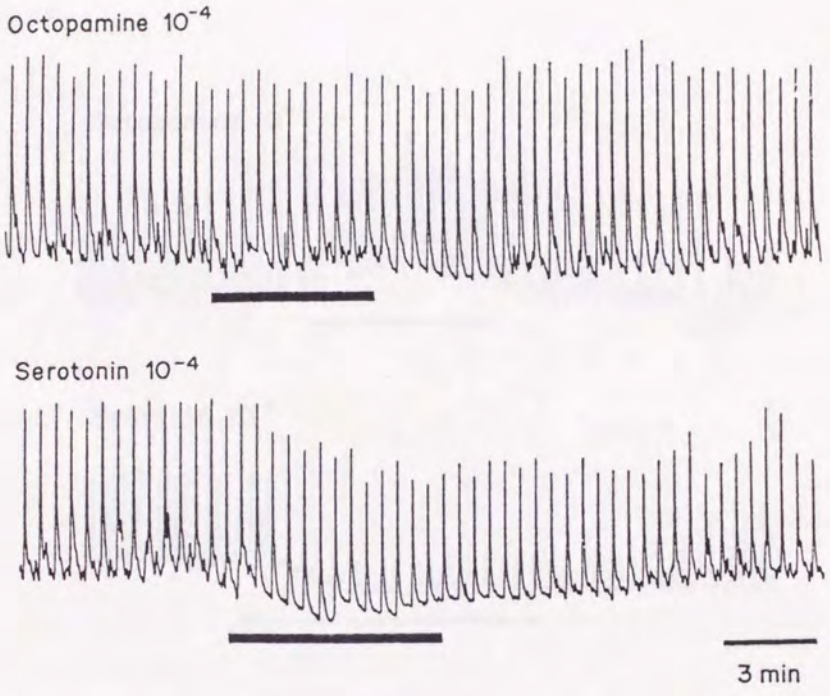

Fig. 18. Effects of 10^{-4} M octopamine and 10^{-4} M serotonin on neurally evoked contractions of the accessory gland. One of the Br3s innervating the accessory gland was antidromically stimulated at 5 Hz for 2 s every 30 s. Each horizontal line denotes the application of drug.

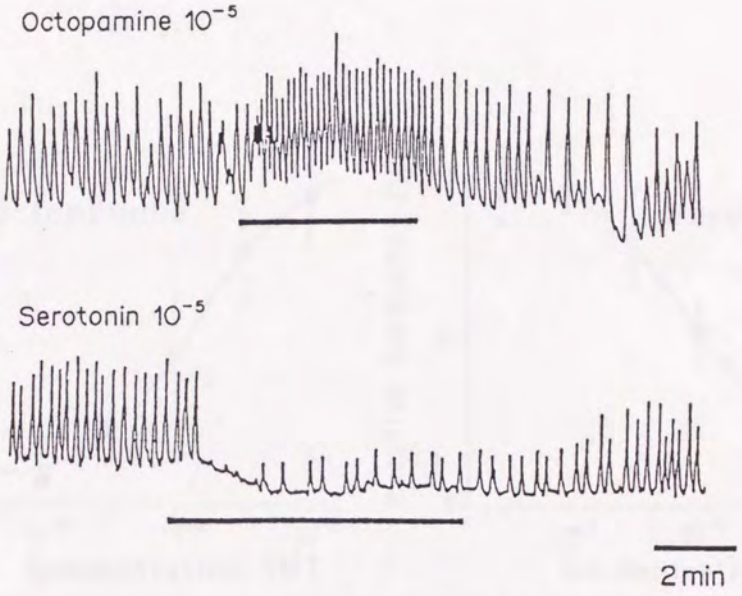

Fig. 19. Effects of 10^{-5} M octopamine and 10^{-5} M serotonin on myogenically evoked contractions of the accessory gland. See Fig. 18 for further explanation.

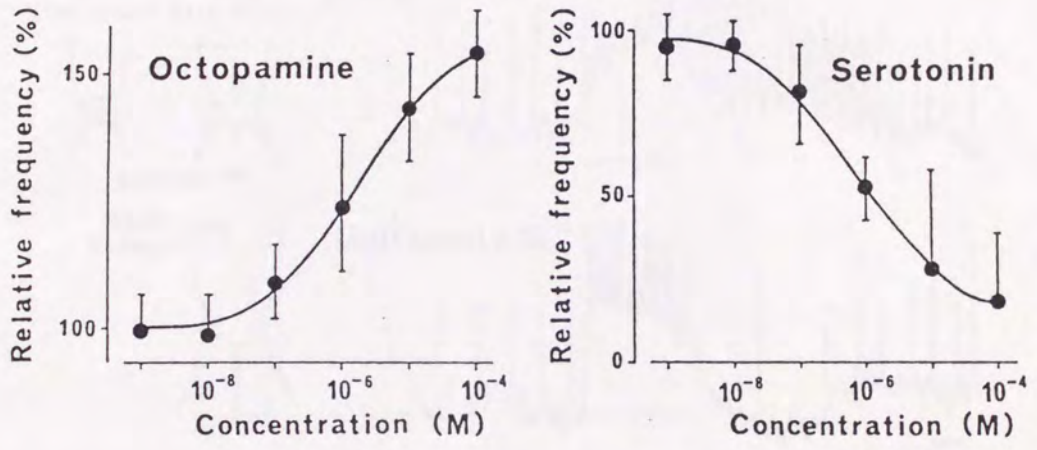

Fig. 20. Dose-response curves of octopamine and serotonin. Each ordinate represents the relative frequency of myogenically evoked contractions: 100% corresponds to the frequency of myogenically evoked contractions before the application of drug.

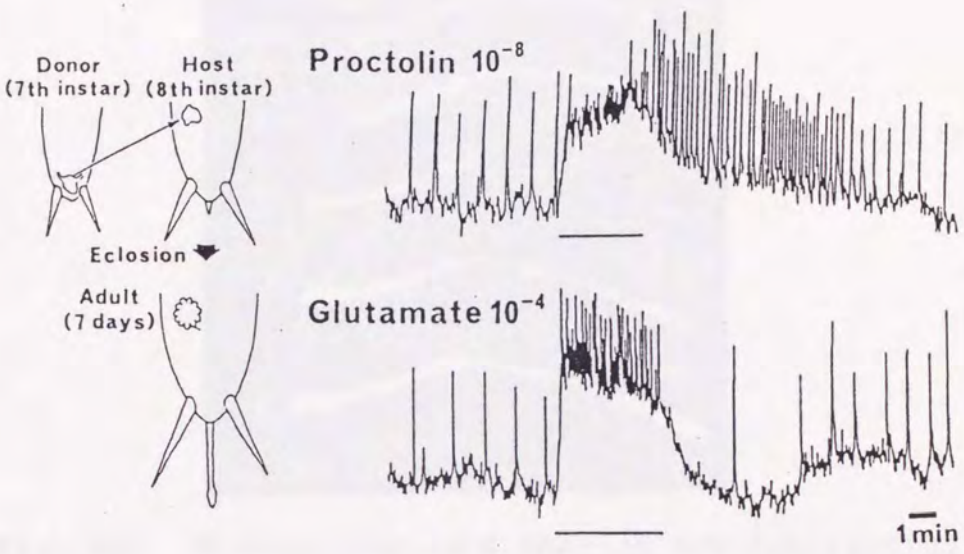

Fig. 21. Effects of 10^{-8} M proctolin and 10^{-4} M glutamate on the transplanted accessory gland which developed without innervation in a host. The insect illustrates the transplantation of the anlage of accessory gland from a donor to the abdomen of the host and the development of transplanted accessory gland in it. The horizontal lines indicate the duration of application of drugs.

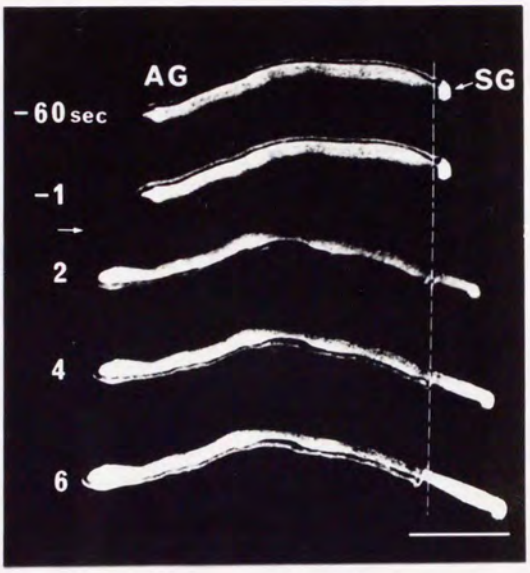

Fig. 22. Microphotographs showing the contraction of an isolated glandular tubule (AG), which has a milky white appearance, evoked by the application of 10^{-7} M proctolin. Negative and positive numerals indicate the time (s) before and after the application of proctolin. The vertical broken line represents the cut end of the tubule. Note that the elongation of the tubule and the release of secretory granules (SG) from its cut end to the bath were accompanied by contraction of the tubule. Length scale, 50 μm.

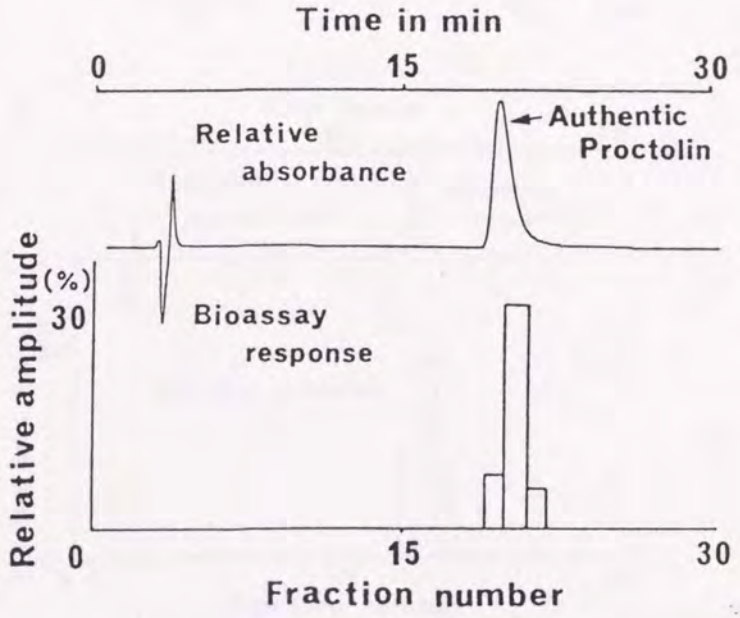

Fig. 23. HPLC analysis of accessory gland-stimulating substance extracted from the accessory glands of 20 males, compared with the standard solution (200 μl) containing 1.54×10^{-4} M authentic proctolin. Samples were applied to a reverse phase column equilibrated with 15% acetonitrile, 0.1% TFA, and eluted with a solvent flow rate of 1 ml/min. One-minute fractions were collected from 0 to 30 min. The accessory gland-stimulating substance in each fraction was determined by bioassay (see Chapter 2, Materials and Methods). On the ordinate, 100% of relative amplitude corresponds to the amplitude of contraction of the accessory gland induced by the application of 10^{-7} M proctolin.

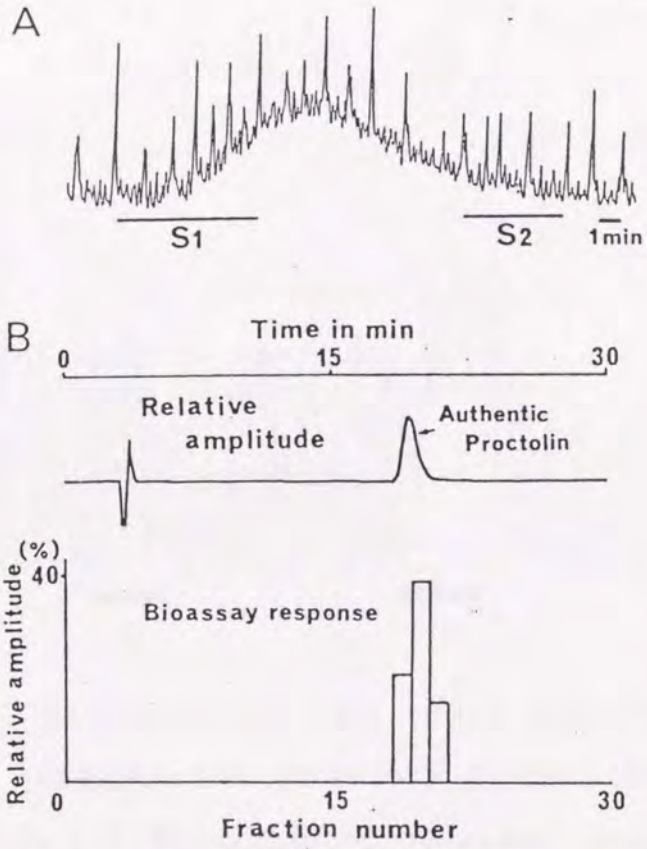

Fig. 24A,B. Stimulating effect and HPLC analysis of accessory gland-stimulating substance which was released from the accessory glands in response to high K^+ saline. (A) Bioassays of Sep-Pak extracts which were obtained from the surrounding media (normal saline and high K^+ saline) of the accessory gland. Eighty accessory glands were immersed in each medium for 15 min. The lines, S_1 and S_2, under the record indicate the duration of application of the extract of high K^+ saline and that of normal saline to the accessory gland, respectively. (B) HPLC analysis of the extract obtained from the surrounding medium, high K^+ saline, compared with the standard solution containing authentic proctolin. For further explanation see Fig. 23 and Chapter 2 (Materials and Methods).

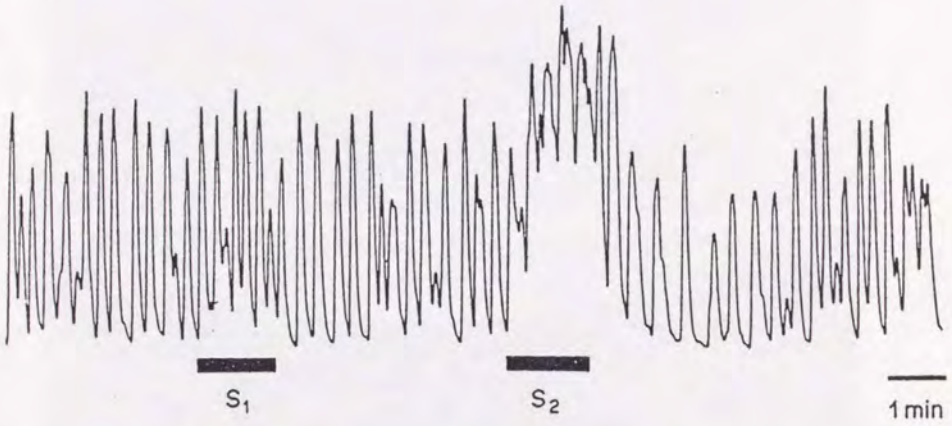

Fig. 25. Bioassays of the crude Sep-Pak extracts of normal and transplanted accessory glands. Both the extract obtained from six transplanted accessory glands (wet weight in total, 68 mg) and the extract from one normal accessory gland (wet weight, 36 mg) were applied to an isolated accessory gland preparation during the periods, S_1 and S_2, respectively. The anlagen of accessory glands of 8th insters were transplanted into the hosts of the same inster, and the accessory glands developing in the hosts were isolated from them 10 days after their imaginal moult. Note that the extract of normal accessory gland was the only extract to produce the contraction.

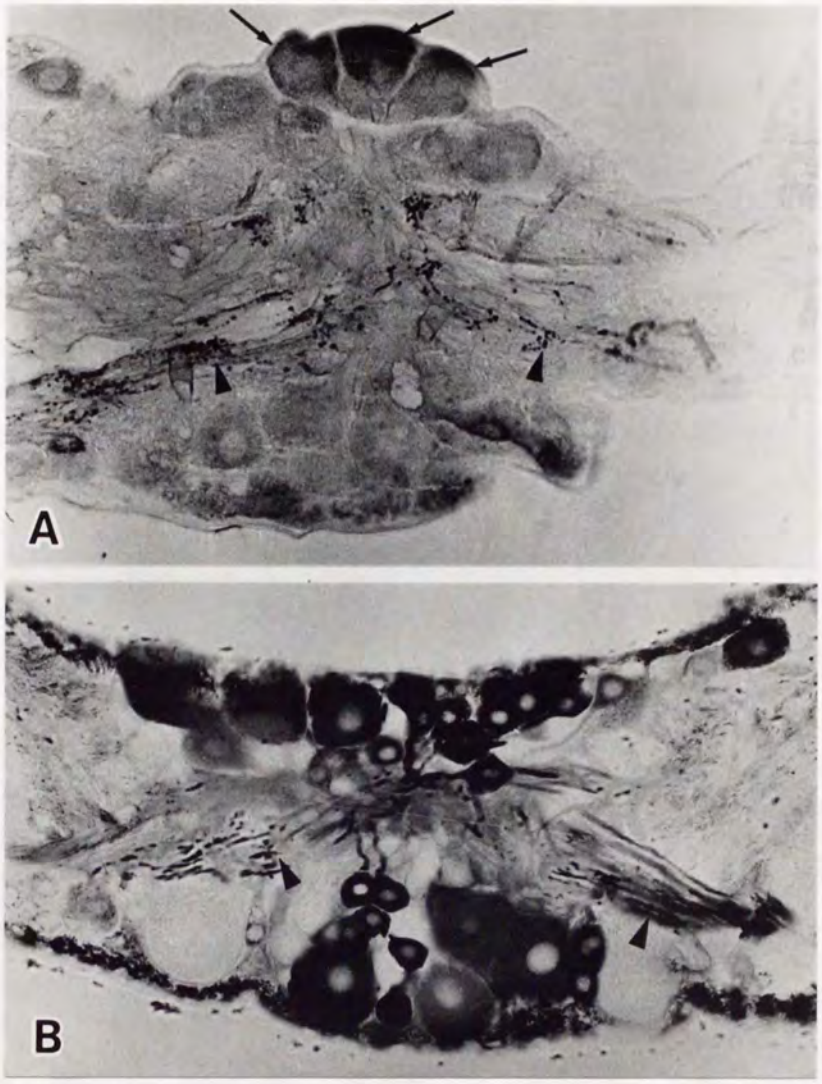

Fig. 26A,B. Light microphotographs of cross sections of the terminal abdominal ganglion of the cricket. (A) Proctolin-like immunoreactive, dorsal medial neurons (arrows) in the caudal region of the terminal abdominal ganglion; X230. Immunoreactive fiber profiles are also seen in the ventral commissure (arrow heads). (B) HRP-labeled DUMR7 neurons in the caudal region of the terminal abdominal ganglion; X230. HRP back-filling through the right Br3 reveals a number of DUMR7 somata and their fibers (arrow heads) running through the ventral commissure in the caudal region. The sectioning level of this section corresponds to that of the section in (A).

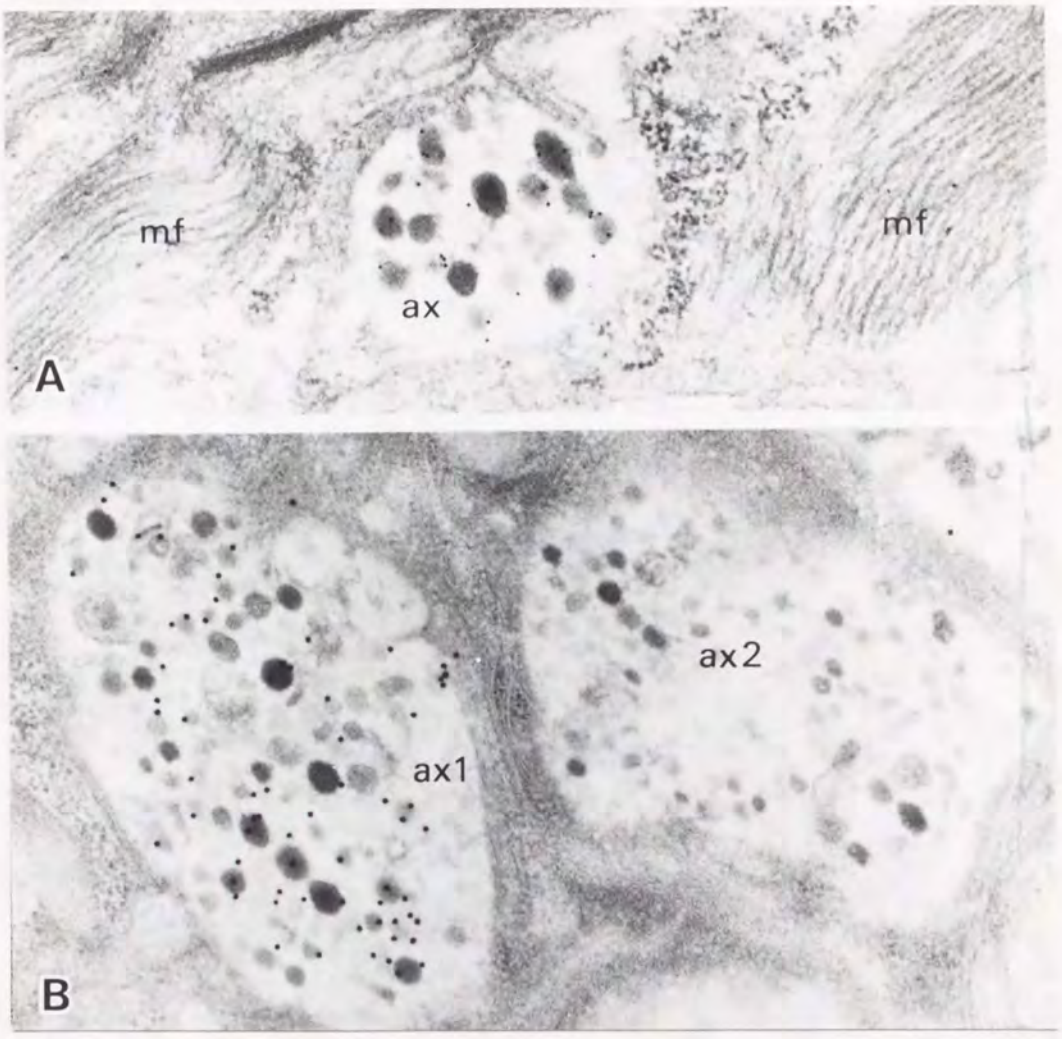

Fig. 27A,B. Electron microphotographs of immunogold-labeled sections treated with proctolin antiserum. (**A**) An axon profile (ax) containing proctolin-like immunoreactive, electron-dense granular vesicles, within the proximal part of the accessory gland tubules where multilayered muscle fibers (mf) are located; X50,000. Gold-labeling is restricted to the vesicles. HRP-labeling was not applied. (**B**) A part of transverse section of a ligated Br3. Numerous vesicles are accumulated in the axons (ax1, ax2) by the ligation; X48,000. The axon (ax1) containing proctolin-like immunoreactive, large, electron-dense granular vesicles is seen, together with the axon (ax2) having non-immunoreactive,

smaller vesicles. HRP-labeling was not applied. The section was stained in uranyl acetate for 5 min, and in lead citrate for 1 min.

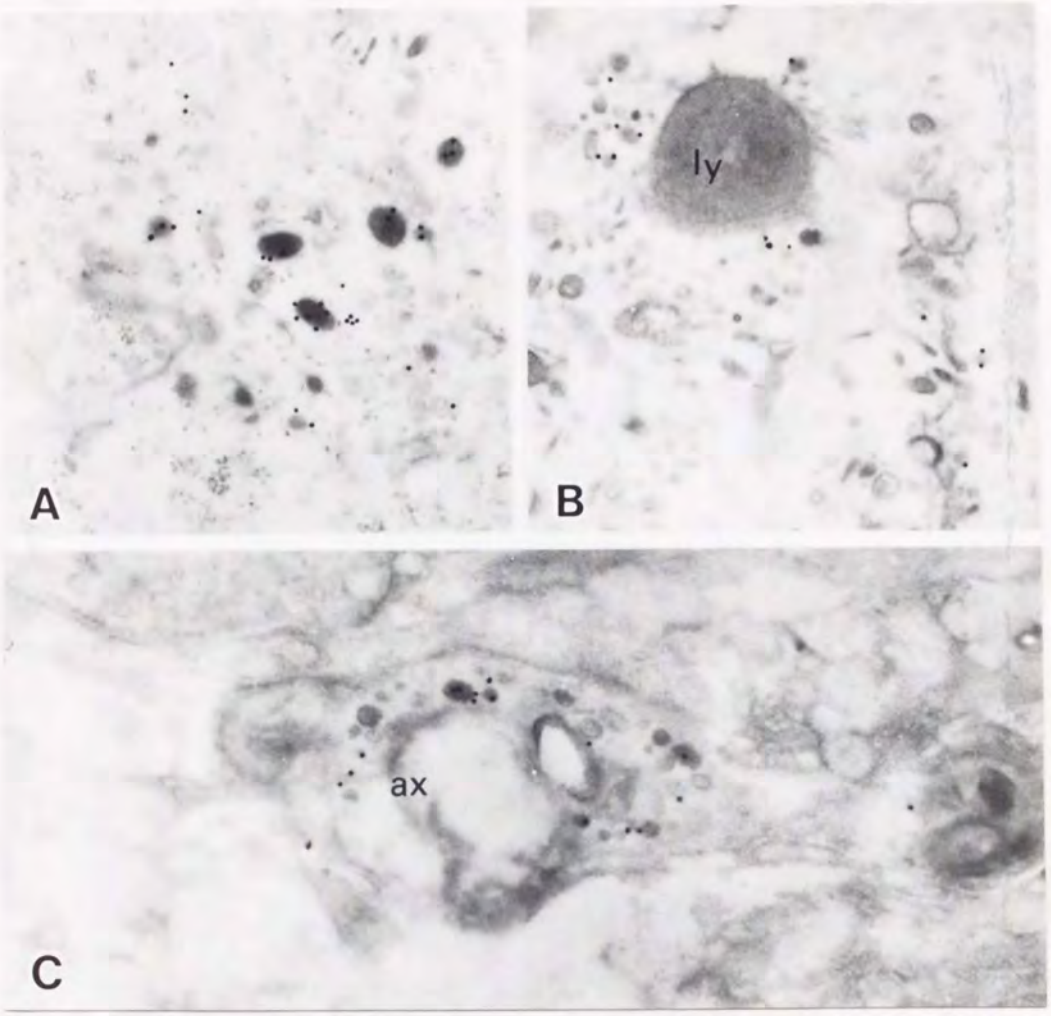

Fig. 28A-C. (A, B) Parts of HRP-labeled somata of DUMR7 neurons in the caudal region of the terminal abdominal ganglion; X38,000. Proctolin-like immunoreactive, electron-dense granular vesicles were found dispersively in the cytoplasm (A). In close to lysosome-like structure (ly), immunoreactive vesicles were seen (B). (C) Proctolin-like immunoreactive vesicles in the HRP-labeled axon (ax) within the ligated Br3; X38,000. This section was prepared from the preparation of which one of Br3 was ligated and the other Br3 was back-filled with HRP. HRP labeled the axons with accumulated vesicles in the ligated Br3. Gold-labeling is seen within the electron-dense, granular vesicles. The

sections were only stained in uranyl acetate for 1 min.

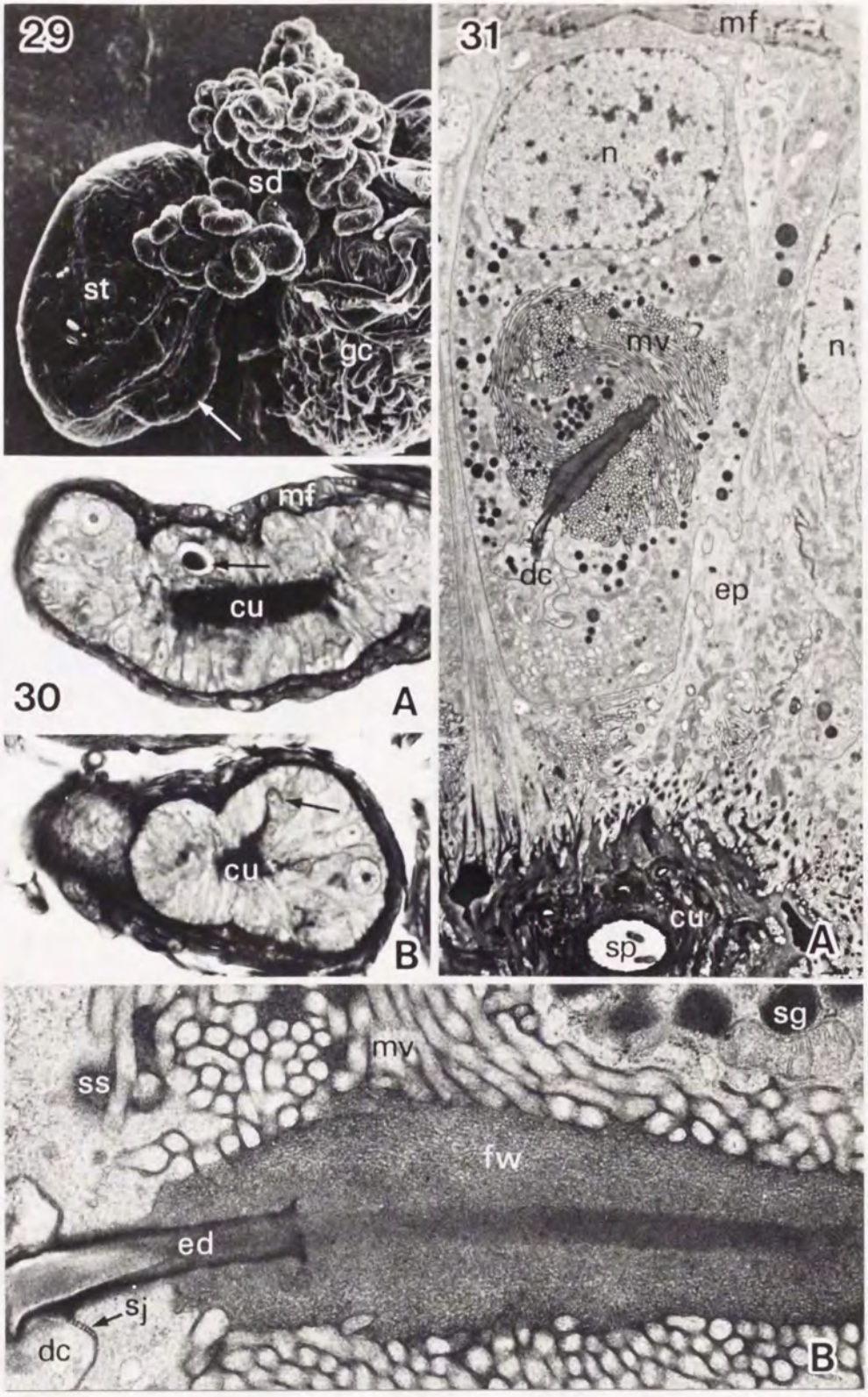

Fig. 29. Scanning electron microphotographs of the female reproductive organs, spermathecal duct (sd) and spermathecal bulb (st); X60. The spermathecal duct is thick in the proximal region near the junction with the spermathecal bulb (arrow). gc, spermathecal pouch of the genital cham-

ber.

Fig. 30A,B. Oblique sections through the median region of the spermathecal duct of a mated female; Azan staining. (A) A large vacuole containing a stainable substance (arrow); X440. (B) A tubular structure which extends from the cuticular intima (cu) into the wall of the spermathecal duct (arrow); X440. mf, muscle fiber.

Fig. 31A,B. (A) A longitudinal section through a glandular cell of a spermathecal duct of a mated female; X4,000. The cavity of the glandular cell is filled with many microvilli (mv). (B) An enlarged view of a part of (A) showing the end apparatus within the cavity; X25,000. Closely packed microvilli (mv) abut the felt-work (fw). cu, cuticular intima; dc, ductule forming cell; ed, cuticular efferent ductule; ep, epithelial cell; mf, muscle fiber; n, nucleus; sg, secretory granule; sj, septate junction; sp, sperm; ss, secretory substance.

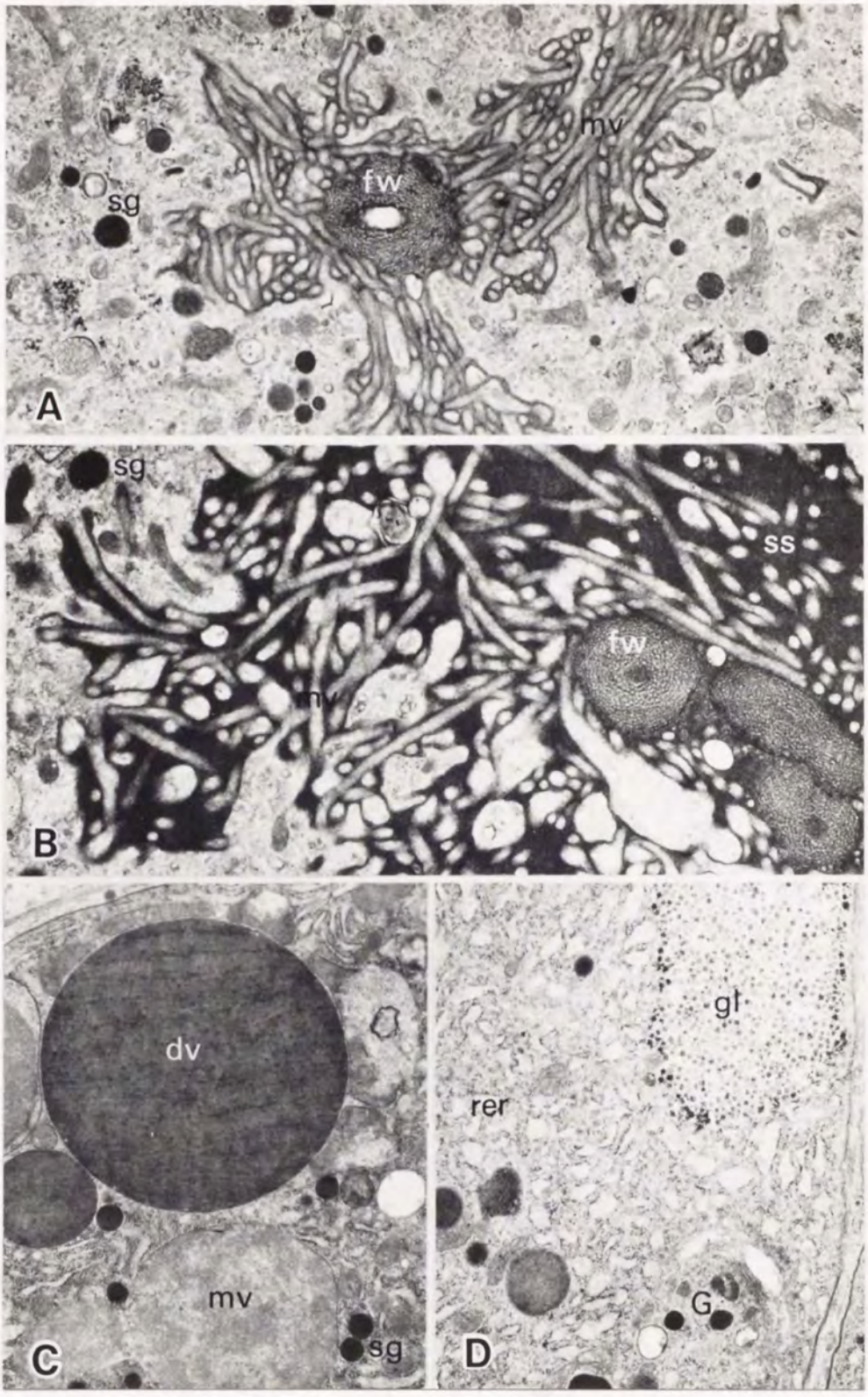

Fig. 32A-D. Electron microphotographs of glandular cell. (A, B) Sections through a central cavity of a glandular cell. (A) A newly emerged, virgin female; X15,000. Microvilli (mv) are in close contact with the felt-work (fw). No secretory substance is visible between the microvilli. (B) A

mated female; X15,000. Microvilli (mv) are separated from each other by the secretory substance (ss), which fills the cavity. (C, D) Glandular cells of a spermathecal duct in a mated female. (C) Large spherical, electron-dense vesicles (dv) and rather irregularly shaped, moderately electron-dense vesicles (dv) are visible, in addition to small secretory granules (sg); X9,000. (D) Dilated cisternae of rough endoplasmic reticulum (rer), a Golgi body (G), and glycogen granules (gl) are visible; X12,000.

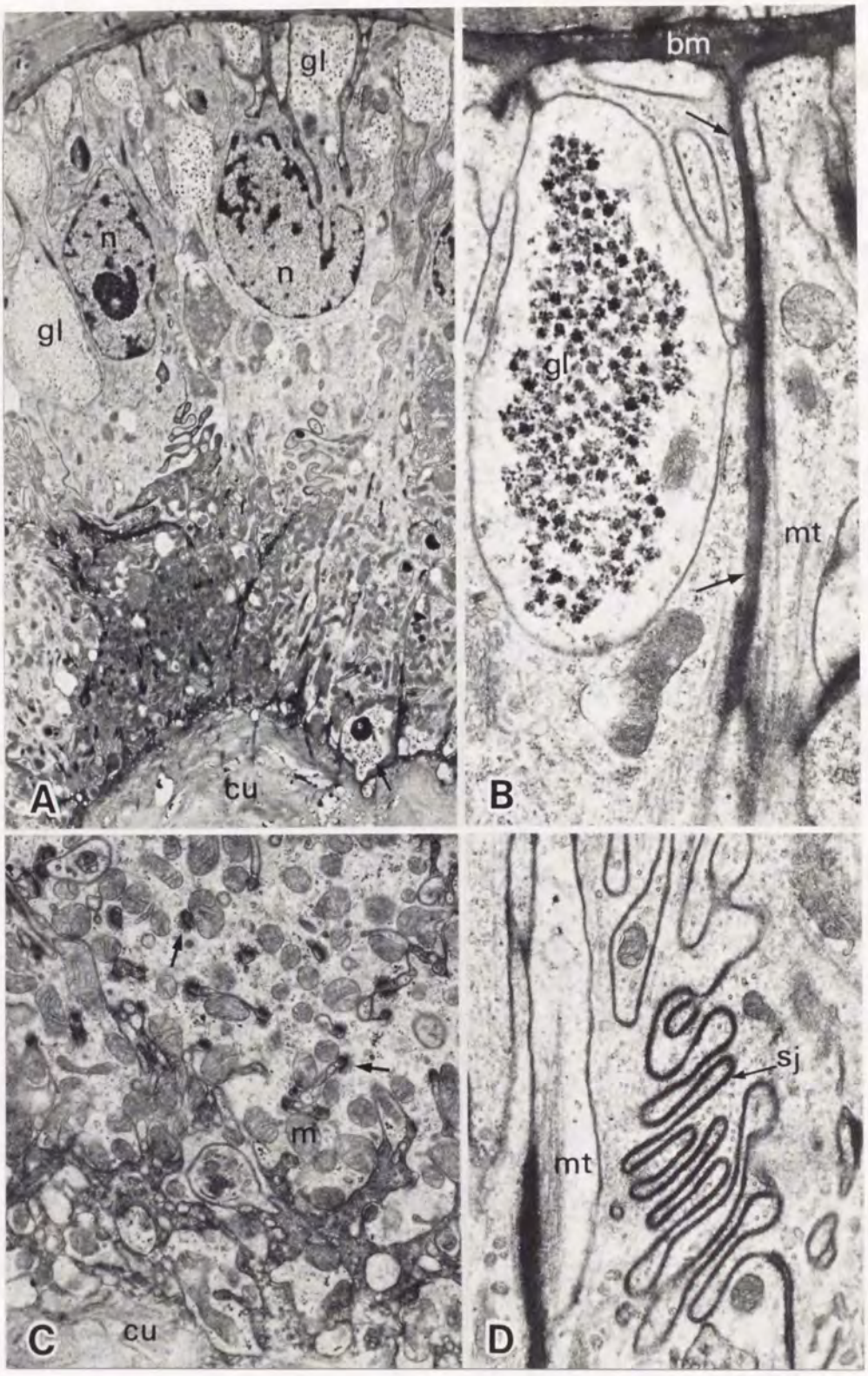

Fig. 33A-D. Electron microphotographs of epithelial cell. (A) A longitudinal section through epithelial cells in the proximal region of a spermathecal duct of mated female; X4,000. Large glycogen areas (gl) are visible. The arrow points to the apical cytoplasm containing glycogen granules.

(B) A longitudinal section through the basal portion of epithelial cells in the distal region of a spermathecal duct of a mated female; X25,000. An intercellular space (arrows) invades deeply. (C) An oblique section through the apical portion of an epithelial cell in the proximal region of a spermathecal duct of a mated female; X14,000. Microtubules surround cuticular processes (arrows). Note the presence of many mitochondria (m). (D) A longitudinal section through the apical portion of epithelial cells in the median region of the spermathecal duct of a newly emerged, virgin female; X26,000. The cytoplasmic membrane possesses a dense area associated with tufts of microtubules (mt). bm, basement membrane; cu, cuticular intima; n, nucleus; sj, septate junction.

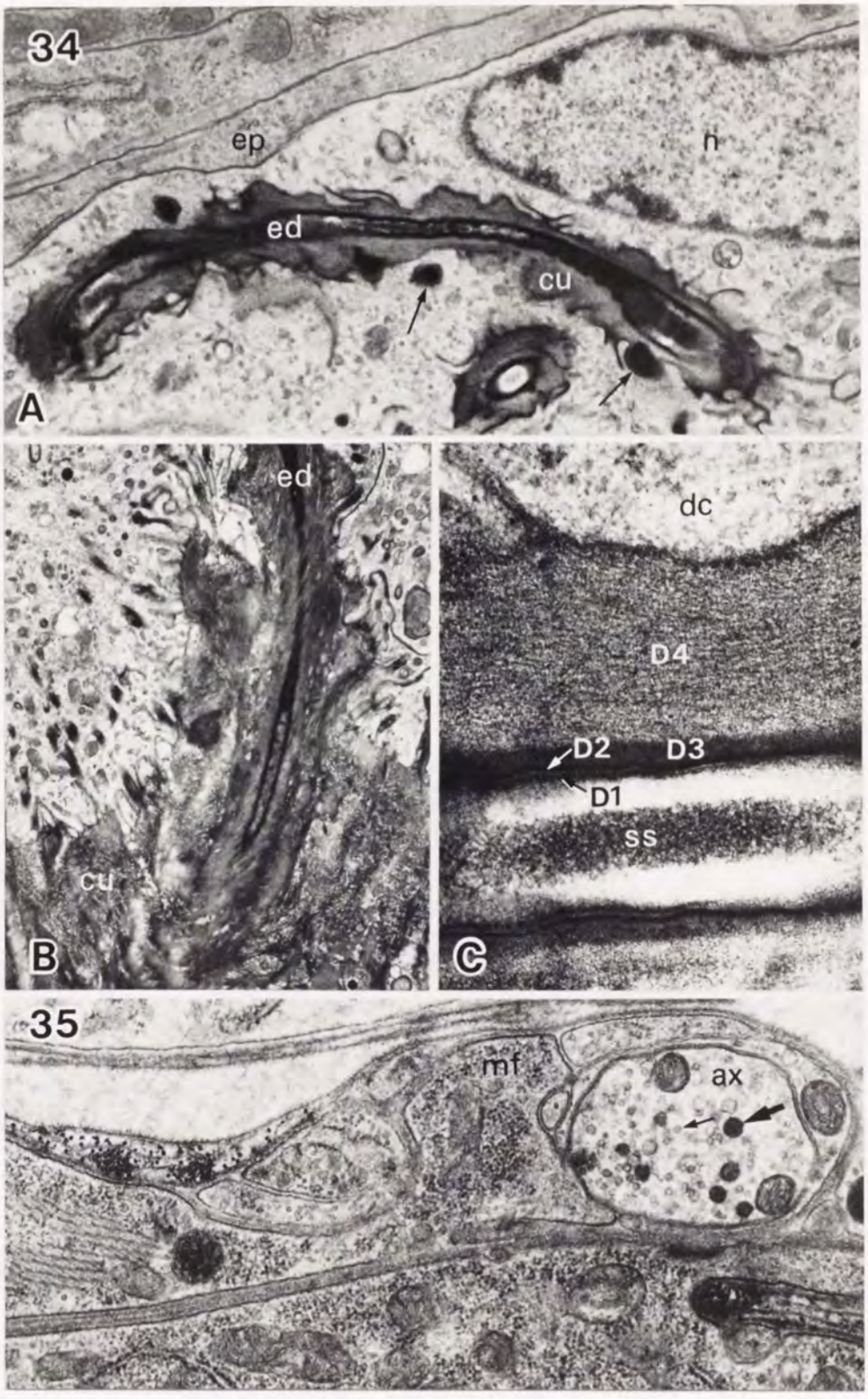

Fig. 34A-C. Electron microphotographs of ductule forming cell. (A) A ductule forming cell of a mated female; X14,000. Electron-dense deposits (arrows) are present in the cuticular intima (cu) of the cuticular efferent ductule (ed). (B) A longitudinal section of an cuticular efferent ductule (ed)

in the cuticular intima (cu) of a spermathecal duct of a newly emerged virgin female; X9,300. (C) A longitudinal section of an cuticular efferent ductule of a mated female; X98,000. The intima of the ductule consists of a pair of superficial electron-dense regions (D1, D2), a homogeneous layer (D3) and a fibrous region (D4). dc, ductule forming cell; ep, epithelial cell; n, nucleus. ss, secretory substance.

Fig. 35. An axon terminal (ax) forming a neuromuscular junction with a muscle fiber (mf) which envelops the spermathecal duct; X26,000. Large dense (large arrow) and small clear (small arrow) vesicles are present in the axon terminal.

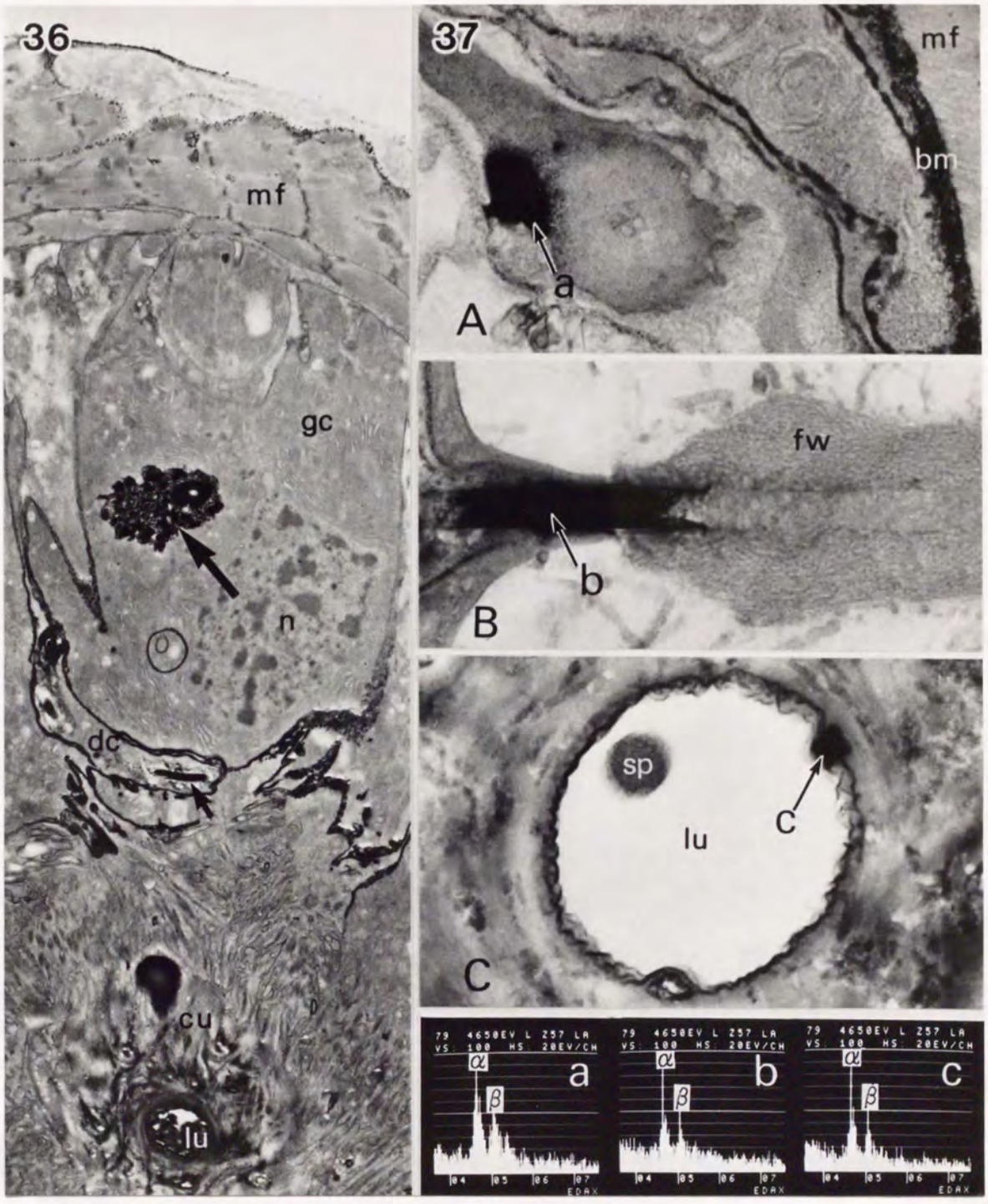

Fig. 36. A transverse section of the median region of the spermathecal duct which was incubated in 20 mM lanthanum solution for 2 hr; X42,000. The lanthanum is visible as electron-dense deposits in the cavity (large arrow) of the glandular cell (gc), and in the lumen of the cuticular ductule (small arrow) carried by the ductule forming cell (dc). The electron-dense deposits are visible also on the

surface of the muscle fibers (mf) and the intercellular space. cu, cuticular intima; lu, lumen of the spermathecal duct; n, nucleus. Unstained.

Fig. 37A-C. Deposits of lanthanum in the basal tip of the cuticular ductule near the muscle layer (**A**), in the tip of the cuticular ductule within the secretory cavity of the glandular cell (**B**), and in the opening of the cuticular ductule to the lumen of the spermathecal duct (**C**) incubated in 5 mM lanthanum solution; X26,500. Three histograms at the bottom (**a-c**) show the counts attributable to the presence of lanthanum in the areas indicated as **a-c** in **A-C**, respectively. The cursors shown as α and β in each histogram reveal the L_α and L_β peaks of lanthanum. bm, basement membrane; cu, cuticular intima; fw, felt-work structure; lu, lumen of the spermathecal duct; mf, muscle fiber; sp, sperm.

11 Bibliography

Papers

1) Satoh K, Masuda Y, Matsumoto K, Ohno N, Yasuyama K (1982) Normal stages in early development of a loach, Cobitis (Misgurnus) anguillicaudatus CANTOR. Kawasaki Igakkai Shi 8:41-48

2) Yasuyama K, Satoh K, Matsumoto K (1982) Inhibition of the leg regeneration and molting by cutting the optic tracts in a brackish water crab, Sesarmops intermedium. Kawasaki Igakkai Shi 8:49-58

3) Satoh K, Matsumoto K, Yasuyama K (1983) On the molting after removal of five legs in the Y-organ-ectomized Sesarmops intermedium. Kawasaki Igakkai Shi 9:63-69

4) Yasuyama K (1987) Fine structure of the spermathecal duct of the cricket, Gryllus bimaculatus. Kawasaki Igakkai Shi 13:81-93

5) Yasuyama K, Kimura T, Yamaguchi T (1988) Musculature and innervation of the internal reproductive organs in the male cricket, with special reference to the projection of unpaired median neurons of the terminal abdominal ganglion. Zool Sci 5:767-780

6) Kimura T, Yasuyama K, Yamaguchi T (1989) Proctolinergic innervation of the accessory gland in male crickets (Gryllus bimaculatus): detection of proctolin and some pharmacological properties of myogenically and neurogenically evoked contractions. J Insect Physiol 35:251-264

7) Shiga S, Kogawauchi S, Yasuyama K, Yamaguchi T (1991) Flight behaviour and selective degeneration of flight

muscles in the adult cricket (Gryllus bimaculatus). J Exp Biol 155:661-667.

8) Yasuyama K, Kimura T, Yamaguchi T (1991) Transverse ionic passages through the cuticular ductules from the haemocoel to the glandular cavities and the lumen of the spermathecal duct in female crickets (Gryllus bimaculatus). J Exp Zool 258:325-328

9) Yasuyama K, Kimura T, Yamaguchi T (1991) Proctolin-like immunoreactivity in the dorsal unpaired median neurons innervating the accessory gland of male crickets (Gryllus bimaculatus). (in preparation)

10) Kimura T, Yasuyama K, Yamaguchi T (1991) Local differentiation of the responsibility to the spermatophore contents and some drugs in the spermathecal duct of female cricket. (in preparation)

Oral Presentations

1) Yasuyama K, Yamaguchi T (1979) Some evidence for functional coupling between crayfish retinula cells in relation to polarized light response. Zool Mag 88:494

2) Yasuyama K, Yamaguchi T (1980) Response characteristics of retinula cells to polarized light in the crayfish. Zool Mag 89:471

3) Yasuyama K, Yamaguchi T (1980) Functional coupling and morphological identification of retinular cells in the crayfish, Procambarus clarkii. Proceedings of the Second Annual Meeting of the Japan Soc for Gen and Comp Physiol pp 16

4) Yasuyama K, Satoh K, Matsumoto K (1982) Effects of

cutting of the optic tracts on the growth of regenerated limb buds in a brackish-water crab (Sesarmops intermedium). Proceedings of the Chugoku- Shikoku Branch of the Zool Soc of Japan 34:20

5) Satoh K, Matsumoto K, Yasuyama K (1983) On the molting after removal of five legs in the Y-organ-ectomized Sesarmops intermedium. Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 35:10

6) Yasuyama K, Satoh K, Matsumoto K (1983) Interruption of the leg regeneration and molting by cutting the optic tracts in a brackish-water crab, Sesarmops intermedium. Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 35:11

7) Tanabe S, Yamaguchi T, Yasuyama K (1983) Functional and morphological coupling between photoreceptor cells in the crayfish retina. Proceedings of the Fifth Annual Meeting of the Japan Soc for Gen and Comp Physiol pp 70

8) Yasuyama K, Yamaguchi T, Kimura T (1984) Morphological differentiation of transplanted male accessory glands and their mechanical activity in the cricket. Dobutu seiri 1:164

9) Yasuyama K, Kimura T, Yamaguchi T (1985) Fine structure of dorsal unpaired median neurons innervating the muscle fibers of the accessory glands, epididymis, and ejaculatory duct of male cricket (Gryllus bimaculatus). Dobutu seiri 2:173

10) Kimura T, Yamaguchi T, Yasuyama K (1984) Movement of male accessory glands and its control by dorsal

unpaired median neurons. Zool Sci 1:883

11) Yasuyama K, Yamaguchi T (1985) Electron microscopic observation on the accessory glands and ejaculatory duct of the male cricket (Gryllus bimaculatus). Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 37:25

12) Kimura T, Yamaguchi T, Yasuyama K (1986) Change of proctolin level in male crickets during postembryonic development. Zool Sci 3:964

13) Yasuyama K, Kimura T, Yamaguchi T (1986) Proctolinergic and serotonergic neurons innervating the male accessory reproductive glands in the cricket. Abstracts of First International Congress of Neuroethology pp 92

14) Kimura T, Yasuyama K (1986) Neural control system of cricket visceral muscles. Dobutu seiri 3:180

15) Kimura T, Yamaguchi T, Yasuyama K (1986) Postembryonic development of the accessory glands and proctolinergic neurons in the male cricket. Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 38:21

16) Shiga S, Yamada K, Yamaguchi T, Yasuyama K (1988) Degeneration of flight muscles in the cricket Gryllus bimaculatus. Dobutu seiri 5:110

17) Yasuyama K, Kimura T, Yamaguchi T (1988) Ultrastructural and immunohistological investigation of the spermathecal duct of the female cricket, Gryllus bimaculatus. Zool Sci 5:1196

18) Shiga S, Yamada K, Yamaguchi T, Yasuyama K (1988)

Degeneration of flight motor system in cricket (Gryllus bimaculatus). Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 40:3

19) Kimura T, Yasuyama K, Yamaguchi T (1988) Effect of some drugs and the bioactive substance derived from the spermatophore on the mechanical activity of the spermathecal duct in the female cricket. Zool Sci 5:1197

20) Yasuyama K, Yamaguchi T (1989) Light and electron microscopic characterization of proctolin-like immunoreactive neurons in the abdominal ganglion of the male cricket, with special reference to the projection of unpaired median neurons to the accessory gland. Dobutu seiri 6:202

21) Shiga S, Yamaguchi T, Yasuyama K (1990) Degeneration of the indirect flight muscle M112a in adult crickets. Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 42:14

22) Yasuyma K, and Yamaguchi T (1991) Electron-microscopic immunocytochemistry of proctolinergic neurons innervating the accessory gland of male cricket. Proceedings of the Chugoku-Shikoku Branch of the Zool Soc of Japan 43:3