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REPRODUCTIVE BEHAVIOUR IN THE MALE CRICKET *GRYLLUS BIMACULATUS* DeGEER

II. NEURAL CONTROL OF THE GENITALIA

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Accepted 7 December 2000; published on WWW 26 February 2001

Summary

To understand the neural mechanisms of reproductive behaviour in the male cricket, we identified motor neurones innervating the muscles in each genital organ by backfilling with cobalt/nickel and recording their extracellular spike activity from nerve bundles of the terminal abdominal ganglion during tethered copulation and spermatophore formation. During tethered copulation, at least two motor neurones innervating two ipsilateral muscles were activated during projection of the guiding rod of the phallic dorsal pouch. Only one motor neurone, innervating four ipsilateral muscles of the dorsal pouch, was responsible for spermatophore extrusion by deforming the dorsal pouch. For spermatophore transfer, three motor neurones, singly innervating three epiphallus muscles, played a major role in opening passages for haemolymph to enter the ventral lobes and median pouch by bending the epiphallus. Two ventral lobe and 3–5 median pouch motor neurones seemed to play a role in expanding or folding the two membranous

structures by relaxing or contracting their muscle fibres. After spermatophore transfer, most of the genital motor neurones exhibited a rhythmic burst of action potentials causing movement of the phallic complex coupled with strong abdominal contractions. For spermatophore formation, the genital motor neurones began to accelerate their rhythmic bursts approximately 30 s prior to subgenital plate opening and then changed their activity to tonic bursting or silence. The results have allowed us to describe the timing of the onset and termination of genital muscle contraction more precisely than before, to examine the neural mechanisms of copulatory motor control and to speculate on the neural organization of the reproductive centre for spermatophore extrusion and protrusion.

Key words: male, cricket, *Gryllus bimaculatus*, reproductive behaviour, neural activity, spermatophore extrusion, spermatophore protrusion.

Introduction

In our companion paper (Kumashiro and Sakai, 2001), we characterized the structure and function of the genitalia in the male cricket and established the sequence of motor actions during copulation and spermatophore formation. To understand the mechanisms of male reproductive behaviour more fully, recordings of neural activity in the nerve innervating the genital organ need to be made under behavioural conditions. Previous work on the neural control of male reproductive behaviour in insects has been confined to studies of the influence of the head ganglia and sensory afferents on the phallic nervous system in the praying mantis and cockroach (Roeder et al., 1960; Grossman and Parnas, 1973). There are a number of reasons for the scarcity of studies using the neuroethological approach. First, the male genital system is relatively small and its innervation is intricate. Second, copulatory actions consist of the coordinated movements of various genital organs and are therefore complex. Third, two main reproductive events, spermatophore formation and spermatophore extrusion, occur just once an

hour and last for only a few seconds. Furthermore, artificial induction of spermatophore extrusion or spermatophore protrusion has not been possible under dissected conditions.

Recently, we developed methods to induce spermatophore extrusion and spermatophore protrusion not only in restrained but also in abdomen-opened males (Kumashiro and Sakai, 2001). This new method has allowed us to characterize the activity of genital motor neurones during copulation and spermatophore formation and to elucidate mechanisms of neural control over muscles and to speculate on the neural organization of the reproductive centre for spermatophore extrusion and protrusion. Preliminary results have been presented elsewhere (Kumashiro and Sakai, 1997).

Materials and methods

Animals

Male crickets, *Gryllus bimaculatus* DeGeer, were used 1–2 weeks after their final moult. To facilitate the induction of

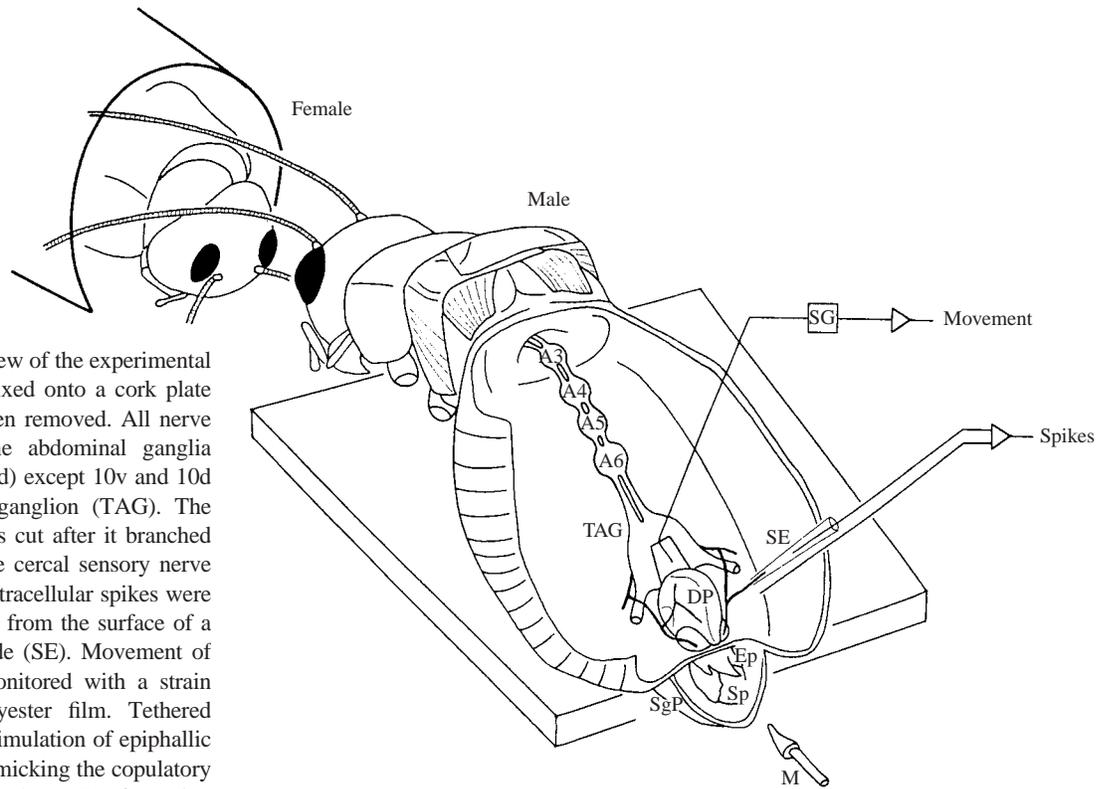


Fig. 1. Semi-diagrammatic view of the experimental preparation. The male was fixed onto a cork plate after its legs and gut had been removed. All nerve bundles emanating from the abdominal ganglia were left intact (not illustrated) except 10v and 10d of the terminal abdominal ganglion (TAG). The cercal motor nerve (10v) was cut after it branched off the genital nerve, and the cercal sensory nerve (10d) was cut proximally. Extracellular spikes were recorded from the cut end or from the surface of a nerve with a suction electrode (SE). Movement of the phallic complex was monitored with a strain gauge *via* a piece of polyester film. Tethered copulation was induced by stimulation of epiphallic sensilla with a model (M) mimicking the copulatory papilla of a female. Spermatophore (Sp) formation was induced by antennal contact with a female held in a polyethylene tube. A3–A6, third to sixth abdominal ganglia; DP, dorsal pouch of the phallus; Ep, epiphallus; SgP, subgenital plate (last abdominal sternite).

copulatory actions, they were isolated from females for 1 week before use.

Backfilling and histology

The abdominal nervous system of the cricket is composed of two fused ganglia (first and second) attached to the metathoracic ganglion, four isolated ganglia (third to sixth) and five fused ganglia (seventh to eleventh) forming the terminal abdominal ganglion (Jacobs and Murphey, 1987). For retrograde staining of neurones by backfilling, the cut end of a nerve was immersed in the tip of a polyethylene tube filled with a 0.5 mol l^{-1} cobalt and nickel mixture (Sakai and Yamaguchi, 1983). Preparations were left in a refrigerator at 4°C for approximately 48 h. The terminal abdominal ganglion was then cut out, reacted with rubeanic acid, fixed in 10% formaldehyde and cleared in methyl salicylate after dehydration in an ethanol series. Preparations were intensified (according to the method of Bacon and Altman, 1977). Nerve sections were made from excised nerve bundles fixed in formaldehyde, mounted in Spurr's resin and cut at $1 \mu\text{m}$ thickness. Sections were stained with Toluidine Blue.

Recording

For extracellular recording, the male was fixed onto a cork plate after removal of the wings and legs (Fig. 1). The abdomen was opened along the dorsal midline, the intestine, paraprocts and cerci were removed and the cavity was filled

with insect saline. In this condition, the male lived for at least 6 h. Suction electrode tips were strictly adjusted to the diameter of the recorded nerve. The movement of the genitalia was monitored with a strain gauge attached to a piece of polyester film slipped under the dorsal pouch of the phallic complex. Conventional methods were used for the amplification and recording of electrical signals. All data were printed out with a thermal array recorder (RTA-1100, Nihon Kohden).

Stimulation and response

The methods used to induce tethered copulation and spermatophore protrusion have been described in detail in our companion paper and are summarized here (Kumashiro and Sakai, 2001). To induce tethered copulation, including guiding rod projection and spermatophore extrusion, the genital cavity hair sensilla and associated external sensilla were simultaneously stimulated with a model (test stimulus) mimicking the female copulatory papilla (Fig. 1). In abdomen-opened males, spermatophore transfer resulting from ventral lobe and median pouch expansion was not actually induced because of the absence of hydrostatic pressure in the haemocoel. However, the end of fictive spermatophore transfer was determined by timing the return movement of the phallic complex to its normal position.

To induce the series of motor actions causing spermatophore formation, including pouch (subgenital plate) opening, secretion of the spermatophore material into the ejaculatory

duct, spermatophore transport and protrusion, the male was allowed make antennal contact with the body of a female after copulation (Fig. 1). Although pouch opening was not actually induced in abdomen-opened males, its timing was retrospectively determined by the timing of spermatophore protrusion.

Head cooling

To inactivate the head ganglia, the head was dipped into ice-cold water in a plastic container. The temperature inside the head dropped to below 10°C within 2 min, and this caused considerable blocking of descending neuronal activity from the head ganglia (Sakai et al., 1995).

Connective cuts

To discriminate the effects of the brain from those of the rest of the central nervous system, the connectives were cut between the brain and the suboesophageal ganglion in pre-copulatory males. In addition, the connectives between the sixth abdominal ganglion and the terminal abdominal ganglion were cut immediately after spermatophore extrusion or pouch opening to examine the roles of the terminal abdominal ganglion in generating post-copulatory rhythmic bursting and spermatophore formation.

Results

Genital organs and their motor neurones

Dorsal pouch

The dorsal pouch (Fig. 2A,B) serves to mould and project the spermatophore attachment plate. It consists of four muscles that contract simultaneously to cause spermatophore extrusion.

The dorsal pouch nerve (dpn, Fig. 2B) branches from the genital nerve and ramifies extensively to innervate muscles DP1–DP4 on the ipsilateral side of the dorsal pouch (Fig. 2A,B). Some nerves fuse in the midline region with those from the other side (Fig. 2B). Although the arrangement

shown in Fig. 2A,B is representative of the basic pattern of dorsal pouch innervation, there were many variations in the branching pattern. Axonal backfilling through the cut end of the dorsal pouch nerve [at the arrowhead labelled (DP) in Fig. 2B] revealed a single stained motor neurone in the terminal abdominal ganglion. The soma is located postero-laterally on the ventral side and the dendrites arborize dorsally (Fig. 3, DP). Sections of the dorsal pouch nerve show that it contains a single large motor axon surrounded by thick glial cells (see Fig. 4B).

Guiding rod

The guiding rod of the median grooved fold in the dorsal pouch serves as a mould for the spermatophore tube. Its posterior portion is used as a guide to project the

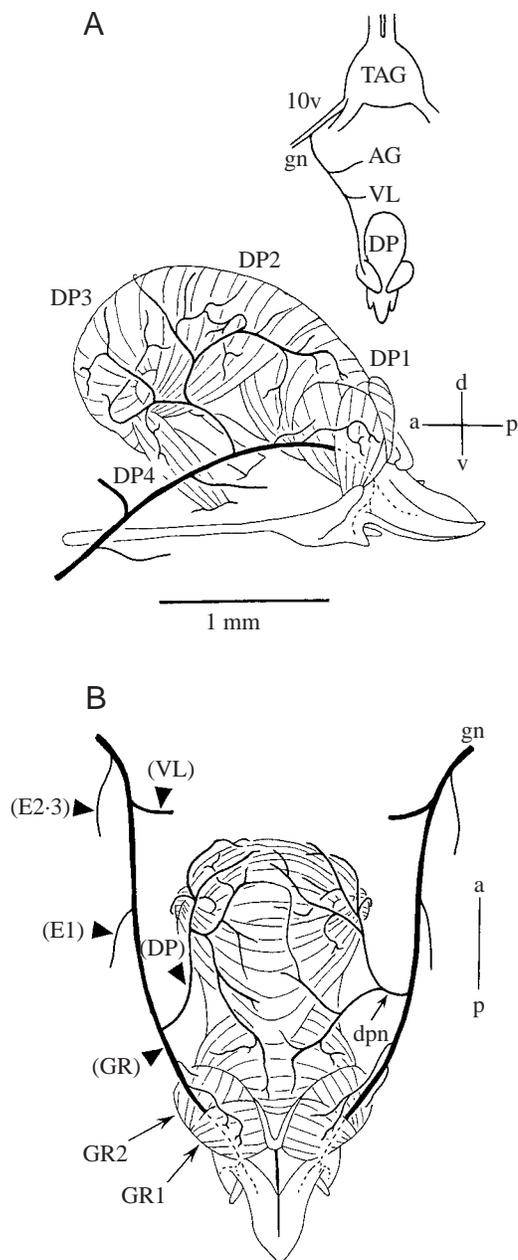


Fig. 2. Innervation of the genital organs. (A) Lateral view. (B) Dorsal view. The inset shows a schematic diagram of the genital nerve (gn) innervating three genital organs (AG, VL and DP). The gn gives rise to the dorsal pouch nerve (dpn in B) which ramifies into many smaller branches ipsilaterally on the four muscles (DP1–DP4) of the dorsal pouch. Other peripheral nerves branch off gn and ramify into the guiding rod muscles (GR1 and GR2). The most peripheral region of gn consists of three sensory nerves innervating sensilla on a median and two lateral processes of the epiphallus and on the wall of the genital cavity. In B, arrowheads show the location of nerve transections for backfilling or extracellular recording, and labelling in parentheses indicates the muscles or sensilla of organs shown by abbreviations. AG, accessory gland; DP, dorsal pouch; DP1–DP4, dorsal pouch muscle; (E1), motor nerve innervating muscle E1; (E2-3), motor nerve differentially innervating muscles E2 and E3; (GR), motor nerve innervating guiding rod; GR1 and GR2; guiding rod muscles; TAG, terminal abdominal ganglion; VL, ventral lobe. a, anterior; p, posterior; d, dorsal; v, ventral. These conventions are adopted in the following figures.

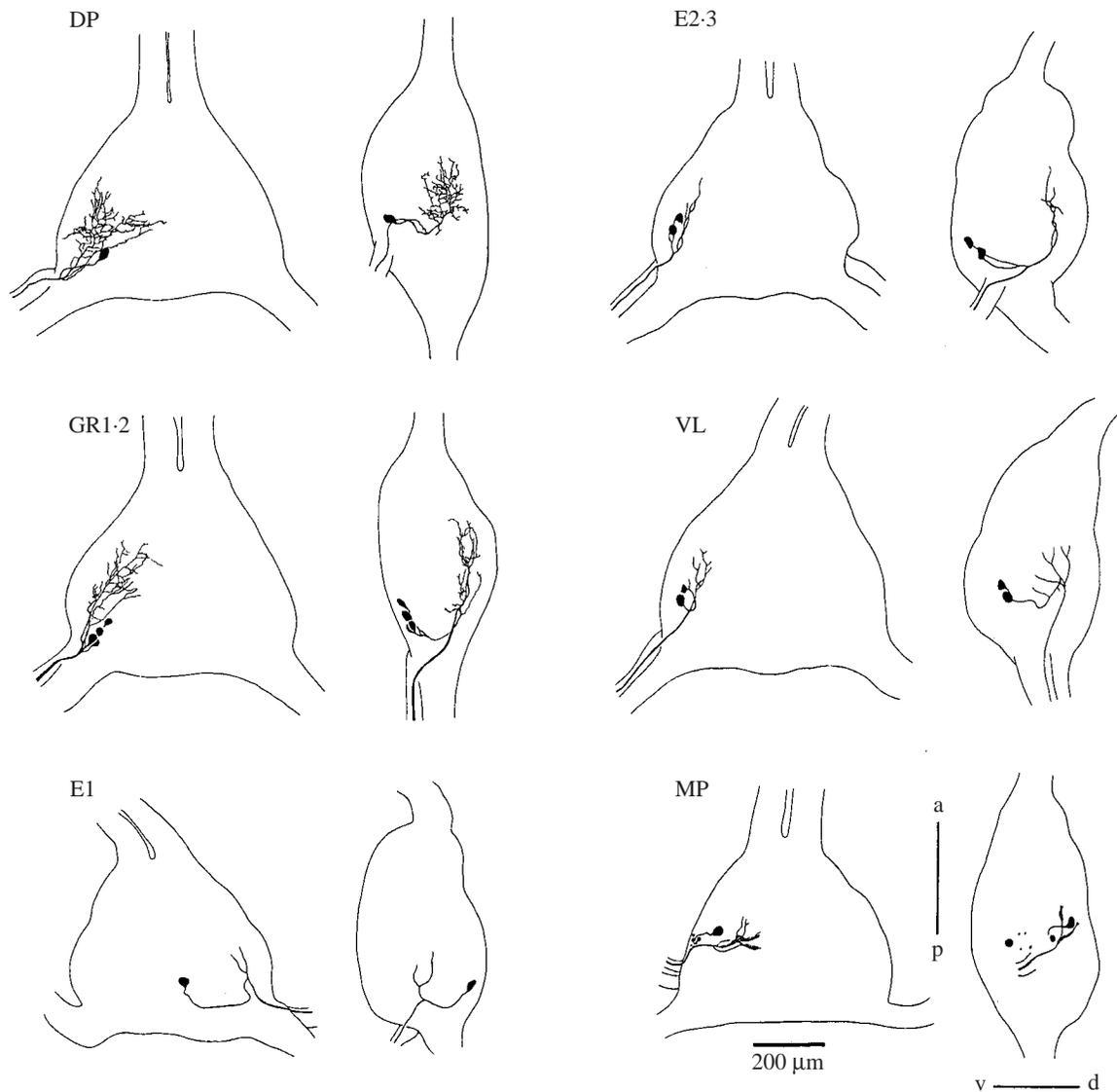


Fig. 3. Retrogradely stained genital motor neurones in the terminal abdominal ganglion. Diagrams DP–MP show the somata of motor neurones innervating six different muscles. DP, dorsal pouch motor neurone innervating muscles DP1–DP4; GR1-2, guiding rod motor neurones differentially innervating muscles GR1 and GR2; E1, epiphallus motor neurone innervating muscle E1; E2-3, epiphallus motor neurones differentially innervating muscles E2 and E3; VL, ventral lobe motor neurones innervating ventral lobe muscles VL; MP, median pouch motor neurones innervating median pouch muscles MP. Left, dorsal view; right, lateral view. a, anterior; p, posterior; d, dorsal; v, ventral.

spermatophore tube into the spermathecal duct of the female copulatory papilla through its aperture (i.e. threading). Both muscles (GR1 and GR2) associated with the guiding rod are innervated by a nerve branch [labelled (GR) in Fig. 2B] containing four axons. Backfilling showed that their somata were located postero-laterally on the ventral side of the terminal abdominal ganglion, with dendrites arborizing dorsally (Fig. 3, GR1-2).

Epiphallus

The epiphallus is used for hooking onto the female subgenital plate with its median process, and its cavity is used for coupling with the female copulatory papilla. Of the three epiphallus muscles (E1–E3, see Fig. 3 in Kumashiro and Sakai, 2001), E1

and E2 attach to the proximal region of the epiphallus at one end, and E1 is attached to the lateral arms and E2 to last sternite apodeme at their other end. E3 connects the lateral arms of the phallic complex with the sternite apodeme. Although they are termed epiphallus muscles here, they are involved not only in movement of the epiphallus but also in movement of the phallic complex, by regulating haemolymph flow into the ventral lobes and median pouch *via* bending of the epiphallic cuticle. For example, contraction of E1 causes movement of the lateral arms to initiate spermatophore transfer, while contraction of E2 and E3 causes the return movement of the phallic complex at the end of the transfer. They also contribute to the production of the rhythmic movement of the epiphallus that occurs after spermatophore transfer.

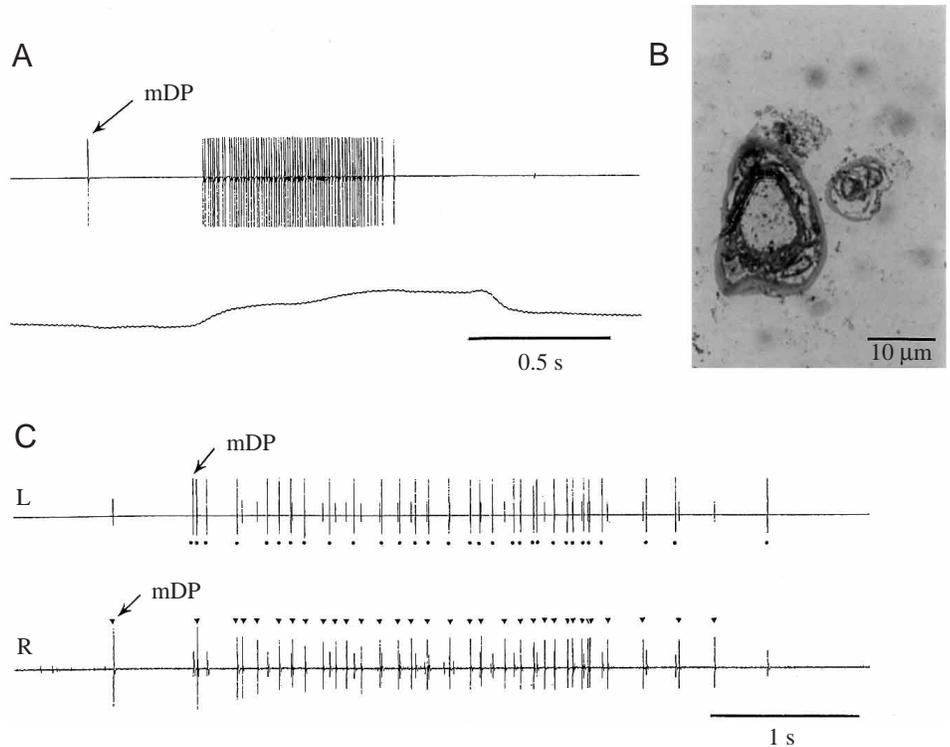


Fig. 4. Spike activity in the dorsal pouch motor neurone (mDP). (A) Spike activity in mDP, recorded between spermatophore extrusion and spermatophore protrusion. Upper trace, spike activity; lower trace, epiphallal complex movement. (B) Transverse section of the dorsal pouch nerve (dpn). The larger section to the left is the dpn. Only one axon is present, and it is surrounded by a glial sheath. (C) Spike activity of the left (L) dpn and mDP (circles) recorded simultaneously with activity of right (R) dpn and mDP (triangles). Unmarked smaller spikes in L and R reflect activity in the contralateral mDP recorded because of crosstalk. This recording was made during a contraction of the dorsal pouch as shown in A.

Backfilling of the nerve branch innervating E1 [indicated by the arrowhead labelled (E1) in Fig. 2] stained one motor neurone with its soma located dorso-medially in the terminal abdominal ganglion (Fig. 3, E1). The very short nerve branches to muscles E2 and E3 precluded independent backfilling (see Fig. 9 in Kumashiro and Sakai, 2001). However, backfilling through the cut end of the common nerve [labelled (E2:3) in Fig. 2B] stained two motor axons with somata located laterally in the terminal abdominal ganglion with dendrites arborizing dorsally (Fig. 3, E2:3).

Ventral lobes

The ventral lobes serve as a receptacle and a constrictor for spermatophore materials and as a mould for the ampulla of the spermatophore. They are also used to lift the spermatophore towards the genital chamber of the female during spermatophore transfer.

Two motor neurones were stained by backfilling through the cut end of the nerve innervating the ventral lobes at the site marked by the arrowhead labelled (VL) in Fig. 2B. Their somata are located laterally on the ventral side, and the dendrites arborize dorsally (Fig. 3, VL).

Median pouch

The median pouch is used for the eversion of the phallic complex by which the male performs hooking. Following spermatophore extrusion, the median pouch serves as a pusher to elevate the spermatophore from below the ventral lobes. This membranous organ moves spontaneously to the left and right throughout the male reproductive cycle, even while it is

folded under the dorsal pouch or its convolution is plugging the dorsal cavity.

Three to four large and three to five small motor axons were stained when the cut end of the nerve branch emanating from 9v was backfilled (see Fig. 9 in Kumashiro and Sakai, 2001). Their somata are located laterally in the terminal abdominal ganglion and their dendrites arborize dorsally (Fig. 3, MP). Anterograde staining of the genital nerve demonstrated that efferent axons innervate the ipsilateral side of the median pouch (not shown) in a manner similar to those innervating the dorsal pouch.

Motor neurone activity during tethered copulation

Dorsal pouch

Recording proximally from the cut end [(DP) in Fig. 2B] of the dorsal pouch nerve revealed only large spikes from a single motor neurone, here termed the dorsal pouch motor neurone (mDP), which showed no spontaneous activity. Fig. 4A shows a single burst of the mDP, at a spike rate of 120 Hz with a burst duration of 0.6 s, recorded in the period between spermatophore extrusion and spermatophore protrusion. Each burst caused a contraction of the dorsal pouch (lower trace). *En passant* recordings from any branch of the intact dorsal pouch nerve (Fig. 2A,B) showed the same spike and burst format as seen in Fig. 4A. Simultaneous recordings from the left and right dorsal pouch nerves indicated that both motor neurones discharged together during dorsal pouch deformation, but not exactly in synchrony (Fig. 4C), suggesting a possible common input but not electrical coupling.

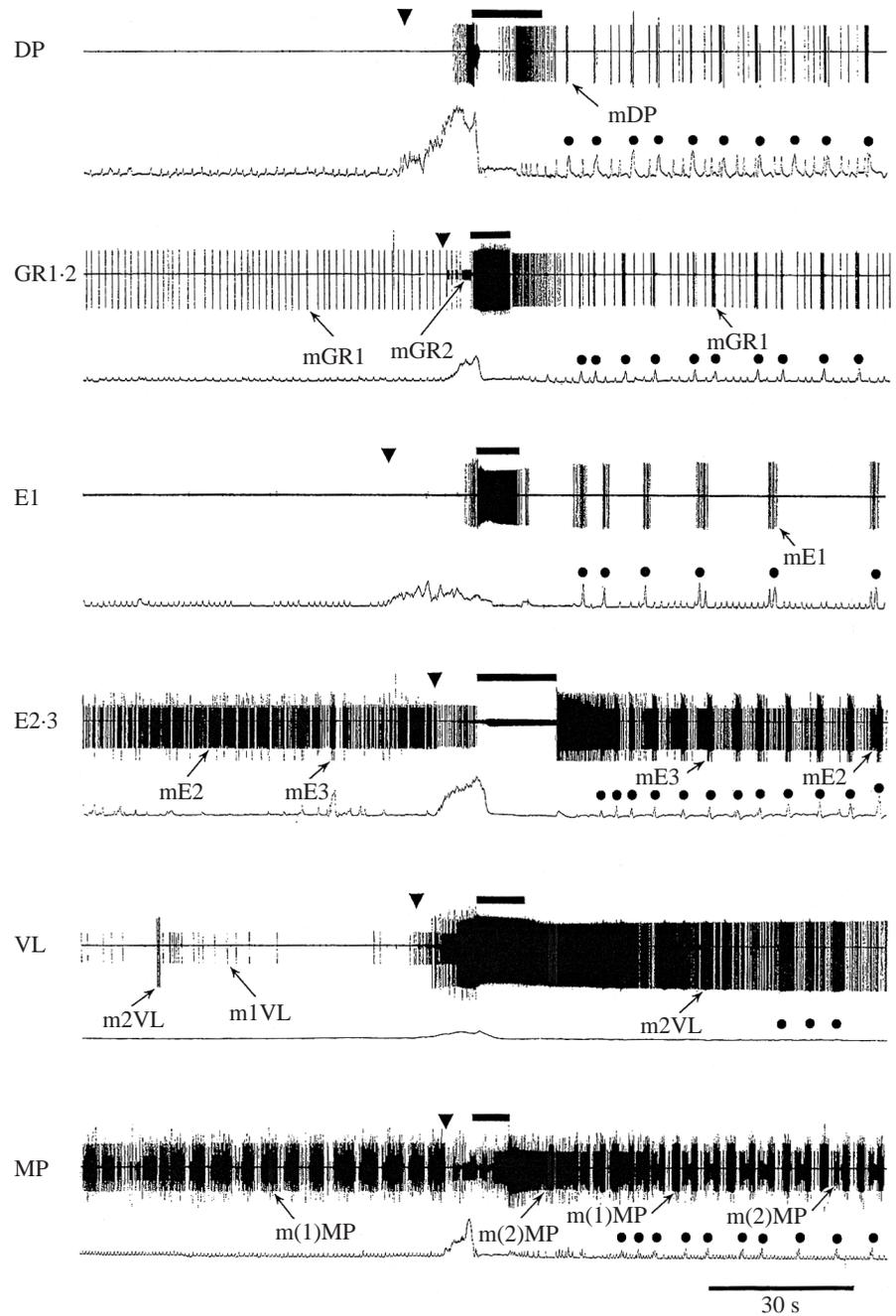


Fig. 5. Spike activity in the genital motor neurones during tethered copulation. For each pair of recordings, the upper trace is spike activity and the lower trace is the movement monitor. The black arrowhead indicates the onset of test stimulation of the epiphallallic sensilla. The horizontal black bar indicates the time from spermatophore extrusion to the end of spermatophore transfer. Spermatophore transfer itself was not actually induced because there was no abdominal hydrostatic pressure in abdomen-opened males. Black circles above the lower traces indicate post-copulatory rhythmic movement of the dorsal pouch. Diagrams DP–MP show the activity of motor neurones innervating six different muscles. DP, activity of a dorsal pouch motor neurone (mDP). In the lower trace, a sharp decline in tension following spermatophore extrusion is caused by the removal of the test stimulus. GR1·2, guiding rod motor neurones (mGR1 and mGR2). E1 and E2·3, epiphallus motor neurones. One unit (mE1) was recorded from the nerve innervating muscle E1 and two units (mE2, mE3) were recorded from the nerve innervating muscles E2 and E3. VL, ventral lobe motor neurones (m1VL and m2VL). MP, median pouch motor neurones, which tended to fire in groups. m(1)MP consists of 2–3 different spikes and m(2)MP consists of 1–2 different spikes.

During tethered copulation (Fig. 5, DP), mDP started to discharge 12 s after the onset of the test stimulus. Soon (2 s) after the peak rate of discharge at 120 Hz, the spermatophore was extruded by strong contraction of the dorsal pouch muscles. The discharge rate subsequently fell to 0 Hz for 5 s and then increased to 65 Hz. The discharge rates in the first and second bursting periods in different males were 137 Hz [median; 95% confidence interval (C.I.) 110–185 Hz; $N=16$] and 63 Hz (C.I. 54–74 Hz; $N=12$) respectively. Following spermatophore transfer, the motor neurone fired rhythmically at a discharge rate of 110 Hz with a burst duration of 0.5–0.8 s. This bursting was the source of the cyclical deformation of the dorsal pouch. The intraburst discharge rate of mDP in different

males was 95 Hz (C.I. 94–115 Hz; $N=11$) and the cycle rate was 0.13 Hz (C.I. 0.10–0.14 Hz; $N=11$).

Guiding rod

A larger and a smaller spike were regularly present in recordings from the guiding rod nerve, with much smaller spikes occurring occasionally. Although the short nerves to each muscle prevented individual recordings during reproductive actions (Fig. 5, GR1·2), separate experiments confirmed that neurone mGR1 (large spike) innervated muscle GR1 and that mGR2 (small spike) innervated muscle GR2.

During tethered copulation (Fig. 5, GR1·2), mGR1 discharged one spike per respiratory movement before application of the test

stimulus. Shortly before spermatophore extrusion, this motor neurone fired vigorously at up to 170 Hz, and it maintained this rate of discharge during spermatophore transfer. This activity caused tonic contraction of muscle GR1 to keep the guiding rod inside the copulatory papilla of the female. The mean discharge rate of mGR1 in males was 190 Hz (C.I. 165–215 Hz; $N=13$). Following spermatophore transfer, the activity decreased and became rhythmic in synchrony with the movement of the dorsal pouch. In contrast, mGR2 responded to test stimulation by discharging earlier than mGR1 to cause repeated projection of the guiding rod. Both motor neurones fired together during spermatophore extrusion and transfer, although this is not clear in the recording shown (Fig. 5, GR1·2).

Epiphallus

Three motor neurones activated the epiphallic muscles during copulation. A single motor unit (mE1) fired in the nerve innervating muscle E1 (Fig. 5, E1), while two motor units fired in the nerve innervating muscles E2 and E3 (Fig. 5, E2·3). In separate experiments, it was shown that one unit innervates muscle E2 and is therefore designated mE2, and that the other innervates muscle E3 and is therefore designated mE3.

During tethered copulation, mE1 was silent before spermatophore extrusion (Fig. 5, E1). Approximately 3 s before spermatophore ejection, it began firing at up to 88 Hz. This activity caused the lateral arms of the phallic complex to be pulled backwards, which opened passages for haemolymph to enter the ventral lobes (see Fig. 2 in Kumashiro and Sakai, 2001). The mean discharge rate of mE1 in different males was 160 Hz (C.I. 150–175 Hz; $N=7$). After spermatophore transfer, the activity suddenly stopped. Shortly thereafter, mE1 burst repeatedly to drive a rhythmic movement of the epiphallus in synchrony with dorsal pouch contraction (black circles, lower trace, Fig. 5).

The activity of mE2 and mE3 was generally reciprocal to that of mE1. Before the test stimulation, mE3 was sporadically active while mE2 fired tonically (Fig. 5, E2·3). During spermatophore extrusion and transfer, both neurones completely stopped discharging. After spermatophore transfer, they became transiently active to cause retraction of the phallic complex. These two motor neurones fired rhythmically in synchrony with dorsal pouch contraction.

Ventral lobes

Recordings from the cut end of the nerve to the ventral lobes [(VL) in Fig. 2B] showed that the two ventral lobe motor neurones fired in an essentially reciprocal fashion (Fig. 5, VL). Motor neurone m1VL was spontaneously active throughout the mating stage, while m2VL was largely inactive (Fig. 5, VL). During the test stimulation, m1VL increased its firing rate at 68 Hz, but it then became silent after spermatophore extrusion. In contrast, m2VL began to discharge vigorously approximately 5 s before spermatophore extrusion at up to 166 Hz. The mean discharge rates among males were 70 Hz for m1VL (C.I. 62–82 Hz; $N=16$) and 203 Hz for m2VL (C.I. 190–265 Hz; $N=20$). The activity of m2VL continued during

and after spermatophore transfer, which may be responsible for controlling ventral lobe tonus. The activity gradually declined and finally changed to a bursting mode in synchrony with dorsal pouch deformation.

Median pouch

In the median pouch, 6–9 motor units were active. These formed two groups, m(1)MP and m(2)MP. Simultaneous bilateral recordings showed left and right reciprocity in the firing of these groups (results not shown). Before test stimulation, m(1)MP motor neurones burst regularly at approximately 0.2 Hz, while m(2)MP motor neurones were sporadically active (Fig. 5, MP). As soon as the epiphallic sensilla were stimulated, the m(1)MP group decreased their activity while the m(2)MP group continued firing. The discharge rate of the latter increased markedly after spermatophore transfer, and m(2)MP may therefore be responsible for folding the proximal region of the median pouch. Soon (30 s) after spermatophore extrusion, both motor neurone groups entered a bursting mode at approximately 0.3 Hz: m(1)MP fired during the rhythmic movement of the median pouch itself, while m(2)MP fired during dorsal pouch contraction.

Motor neurone activity during spermatophore formation

Dorsal pouch

Approximately 30 s before opening of the subgenital plate, the brief high-frequency bursts of mDP gradually accelerated and then stopped at the onset of subgenital plate opening (Fig. 6, DP).

Guiding rod

Both motor neurones (mGR1 and mGR2) fired during spermatophore formation (Fig. 6, GR1·2) although, in the recording shown, activity in some other neurones, producing spikes of a much smaller size, is also visible. Thirty seconds before opening of the subgenital plate, mGR1 increased its burst rate and then changed to tonic firing. After opening of the subgenital plate, it again became rhythmically active and burst duration decreased. The firing of mGR2 appeared to be synchronised with that of mGR1.

Epiphallus

The firing pattern of mE1 resembled that of the guiding rod motor neurones before and during subgenital plate opening (Fig. 6, E1). Unlike the former, mE1 stopped firing after spermatophore protrusion. In contrast, mE2 and mE3 increased their burst rates before subgenital plate opening, and activity then dropped to zero at the onset of opening (Fig. 6, E2·3). Twenty seconds later, only mE2 recovered a pattern of tonic firing, which became cyclically inhibited at each occurrence of guiding rod movement (lower trace) after spermatophore protrusion.

Ventral lobes

Weak bursting of the m1VL motor neurone commenced 1 min before subgenital plate opening and increased gradually

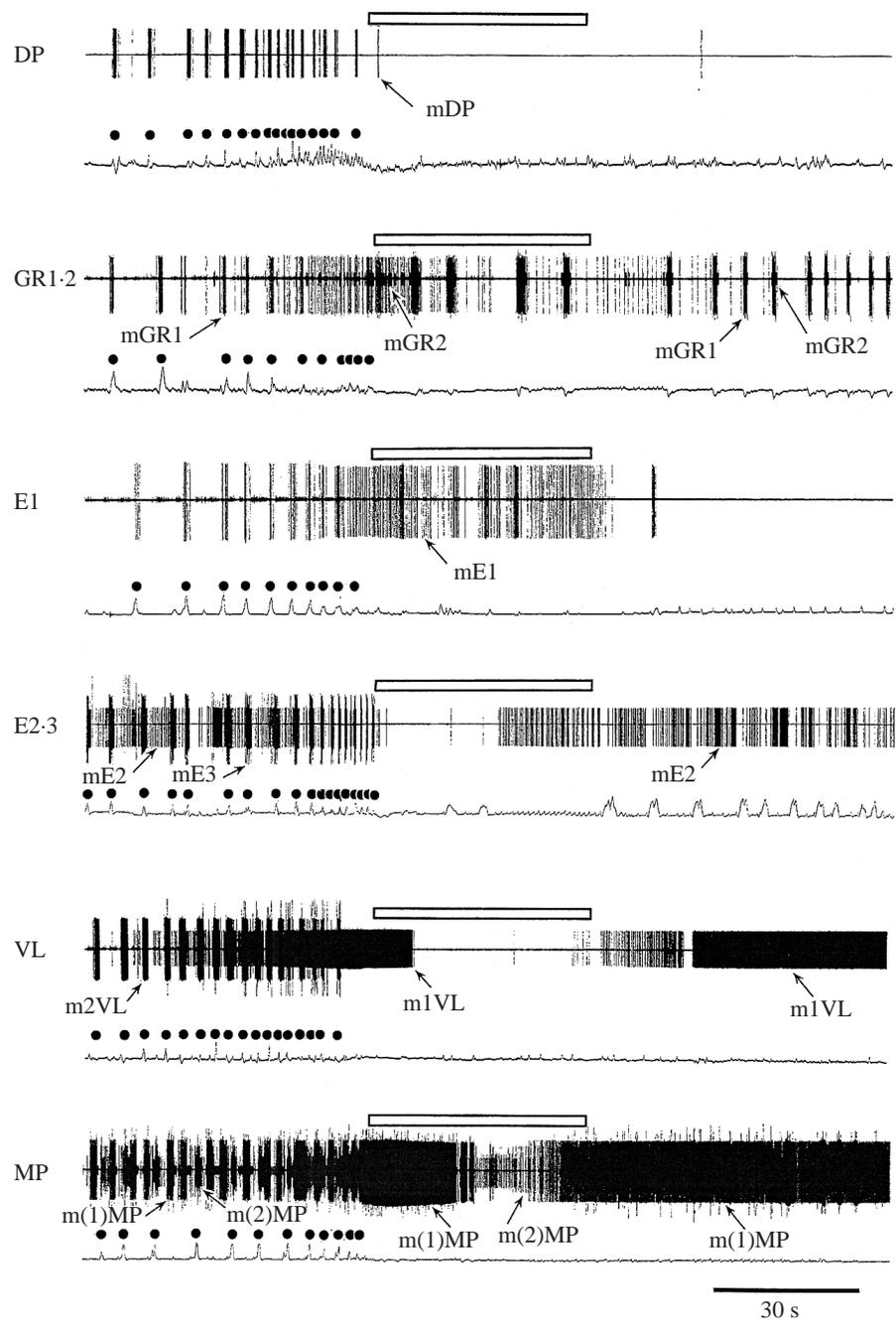


Fig. 6. Spike activity in the genital motor neurones during spermatophore formation. Data for DP, GR1·2 and VL were obtained from different males, while those in E1, E2·3 and MP were from the same males as for Fig. 5. The horizontal white bar indicates the time from opening of the subgenital plate to spermatophore protrusion. During this period, secretion and transport of spermatophore materials are carried out in the ejaculatory duct. Black circles indicate spontaneous movements of the dorsal pouch. See Fig. 5 for an explanation of the abbreviations.

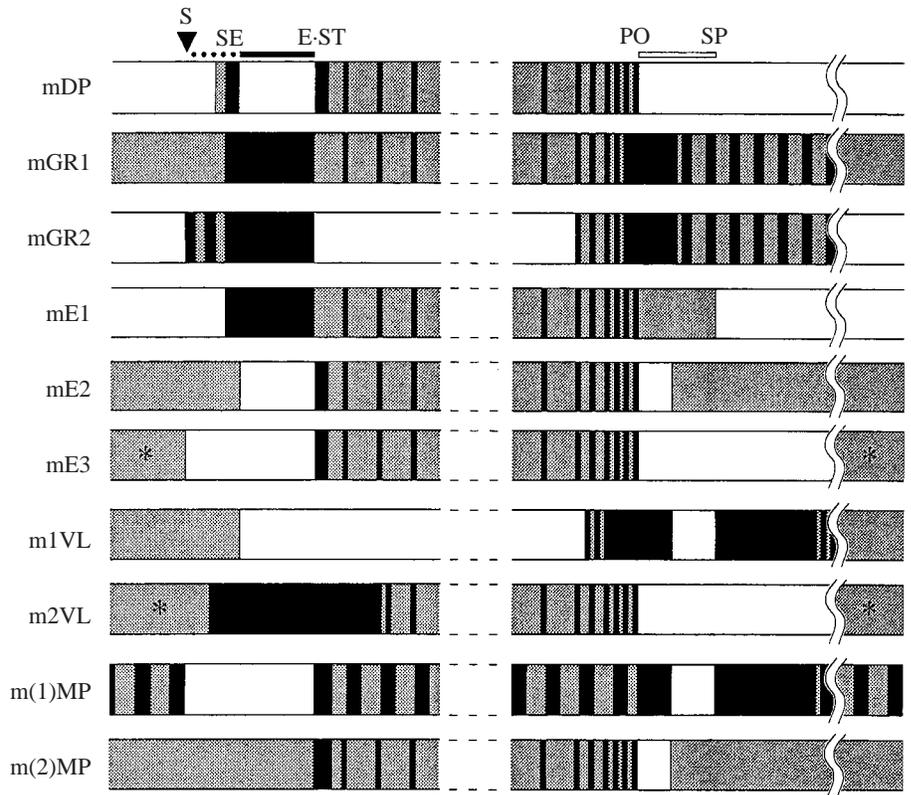
to tonic firing at 68 Hz. This was maintained until 20 s after opening (Fig. 6, VL). Firing then abruptly ceased for approximately 30 s but gradually recommenced after spermatophore protrusion. During this latter period, the action of m1VL seemed to provide an increase in muscle tonus of the ventral lobes in preparation for the receipt of the jelly-like spermatophore material. After the spermatophore materials had been ejected through the genital cavity, m1VL began to burst again at 0.6 Hz (not clearly visible in Fig. 6, VL), driving vigorous constriction movements of the ventral lobes. This robust motor neurone activity decreased gradually to the level recorded before tethered copulation. m2VL, however, accelerated its burst rate as subgenital plate opening

approached, ceased to discharge before the onset of subgenital plate opening and remained silent after spermatophore protrusion.

Median pouch

Prior to subgenital plate opening, the m(1)MP and m(2)MP motor neurone groups gradually increased their bursting rates (Fig. 6, MP). Thereafter, m(1)MP neurones showed tonic activity that corresponded to the timing of median pouch folding. Approximately 20 s later, they stopped discharging for 10 s concomitant with the interruption of the spontaneous phallic complex movement during which spermatophore materials are transported through the ejaculatory duct.

Fig. 7. The activity of each genital motor neurone during tethered copulation and spermatophore formation shown in diagrammatic form as horizontal bands. Top to bottom, mDP, dorsal pouch motor neurone; mGR1, the guiding rod motor neurone innervating guiding rod muscle mGR1; mGR2, the guiding rod motor neurone innervating guiding rod muscle mGR2; mE1, mE2 and mE3, epiphallus motor neurones innervating epiphallus muscles E1, E2 and E3, respectively; m1VL and m2VL, ventral lobe motor neurones innervating muscles of the ventral lobes; m(1)MP and m(2)MP, median pouch motor neurone groups innervating muscles of the median pouch. For each band, white sections represent no activity, stippled sections represent intermediate activity, stippled sections with an asterisk represent sporadic activity and black sections represent intense activity. During natural copulation, the approximate times between events are as follows: 4 s between the onset of the test stimulus (S) and spermatophore extrusion (SE), 9 s between spermatophore extrusion (SE) and the end of spermatophore transfer (E:ST), 4 min between the end of spermatophore transfer (E:ST) and pouch opening (PO) and 45 s between pouch opening (PO) and spermatophore protrusion (SP).



However, after spermatophore protrusion, both groups of motor neurone resumed tonic firing, which soon changed into the bursting pattern (Fig. 6, MP).

Summary of motor neurone activity

Genital motor neurone activity during tethered copulation and spermatophore formation is summarized diagrammatically in Fig. 7. Most of the motor neurones changed their activity in synchrony prior to or during the two main reproductive events. During copulation (Fig. 7, left), one (mGR2) of the guiding rod motor neurones becomes active during test stimulation (S), causing the projection movement of the guiding rod into the spermathecal duct of the female. When the other motor neurone (mGR1) discharges vigorously together with mGR2, the guiding rod is inserted firmly into the spermathecal duct by the tonic contraction of muscles GR1 and GR2. At the same time, mDP is suddenly activated, causing contraction of the dorsal pouch muscles and spermatophore extrusion (SE). For spermatophore transfer (ST), one (mE1) of the epiphallus motor neurones is co-activated with mDP. As a result, the epiphallus muscle E1 contracts, jerking the lateral arms of the phallic complex backwards. At the same time, motor neurones mE2 and mE3 are inactivated, which helps to protract the phallic complex by relaxing muscles E2 and E3. The firing patterns in mE1–mE3 cause the opening of passages that allow haemolymph inflation of the ventral lobes and median pouch.

The inactivation of m1VL and m(1)MP during spermatophore transfer may cause relaxation of the ventral lobes and median pouch. During spermatophore transfer, m2VL shows strong tonic activity followed by burst activity. At the end of spermatophore transfer (E:ST), both the ventral lobes and the proximal part of the median pouch quickly shrink and fold under the dorsal pouch, and the phallic complex is also retracted to its original position. These movements are caused by an increase in activity in mE2, mE3, m(1)MP and m(2)MP. During natural copulation, they are observed at the moment when the male unhooks following spermatophore transfer.

Soon (20–30 s) after spermatophore transfer, all the genital motor neurones except mGR2 and m1VL assume rhythmic bursting at a rate of 0.17 Hz (Fig. 7, middle). The rhythmic activity is accelerated approximately 30 s before pouch opening (PO) and then suddenly changes prior to and during pouch opening: motor neurones mGR1, mGR2, m1VL and m(1)MP become tonically active, while mDP, mE2, mE3, m2VL and m(2)MP become silent (Fig. 7, right). At the opening of the subgenital plate, mGR1 and mGR2 begin to fire, causing the dorsal pouch to be pulled backwards by the contraction the guiding rod muscles GR1 and GR2. The moderate activation of mE1 and absence of activity in mE2 and mE3 allow haemolymph pressure to extend into the ventral lobes *via* downward eversion of the lateral arms of the phallic skeleton. The activity of m1VL may generate ventral lobe

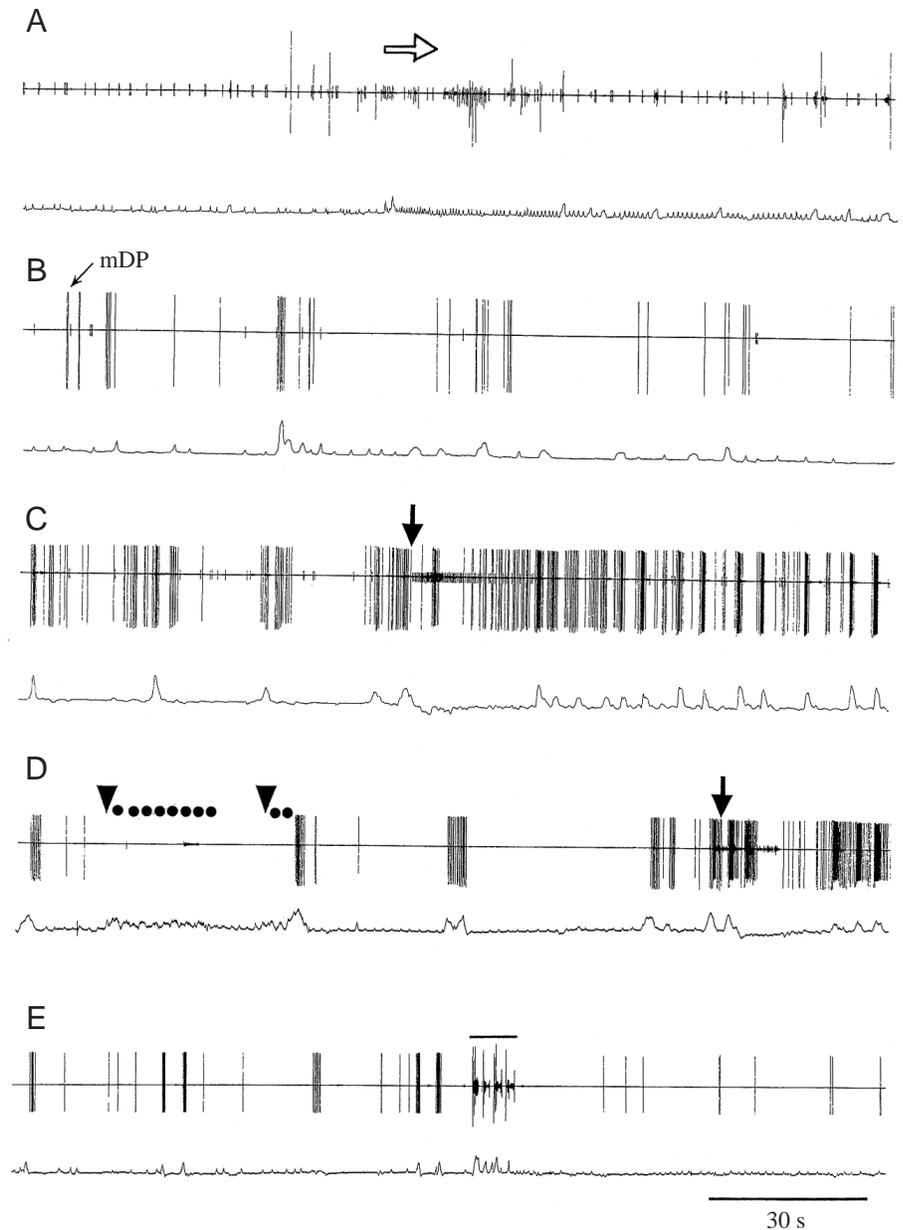


Fig. 8. Effects of inactivation of the head ganglia on the activity of a dorsal pouch motor neurone (mDP). Upper traces, spike activity in mDP; lower traces, contractility of the dorsal pouch. (A) Initiation of head cooling (horizontal arrow). Some large and medium-sized vertical lines are artefacts caused by the cooling treatment. (B) Activity 10 min after cooling was initiated. (C) Activity 20 min after cooling was initiated. The vertical arrow indicates ejection of the spermatophore. Note that rhythmic bursting appeared after spermatophore extrusion, during inactivation of the head ganglia. (D) Response of mDP to test stimulation during head cooling in a different male. This stimulus was applied twice (arrowheads, onset of stimulation; circles, period of stimulation). The mDP responded weakly to the second stimulus, but there was no spermatophore extrusion. The spermatophore was ejected spontaneously at the arrow. (E) Effects of noxious stimulation on post-copulatory rhythmic bursting of mDP under control conditions in a different male. The left and right antennae were pinched twice each with forceps (below the horizontal bar). Note the inhibition of bursting after this stimulus.

tension to prepare for the receipt of the spermatophore material. Finally, resumed activity in m(1)MP and m(2)MP contributes to the folding of the entire median pouch under the ventral lobes by contraction of the median pouch muscles.

The effect of head cooling

To examine the influence of the head ganglia on spermatophore extrusion, the activity of mDP (which is responsible for dorsal pouch deformation) was recorded while the head of a sexually active male was cooled. Before cooling, mDP was silent (Fig. 8A), but 10 min after cooling it began weak bursting (Fig. 8B). After 20 min (Fig. 8C), mDP fired more often with occasional bursts at 40 Hz. At every burst, the dorsal pouch contracted slightly. When a relatively stronger burst (60 Hz) occurred, the spermatophore was extruded by

dorsal pouch deformation (Fig. 8C, black arrow). The burst was less intense, however, than that seen in response to the test stimulus under normal conditions (Fig. 5, DP). After spermatophore extrusion (Fig. 8C), the spike discharge assumed the typical post-copulatory rhythmicity (Fig. 5, DP). To examine the effects of test stimulation during head cooling on spermatophore extrusion, epiphallallic sensilla were stimulated (Fig. 8D, circles). mDP did not yet respond to the test stimulus (Fig. 8D, arrowheads), but after approximately 1 min, mDP exhibited spike bursts that caused the spermatophore to be slowly extruded (Fig. 8D, black arrow). Under control conditions, the post-copulatory rhythmic burst was inhibited (Fig. 8E) when noxious stimulation (antennal pinching) was applied (Fig. 8E, bar). In contrast, mDP, which for unknown reasons failed to show rhythmic bursting in the

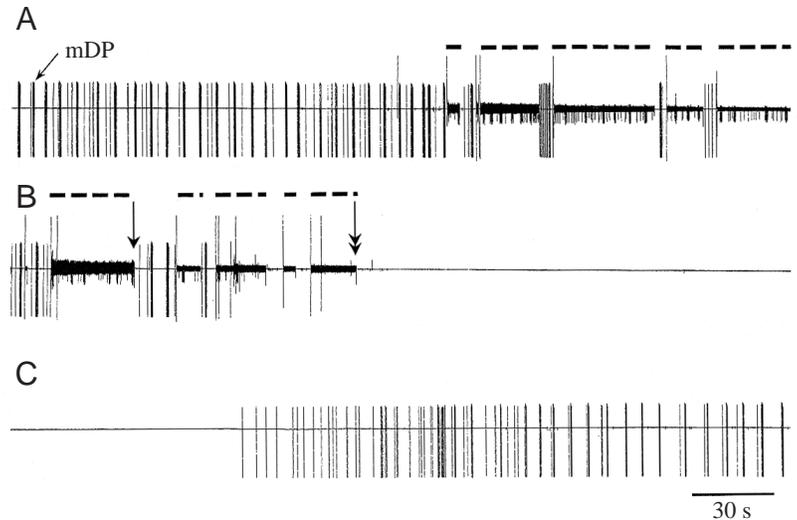


Fig. 9. Effects of transecting the ventral lobe nerve on activity in a dorsal pouch motor neurone (mDP). (A,B) Continuous recording of mDP after spermatophore extrusion. In B, the single-headed arrow indicates a nerve cut ipsilateral to the recorded side and the double-headed arrow indicates a contralateral nerve transection. Broken lines in A and B show recording disturbances during surgical treatment. Note that rhythmic bursting of mDP is present after the ipsilateral nerve cut, but disappears after an additional contralateral nerve cut. (C) Recovery of rhythmic burst approximately 4 min after the second nerve cut.

control, began to exhibit it as soon as the head was cooled (results not shown).

Effects of cutting the connectives

To clarify the results obtained by cooling, a bilateral connective cut was performed while mDP activity was being recorded from a pre-copulatory male. When the connectives were cut between the brain and the suboesophageal ganglion, the hitherto silent mDP immediately began to discharge. The pattern of spontaneous activity was nearly the same as that seen during head cooling, indicating that the effects of head cooling on mDP (Fig. 8A–C) were due to the inactivation of the brain. In the post-copulatory male, rhythmic bursting occurred during cooling of the head ganglia, but it was not clear where the rhythm was generated. When the connectives between the sixth abdominal ganglion and the terminal abdominal ganglion were cut following head cooling, the mean intraburst discharge rate increased from 83 Hz (median; C.I. 75–100 Hz; $N=12$) to 108 Hz (C.I. 100–120 Hz; $N=10$), while the mean cycle rate did not change from 0.11 Hz (C.I. 0.09–0.12 Hz; $N=13$, before; C.I. 0.10–0.15 Hz; $N=11$, after). Thus, the pattern of post-copulatory rhythmic activity was not essentially different between males with a cooled head and those with their connectives cut, although spike bursting was more robust in the latter. This indicates that the rhythm was intrinsic to the terminal abdominal ganglion. For spermatophore protrusion, when the sixth abdominal ganglion/terminal abdominal ganglion connectives were cut immediately after pouch opening, spermatophore protrusion followed pouch opening after approximately 50 s (median; C.I. 45–55 s; $N=25$).

These results reveal that, among the genital motor neurones, at least mDP is inhibited by the brain during the pre-copulatory stage and that both post-copulatory rhythm generation and spermatophore protrusion are carried out totally under the control of the terminal abdominal ganglion.

The effects of deafferentation

It has previously been observed that the ease of artificially

eliciting spermatophore extrusion by applied epiphallallic cavity hair stimulation is proportional to the vigour of the copulatory response of the male to artificial stimuli (Sakai et al., 1991). This suggested that afferent feedback derived from an increase in body tonus may help to facilitate the elicitation of spermatophore extrusion. To test this hypothesis, all the nerve bundles emanating from the third to the sixth abdominal ganglia and the terminal abdominal ganglion, except the genital nerve, were cut. Two of the seven males ejected their spermatophore during this operation. In the five remaining males, mDP showed a strong burst of activity in response to the test stimulus, which resulted in spermatophore extrusion. This indicated that the elimination of sensory feedback from the abdomen is not necessarily required for the ejection of the spermatophore.

Deafferentation was also used to examine whether the genital motor neurones required peripheral feedback to show post-copulatory rhythmic bursting. The abdominal nerve bundles were first cut as described above, and different nerve branches of the genital nerve were then cut singly or in combination. The effects of the operation were detected only in males in which the innervation to the ventral lobes had been cut: rhythmic bursting ceased after a bilateral nerve cut (Fig. 9A,B). However, this effect was temporary, and the rhythm recovered several minutes later (Fig. 9C), suggesting that feedback input from sensilla on the ventral lobes (see Fig. 7A in Kumashiro and Sakai, 2001) serves only a reinforcing role in rhythm generation.

Discussion

Neuroethological studies of instinctive behaviour have often focused on mechanisms for triggering patterned motor actions such as startle responses (Wine and Krasne, 1982; Camhi, 1984; Pearson and O'Shea, 1984; Ritzmann, 1984; Hoy, 1989; Burrows, 1995). Copulatory behaviour incorporates an apparent trigger mechanism in the sperm ejaculation or spermatophore extrusion that occurs at the culmination of

mating. However, this trigger mechanism differs from that seen in startle responses. The former requires a fixed duration of stimulation and cannot recommence soon after occurring. Moreover, male behaviour often changes from courting to resting, guarding or occasionally dying after copulation. In contrast, startle responses operate rapidly at any time and can recommence very quickly. These unique aspects of the trigger mechanism of copulatory behaviour may provide new insights into our understanding of behavioural switching.

So far, however, copulatory behaviour has scarcely been studied at the cellular level. Recently, neural recording from a freely moving snail *Lymnaea stagnalis* (De Boer et al., 1997) has shown that electrical activity in neurones of the cerebral ganglion (Chase, 1986) can induce eversion of the preputium (penis-carrying structure), which occurs during male copulation, by release of a peptide. In insects, however, a neuroethological approach has not yet been adopted for the analysis of the neural control of mating.

In the present paper, we identify motor neurones innervating five genital organs in the male cricket and characterize their spike activity during tethered copulation and spermatophore formation. The influences of the brain and peripheral feedback on spermatophore extrusion have also been examined by cooling and by nerve transection. The results provide a basis for investigating neural mechanisms of reproductive behaviour in male insects.

Spermatophore extrusion

Spermatophore extrusion is one of the final motor actions carried out by dorsal pouch contraction. During natural copulation, it occurs 3.8 s (median; 95 % C.I. 2.5–5.0 s; $N=20$) after the male ceases to pull down the subgenital plate of the female, following insertion of the copulatory papilla of the female into the genital cavity of the male (Sakai et al., 1991). Although this delay reveals that dorsal pouch contraction is not easily triggered, even by the key stimulus, its occurrence is time-fixed as shown by the small value of the confidence interval. In abdomen-opened males, however, more than 10 s of epiphallallic stimulation was needed to elicit dorsal pouch contraction. The difference in delay between natural and artificial ejection of the spermatophore attachment plate may indicate that the model is less efficient in stimulating sensilla than the copulatory papilla. However, the artificial method revealed a gradual increase in movement of the abdomen of the male during the prolonged test stimulation (Kumashiro and Sakai, 2001), suggesting a mechanism that builds sexual excitation up towards a culmination, by recruiting and augmenting the state of the reproductive centre, which is a prerequisite for strong contraction of the dorsal pouch. The 4 s latency for spermatophore extrusion may provide a behaviourally adaptive advantage, allowing strengthening of genital coupling or rendering the female completely immobile to ensure subsequent spermatophore transfer.

Our extracellular recordings indicated that mDP showed no spontaneous discharge and no response to test stimulation. However, once it had been activated, the discharge continued

for more than 5 s, at a frequency of 137 Hz, and was responsible for the strong contraction of the dorsal pouch. Two guiding rod motor neurones (mGR1 and mGR2), one (mE1) of the epiphallus motor neurones and two ventral lobe motor neurones (m1VL and m2VL) also fired synchronously with mDP (Fig. 7). Such a time-locked activation of functionally different motor neurones suggests the presence of a common premotor neurone driving this motor sequence.

A prolonged triggering delay is unusual in motor control circuitry. Although another well-studied behaviour, jumping in locusts, also involves an initial long delay, the underlying skeleto-muscular actions are quite different from those in cricket copulation. The former involves antagonist co-contraction during the delay and sudden flexor inhibition to release the ballistic leg extension, while in the latter, no dorsal pouch motor output occurs during the delay. However, the neural circuitry for both behavioural delays could share a common feature, namely high-threshold premotor trigger neurones. This was thought to occur in the jumping circuit (Pearson et al., 1980), but its trigger mechanism is now being questioned (Burrows, 1996). It may well occur in the cricket copulatory circuit, where excitation build-up, or interaction between tonic excitation and critically timed inhibition, could drive a high-threshold element.

In the other recent recordings of genital nerve activity in crickets, Snell and Killian (Snell and Killian, 2000) reported rhythmic discharges of six motor units during spontaneous movements of the genitalia. These were identified as DP (dorsal protractor), EcL1 and EcL2 (levators of the ectoparamere), DL (dorsal pouch levator), EpAb (epiphallallic abductor) and EpAd (epiphallallic adductor) in *Acheta domesticus*. These motor units appear to correspond, respectively, to mDP, mGR2, mGR1, mE1, mE2 and mE3 in *G. bimaculatus* (for muscle correspondence, see Kumashiro and Sakai, 2001). The prolonged spontaneous rhythmic discharges reported for *A. domesticus* were released by decapitation of dissected males. In our recordings, however, the burst discharges of mDP responsible for spermatophore extrusion occur only once in response to key stimulation to the epiphallallic sensilla. Repeated burst discharges do not seem to be associated with spermatophore extrusion during copulation. In Fig. 8A–C, a change in the activity of mDP after head cooling (equivalent to decapitation) is shown in which spontaneous discharges appeared several minutes after inactivation of the head ganglia, and the burst then became faster and more regular after spontaneous ejection of the spermatophore. The rhythmic bursts seen here before and after spontaneous ejection of the spermatophore during cooling may correspond to those recorded by Snell and Killian (Snell and Killian, 2000). As will be described for the role of the brain in spermatophore extrusion, a sexually excited male occasionally shows self-spermatophore extrusion several minutes after the cessation of copulation attempts. At that time, the male has apparently lost motivation to copulate and ejects the spermatophore himself ('abortion', Sakai et al., 1991) as if it were an excretion. Two to three minutes before abortion, the

male begins to exhibit repeated contractions of the terminal abdominal segment associated with strong abdominal movement that resemble those observed after decapitation or head cooling. From these observations, it seems likely that the spontaneous rhythmic bursts of the genital motor units in decapitated males reported by Snell and Killian (Snell and Killian, 2000) may be involved in the movements preceding abortion or in those after spermatophore extrusion.

The role of the brain and peripheral feedback in spermatophore extrusion

It is well known that transection of the ventral nerve cord causes spontaneous spike discharge in the phallic nerve of the praying mantis and cockroach (Roeder et al., 1960). Similar discharges can be inhibited by electrical stimulation of the ventral cord in the cockroach (Grossman and Parnas, 1973). These observations suggest that the anterior ganglia may play a role in inhibiting the phallic neural circuitry in the terminal abdominal ganglion. We previously found that the spermatophore was spontaneously ejected after decerebration of males in the mating stage (Matsumoto and Sakai, 2000a) and, here, that head cooling and cutting the connectives between the brain and the suboesophageal ganglion induced spontaneous discharges in mDP, which eventually formed weak bursts, resulting in dorsal pouch deformation. This strongly suggests that the brain inhibits mDP even during the mating stage. However, the spike burst induced with the test stimulus during head cooling was less intense than the spike burst evoked under control conditions, and the spermatophores were ejected slowly, as in the case of spermatophore abortion discussed above. These observations suggest that brain inhibition prior to spermatophore extrusion may be necessary for mDP to discharge vigorously at the ejection of the spermatophore.

Peripheral feedback provided by normal body tonus before spermatophore extrusion appears not to be necessary for triggering dorsal pouch deformation, since transection of sensory nerves to the abdominal ganglia had little effect on the efficacy of spike burst activation in mDP. It should be noted that some males ejected their spermatophore during the operation. Peripheral feedback may provide some tonic inhibition until sexual excitation reaches its culmination, as proposed for descending inhibition from the brain. At present, it is premature to draw any conclusions about the functional role of reafference from the periphery in spermatophore extrusion.

Comparison with ejaculation in mammals

Spermatophore extrusion in the cricket may be likened to ejaculation in rats, during which seminal fluid is ejected in the course of 5–10 intromissions (Hart and Odell, 1981). During intromission, penis receptors are mechanically stimulated by the female vagina, which facilitates copulatory actions, such as erection and flips, before triggering strong contraction of the genital muscles including sphincter muscles. It is plausible that afferent information from the mechanoreceptors on the penis

stimulates ejaculation control centres, one in the spinal cord and the other in the cerebrum (brain stem, hypothalamus and forebrain) through the build-up of sexual excitation. It is known, however, that the same afferent information from the penis inhibits ejaculation, which may represent spinal (Hart and Odell, 1981) and cerebral (Marson and MaKenna, 1990; Sachs and Meisel, 1988) mechanisms responsible for the obligatory copulatory intromissions that precede ejaculation. These excitatory and inhibitory controls over ejaculation in the lower and higher centres parallel those shared by the terminal abdominal ganglion and brain mechanisms controlling spermatophore extrusion in the male cricket.

Post-copulatory rhythmic burst

One of the conspicuous features of the genital motor neurones is the rhythmic activity that appears during the period between spermatophore extrusion and protrusion. This was observed almost synchronously in all the motor neurones in the five genital organs. Although we have discussed the functional roles of the rhythmic movement in relation to the preparatory actions for subsequent spermatophore protrusion (Kumashiro and Sakai, 2001), the discussion was speculative. It may simply reflect a state of functional decoupling of genital motor neurones from the reproductive centre (Sakai et al., 1995). It is certain, at least, that the rhythm is generated within the terminal abdominal ganglion because it was retained during head cooling and after transection of the connectives between the sixth abdominal ganglion and the terminal abdominal ganglion. Furthermore, it persisted after transection of the nerve bundles to the terminal abdominal ganglion. However, the rhythm was temporarily inhibited by noxious stimulation to the antenna (just as copulatory actions were inhibited by the same stimulation *via* the brain; Matsumoto and Sakai, 2000b), suggesting that the brain is involved in inhibiting the post-copulatory rhythm. These observations reveal that the neural circuits for rhythmic movement are intrinsic to the terminal abdominal ganglion and function in the absence of brain inhibition.

Spermatophore formation

Spermatophore formation starts with subgenital plate (pouch) opening, followed by secretion of the spermatophore materials, transport through the ejaculatory duct and ejection onto the ventral lobes. The ejected jelly-like spermatophore material is then divided into two parts. These steps proceed fully automatically under the control of the terminal abdominal ganglion once the initial step has been triggered by the brain, since connective transection just anterior to the terminal abdominal ganglion immediately after pouch opening does not hinder the occurrence of spermatophore protrusion, while decerebration before pouch opening abolishes the occurrence of spermatophore protrusion (Ootsubo and Sakai, 1992).

Our recordings indicated that post-copulatory rhythmic bursting was accelerated in almost all the genital motor neurones approximately 30 s before opening of the subgenital plate. This acceleration may reflect the arrival of descending

brain commands to initiate the motor actions for spermatophore formation. This descending input may alter the state of the terminal abdominal ganglion reproductive centre or functionally couple the motor neurones to a different centre, either or both of which serve to maintain tonic discharges or silence in many motor neurones. After spermatophore protrusion, m1VL exhibits the vigorous activity involved in partitioning the spermatophore material and compressing the anterior part into the dorsal cavity. Only this movement was found to be aided by sensory feedback from the sensilla on the ventral lobes (Kumashiro and Sakai, 2001).

In conclusion, our results demonstrate that spatially and temporally coordinated activity in the genital motor neurones is responsible for copulation and spermatophore formation, suggesting the presence of a terminal abdominal ganglion pattern generator linked to a long-delay trigger element. The present study sets a framework for neuroethological analysis of copulatory behaviour in insects by proposing neuronal mechanisms amenable to intracellular recording techniques.

We thank Dr S. Takahashi for nerve sectioning and Dr L. H. Field for his valuable comments and critical reading of the manuscript. This project was supported by a Grant-in-Aid for Scientific Research (11640680) from the Japanese Ministry of Education, Science, Sports and Culture to M.S.

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