The Journal of Experimental Biology 202, 103–113 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1738

PATTERNED ACTIVATION OF UNPAIRED MEDIAN NEURONS DURING FICTIVE CRAWLING IN *MANDUCA SEXTA* LARVAE

R. M. JOHNSTON*, C. CONSOULAS, H.-J. PFLÜGER‡ AND R. B. LEVINE§

Division of Neurobiology, University of Arizona, Tucson, AZ 85721, USA

*Present address: Department of Integrative Biology, University of California, Berkeley, CA 94720, USA
‡Permanent address: Freie Universität Berlin, Institut für Neurobiologie, Königin-Luise-Strasse 28–30, D-14195 Berlin, Germany
§Author for correspondence at: Neurobiology, Room 611 Gould Simpson, PO Box 210077, University of Arizona, Tucson, AZ 85721, USA (e-mail: rbl@neurobio.arizona.edu)

Accepted 29 October; published on WWW 14 December 1998

Summary

The unpaired median neurons are common to the segmental ganglia of many insects. Although some of the functional consequences of their activation, among them the release of octopamine to modulate muscle contraction, have been described, less is understood about how and when these neurons are recruited during movement. The present study demonstrates that peripherally projecting unpaired median neurons in the abdominal and thoracic ganglia of the larval tobacco hornworm *Manduca sexta* are recruited rhythmically during the fictive crawling motor activity that is produced by the isolated central nervous system in response to pilocarpine. Regardless of the muscles to which they project, the efferent unpaired median neurons in all segmental ganglia are depolarized together during the phase of the crawling cycle when the thoracic leg levator motoneurons are active. During fictive crawling, therefore, the unpaired median neurons are not necessarily active in synchrony with the muscles to which they project. The rhythmical synaptic drive of the efferent unpaired median neurons is derived, at least in part, from a source within the subesophageal ganglion, even when the motor pattern is evoked by exposing only the more posterior ganglia to pilocarpine. In pairwise intracellular recordings from unpaired median neurons in different ganglia, prominent excitatory postsynaptic potentials, which occur with an anterior-to-posterior delay in both neurons, are seen to underlie the rhythmic depolarizations. One model consistent with these findings is that one or more neurons within the subesophageal ganglion, which project posteriorly to the segmental ganglia and ordinarily provide unpatterned synaptic inputs to all efferent unpaired median neurons, become rhythmically active during fictive crawling in response to ascending information from the segmental pattern-generating network.

Key words: *Manduca sexta*, unpaired median neurone, crawling, synaptic drive, subesophageal ganglion.

Introduction

Successful execution of behavioral movements requires flexibility in patterned motor activity and muscle function. Sensory feedback and neuromodulation are two mechanisms that permit flexibility in central circuits and peripheral motor function in the face of changing conditions in the internal or external environment (Burrows, 1996; Harris-Warrick and Marder, 1991; Sombati and Hoyle, 1984; Ramirez and Pearson, 1991a,b). The recruitment of neuromodulatory neurons during patterned motor activity may be essential both for adapting muscle function to meet particular demands and for modulation of the motor pattern itself. One neuromodulator with multiple functions within the insect nervous system is the biogenic amine octopamine (Evans, 1980, 1985).

Biochemical (Evans and O'Shea, 1978) and immunohistochemical (Konings et al., 1988; Stevenson et al., 1992) methods reveal a group of peripherally projecting modulatory neurons in the locust, the efferent unpaired median (UM) neurons, which contain octopamine. These neurons have also been identified in many other insect species (Plotnikova, 1969; Crossman et al., 1971, 1972; Hoyle, 1978; Stevenson and Spörhase-Eichmann, 1995; Brookes, 1988; Brookes and Weevers, 1988) and, from the dorsal or ventral position of their somata, they have been named dorsal unpaired median (DUM) (Hoyle, 1975, 1978) or ventral unpaired median (VUM) (Kondoh and Obara, 1982; Watson, 1984) neurons. These cells have unique bifurcating primary neurites, which give rise to two axons that innervate the same targets on both sides of a segment. Neurons with similar characteristics have been identified in the hawkmoth *Manduca sexta* (Pflüger et al., 1993). Larval efferent UM neurons in *M. sexta* undergo dendritic remodeling, but persist through metamorphosis to innervate targets in the adult (Pflüger et al., 1993).

Efferent UM neuron activity in locusts and other insects modulates the effectiveness of neuromuscular systems. The twitch tension and relaxation rate of skeletal muscle are enhanced (O'Shea and Evans, 1979), whereas myogenic contractions of visceral muscles are inhibited (Lange and Orchard, 1984a,b; Orchard and Lange, 1986; Kalogianni and Pflüger, 1992; Kalogianni and Theophilidis, 1993). Modulatory effects of octopamine on peripheral sense organs have also been reported in locusts (Ramirez and Orchard, 1990; Matheson, 1997). Octopamine is known to affect muscle metabolism (Candy et al., 1997), so UM neurons may also exert metabolic effects on target tissues.

Unpaired median neurons are also suspected to be the mediators of octopaminergic effects within central neuropils. For example, flight-like motor patterns are induced by ionophoretic or pressure injection of octopamine into specific locations within locust thoracic ganglia (Sombati and Hoyle, 1984; Stevenson and Kutsch, 1988). This may be due to the induction of plateau potentials and bursting in certain interneurons of the flight circuit (Ramirez and Pearson, 1991a,b). The possibility that these effects are ordinarily mediated by the release of octopamine from the central processes of efferent UM neurons remains open to question (Pflüger and Watson, 1995; Parker, 1996), but central release from the processes of local or intersegmental octopaminergic cells that lack peripheral axons may occur within the locust pterothoracic ganglia (Stevenson et al., 1994), and good evidence for the release of octopamine by UM neurons within the brain comes from studies of olfactory learning in honeybees (Hammer, 1993).

Efferent UM neurons are activated during the flight motor pattern of locusts (Ramirez and Orchard, 1990), during walking in crickets (Gras et al., 1990) and by ventral giant interneurons in the cockroach (Pollack et al., 1988). Recent studies in the locust provide evidence that specific patterns of recruitment occur during different motor tasks. For example, only three of the nineteen efferent UM neurons in the metathoracic ganglion are strongly activated in parallel with motor circuits during hindleg kicking (Burrows and Pflüger, 1995), whereas another distinct sub-population is inhibited during the same movement. The same sub-population of UM neurons is also inhibited during locust flight (Duch et al., 1997). In another example, efferent UM neurons that project to leg muscles generate bursts of action potentials with each cycle of activity during the pilocarpine-induced rhythmic leg motoneuron pattern in locusts (Baudoux et al., 1998). The sources of UM neuron drive include sensory neurons and unidentified intrasegmental and intersegmental interneurons (Duch and Pflüger, 1995; 1996; Duch et al., 1997). One source of efferent UM neuron drive may be located within the subesophageal ganglion. In the absence of patterned motor activity, synaptic inputs that occur in common in all efferent UM neurons were observed in *M. sexta*, and their source was located to within the subesophageal ganglion (Pflüger et al., 1993). Similar synaptic inputs have been described in the silkmoth (Brookes and Weevers, 1988) and locust (Watson, 1984; Pflüger and Watson, 1995), but their role in activating UM neurons during movement remains unclear.

One strategy for characterizing patterns of modulatory neuron recruitment is to record from identified UM neurons during rhythmic fictive motor patterns that are produced by the perfusion of the muscarinic agonist pilocarpine (Baudoux et al., 1998; Büschges et al., 1995; Chrachri and Clarac, 1990; Ryckebusch and Laurent, 1993, 1994). Pilocarpine induces a pronounced inter- and intrasegmentally coordinated motor pattern, called fictive crawling, in isolated nerve cords from *M. sexta* larvae (Johnston and Levine, 1996). The motor pattern is qualitatively and quantitatively similar to that associated with crawling (Johnston and Levine, 1996). During each cycle of this fictive crawling, the motoneurons that innervate the body wall muscles produce bursts of activity that proceed in a posterior-to-anterior sequence through the abdominal segments. The posterior-to-anterior sequence continues as motoneurons innervating the levator and depressor muscles of the three pairs of thoracic legs sequentially produce alternating bursts of activity during each cycle. The activity is synchronous in motoneurons that innervate the right and left legs or sides of the body. Here, we take advantage of this system in *M. sexta* to characterize the pattern of efferent UM neuron recruitment during fictive crawling. A preliminary account of this work has been presented (Johnston et al., 1997).

Materials and methods

Experimental animals

Manduca sexta larvae, obtained from a colony at the University of Arizona, were reared on an artificial diet (modified from Bell and Joachim, 1976) and maintained on a 17 h:7 h light:dark photoperiod at 26 °C and 50–60 % relative humidity. Larvae were used for experiments on the day following the molt into the fifth (final) larval instar (mass approximately 5–6 g).

Electrophysiological techniques

The techniques employed in this study were similar to those described previously (Johnston and Levine, 1996). Animals were anesthetized on ice for 30–50 min before dissection. All dissections were carried out under chilled saline consisting of (in mmol l^{-1}) 140 NaCl, 5 KCl, 4 $CaCl_2$, 28 glucose, and 5 Hepes; pH 7.4 (Trimmer and Weeks, 1989). Anesthetized larvae were pinned dorsal side up in a dish lined with silicone elastomer (Sylgard; Dow Corning). A dorsal incision was made from the head to the most posterior segment, and the gut was removed. Specific nerve branches of each ganglion were dissected free from the surrounding tissue. The nerve cord from the subesophageal ganglion to the terminal abdominal ganglion was dissected from the body and pinned ventral side up in a second Sylgard-lined dish filled with chilled saline. The ventral surface of each ganglion was treated briefly with 3 % (w/v) collagenase–dispase (Sigma) in saline and desheathed using fine forceps. The isolated nerve cord was perfused continuously with saline or

pilocarpine-HCl (10^{-3} mol l^{-1}; Sigma) dissolved in saline, both at room temperature (21–23 °C) (flow rate approximately 15 ml min^{-1}). During some experiments, the subesophageal ganglion was removed by cutting the interganglionic connectives anterior to the prothoracic ganglion. To accomplish this without disturbing ongoing intracellular recordings in more posterior ganglia, the prothoracic ganglion was pinned to the recording chamber but the subesophageal ganglion remained loose, thereby allowing the connectives to be cut with minimal movement and without loss of intracellular recordings.

To monitor motor activity in the isolated nerve cord, extracellular glass-tipped suction electrodes were used to record from selected nerve branches of specific thoracic and abdominal ganglia. In the thorax, we monitored the motor activity from the two main branches of the leg nerve. The 1b branch carries the axons that innervate the femoral levator and the tibial extensor. The axons in the 2a branch innervate

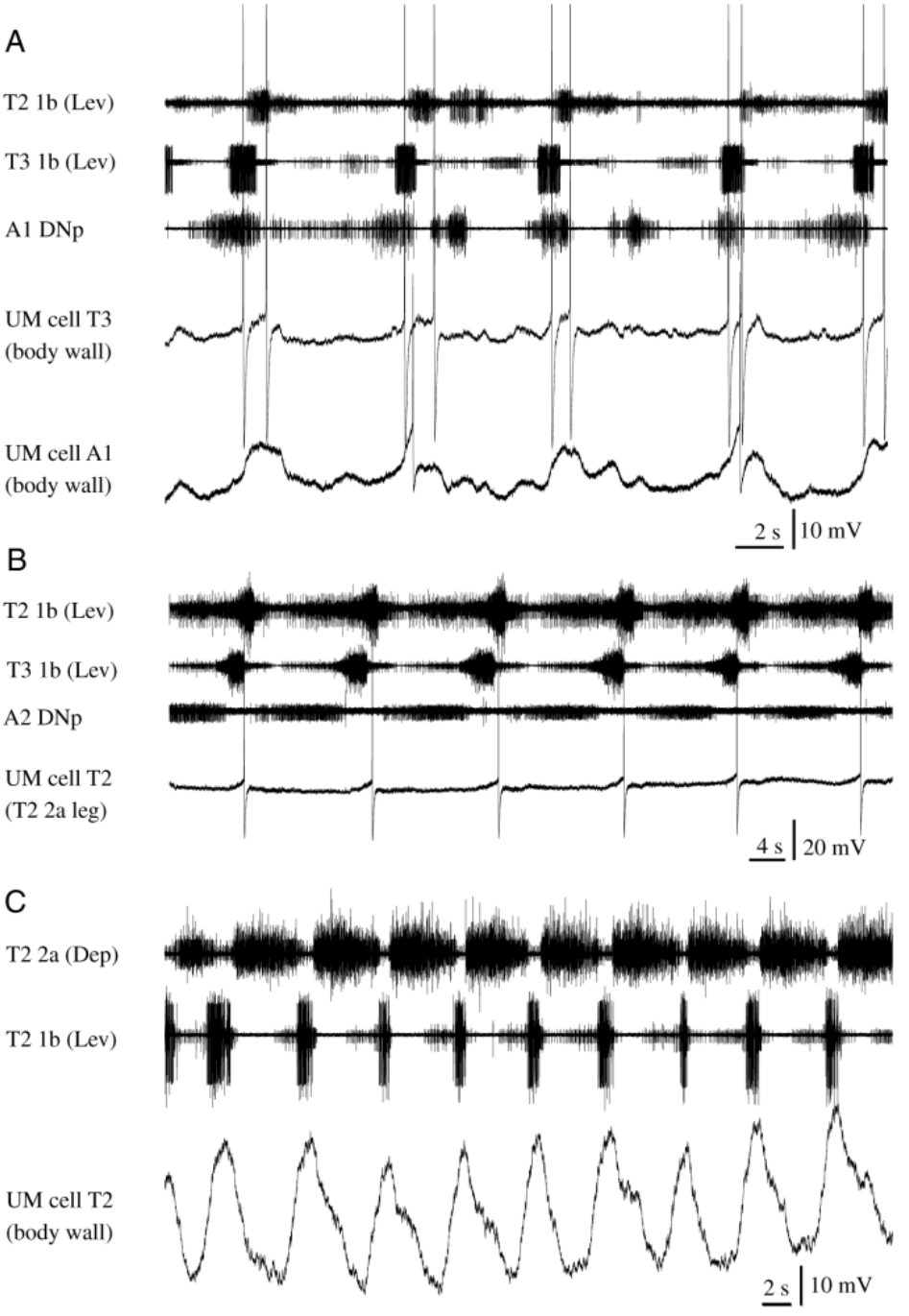

Fig. 1. Rhythmic activity in unpaired median (UM) neurons during pilocarpine-evoked fictive crawling. (A) Simultaneous intracellular recordings from a UM neuron innervating the body wall in the metathoracic segment (T3) and a UM neuron in abdominal ganglion 1 (A1) that projects through the posterior branch of the dorsal nerves (DNp) to innervate body wall muscles. Extracellular recordings are from the nerves innervating levator (Lev) muscles in the mesothoracic and metathoracic segments and from the DNp in the first abdominal segment (A1). Note the posterior-to-anterior progression of activity in the extracellular recordings. Depolarizations in the UM neurons and bursts of activity in the motor roots occurred approximately in phase with thoracic leg levator nerve activity (see also Fig. 2). (B) Intracellular recording from the UM neuron with its axon in the mesothoracic nerve that contains the axons of leg depressor motoneurons. Extracellular recordings are from the nerve innervating levator muscles in the mesothoracic and metathoracic legs and from the posterior branch of the dorsal nerve in the second abdominal ganglion (A2). Note that the UM neuron is depolarized above action potential threshold one-for-one with the bursts of motor activity in the extracellular recordings and approximately in phase with the leg levator nerves. (C) Intracellular recording from a UM neuron in the mesothoracic ganglion that projects to the body wall. Extracellular recordings from mesothoracic leg nerves: nerve T2 2a innervates mesothoracic leg depressor (Dep) muscles, nerve T2 1b innervates mesothoracic leg levator muscles. Note the rhythmic alternation between levator and depressor motoneuron activity in the mesothoracic leg nerves. The UM neuron was hyperpolarized with current injection to below the action potential threshold. Prominent oscillations in membrane potential occur one-for-one with bursts of motoneuron activity in the extracellular recordings, and these peak approximately in phase with levator nerve activity.

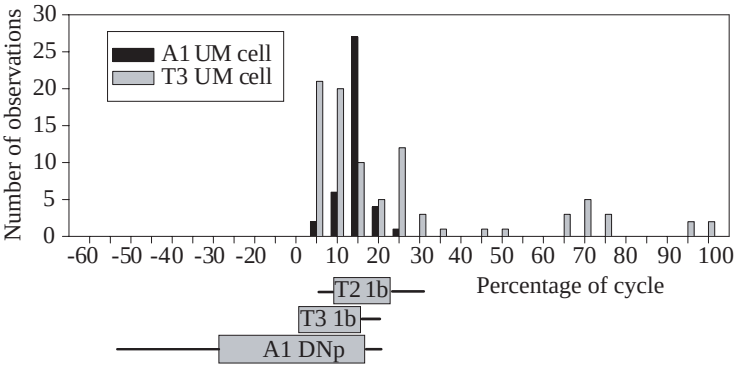

Fig. 2. Phase histogram showing the timing of action potentials in unpaired median (UM) neurons in A1 and T3 relative to the fictive crawling cycle. Data are from the same preparation as that shown in Fig. 1A. To construct the plot, 39 cycles (3.9 min) of fictive crawling activity were used. The cycle period was defined as the interval between the onsets of bursts in segment T3 leg levator motoneurons, as measured from extracellular recordings from nerve T3 1b (mean cycle period 5.9 s; standard deviation 1.1 s; mean T3 1b burst duration 0.9 s; standard deviation 0.2 s). The times for onset and termination of bursts in T2 and T3 leg levator motoneurons (nerves T2 1b and T3 1b) and A1 body wall motoneurons (nerve A1 DNp), relative to the onset of T3 1b bursts, were determined for each cycle and are expressed as a percentage of that cycle period. The mean percentages are shown in the gray bars below the plot, with the solid lines indicating the range of percentage values. To construct the histogram, each cycle was divided into 20 bins representing 5 % of the cycle period. The number of action potentials per bin in UM neurons innervating the body wall in A1 and T3 was counted from the intracellular recordings (segments of which are shown in Fig. 1A). Note that most action potentials in both UM neurons occurred during the portion of the cycle when levator motoneurons were active and that, typical of other preparations, the peak of activity in the A1 UM neuron does not precede that for the T3 UM neuron, as might be expected given the posterior-to-anterior progression of motor activity during fictive crawling.

femoral depressors and the flexors of the more distal leg segments (Johnston and Levine, 1966; Kent and Levine, 1988). In the abdomen, we focused on the posterior branch of the dorsal nerve (DNp), which carries the axons of motoneurons that innervate the lateral, ventral and oblique intersegmental muscles of the body wall (Levine and Truman, 1985). The extracellular signals were amplified with differential a.c. amplifiers (A-M Systems), filtered, and stored on a Vetter PCM eight-channel recorder for subsequent analysis. For intracellular recordings, motoneuron somata were impaled with glass micropipette electrodes (resistance 30–60 MΩ) filled with 2 mol l^{-1} potassium acetate. Intracellular recordings were amplified (Axoclamp 2A; Axon Instruments) and stored on video tape. Both intracellular and extracellular signals were played back onto a chart recorder and subsequently transferred to a computer (acquisition rate 13 kHz per channel) for analysis using Data-Pac II software (Run Technologies).

To reveal the dendritic anatomy of abdominal and thoracic UM neurons, and to confirm their identification by visualizing axonal projection pathways, UM neuron somata were impaled with glass microelectrodes filled with 3 % biocytin (resistance 30–50 MΩ). Depolarizing current pulses of 5–10 nA amplitude and 500 ms duration were injected into the cells at a rate of 1 Hz for 20–60 min. To allow staining of the UM cell axons in the periphery, the preparations were left first under constant saline flow for 60 min and then kept at 7 °C for another 6–8 h. Preparations were processed as described previously (Consoulas et al., 1996), then photographed with a compound microscope or drawn using a *camera lucida* attachment.

Results

The efferent unpaired median neurons are rhythmically active during fictive crawling

The efferent UM neurons within the segmental ganglia of *M. sexta* larvae have prominent cell bodies located on the posterior midline and close to the ventral surface of the ganglion. Three UM neurons are located in every thoracic ganglion. Two of these project in the dorsal segmental nerve to body wall muscles, while the third enters the thoracic legs within nerve 2a (C. Consoulas, R. M. Johnston, H.-J. Pflüger and R. B. Levine, unpublished obervations). Two UM neurons are found within each of the unfused abdominal ganglia. Although they have similar dendritic structures, one projects into the lateral and posterior branches of the dorsal segmental nerve, whereas the other projects into the anterior branch exclusively; however, both innervate body wall muscles (Pflüger et al., 1993). Intracellular recordings from the soma of any of these efferent UM neurons reveal broad overshooting action potentials, a characteristic hyperpolarizing undershoot (see Fig. 1A,B) and prominent excitatory postsynaptic potentials (EPSPs) (see Figs 3–5).

In the presence of the muscarinic agonist pilocarpine, isolated ventral nerve cords from *M. sexta* larvae display a robust motor pattern that is qualitatively and quantitatively similar to that associated with crawling (Johnston and Levine, 1996). The fictive crawling motor pattern is distinguished by bursts of activity in body wall motoneurons that proceed in a posterior-to-anterior sequence through the abdominal segments. Subsequently, during each cycle, the motoneurons innervating the levator and depressor muscles of the three pairs of thoracic legs sequentially produce alternating bursts of activity, which are synchronous in nerves that innervate the right and left legs or sides of the body. Of the 45 isolated nerve cord preparations used for this study, 28 displayed patterned activity in response to pilocarpine that could be identified as fictive crawling using these features.

In 15 of the preparations in which the isolated nerve cords produced a clear fictive crawling pattern, intracellular recordings from several different identified efferent UM neurons revealed patterned activity, with depolarizations and action potential bursts accompanying each cycle of motor activity in the extracellular nerves (Fig. 1). In the remaining 13 preparations, there were rhythmic bouts of depolarizing

synaptic inputs to the UM neurons, but these had a minimal effect on the patterning of action potential activity. Since all the UM neurons monitored within a preparation behaved in a similar manner, the difference was dependent on the preparation rather than on the specific identity of the UM neuron. In the former group of preparations, each depolarization of the membrane potential led to 1–3 action potentials in the UM neurons (Fig. 1A,B). Hyperpolarizing the UM neuron membrane potential with constant-current injection prevented action potential production, but the rhythmic depolarizations increased in amplitude, suggesting that they were dependent on synaptic drive (Fig. 1C).

The peak membrane depolarization and action potential activity in several different types of UM neuron, including those innervating the abdominal and thoracic body-wall muscles (Fig. 1A,C) or the thoracic leg muscles (Fig. 1B), occurred during the phase of the crawling cycle when there were bursts of motoneuron activity in the thoracic leg levator nerves (Figs 1A–C, 2). Furthermore, there was substantial overlap in the timing of membrane depolarization and action

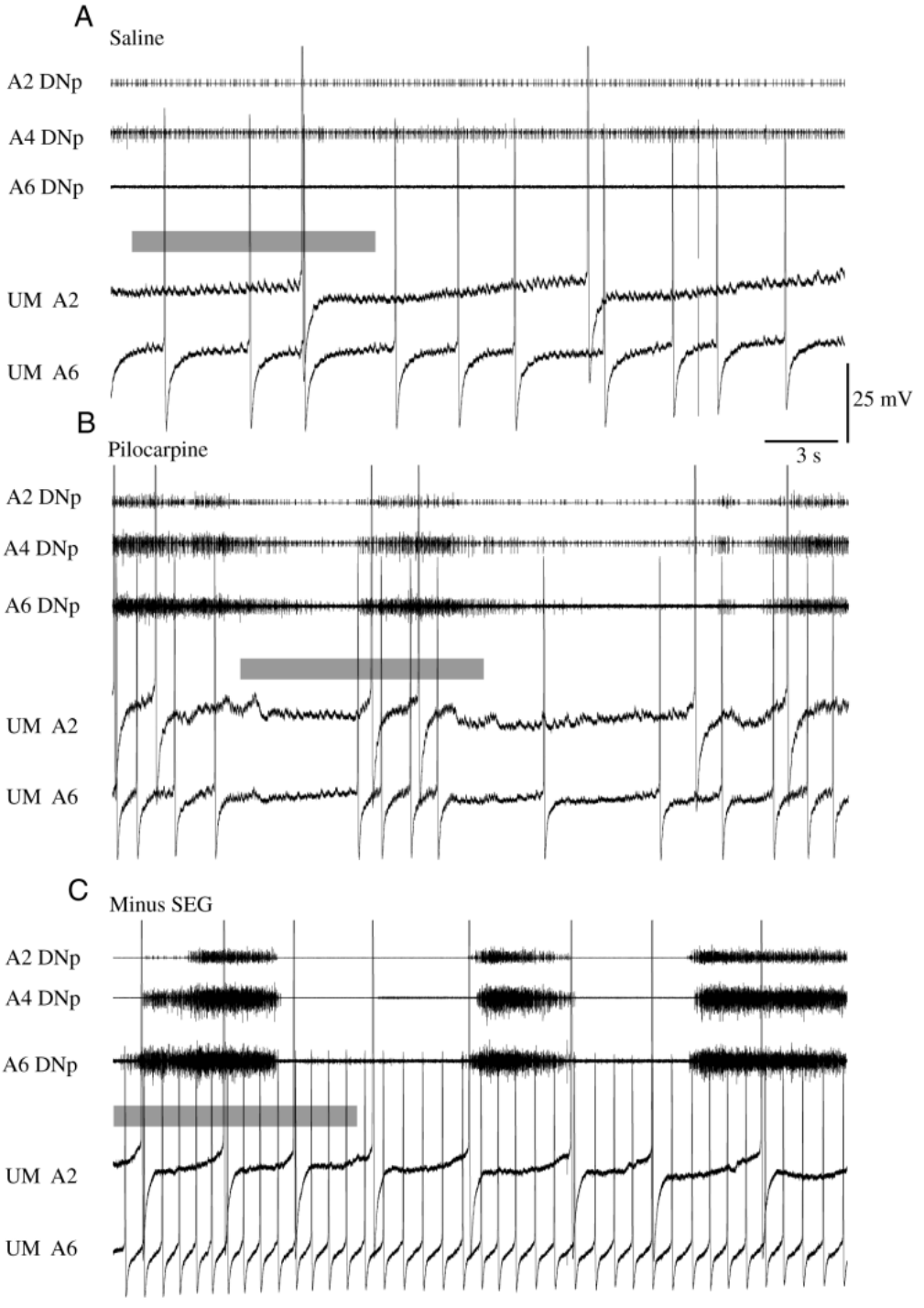

Fig. 3. Characterization of rhythmic activity induced in efferent unpaired median (UM) neurons by pilocarpine. (A) Simultaneous intracellular recordings from efferent UM neurons in A2 and A6 before addition of pilocarpine. Extracellular recordings from body wall motoneurons in abdominal segments 2, 4 and 6. Unpatterned spontaneous activity is present in the motor roots and in both UM neurons. In addition, prominent postsynaptic potentials (EPSPs) occur in both UM neurons (see also Fig. 4A). The gray bar denotes a region of the recording that is shown on an expanded time scale in Fig. 4A. (B) Same preparation and recordings 40 min after the addition of pilocarpine and the onset of fictive crawling. Bursts of EPSPs lead to rhythmic depolarization and the generation of action potentials in both UM neurons. The shaded region is expanded in Fig. 4B to show these EPSPs. (C) Same preparation and recordings 20 s after cutting off the subesophageal ganglion (SEG) in the continued presence of pilocarpine. The UM neuron in A6 became depolarized following the nerve cord cut, but the UM neuron in A2 did not. Note that patterned activity continues in the motor roots, but not in the UM neurons. Also note the absence of the prominent EPSPs, most easily seen in the recording of UM A2 (see also Fig. 4C). DNp, posterior branch of the dorsal nerve.

potential bursts in simultaneous recordings from several different pairs of UM neurons (Figs 1A, 2, 3B), even when they were separated by several segments. Thus, the rhythmic depolarization of efferent UM neurons during fictive crawling did not necessarily occur in phase with activity in the excitatory motoneurons that project to the same target muscles, although there was usually some overlap. For example, a phase plot constructed from 39 cycles of activity (3.9 min) of the preparation shown in Fig. 1A revealed overlapping peaks of action potential activity in UM neurons in A1 and T3 during the phase of the cycle when leg levator motoneurons were active (Fig. 2). Nevertheless, owing to the long duration of abdominal motoneuron bursts, there was overlap between activity in the UM neuron in A1 and the activity of excitatory motoneurons that project to the same muscles.

The subesophageal ganglion as a source of rhythmic synaptic drive to unpaired median neurons

In the isolated ventral nerve cord with the subesophageal ganglion attached, prominent EPSPs occurred in common, but with an anterior-to-posterior delay, in all the efferent UM neurons (Pflüger et al., 1993). In the absence of pilocarpine, these EPSPs occurred continuously, with no apparent patterning, as each UM neuron reached its action potential threshold at different times (Figs 3A, 4A). After the addition of pilocarpine and the onset of fictive crawling, the prominent EPSPs increased in frequency and became clustered into bursts that occurred with each cycle of activity in the motor roots (Figs 3B, 4Bi,ii, 5C,D). These caused compound depolarizations that usually led to action potentials in the UM neurons. As was true of the EPSPs that occurred in the absence of pilocarpine (see also Pflüger et al., 1993), there was an anterior-to-posterior delay between the appearance of the EPSPs in efferent UM neurons of different segments (Fig. 4Bi,ii), but this delay was brief relative to the cycle period. The delay of approximately 40 ms for EPSPs in A2 and A6 yields a conduction velocity of approximately $3\,m\,s^{-1}$, a rate similar to that calculated in a previous study (Pflüger et al., 1993).

The prominent EPSPs that occur in the absence of pilocarpine are not dependent upon the brain, but are lost following removal of the subesophageal ganglion (Pflüger et al., 1993). Along with the anterior-to-posterior delay between their occurrence in

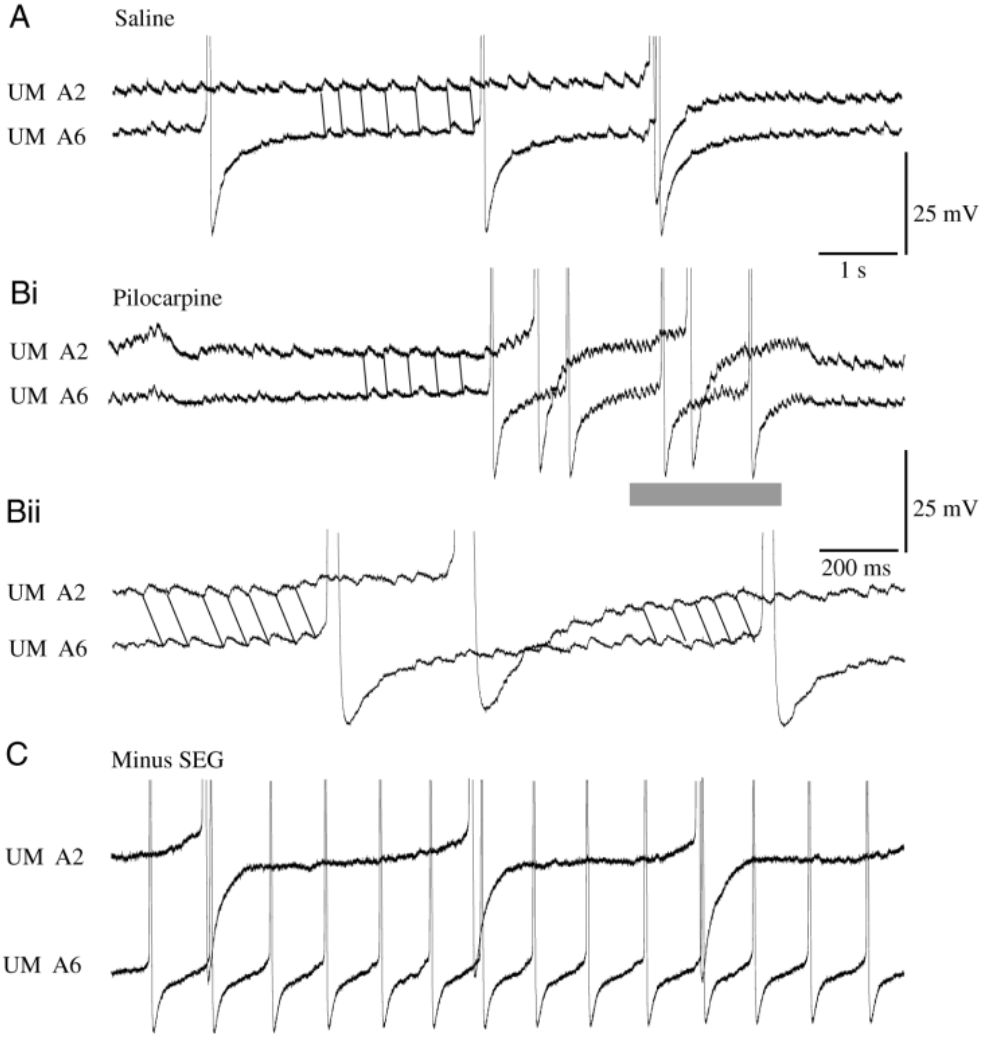

Fig. 4. Portions of the same recordings as those shown in Fig. 3 (see gray bars) shown on an expanded time scale. Action potentials have been clipped for figure preparation. (A) Before the addition of pilocarpine (from Fig. 3A), prominent postsynaptic potentials (EPSPs) occur in both unpaired median (UM) neurons. The dotted lines, which were all drawn at the same angle, indicate some of the EPSPs that occur in common in both neurons, with an anterior-to-posterior delay. (Bi) After the addition of pilocarpine and the onset of fictive crawling (from Fig. 3B), prominent EPSPs occur in high-frequency bursts and lead to action potentials in both UM neurons. Dotted lines indicate some of the EPSPs that occur in common with a constant anterior-to-posterior delay. The gray region is shown on an expanded time scale in Bii. (Bii) On an expanded time scale, the prominent EPSPs that underlie the rhythmic depolarization of UM neurons during fictive crawling are clear. Dotted lines drawn at a constant angle indicate some of the EPSPs that occur in common in both UM neurons. (C) After removal of the subesophageal ganglion (SEG) (from Fig. 3C), the prominent EPSPs are no longer apparent. The time and voltage calibrations in A also refer to Bi and C.

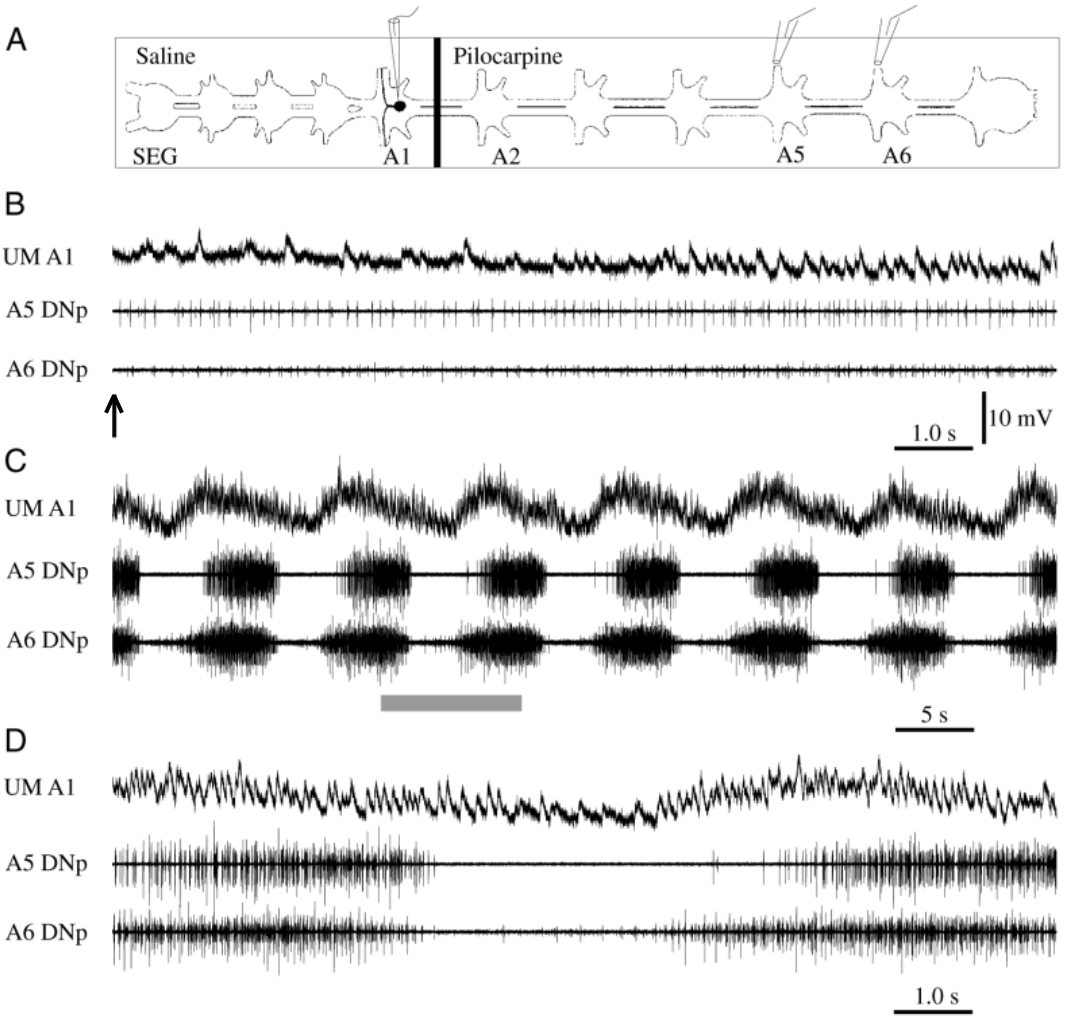

Fig. 5. Increased excitatory postsynaptic potential (EPSP) frequency and membrane potential oscillations in the unpaired median (UM) neurons during fictive crawling are not dependent upon exposure of the subesophageal ganglion (SEG) to pilocarpine. (A) Diagram of the preparation. The isolated nerve cord was placed in a chamber containing a barrier such that only the abdominal ganglia posterior to A1 were exposed to pilocarpine, while the subesophageal ganglion, thoracic ganglia and A1 remained in saline alone. (B) Intracellular recording from a UM neuron in A1. Soon after addition of pilocarpine (arrow) to the posterior chamber, prominent EPSPs began to increase in frequency even though the subesophageal ganglion and A1 had not been exposed to pilocarpine and the fictive crawling pattern had not yet been established. (C) Same preparation after the onset of fictive crawling (4 min after the addition of pilocarpine). Rhythmic depolarizations occurred in the UM neuron, one-for-one with bursts of motoneuron activity recorded extracellularly from the abdominal motor roots. The shaded bar indicates a region of the recording that is expanded in D. (D) Prominent EPSPs, which increase in frequency to cause rhythmic depolarization of the UM neuron, are visible in this expanded recording. DNp, posterior branch of the dorsal nerve.

different segments, this suggests that the EPSPs are generated by neurons that descend from the subesophageal ganglion. Similarly, during pilocarpine-induced fictive crawling, the rhythmic bursts of prominent EPSPs in the efferent UM neurons were dependent upon the presence of the subesophageal ganglion. Following removal of the subesophageal ganglion in preparations that were displaying fictive crawling, the bursts of prominent EPSPs in the UM neurons ceased (N=13; Figs 3C, 4C, 6B,C). In some cases, the UM neurons continued to fire irregularly following removal of the subesophageal ganglion (Fig. 3C), whereas in other cases weak membrane potential oscillations and small EPSPs, perhaps reflecting local segmental inputs, were apparent (Fig. 6B,C).

The patterned motoneuron activity that occurs in the segmental nerves during fictive crawling does not depend upon the subesophageal ganglion, nor does the subesophageal ganglion have to be exposed to pilocarpine for fictive crawling to be evoked (Johnston and Levine, 1996). The prominent EPSPs that caused rhythmic depolarization of the UM neurons during fictive crawling, however, depended on the subesophageal ganglion. This raises the possibility that pilocarpine acts on the subesophageal ganglion to increase the descending drive of the efferent UM neurons.

To test this possibility, isolated nerve cords were placed in chambers containing petroleum jelly barriers that allowed subsets of ganglia to be exposed selectively to pilocarpine (N=2; Fig. 5A). Fictive crawling activity was still evoked in the motor roots following exposure to pilocarpine of only those segmental ganglia posterior to A1 (Fig. 5B,C). Moreover, the increased frequency of prominent EPSPs (Fig. 5B), and their grouping into bursts, still occurred in the efferent UM neurons whether or not they had been exposed directly to pilocarpine (Fig. 5C,D). Following removal of the subesophageal ganglion in this preparation (Fig. 6A), the fictive crawling pattern continued in the motor roots, but became more irregular (Fig. 6B). The prominent EPSPs disappeared, although small EPSPs and membrane potential depolarizations of the UM neuron still occurred and were often correlated with bursts of activity in the

motor roots (Fig. 6B,C). Although the UM neuron gradually depolarized by approximately 10 mV following removal of the subesophageal ganglion in this preparation, this did not account for the absence of the prominent EPSPs. As with the preparation shown in Figs 3 and 4, the prominent EPSPs disappeared immediately after removal of the subesophageal ganglion and were not revealed when the membrane potential was hyperpolarized with current injection. Thus, although a significant source of rhythmic synaptic drive to efferent UM neurons is derived from the subesophageal ganglion during fictive crawling, the activation of this drive does not require the direct exposure of the subesophageal ganglion to pilocarpine.

Discussion

Efferent UM neurons are activated rhythmically during fictive crawling so that, in most preparations, depolarizations and action potentials occur with each cycle of activity in the body-wall and thoracic leg motoneurons. This activity is relatively synchronous in all the segmental efferent UM neurons regardless of the muscles innervated by their peripheral axons. Thus, the two efferent UM neurons within each abdominal ganglion are active together with those in the thoracic ganglia, including the one that projects to the muscles of the thoracic legs. The efferent UM neurons were depolarized and produced action potentials during the portion of the crawling cycle when the thoracic leg levator motoneurons were active. Similarly, in the presence of pilocarpine, UM neurons that innervate leg muscles in the locust were driven rhythmically to produce bursts of action potentials during bursts of activity in the levator motoneurons (Baudoux et al., 1998).

The functional significance of this phasing is unknown at present, but the efferent UM neurons were not necessarily activated together with the excitatory motoneurons that project to the same muscles (Johnston and Levine, 1996). Although the thoracic leg UM neuron projects in nerve 2a (C. Consoulas, R. M. Johnston, H. J. Pflüger and R. B. Levine, unpublished obervations), which innervates the depressor muscles of the larval leg, it was depolarized during the phase of the fictive crawling cycle when all leg levator motoneurons were active. Nevertheless, octopamine that is released peripherally into the vicinity of the target muscles as a result of UM neuron activity may have relatively long-term effects on both the contraction amplitude and relaxation rate of the muscle (Evans and O'Shea, 1978), although these effects have yet to be demonstrated in *M. sexta*. Thus, precise coupling of UM neuron activity to that of the muscles that they innervate may be unnecessary, as long as octopamine release is ensured during periods of crawling. Alternatively, the coupling observed in the isolated central nervous system (CNS) preparation may be different from that in an intact animal during crawling. For example, segmental sensory input may force efferent UM neuron activity into a clear phase relationship with the motoneurons innervating specific muscles. Another possibility is that UM neuron activity during crawling is important for the release of octopamine into the CNS, which may augment rhythmic motor activity. Although few central output sites were identified in an ultrastructural analysis of efferent UM neurons in the locust (Pflüger and Watson, 1995), central effects

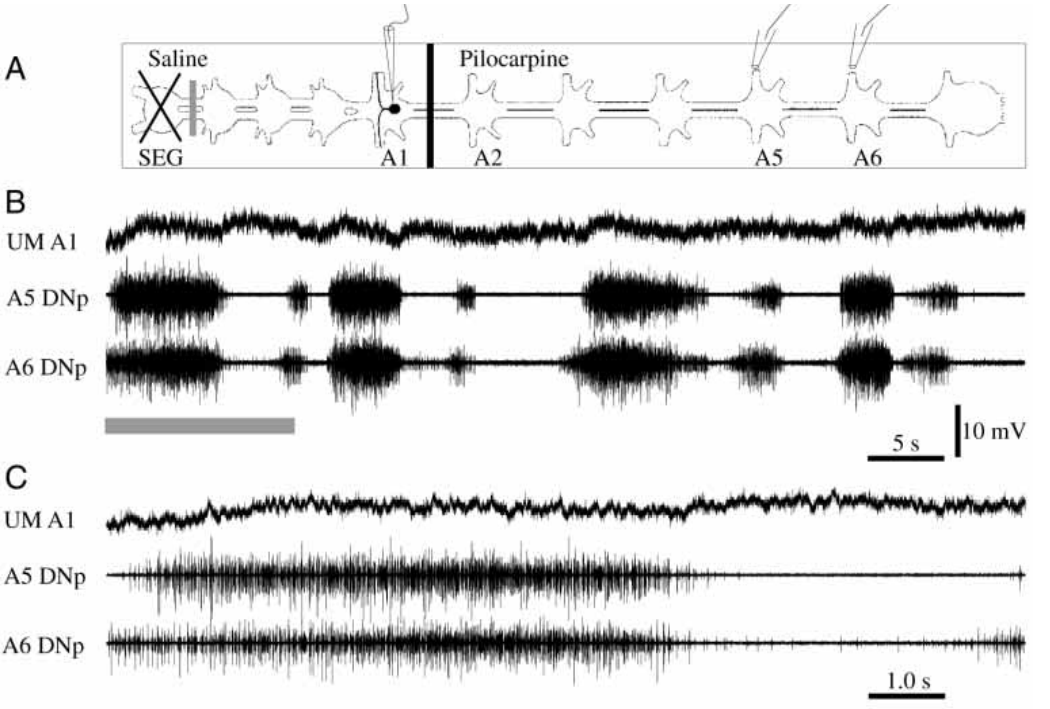

Fig. 6. Prominent excitatory postsynaptic potentials (EPSPs) that occur in the unpaired median (UM) neurons during fictive crawling are dependent upon the subesophageal ganglion (SEG) even though the subesophageal ganglion had not been exposed to pilocarpine. (A) Diagram of the preparation. The recordings are from the same preparation as in Fig. 5, but the subesophageal ganglion had been removed. Both intracellular and extracellular recordings were maintained during removal of the subesophageal ganglion. (B) After removal of the subesophageal ganglion (1.75 min), rhythmic fictive crawling activity persists in the motor roots, but the pattern is somewhat disrupted as extra bursts appear. The prominent EPSPs that caused rhythmic depolarizations in the UM neuron are no longer apparent (compare with Fig. 5C), but small depolarizations occur together with activity in the motor roots. The shaded bar indicates a region of the recording that is expanded in C. (C) On an expanded time scale, the prominent EPSPs that were present before removal of the subesophageal ganglion (see Fig. 5D) are absent, but small EPSPs, which cause weak depolarization of the UM neuron, are apparent. DNp, posterior branch of the dorsal nerve.

on motoneurons in this preparation have been described (Parker, 1996). However, current injection into UM neurons did not alter the rhythmic motor activity induced in locust leg motoneurons by pilocarpine (Baudoux et al., 1998).

The rhythmic depolarizations during fictive crawling in *M. sexta* are evoked primarily by prominent EPSPs that occur in common in abdominal and thoracic efferent UM neurons during pairwise recordings. These may be generated by the same unidentified presynaptic neurons that are the source of the EPSPs that occur in common during pairwise UM neuron recordings in the absence of fictive crawling (Pflüger et al., 1993). In both cases, the EPSPs are dependent upon the presence of the subesophageal ganglion and occur with an anterior-to-posterior delay. In the presence of pilocarpine, these prominent EPSPs increase in frequency and become grouped into bursts that cause membrane potential depolarization and spiking of the efferent UM neurons. However, the rhythmic activity of UM neurons during fictive crawling does not depend exclusively on drive from the subesophageal ganglion. Following removal of the subesophageal ganglion, weak depolarizations of the UM neurons, which may reflect segmental inputs, occurred with bursts of motoneuron activity.

Although the prominent EPSPs in efferent UM neurons are dependent upon the subesophageal ganglion, their increase in frequency and grouping into bursts during fictive crawling do not depend upon exposure of the subesophageal ganglion to pilocarpine. Both occur upon exposure of more posterior ganglia, which also evokes fictive crawling. One hypothesis that is consistent with this observation is that descending interneurons within the subesophageal ganglion, which evoke EPSPs in all efferent UM neurons, receive phasic excitatory or inhibitory drive from the crawling central pattern generator. The anterior-to-posterior delay between the onset of EPSPs in UM neurons in two different segments is constant and is consistent with that expected as a result of action potential conduction between segments (Pflüger et al., 1993). Nevertheless, there may be multiple descending interneurons from the subesophageal ganglion that mediate these prominent EPSPs, and they need not synapse directly onto the UM neurons within the segmental ganglia.

In this context, it is noteworthy that the subesophageal ganglion has been regarded as an important integrative center for locomotory control, although the experimental basis for this idea is rather limited (see Burrows, 1996). Decerebrated stick insects with both circumesophageal connectives cut exhibit long bouts of walking activity, suggesting an inhibitory influence of the brain onto locomotory centers in the subesophageal ganglion. In contrast, stick insects with the neck connectives (i.e. just posterior to the subesophageal ganglion) cut do not express any walking activity (Bässler, 1983). From electrical stimulation of fiber populations in the circumesophageal and neck connectives, Kien (1983) postulated effects on many different aspects of walking such as initiation, speed, step length and coordination. In a subsequent paper, a few descending interneurons of the subesophageal ganglion were filled with dye and their structure was revealed (Kien and Altman, 1984). Descending interneurons of the subesophageal ganglion are also involved in the control of locust flight (Ramirez, 1988), of locust (Ramirez and Pearson, 1989) and cricket (Otto and Januszewski, 1989) ventilation and of stridulation in the cricket (Otto and Hennig, 1993) and grasshopper (Hedwig, 1986). Our results suggest that the subesophageal ganglion may also function to control the excitability of neuromodulatory neurons.

The existence of EPSPs that occur in common in all efferent UM neurons in larval *M. sexta*, and their role in providing common drive during fictive crawling, is in contrast to results from the adult locust, where the total population of UM neurons is divided into sub-populations that are only active during specific patterns of motor behavior (Burrows and Pflüger, 1995; Duch and Pflüger, 1995, 1996; Baudoux et al., 1998) and where sub-populations exist that are specifically inhibited during ongoing motor behavior such as flight (Duch et al., 1997). In the locust, these sub-populations are defined by the presence (or absence) of common EPSPs, which come either from local sources within the same ganglion or from the subesophageal ganglion (Duch and Pflüger, 1995, 1996). In contrast to *M. sexta*, sub-populations of UM neurons exist in the locust that do not rely on intersegmental inputs from the subesophageal ganglion for patterned activation. For example, in a completely isolated metathoracic ganglion, only UM neurons innervating the leg muscles were coupled to the rhythmic leg motoneuron activity induced by pilocarpine (Baudoux et al., 1998).

There are several possible reasons for the more specific recruitment strategies in the locust and for the greater importance of local segmental drive of efferent UM neurons during pilocarpine-induced rhythmic leg motor activity. First, the metathoracic ganglion of the adult locust is involved in several distinct types of behavior, which may require unique patterns of UM neuron activity or specific sets of UM neurons. Second, fictive crawling in *M. sexta* shows well-structured intersegmental coordination that, unlike adult walking, includes the abdominal segments. Finally, the pilocarpine-induced pattern in the locust leg motoneurons involves bilateral alternation (Ryckebusch and Laurent, 1993), rather than synchronous activation across the ganglion as in fictive crawling in *M. sexta* (Johnston and Levine, 1996). Locust DUM neurons, which project bilaterally to innervate both legs, couple to the rhythmic leg motoneuron pattern in both hemiganglia (Baudoux et al., 1998). Although common drive to all efferent UM neurons from the subesophageal ganglion remains in *M. sexta* adults (Pflüger et al., 1993), it will be interesting to determine whether other sources of synaptic input cause the UM neurons that project to different targets to display unique activity patterns during adult walking and flight behavior.

References

Bässler, U. (1983). *Neural Basis of Elementary Behavior in Stick Insects (Studies of Brain Function)*. Berlin: Springer.

Baudoux, S., Duch, C. and Morris, O. (1998). Coupling of efferent neuromodulatory neurons to rhythmic motor activity in the locust. *J. Neurophysiol.* **79**, 361–370.

Bell, R. A. and Joachim, F. A. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Am.* **69**, 365–373.

Brookes, S. J. H. (1988). Unpaired median neurones in a lepidopteran larva (*Antheraea pernyi*). II. Peripheral effects and pharmacology. *J. Exp. Biol.* **136**, 333–350.

Brookes, S. J. H. and Weevers, R. deG. (1988). Unpaired median neurones in a lepidopteran larva (*Antheraea pernyi*). I. Anatomy and physiology. *J. Exp. Biol.* **136**, 311–332.

Burrows, M. (1996). *The Neurobiology of an Insect Brain*. Oxford: Oxford University Press.

Burrows, M. and Pflüger, H.-J. (1995). The action of locust neuromodulatory neurons is coupled to specific motor patterns. *J. Neurophysiol.* **74**, 347–357.

Büschges, A., Schmitz, J. and Bässler, U. (1995). Rhythmic patterns in the thoracic nerve cord of the stick insect induced by pilocarpine. *J. Exp. Biol.* **198**, 435–456.

Candy, D. J., Becker, A. and Wegner, G. (1997). Coordination and integration of metabolism in insect flight. *Comp. Biochem. Physiol.* **117**B, 497–512.

Chrachri, A. and Clarac, F. (1990). Fictive locomotion in the fourth thoracic ganglion of the crayfish, *Procambarus clarkii*. *J. Neurosci.* **10**, 707–719.

Consoulas, C., Kent, K. S. and Levine, R. B. (1996). Remodeling of the peripheral processes and presynaptic terminals of leg motoneurons during metamorphosis of the hawk moth, *Manduca sexta*. *J. Comp. Neurol.* **372**, 415–434.

Crossman, A. R., Kerkut, G. A., Pitman, R. M. and Walker, R. J. (1971). Electrically excitable nerve cell bodies in the central ganglia of two insect species, *Periplaneta americana* and *Schistocerca gregaria*: investigation of cell geometry and morphology by intracellular dye injection. *Comp. Biochem. Physiol.* **40**, 579–596.

Crossman, A. R., Kerkut, G. A. and Walker, R. J. (1972). Electrophysiological studies on the axon pathways of specialized nerve cells in the central ganglia of two insect species, *Periplaneta americana* and *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **43**, 393–415.

Duch, C., Büschges, A. and Pflüger, H.-J. (1997). Differential activation of octopaminergic DUM-neurons during flight. In *Proceedings of the 25th Göttingen Neurobiology Conference*, vol. II (ed. N. Elsner and H. Wässle), p. 273. Stuttgart, New York: Thieme Verlag.

Duch, C. and Pflüger, H.-J. (1995). Common drive of thoracic DUM-neurones in the locust, *Schistocerca gregaria*. In *Neural Systems and Behaviour. Proceedings of the Fourth International Congress on Neuroethology* (ed. M. Burrows, T. Matheson, P. L. Newland and H.Schuppe), p. 489. Stuttgart, New York: Thieme Verlag.

Duch, C. and Pflüger, H.-J. (1996). Common input to distinct subpopulations of thoracic DUM-neurones in the locust. *Proceedings of the 24th Göttingen Neurobiology Conference*, vol. II (ed. N. Elsner and H.-U. Schnitzler), p. 110. Stuttgart, New York: Thieme Verlag.

Evans, P. D. (1980). Biogenic amines in the insect nervous system. *Adv. Insect Physiol.* **15**, 317–473.

Evans, P. D. (1985). Octopamine. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 11, *Pharmacology* (ed. G. A. Kerkut and L. I. Gilbert), pp. 499–530. Oxford: Pergamon Press.

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

Gras, H., Hörner, M., Runge, L. and Schürmann, F.-W. (1990). Prothoracic DUM neurons of the cricket, *Gryllus bimaculatus*: Responses to natural stimuli and activity in walking behavior. *J. Comp. Physiol.* A **166**, 901–914.

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**, 59–63.

Harris-Warrick, R. M. and Marder, E. (1991). Modulation of neural networks for behavior. *Annu. Rev. Neurosci.* **14**, 39–57.

Hedwig, B. (1986). On the role in stridulation of plurisegmental interneurons of the acridid grasshopper *Omocestus viridulus* L. I. Anatomy and physiology of descending cephalothoracic interneurons. *J. Comp. Physiol.* **158**A, 413–427.

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 neurones of the locust metathoracic ganglion. *J. Neurobiol.* **9**, 43–57.

Johnston, R. M., Consoulas, C., Pflüger, H.-J. and Levine, R. B. (1997). Activity during fictive crawling and peripheral release sites of modulatory neurons in *Manduca*. *Soc. Neurosci. Abstr.* **23**, 766.

Johnston, R. M. and Levine, R. B. (1996). Crawling motor patterns induced by pilocarpine in isolated larval nerve cords of *Manduca sexta*. *J. Neurophysiol.* **76**, 3178–3195.

Kalogianni, E. and Pflüger, H.-J. (1992). The identification of motor and unpaired median neurones innervating the locust oviduct. *J. Exp. Biol.* **168**, 177–198.

Kalogianni, E. and Theophilidis, G. (1993). Centrally generated rhythmic activity and modulatory function of the oviductal dorsal unpaired median (DUM) neurones in two orthopteran species *Calliptamus* and *Decticus albifrons*. *J. Exp. Biol.* **174**, 123–138.

Kent, K. S. and Levine, R. B. (1998). Neural control of leg movements in a metamorphic insect: sensory and motor elements of the larval thoracic legs in *Manduca sexta*. *J. Comp. Neurol.* **271**, 559–576.

Kien, J. (1983). The initiation and maintenance of walking in the locust: an alternative to the command concept. *Proc. R. Soc. Lond. B* **219**, 137–174.

Kien, J. and Altman, J. S. (1984). Descending interneurones from the brain and suboesophageal ganglia and their role in the control of locust behaviour. *J. Insect Physiol.* **30**, 59–72.

Kondoh, Y. and Obara, Y. (1982). Anatomy of motoneurons innervating mesothoracic indirect flight muscles in the silk moth, *Bombyx mori*. *J. Exp. Biol.* **98**, 23–37.

Konings, P. N. M., Vullings, H. G. B., Geffard, M., Buijs, R. M., Diederen, J. H. B. and Jansen, W. F. (1988). Immunocytochemical demonstration of octopamine-immunoreactive cells in the nervous system of *Locusta migratoria* and *Schistocerca gregaria*. *Cell Tissue Res.* **251**, 371–379.

Lange, A. B. and 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, A. B. and Orchard, I. (1984b). Some pharmacological properties of neuromuscular transmission in the oviduct of the locust, *Locusta migratoria*. *Arch. Insect Biochem. Physiol.* **1**, 231–241.

Levine, R. B. and Truman, J. W. (1985). Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci.* **5**, 2424–2431.

Matheson, T. (1997). Octopamine modulates the response and

presynaptic inhibition of proprioceptive sensory neurons in the locust *Schistocerca gregaria*. *J. Exp. Biol.* **200**, 1317–1325.

Orchard, I. and Lange, A. B. (1986). Pharmacological profile of octopamine receptors on the locust, *Locusta migratoria*. *J. Insect Physiol.* **32**, 741–745.

O'Shea, M. and Evans, P. D. (1979). Potentiation of neuromuscular transmission by an octopaminergic neuron in the locust. *J. Exp. Biol.* **79**, 169–190.

Otto, D. and Hennig, R. M. (1993). Interneurons descending from the cricket suboesophageal ganglion control stridulation and ventilation. *Naturwissenschaften* **80**, 36–38.

Otto, D. and Janiszewski, J. (1989). Interneurons originating in the suboesophageal ganglion that control ventilation in two cricket species: effect of the interneurones (SD-AE neurones) on the motor output. *J. Insect Physiol.* **35**, 483–491.

Parker, D. (1996). Octopaminergic modulation of locust motor neurons. *J. Comp. Physiol.* **178**, 243–252.

Pflüger, H.-J. and Watson, A. H. D. (1995). GABA- and glutamate-like immunoreactivity in central synapses of dorsal unpaired median neurones in the abdominal nerve cord of locusts. *Cell Tissue Res.* **280**, 325–333.

Pflüger, H.-J., Witten, J. L. and Levine, R. B. (1993). Fate of abdominal ventral unpaired median cells during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* **335**, 508–522.

Plotnikova, S. I. (1969). Effector neurons with several axons in the ventral nerve cord of the Asian grasshopper *Locusta migratoria*. *J. Evol. Biochem. Physiol.* (English translation) **5**, 276–277.

Pollack, A. J., Ritzmann, R. E. and Westin, J. (1988). Activation of DUM cell interneurons by ventral giant interneurons in the cockroach, *Periplaneta americana*. *J. Neurobiol.* **19**, 489–497.

Ramirez, J.-M. (1988). Interneurons in the suboesophageal ganglion of the locust associated with flight initiation. *J. Comp. Physiol.* **162**A, 669–686.

Ramirez, J. M. and Orchard, I. (1990). Octopaminergic modulation of the forewing stretch receptor in the locust *Locusta migratoria*. *J. Exp. Biol.* **149**, 255–279.

Ramirez, J.-M. and Pearson, K. G. (1989). Distribution of intersegmental interneurons that can reset the respiratory rhythm of the locust. *J. Exp. Biol.* **141**, 151–176.

Ramirez, J.-M. and Pearson, K. G. (1991a). Octopamine induces bursting and plateau potentials in insect neurones. *Brain Res.* **549**, 332–337.

Ramirez, J.-M. and Pearson, K. G. (1991b). Octopaminergic modulation of interneurons in the flight system of the locust. *J. Neurophysiol.* **66**, 1522–1537.

Ryckebusch, S. and Laurent, G. (1993). Rhythmic patterns evoked in locust leg motor neurons by the muscarinic agonist pilocarpine. *J. Neurophysiol.* **69**, 1583–1595.

Ryckebusch, S. and Laurent, G. (1994). Interactions between segmental leg central pattern generators during fictive rhythms in the locust. *J. Neurophysiol.* **72**, 2771–2785.

Sombati, S. and Hoyle, G. (1984). Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. *J. Neurobiol.* **15**, 481–506.

Stevenson, P. A. and Kutsch, W. (1988). Demonstration of functional connectivity of the flight motor system in all stages of the locust. *J. Comp. Physiol.* A **162**, 247–259.

Stevenson, P. A., Pflüger, H.-J., Eckert, M. and Rapus, J. (1992). Octopamine immunoreactive cell populations in the locust thoracic–abdominal nervous system. *J. Comp. Neurol.* **315**, 382–397.

Stevenson, P. A., Pflüger, H.-J., Eckert, M. and Rapus, J. (1994). Octopamine-like immunoreactive cell populations in the locust genital abdominal ganglia. *Cell Tissue Res.* **275**, 299–308.

Stevenson, P. A. and Spörhase-Eichmann, U. (1995). Localization of octopaminergic neurons in insects. *Comp. Biochem. Physiol.* **110**A, 203–215.

Trimmer, B. A. and Weeks, J. C. (1989). Effects of nicotinic and muscarinic agents on an identified motoneurone and its direct afferent inputs in larval *Manduca sexta*. *J. Exp. Biol.* **144**, 303–337.

Watson, A. H. D. (1984). The dorsal unpaired median neurons of the locust metathoracic ganglion: neuronal structure and diversity and synapse distribution. *J. Neurocytol.* **13**, 303–327.