Interneurons in the Flight System of the Locust: Distribution, Connections, and Resetting Properties

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ABSTRACT

The organization and functional properties of interneurons in the flight system of the locust, Locusta migratoria, were investigated by using intracellular recording and staining techniques. Interneurons were found to be distributed within the three thoracic and the first three abdominal ganglia, and they could be subdivided into three organizational categories: (1) members of one of two serially homologous groups controlling either the forewing or the hindwing, (2) unique individuals with no known homologues in other ganglia, and (3) members of a set of serial homologues in the metathoracic and first three abdominal ganglia. Interneurons in the last two categories influenced both forewing and hindwing motoneurons in a similar manner. Thus interneuronal organization is not characterized by two distinct homologous groups of interneurons for the separate control of forewing and hindwing motor activity.

Flight interneurons may also form two separate functional categories: (1) those making short latency connections to motoneurons (premotor interneurons), and (2) those which reset the flight rhythm when depolarized by brief current pulses (pattern generator interneurons). None of the ten premotor interneurons we identified influenced the flight rhythm when depolarized and none of the three groups of pattern generator interneurons were found to form short latency connections with motoneurons. This separation of function may allow phase-shifts in motor output for flight control without changes in wingbeat frequency. Pattern generator interneurons influence motor output to both forewings and hindwings. Thus we conclude that the flight rhythm is generated in a distributed neuronal oscillator driving both pairs of wings.

The organization of flight interneurons is considerably more complex than predicted from existing models of the flight system, or anticipated from the relative simplicity of the motor output. Our finding of homologous sets of interneurons in the abdominal ganglia supports the notion that insect flight evolved from a behavior using appendages distributed along the thorax and the abdomen. Thus the organization of flight interneurons may reflect an interneuronal system which controlled the behavior from which flight evolved.

Key words: invertebrate, insect, behavior, pattern generation, neuronal structure

The organization and functional properties of interneurons in motor systems are poorly understood. This deficiency is due to the difficulty of recording intracellularly from interneurons in behaving animals and to the small number of systems where this is even possible. One such system is locust flight. Although flight in the locust is a complex behaviour, it is controlled by a limited number of appendages, muscles, and neurons, all of which are accessible. As a result a large body of data on many aspects of flight has accumulated (for reviews see Wilson, '68; Burrows, '76, '77). Furthermore, it is possible to record intracellularly from motoneurons and interneurons in the flight system during the expression of the flight rhythm (Robertson and Pearson, '81, '82).

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The flight system in the locust is characterized by strict serial homology at least to the level to which it has been described. Two pairs of wings on the mesothoracic and metathoracic segments are powered by two sets of thoracic muscles which in turn are driven by two apparently identical sets of motoneurons. There are about 40 identifiable motoneurons for each pair of wings (Bentley, '70; Burrows, '77). One question we addressed in this study was whether this strict serial homology extends to an interneuronal level. One possibility is that there are two identical sets of interneurons. This arrangement would result in a two oscillator system, one for each wing pair, such as has been proposed by Wilson and Waldron ('68). On the other hand, there may be a single flight oscillator whose output is distributed to both pairs of wings. Another question was whether the interneurons driving motoneurons are separate from those generating the rhythm. By locating and describing the structure and physiology of interneurons in the flight system we hoped to answer these questions and to gain some understanding of the cellular basis for the generation of a rhythmical motor program.

To date we have identified more than 50 interneurons which are phasically active with the flight rhythm. Some of those for which we have stronger evidence for their involvement in flight are described in this paper. The organization and distribution of these interneurons demonstrate that the locust flight motor is a complex circuit distributed within at least six segmental ganglia and functioning as a unit with a single neural oscillator. This interneuronal organization could not be predicted from existing models of the flight system (Wilson and Waldron, '68). Furthermore it is clear from the present work that some organizational features of the nervous system can be explained only after consideration of the evolutionary history of the system. Our findings support a proposal that wings evolved from originally jointed appendages of the thorax and the abdomen and that they were preadapted for a prior motor behaviour (Kukalova-Peck, '78).

MATERIALS AND METHODS

Male and female Locusta migratoria were used in all experiments and were obtained from a long-established colony at the University of Alberta. Experiments were performed at room temperature (22–24°C).

Preparation

The preparation has been described in detail in a previous paper (Robertson and Pearson, '82). The thoracic ganglia were exposed by a dorsal dissection and stabilized on a stainless steel plate for microelectrode penetration. The nerve roots of the thoracic ganglia were cut, except for those nerves innervating the dorsal longitudinal (DL) muscles (wing depressors). An electromyographic monitor of DL activity during flight sequences was obtained by inserting fine copper wire electrodes, insulated except at the tips, into a single DL muscle. The flight rhythm was induced by wind stimulation of the sensory hairs on the head (Weis-Fogh, '56). The preparation was bathed with saline (NaCl 147 mM; KCl 10 mM; CaCl₂ 4 mM; NaOH 3 mM; HEPES buffer 10 mM) at room temperature.

The motoneuronal processes of neurons in the thoracic ganglia were penetrated by glass capillary microelectrodes pulled such that their resistance if filled with 1 M potassium acetate was approximately 50 MΩ. The tips of these were filled with 4% Lucifer Yellow (Stewart, '78) and the shafts filled with 0.5 M lithium chloride. Filled in this way the electrodes had resistances of over 100 MΩ. Lucifer Yellow was passed into impaled neurons by the passage of about 5 namps of constant hyperpolarizing current for 5–10 minutes. Ganglia were fixed in 4% paraformaldehyde (pH 7.2, 30 minutes), dehydrated in absolute alcohol (20 minutes), and cleared in methyl salicylate (10 minutes). Ganglia treated in this way were viewed on a Leitz fluorescence microscope and dye-filled neurons were immediately photographed and drawn. Not all fine branches of neurons are shown in the drawings presented in this paper. However, these drawings are sufficient to illustrate the basic morphology of each neuron. Also the drawings show the neurons from only a dorsal aspect because their processes were predominantly confined to a twodimensional sheet just under the ganglionic sheath.

Identification

Our criteria for identifying interneurons in different ganglia as serial homologues were both morphological and physiological. The morphological features that were particularly noted were location of the cell soma, paths of the primary neurite and neuropile segment, major dendritic branching patterns (i.e., branching off the main neuropile segment), the path of the axon in the ganglion, and the location of the axon in the connective. Physiologically the neurons at least had to show the same response to the onset of wind stimulation (depolarization or hyperpolarization) and similar activity patterns during flight sequences (phase, burst length, intraburst frequency).

Classification of interneurons

There is a relatively large number of interneurons involved in flight. Thus it was necessary to devise a numbering scheme for referring to the different interneurons. This scheme made the retrieval of information about particular interneurons much easier and in addition gave each interneuron an identity. The scheme is a three-digit number, the first numeral indicating the course of the axon in the thoracic nerve cord and the subsequent two numerals specifying the interneuron within that class. The general classes are: 100-199 for local neurons; 200-299 for interneurons with axons descending and ipsilateral to the cell soma; 300-399 for interneurons with axons descending and contralateral to the cell soma; 400-499 for interneurons with axons descending and contralateral to the cell soma; 500-599 for interneurons with axons ascending and ipsilateral to the cell soma; 600-699 for interneurons with axons descending and ascending and ipsilateral to the cell soma; 700-799 for interneurons with axons descending and ascending and contralateral to the cell soma. Serially homologous interneurons which have the same identity were given the same number and the ganglion in which the cell soma was located is indicated parenthetically when it is necessary to distinguish them. For example 201(T1) and 201(T2) are homologous interneurons in the first thoracic and second thoracic ganglia, respectively, and their axons descend the ventral nerve cord in the connectives ipsilateral to their cell somas.

RESULTS

The locust flight pattern consists of alternating bursts of activity in elevator and depressor motoneurons, with
the latency from depressor to elevator being longer than the latency from elevator to depressor (Wilson and Weis-Fogh, '62; Waldron, '67; Wilson, '68). The pattern is variable at the start of flight (Pond, '72) but soon settles into a regularly repeating cycle at about 20 Hz. In our preparations the cycle frequency is approximately a half (10 Hz) of that seen in intact animals but the basic pattern is similar. Elevator motoneurons fire first and are followed by depressor motoneurons, followed by a variable silent period (Robertson and Pearson, '82). Because the motor pattern we record is clearly a manifestation of the flight rhythm we feel confident that an understanding of interneuronal activity and interneuronal organization in our preparation will give insight into the neural generation of flight activity.

Our strategy in studying the functional organization was first to identify interneurons which were phasically active with the flight rhythm. Then by simultaneously penetrating interneurons and motoneurons and by perturbing their activity with depolarizing current pulses we attempted to determine whether or not an interneuron had synaptic connections with motoneurons and whether or not the activity of each is almost identical (compare 201 trace in c and d). In this and all subsequent figures an arrow under the trace indicates the onset of wind stimulation. In both c and d the depressor motoneuron (D) recorded is the second basalar motoneuron drawn in b. e. EPSP recorded in the same second basalar motoneuron (D) a short and constant latency after each spike of the 201 whose activity is recorded in d. The oscilloscope sweep was triggered off the rising phase of each 201 action potential. The EPSP from the other 201 was similar.
not it was part of the rhythm pattern generator. The interneuron to motoneuron connections that we have found have not been demonstrated to be monosynaptic by the stringent criteria used in molluscan studies (Berry and Pentreath, '76; Getting, '81). However, because of the short and constant latency from the interneuronal action potential to the synaptic potential in the motoneuron and because each action potential was always followed by the postsynaptic potential we conclude that the connections are most likely monosynaptic. The criteria used to establish that a neuron is part of a rhythm generator were first that it was phasically active with the rhythmic output and second that transient perturbations of its activity reset the rhythm (see e.g., Friessen et al., '76; Kristan et al., '77; Weeks, '81). The ability of neurons to reset the flight rhythm was tested by passing depolarizing current pulses (up to 10 namps) of varying duration (100 msec–1 second) into the neuropile processes of these neurons during the expression of the flight rhythm. The duration of the depolarizing current pulses was adjusted to overlap with the spiking phase of the neuron’s oscillation whenever they were presented in the cycle. This resulted in intense activity during at least part of the depolarizing pulse and thus helped greatly in obtaining data from short-term penetrations.

The interneurons we have found show an apparent separation of function such that the function of motoneuron driving is mutually exclusive with the function of rhythm generation. Thus the results are presented in two groups: premotor interneurons and pattern generator interneurons. Within these broad categories the interneurons had three different types of organization. First, there are those which were organized in two serially homologous sets, each of which controls either the forewing or the hindwing—that is to say, in the same way as motoneurons. Second, some interneurons appear to be unique individuals with no known serial homologues in other ganglia. Finally, interneurons could be organized as a set of serial homologues within the metathoracic ganglion and the three abdominal ganglia which are fused with the metathoracic.

**Premotor interneurons**

Homologous forewing and hindwing sets.

*Interneuron 201 (Figs. 1–3).* There was more than one interneuron in a single hemiganglion each of which had the morphological and physiological characteristics of an interneuron 201 (e.g., Fig. 1). Thus 201 refers to a class of an unknown number of interneurons which, using our present criteria, cannot reliably be distinguished one from another.

In the mesothoracic ganglion (Fig. 1a) each 201 had a small (25–30 μm diameter) cell body which lay close to the ganglionic midline in the ventral portion of the ganglion. The primary neurite ran dorsally to the thicker neuropile segment which was orientated longitudinally in the neuropile. As with most of the flight neurons we have found so far, the dendritic arbour was predominantly confined to the dorsal neuropile just under the ganglionic sheath. The axon arose from the posterior end of the neuropile segment and had a characteristic kink before running into the ipsilateral connective. As the axon left the ganglion, just posterior to the kink, it gave off a branch which crossed the midline to ramify in a limited region of the posterior dorsal neuropile of the contralateral hemiganglion. The main axon travelled down the midline of the mesomethathoracic connective and terminated in fine branches in the regions of the dendritic arborizations of flight motoneurons of both the right and left sides of the metathoracic ganglion.

Interneurons with this structure fired with brief bursts in phase with depressor activity during flight (Fig. 1c,d). The onset of wind stimulation usually hyperpolarized 201 (Fig. 1d). By recording simultaneously from a 201 and different wing depressor motoneurons we established that 201 had short constant latency connections with both first and second basalar motoneurons of the left and right hindwings. Figure 1e shows as an example the excitatory postsynaptic potential (EPSP) recorded in a metathoracic second basalar motoneuron (Fig. 1b) following each spike of a 201. The EPSPs varied in amplitude between 1 mV and 3 mV. We have not yet established that 201 connects to other hindwing motoneurons, i.e., metathoracic subalar motoneurons or mesothoracic dorsal longitudinal motoneurons. This seems likely because of the axonal projections of 201 in the metathoracic and the metathoracic ganglia. Depressor motoneurons for the hindwing are located in the posterior region of the mesothoracic ganglion as...
Fig. 3. Interneuron 201 in the prothoracic ganglion. a. The structure of 201(T1). Note the similarity with the structure of 201 in the mesothoracic ganglion (Fig. 1a). b. Intracellular recording from 201(T1) during a flight sequence. The depressor motoneuron (D) is the ipsilateral first basalar motoneuron in the mesothoracic ganglion. The recorded spikes of 201 are small due to the location of the penetration far from the spike initiation site. Note that 201(T1) fires in phase with depressor motoneurons and is hyperpolarized by the onset of wind stimulation. c. EPSP recorded in the ipsilateral mesothoracic first basalar motoneuron (D) a short and constant latency after each spike of 201(T1) which was used to trigger the oscilloscope sweep.

As with 201(T2), we have found more than one 201(T1) in a single hemiganglion. The fact that 201 represents a class of interneurons and not an individual raises several questions which will need to be answered before a full understanding of their role is possible. Of primary importance is to determine the absolute number in the class. Also to be determined is how many members of the class project to a single motoneuron and whether a single interneuron projects to more than one motoneuron.

We conclude that there are two sets of premotor interneurons which are serially homologous and each set is responsible in part for driving either forewing or hindwing depressor motoneurons. But as the results which follow will testify, this organization was not common for all the premotor interneurons that we have found. There were other interneurons which were excitatory to motoneurons and yet others which were inhibitory to motoneurons and these appeared to have no serially homologous counterparts.

Individuals in the mesothoracic ganglion and metathoracic ganglionic mass. The justification for referring to any interneuron as an individual is that after extensive exploration of the mesothoracic ganglion and the metathoracic ganglionic mass (over 175 separate single and multiple fills of interneurons) we have no evidence to suggest that there was any other interneuron with the same structure and physiology apart from its contralateral homologue. Of course this does not prove that homologues...
do not exist for logically this cannot be excluded until the entire interneuronal organization is known. But until there is positive evidence for the existence of homologues these interneurons are most conveniently treated as unique individuals with no serial homologues in the mesothoracic ganglion and metathoracic ganglionic mass.

**Interneuron 701** (Fig. 4). The cell body of 701 (35–40 μm diameter) was located on the posterior lateral margin of the mesothoracic ganglion (Fig. 4a). It lay approximately midway between the dorsal and ventral surfaces of the ganglion with a tendency to be more dorsally situated. The primary neurite ran almost directly medially and thickened into the neuropile segment which lay approximately 100 μm beneath the dorsal surface of the ganglion. At two points along the neuropile segment thick dendritic branches arose, ran up to the dorsal neuropile, and ramified profusely in a limited area. At the end of the neuropile segment in the ganglionic midline a process rose to the dorsal surface and gave off numerous long branches which extended throughout the ipsilateral hemiganglion and some branches which ran a short way into the contralateral hemiganglion. All of these lay just under the ganglionic sheath. The axon arose at the midline, ran laterally into the contralateral hemiganglion, and bifurcated. One branch proceeded anteriorly, sending branches into the dorsal neuropile in the region of flight motoneurons and then passed out of the ganglion laterally in the contralateral connective. The other branch proceeded posteriorly and headed for the metathoracic ganglion in the midline of the connective. The terminals of the axon in the prothoracic and metathoracic ganglia are unknown at present but there is some evidence that in the metathoracic ganglion branches are given off which cross the midline. Thus both sides of the metathoracic ganglion would be innervated.

Every spike of a 701 was followed after a short and constant latency by small EPSPs (1–2 mV) in depressor motoneurons of the hindwing. Figure 4b shows the connection between 701 and a posterior mesothoracic dorsal longitudinal motoneuron contralateral to the cell body of 701. This connection is consistent with the firing pattern of 701. The 701 fired in short high-frequency bursts in phase with depressor motoneurons during flight activity and it was hyperpolarized by the onset of wind stimulation (fig. 4c). Judging by the axonal branching of 701 in the region of forewing depressor motoneurons and its passage toward the prothoracic ganglion where other forewing depressor motoneurons are located we predict that 701 also has excitatory connections to forewing depressor motoneurons.

**Interneuron 514** (Fig. 5). This interneuron had its cell body (25–30 μm diameter) located on the ventral surface of the metathoracic ganglion in the posterior lateral quadrant of the hemiganglion (Fig. 5a). The primary neurite rose sharply from the cell body to a neuropile segment which was oriented transversely across the ipsilateral hemiganglion approximately 10 μm beneath the dorsal surface of the ganglion. The dendrites branched dorsally from the middle of the neuropile segment in a complex fashion and were confined to the ipsilateral hemiganglion.
Fig. 5. Interneuron 514. a. Structure of 514 in the metathoracic ganglionic mass. b, c. Facilitatory EPSP recorded in a contralateral mesothoracic tergosternal motoneuron (E) a short and constant latency after each spike of 514. In b, 514 is firing at 20 impulses/second and the amplitude of the EPSP is about 1 mV. In c, 514 is firing at 50 impulses/second and the amplitude of the EPSP is about 2 mV. d. Intracellular recording from 514 and the same tergosternal motoneuron (E) during a flight sequence. During each burst 514 fires at about 200 impulses/second.

The neuropile segment was constricted at the ganglionic midline and the axon arose from this constriction. The axon turned anteriorly almost immediately and passed out of the ganglion in the medial edge of the connective contralateral to the cell body. While still in the metathoracic ganglion the axon gave off two sizable branches which ramified extensively in the dorsal neuropile region of flight motoneurons. The course and terminals of the axon anterior to the metathoracic ganglion are unknown at present although there is physiological evidence that the axon extended as far as the anterior region of the mesothoracic ganglion where it connected to flight motoneurons (see below).

Each spike of 514 was followed after a short and constant latency by an EPSP in tergosternal elevator motoneurons in the mesothoracic ganglion (Fig. 5b). An interesting aspect of this connection is that it was facilitatory. A change in the firing frequency of 514 from 20 impulses/second (Fig. 5b) to 50 impulses/second (Fig. 5c) resulted in a doubling of the amplitude of the EPSP from about 1 mV to about 2 mV. During flight sequences 514 fired in bursts in phase with elevator motoneurons and within a burst the firing frequency was about 200 impulses/second (Fig. 5d). In response to the onset of wind stimulation 514 was depolarized past threshold (Fig. 5d).

Interneuron 511 (Fig. 6). The cell body of 511 was about 35 μm in diameter and was located on the ventral midline of the posterior region of the metathoracic ganglion (Fig. 6a). The primary neurite rose dorsally and laterally from the cell body, then abruptly shifted direction, crossing the ganglionic midline. Just past the midline the primary neurite joined a thick neuropile segment about 100 μm beneath the dorsal surface of the ganglion. There were numerous dendritic branches which rose to the dorsal neuropile and extended throughout the hemiganglion contralateral to the cell body. A limited number of branches crossed back to the ipsilateral hemiganglion. The axon arose at the anterior end of the neuropile segment, ran directly to the dorsal surface, and entered the mesometathoracic connective. Before leaving the ganglion the axon sent branches into the neuropile region of flight motoneurons on the same side as the axon and it also sent a branch to the equivalent area on the side opposite that of the axon. The axon ascended the nerve cord in the medial portion of the connective contralateral to the cell body. Its ultimate destination is unknown.

Interneuron 511 fired low-frequency bursts of spikes in phase with elevator motoneurons during flight sequences and the onset of wind stimulation depolarized the neuron and caused it to fire (Fig. 6b). Spikes in 511 were followed after a short and constant latency by inhibitory postsynaptic potentials (IPSPs) in depressor motoneurons of the hindwing. The example shown is a small (1 mV) IPSP in a dorsal longitudinal motoneuron in the posterior region of the mesothoracic ganglion (Fig. 6c).

Interneuron 302 (Fig. 7). The cell body of 302 (35 μm diameter) was situated ventrally in the posterior medial quadrant of a mesothoracic hemiganglion (Fig. 7a). The primary neurite rose dorsally to the lateral end of the neuropile segment (depth 30–50 μm). The neurite seg-
Fig. 6. Interneuron 511. a. Structure of 511 in the metathoracic ganglion. b. Intracellular recording from 511 and a contralateral mesothoracic dorsal longitudinal motoneuron (D) during a flight sequence. c. IPSP recorded in the same dorsal longitudinal motoneuron (D) a short and constant latency after each spike of 511.

ment gave off a number of dendritic branches which were confined to the hemiganglion ipsilateral to the cell body before crossing the midline. The axon arose close to the midline and ran laterally for a short distance before bifurcating. One branch ramified in the dorsal neuropile region of flight motoneurons in the mesothoracic ganglion contralateral to the cell body of 302. The other branch left the mesothoracic ganglion in the midline of the connective contralateral to the cell body and branched in the equivalent area of dorsal neuropile in the metathoracic ganglion. There were no axon branches to regions of flight neuropile ipsilateral to the cell body in either the meso- or the metathoracic ganglion.

Interneuron 302 had short latency inhibitory connections with hindwing elevator motoneurons (Fig. 7b). These IPSPs varied in amplitude from 1 mV to 3 mV. During flight sequences 302 tended to be hyperpolarized by the onset of wind stimulation and fired short high-frequency bursts in phase with depressor activity (Fig. 7c). Interneuron 302 was also affected by auditory and visual input and fired in response to a hissing sound and to a sudden decrease in the light intensity (data not shown).

Sets of serial homologues. The metathoracic ganglionic mass in the adult locust is composed of the fused metathoracic and first three abdominal ganglia. For some interneurons we found homologues in all or most of these ganglia (Fig. 8, Robertson et al., '82) and they are described fully in this paper. Some are pattern generator interneurons and are included in the next section.

Interneuron 504 (Figs. 8, 9). Serial homologues of this interneuron were found in the metathoracic and first three abdominal ganglia (Fig. 8). The posterior portion of the metathoracic ganglionic mass has three pairs of lateral swellings, one for each of the three fused abdominal ganglia. The cell body of a 504 (25 μm diameter) lay ventrally, in or very close to one of these swellings (Fig. 9a). The primary neurite ascended from the cell body to the dorsal neuropile where the neuropile segment was orientated transversely across the ganglion on both sides of the midline. There was a characteristic longitudinal array of dendrites from the neuropile segment. These were directed primarily anteriorly but a number of finer processes ran posteriorly. The dendrites lay just under the dorsal sheath of the ganglion. The axon arose at the contralateral end of the neuropile segment, ran anteriorly, and passed out of the metathoracic ganglion in the extreme lateral edge of the connective. Along the axon were small branches which projected into the dorsal neuropile. The pathway of
LOCUST FLIGHT INTERNEURONS

Fig. 7. Interneuron 302. a. Structure of 302 in the mesothoracic ganglion. b. IPSP recorded in an unidentified contralateral metathoracic elevator motoneuron (E) a short and constant latency after each spike of 302. c. Intracellular recording from 302 and a contralateral metathoracic tergosternal motoneuron during a flight sequence. Note the compound IPSPs in the elevator motoneuron in phase with bursts in 302. The penetration of 302 was close to the axon and far from the spike initiation site resulting in a relative lack of oscillation in the membrane potential of 302.

the axon is unknown anterior to the metathoracic ganglion. Interneuron 504 had an excitatory connection with elevator motoneurons and fired in phase with elevators (Fig. 9b,c). Each spike of a 504 was followed after a short and constant latency by an EPSP of variable small amplitude (1–2 mV) in mesothoracic tergosternal motoneurons (Fig. 9b).

Interneuron 503 (Figs. 8, 10). The structure of 503 (Fig. 10a) was almost identical to that of 504 (Fig. 9a), and 503 also had homologues in each of the first three abdominal ganglia (Fig. 8). By comparison with the other sets of homologues it is likely that a metathoracic 503 exists but this has not yet been found. A verbal description of 503 is unnecessary as it would be the same as that for 504. It is possible that a more refined examination of the structures of the two interneurons would be able to distinguish them; however, their physiology effectively separates them.

Interneuron 504 had an excitatory connection to elevator motoneurons and fired in phase with elevators (Fig. 9b,c). On the other hand, 503 had short constant latency excitatory connections to 201(T1) and 701 and fired in phase with depressors (Fig. 10b,c). Although there is some evidence that 503 had no direct connections to depressor motoneurons we cannot yet be sure that this is the case. Strictly speaking 503 is therefore not a premotor interneuron. It is included in this section because evidence indicates that it is not a pattern generator interneuron and because of its great similarity to 504, which is a premotor interneuron. The EPSPs recorded were large (4–5 mV) in both cases. During flight sequences 503 fired high-frequency bursts of spikes and it tended to be depolarized by the onset of wind stimulation. It has already been shown that 201(T1) projected to depressor motoneurons of the forewing and that 701 projected to depressor motoneurons of the hindwing. Thus 503 had a disynaptic excitatory link with depressor motoneurons of both the forewing and the hindwing.

Pattern generator interneurons

Individuals in the mesothoracic ganglion and metathoracic ganglionic mass.

Interneuron 301 (Fig. 11). To date this is the only pattern generator interneuron we have found in the mesothoracic ganglion. As far as is known 301 has no homologue in the metathoracic or the first three abdominal ganglia. The cell body (35–40 μm diameter) of 301 was found in the posterior medial region of a mesothoracic hemiganglion (Fig. 11a). It was located on the ventral surface of the ganglion. The primary neurite ran laterally to the dorsal neuropile where it joined the neuropile segment (depth 60–70 μm). Branching from the neuropile segment in the ipsilateral hemiganglion was extensive especially in the lateral dorsal neuropile. At the midline the neuropile segment bifurcated. The more anterior branch extended into the lateral dorsal neuropile of the contralateral side. The more posterior branch left the mesothoracic ganglion as the axon in the midline of the contralateral connective. The structure of the axon in the metathoracic ganglionic mass is uncertain but one exceptionally good fill of 301 showed profuse branching along
Fig. 8. Sets of homologous flight interneurons in the metathoracic and first three abdominal ganglia. The interneurons are assigned to sets on the basis of similarities in structure and physiology. The metathoracic ganglionic mass can be divided into separate regions corresponding to the metathoracic (T3) and first three abdominal (A1, A2, A3) ganglia. For clarity only the interneurons on the right side are shown. The axon of each interneuron is shown in full but the dendritic branching of each is shown only in that area of the metathoracic ganglionic mass corresponding to the ganglion containing the cell body of the interneuron unless the adjacent area is unoccupied (e.g., c, d). These interneurons are described more fully in the text and in subsequent figures.

Fig. 9. Interneuron 504. a. Structure of a 504 in the metathoracic ganglionic mass. b. EPSP recorded in a contralateral mesothoracic tergosternal motoneuron (E) a short and constant latency after each spike of 504. c. Intracellular recording from a 504 during a flight sequence.

Fig. 10. Interneuron 503. a. Structure of a 503 in the metathoracic ganglionic mass. b, c. Each spike of 503 is followed after a short and constant latency by an EPSP in 701 (b), and by an EPSP in 201(T1) (c). The latency is longer in c because of the greater path length to the prothoracic ganglion, location of 201(T1), than to the mesothoracic ganglion, location of 701. Note also that the amplitude scale is different from previous figures. Both EPSPs are large at about 4 mV. d. Intracellular recording from a 503 and the same 201(T1) as in c during a flight sequence.
LOCUST FLIGHT INTERNEURONS

Figures 9 and 10
Interneuron 301. a. Structure of 301 in the mesothoracic ganglion. b. Postsynaptic potential recorded in interneuron 501 (see below) a long latency after each spike of 301. c. Intracellular recording from 301 and 501 during a flight sequence. Note the characteristic membrane potential wave form of 301 (description in text). d. A pulse of depolarizing current (about 10 namps) delivered to 301 causes 501 to fire. The firing of 501 is patterned into an initial burst similar to its burst during flight. Note that during this burst dorsal longitudinal motoneurons (DL) also fire and firing in a 501 (Fig. 11d), presumably via the long latency pathway described above (Fig. 11b). The interesting feature of this effect is that the firing of 501 was patterned such that it gave a burst similar to its burst during flight activity. It appeared therefore that depolarization of 301 was capable of initiating flight activity. This is corroborated by the fact that during the burst of 501 dorsal longitudinal motoneurons fired at the appropriate phase of the cycle and were recorded electromyographically (Fig. 11d). There is evidence (not shown) that 301 had a long latency connection to mesothoracic first basalar motoneurons similar to its connection to 501 (Fig. 11b). When pulses of depolarizing current were passed into 301 during flight sequences the frequency of the flight rhythm was altered. The example shown (Fig. 11e) is an increase in the frequency of the rhythm from 10 Hz to 12 Hz only during the stimulus. However, in different experiments similar depolarizing pulses could decrease the frequency of the flight rhythm.

Sets of serial homologues. Interneuron 501 (Figs. 8, 12). There were homologues of 501 in all four of the ganglia of the metathoracic ganglionic mass (Fig. 8) but we never found a similar interneuron in other ganglia. Each 501 had a deep ventral cell body (25–30 μm diameter) situated laterally in the ganglion (Fig. 12a). The primary neurite ran dorsally to connect with a short neuropile segment orientated trans-
Interneuron 501 was hyperpolarized by the onset of wind stimulation and fired bursts of spikes at a high frequency in phase with depressor motoneuron activity during flight sequences (Fig. 12b). Passage of depolarizing current dramatically slowed the frequency of the rhythm which returned to the prestimulation frequency immediately after the stimulus (Fig. 12c). For this interneuron the effect of depolarizing current was less variable and never induced an increase in the frequency of bursting. In some experiments the flight frequency returns to normal. A similar pulse of current delivered to 501 in a different experiment has equal effects on frequency of bursting of forewing depressor motoneurons (meso DL) and hindwing depressor motoneurons (meta DL). The effect lasts only as long as the stimulus.
ments we monitored both forewing and hindwing motoneurons by implanting electrodes into the dorsal longitudinal muscles of both the meso- and the metathoracic segments. In these cases it was apparent that the effect of 501 stimulation was the same for both the forewing and the hindwing (Fig. 12d). Similar results were obtained across a ganglion (i.e., right and left affected equally). At present we have no knowledge of the output connections of 501.

For the two pattern generator interneurons described above we tested their ability to reset the flight rhythm with short pulses of depolarizing current (Fig. 13). For both 501 (Fig. 13a) and 301 (Fig. 13b) a 100-msec pulse of depolarizing current would increase the period of a single cycle, thus resetting the phase of the flight rhythm. It is interesting that the ability of 301 to reset the flight rhythm did not appear to be mediated via its polysynaptic excitatory connection to 501 as the firing pattern of this neuron was not substantially altered by the pulse of current to 301 (Fig. 13b).

Interneuron 401 (Figs. 8, 14). We found homologues of this interneuron in the metathoracic and first two abdominal ganglia (Fig. 8). It is probable that a homologue exists in the third abdominal ganglion also. Each 401 had a ventrally located cell body (30 μm diameter) located in the midline of the metathoracic ganglionic mass (Fig. 14a). The primary neurite ran directly dorsally to the neuropile segment (40–60 μm) which looped back on itself and gave rise to an axon which ran anteriorly up the midline of the ganglionic mass. There were dendritic branches bilaterally in the dorsal neuropile although the predominant branching was ipsilateral and the more anterior 401s had fewer branches which ran contralaterally. The major feature of the dendritic arbour was a thick process which arose at the lateral end of the neuropile segment and ramified profusely in the lateral dorsal neuropile. The axons gave off several laterally directed branches on both sides along its length and exited the metathoracic ganglionic mass medially in the midline ipsilateral connective heading toward the mesothoracic ganglion. Its structure anterior to this is unknown.

During flight sequences 401 fired short bursts of spikes immediately prior to depressor motoneuron activity (Fig. 14b,c). On occasions the burst could overlap the start of the depressor phase but not by a substantial amount. The onset of wind stimulation tended to depolarize 401 (data not shown). Passing pulses of depolarizing current into the neuropile segment of 401 during flight had a variable effect similar to the response of 301 (see above). In some experiments the stimulus caused the frequency of the flight rhythm to increase (Fig. 14b). More commonly, though, there was a decrease in the frequency during the stimulus (Fig. 14c). In all cases the frequency of the flight rhythm returned to its prestimulation frequency immediately on cessation of the stimulus (Fig. 14b,c). So far we have not established which neurons are postsynaptic to 401. From several double penetrations of 401 and various motoneurons it appeared not to have short latency connections with motoneurons. However, on two occasions strong depolarization of 401 causing it to fire at a high frequency resulted in depolarization and firing of identified depressor motoneurons (ipsilateral hindwing dorsal longitudinal motoneuron and ipsilateral forewing first basalar motoneuron) (data not shown). The latency from the first spike of 401 to the first sign of depolarization in each motoneuron was long and variable (20–30 msec) so this effect was presumably mediated polysynaptically.

**DISCUSSION**

This paper describes a number of interneurons in the flight system of the locust for which we have been able to characterize some important physiological features. Since these units are but a fraction of the interneurons which we believe to be involved in the control of flight (to date we have determined the structure of more than 50 different interneurons) we are unable to construct a circuit diagram of the flight system or even to speculate on the cellular mechanisms underlying the generation of the rhythm. However, our results do provide a basis for classifying flight interneurons and do provide considerable insight into the organization of interneurons on the flight system. This discussion is divided into two parts: comments on particular features of the structure and physiology of the interneurons including a consideration of what can be inferred about the nature of the flight pattern generator, and some deliberation on the evolutionary significance of such an organization.

**Functional organization of flight interneurons**

The most striking feature of the flight interneurons that we have found to date is their number and diversity. Despite this it is possible to separate them into two functional classes—those driving motoneurons and those that are part of the pattern generator. As more flight interneurons are identified it may be necessary to modify this conclusion but at the moment the division is justified for the reasons that perturbation of the premotor interneurons has not yet been seen to affect the flight rhythm and that the pattern generator interneurons have not yet been found to have short constant latency connections to motoneurons. The
Fig. 14. Interneuron 401. a. Structure of a 401 in the metathoracic ganglionic mass. b. A pulse of depolarizing current delivered to 401 during a flight sequence causes the flight frequency to increase transiently and then return to normal. DL activity is also increased. c. A similar pulse of depolarizing current delivered to 401 in a different experiment causes the flight frequency to decrease dramatically. These effects are evident only during the stimulus.

It is reasonable to ask whether the number and variety of premotor interneurons have any functional significance. A large number of premotor interneurons each having a small effect can exert a finer control over the phasing of the motor output. Different premotor interneurons to the same motoneurons tend to fire at slightly different phases of the flight cycle. For example, 701 (Fig. 4c) tends to fire slightly earlier in the cycle (relative to DL activity) than 201(T2) (Fig. 1c). Thus an input which selectively acts on one of these, causing it to give a stronger burst, would shift the phase of the following motoneuron relative to any motoneuron (e.g., an elevator) which was not postsynaptic to the same interneuron. Preliminary experiments show that differential access to sensory information is common in the flight system. Only some of the premotor interneurons described here receive input from the wing stretch receptors (Robertson and Pearson, unpublished) or from identified wind-sensitive interneurons (Bicker, unpublished). Similarly, some, but not all, are sensitive to auditory stimulation, or to visual stimulation. Thus the number and diversity of the premotor interneurons may reflect the great manoeuvrability of a flying locust in response to various environmental stimuli.

The finding of a large number of premotor interneurons also accounts for the difference between the size of the
unitary postsynaptic potentials and the amplitude of the membrane potential oscillation of motoneurons. Motoneurons can show oscillations of up to 25 mV during flight (Robertson and Pearson, '82) whereas each unitary EPSP is commonly 2–3 mV and rarely as much as 4–5 mV. Even if the connection from a single interneuron to a motoneuron is facilitatory (e.g., Fig. 5b,c) this would not account for the size of the membrane potential oscillation. We proposed in a previous paper that the smooth changes in the membrane potential of motoneurons are a result of drive from sets of four serial homologues each of which has a relatively weak connection (Robertson and Pearson, '82). To this must be added the observation that interneurons other than members of these sets and whose primary function may be to mediate slight changes in phase under the influence of sensory information also project to motoneurons, both elevators and depressors, and contribute significantly to the membrane potential wave form of motoneurons.

The pattern generator interneurons that are described in this paper are also numerous and varied although relatively less so than the premotor interneurons. Taking account of the sets of serial homologues and bilateral homologues we can propose that there are, at the very least, 18 interneurons involved in pattern generation (eight 401s, eight 501s, two 301s). This estimate is assuredly low, first, because we have not tested all identified flight interneurons (although we have tested all the premotor interneurons described here). Second, further searching will probably reveal other pattern generator interneurons which are not yet identified. However, even accepting this minimal number it is perhaps surprising, and very fortunate, that current injection into single interneurons can reset and modulate the rhythm at all. This finding encourages the belief that the rhythm-generating mechanism can be elucidated using current techniques.

In the beginning of this paper we posed the question: How far into the flight system does the strict serial homology observed at the periphery (e.g., forewing-hindwing, forewing muscles-hindwing muscles, forewing motoneurons-hindwing motoneurons) extend? The flight interneurons that we found fall into three main categories: unique individuals with no known homologues in the mesothoracic ganglion and metathoracic ganglionic mass, members of one of two sets of serial homologues which are concerned either with movements of the forewing or with movements of the hindwing, and members of a set of at least four serial homologues located in the metathoracic and first three abdominal ganglia. This last grouping is particularly interesting. Interneurons which are members of such sets are involved in driving motoneurons indirectly (Figs. 10, 11, 12, 14) or directly (Figs. 9) or are members of the flight pattern generator (Figs. 12, 14). It is clear, therefore, that the flight system is distributed between at least six segmental ganglia; from the prothoracic (e.g., 201(T1), Fig. 3) to the third abdominal. We do not yet have any information about interneurons in the unfused abdominal ganglia. It is possible that these ganglia contain flight interneurons or homologues of the interneurons we have described but these cells cannot be crucial to flight as the flight motor pattern is not obviously altered when the abdominal connectives are severed just posterior to the third abdominal ganglion (Wilson, '61; Robertson and Pearson, '82). Concerning the forewing and hindwing serially homologous sets, we have found this organization at the premotor level (Figs. 1,3) but not yet at the pattern generator level. Even at the premotor level this sort of organization is not common and individuals are readily found (Figs. 4–7). Moreover, drive for depressor motoneurons of both the forewing and the hindwing comes disynaptically from a single set of four serial homologues in the metathoracic ganglionic mass (Fig. 10) with no known counterpart in the mesothoracic ganglion. A very similar set of interneurons (Fig. 9) is known to drive forewing elevator motoneurons, and judging by its axonal projections it would be surprising indeed if it did not also drive hindwing elevator motoneurons. A common source of drive for the motoneurons of both the forewings and the hindwings argues for a single distributed rhythm-generating system. Strong supporting evidence for this idea comes from the observation that the pattern-generating interneurons that we found (Figs. 11, 12, 14) cannot be divided on anatomical grounds (e.g., individual interneurons in the meso- and metathoracic are completely different) or on physiological grounds (perturbation of single interneurons affects forewing motoneurons and hindwing motoneurons equally, Fig. 11d) into two groups associated with either the forewing or the hindwing. We conclude, therefore, that the interneuronal system generating the flight rhythm and patterning motoneuronal activity is distributed as a unit in six segmental ganglia.

Previous models of the locust flight system (Wilson and Waldron, '68) have been relatively simple and do not predict the complex interneuronal organization described in this paper. Also, the flight system is often used as an example of a rhythmical system relying on reciprocal inhibitory connections for its operation (e.g., Kristan, '80). While the present results neither confirm nor deny the role of such connections they do indicate that there is no simple scheme for their use. First, there is no evidence for just two groups of interneurons which reciprocally inhibit each other. Second, a simple reciprocal inhibition would not explain our observation that all of the pattern-generating interneurons promote activity in depressor motoneurons. Even pattern generator interneurons which fire in phase with elevator motoneurons during flight (Figs. 11, 14) will, when depolarized by current injection, cause depressor motoneurons to fire in nonflying preparations. Finally, both Waldron ('67) and Bentley ('69) observed an asymmetry in the system such that depressors were more liable to burst than elevators. These data therefore indicate that previous models of the locust flight pattern generator are inadequate.

Evolutionary considerations

The classical way of determining an animal's evolutionary lineage and its relationships with other animal groupings is by detailed comparison of various aspects of their biology (biochemistry, anatomy, physiology, morphology, behaviour, etc.). It is only recently that it has been possible to extend this study to the level of identified neurons (e.g., Mittenthal and Wine, '78; Bacon, '80; Dickinson, '80a,b; Wohlers and Bacon, '80; Paul, '81). However even from observations on single species it is possible to obtain data in consideration of an animal's evolution because they could either support or refute existing theories which were based on unrelated observations.
There are currently two main theories to account for the evolution of insect flight and the evolutionary origin of the insect wing. These are the paranotal lobe theory in which insect wings derive from rigid thoracic structures preadapted for gliding (e.g., Wootton, '76) and the pleural appendage theory in which insect wings derive from jointed, thoracic and abdominal appendages preadapted for a previous motor behaviour such as swimming or ventilation (Kukalová-Peck, '78). It is our opinion that our finding of important flight interneurons in at least six segmental ganglia and, significantly, that three of these are abdominal in origin supports the latter of these theories—the pleural appendage theory. Our argument for this is presented in detail in another paper (Robertson et al., '82).

Essentially it rests on the observation that in all other described segmental motor systems the segmental nature of the control system is a reflection of the segmental nature of the structures being controlled (e.g., Stein, '74; Thompson and Stent, '76; Weeks, '81). We feel that the most parsimonious explanation for the abdominal distribution of the locust flight motor is that it reflects a prior evolutionary stage when the structures being controlled were distributed serially along the abdomen. This is a cornerstone of the pleural appendage theory.

The finding of flight interneurons in the abdominal ganglia may have additional significance. The concentration of the nervous system by fusion of the segmental ganglia is ubiquitous in insects and it has been suggested that the advantages may lie in reducing the time for communication between related neural circuits (Pipa, '78). Our data associating interneurons in the abdominal ganglia with flight control suggest that the fusion between the metathoracic and the first three abdominal ganglia may have arisen as a result of selection pressure to reduce the conduction time between the components of the flight motor and thus enable flight frequencies to increase dramatically. High flight frequencies are thought to be a latter-day refinement and the vast wings of the early fossil pterygotes are thought to reflect a far slower wingbeat (Ewer, '63). Some support for this idea is given by the observation that there is a trend developmentally and phylogenetically for the acquisition and improvement of flight to coincide with the concentration of the nerve cord. Given that there may be other reasons for fusion of the abdominal ganglia and because of the great variety of insect species within each order this trend is difficult to document. However, the majority of those insect orders which have member species with seven or more separate abdominal ganglia are considered as being poor or weak fliers (Ephemeroptera, Plecoptera, Neuroptera), whereas the majority of the best fliers (Lepidoptera, Hymenoptera, Diptera) have less than six separate abdominal ganglia (Bullock and Horridge, '65; Giliot, '80; Matsuda, '56, '76). It is quite conceivable that investigations of widely different insects will reveal the intimate role of their abdominal ganglia in flight.

In conclusion, it is demonstrated that interneurons originating in the abdominal ganglia are important in the control of locust flight. The presence of this type of neuronal organization to control wings would be difficult to explain if wings evolved according to the paranotal lobe theory. Our data support theories which propose that wings originated from structures which were segmentally repeated along both the thorax and the abdomen, movable and controlled by the central nervous system. These results agree with the fossil record (Kukalová-Peck, '78) but do not preferentially support any of the various proposals for the motor functions of the pro-wings. Finally, once flight was achieved, it is possible that the increased temporal demands of more rapid flight favoured the fusion of the metathoracic and first three abdominal ganglia to reduce the conduction time between the elements of the flight motor.

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LITERATURE CITED


LOCUST FLIGHT INTERNEURONS

Since this paper was written we have discovered that the separation of interneuronal function is not as straightforward as suggested above. Pattern generator interneurons 501 and 401 can form direct inhibitory connections with motoneurons. However, some form of hierarchy is present for we have established the existence of excitatory premotor interneurons at an intermediate level between the pattern generator interneurons and motoneurons.