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Neural Circuits in the Flight System of the Locust

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SUMMARY AND CONCLUSIONS

1. Circuitry in the flight system of the locust, *Locusta migratoria,* was investigated by use of intracellular recording and staining techniques. Neuronal connections were established by recording simultaneously from neuropile segments of pairs of identified interneurons.

2. Brief depolarizing current pulses delivered to interneurons 301 and 501 reset the flight rhythm in a phase-dependent manner, thus establishing the importance of these neurons in rhythm generation. Interneuron 301 was found to make a strong delayed excitatory connection with 501 and to receive a short-latency inhibitory connection from 501. The circuit formed by 301 and 501 appears suited for promoting rhythmicity in the flight system.

3. The delayed excitatory potential recorded in 501 following each spike of 301 was reversed by hyperpolarizing 501. This potential and short-latency inhibitory postsynaptic potentials from 301 to other interneurons were blocked with the application of picrotoxin. We conclude that the delayed excitation is produced via a disynaptic pathway from 301 to 501, with 301 inhibiting in a graded manner the tonic release of transmitter from one or more unidentified intercalated neurons.

4. Interconnections between the 301-501 circuit and other identified interneurons were discovered. This circuitry can account for two features of the flight motor pattern recorded in deafferented preparations. These features are the constant-latency relationship between depolarizations in elevator and depressor motoneurons and the relatively constant duration of depressor motoneuron bursts.

5. The locust flight system shares general features with other described rhythm-generating systems. These include the occurrence of graded interactions, the probability of multiple oscillatory mechanisms, and a predominance of inhibitory connections. Its uniqueness lies in the way that components and processes are assembled and operate.

INTRODUCTION

Data on the neural mechanisms generating patterned motor output continue to accumulate. This is particularly true for rhythmical motor activity produced by deafferented preparations. To a limited degree it has been possible to classify rhythmical motor systems according to parameters of their output with the hope that common neural strategies for control can be inferred (28). However, the extent to which this can be done is limited by the relatively few systems in which the controlling mechanisms are known in any detail. General principles of neuronal function are the goal, but they can become established only by identifying them in different systems.

The flight system of the locust has proved to be a fruitful subject of study, and a great deal of information on various aspects of flight is currently available (see Refs. 6, 7, 49, 66, and 68 for reviews). The flight motor pattern of intact tethered animals is relatively simple and consists of an alternation of antagonistic muscles for each of four wings (paired fore- and hindwings) with hindwing muscle activation preceding that of the homologous forewing muscles by 5–8 ms (69). The motoneurons driving the muscles have been identified (5, 46, 56), and their membrane potential waveforms during the flight sequences expressed by deafferented preparations have recently been analyzed in some detail (24). The motor pattern repeats at ~20 Hz in the intact animal (1). Although the repetition rate drops to ~10 Hz in deafferented preparations, it is quite clear that the

nervous system can produce rhythm in the absence of phasic sensory input (46, 67) even when some features of the pattern are altered (24). It is equally clear that phasically active peripheral elements can modulate and reset the timing of this rhythm and that they are essential for the production of the natural flight motor pattern (25, 35, 41, 65). Here we are concerned solely with the central circuits that produce the basic flight rhythm upon which the sensory elements can act.

In previous papers we have described a functionally deafferented preparation suitable for recording intracellularly from flight interneurons and motoneurons during the expression of the flight rhythm (46), identified some of the flight interneurons (47), and commented on aspects of interneuronal organization in the system (47, 48, 50). This work showed that the flight rhythm is generated by interneurons distributed between the three adult thoracic ganglia, and that a single neuronal oscillator drives both pairs of wings (i.e., there is not a separate oscillator for each of the two pairs of wings) (47). Recently we have been studying the synaptic connections among the flight interneurons to try to discover the nature of this central oscillator. In this paper we describe the connections which may cause the constant latency from elevator to depressor activity seen in deafferented preparations (24) and a circuit which may underlie each burst of activity and help to promote rhythmicity. We also describe a delayed excitatory connection between flight interneurons that appears to result from a disynaptic disinhibitory pathway with tonic and graded release of transmitter, at least at the second synapse. Our previous proposal that flight interneurons may form two separate functional categories (premotor and pattern generator) (47) is modified in light of new data presented here. Finally, the flight system is compared with other rhythmical motor systems.

METHODS

Preparation and recording

Adult male and female *Locusta migratoria* were obtained from a breeding colony at the University of Alberta. Young males were preferred but not used exclusively, and no difference in results attributable to age or sex was observed. All experiments were done at room temperature (22–24°C).

The preparation has been described in detail elsewhere (46). Essentially, the thoracic ganglia were exposed by a dorsal midline incision, and the meso- and metathoracic ganglia were stabilized on a rigid stainless steel plate. The interior of the preparation was flooded with saline at room temperature [in mM: 147 NaCl, 10 KCl, 4 $CaCl_2$, 3 NaOH, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer]. Usually the innervation of the dorsal longitudinal muscles was left intact, and the time of depressor activity was monitored electromyographically from these muscles. In some experiments all the thoracic nerves were cut, and the time of depression was monitored by recording dorsal longitudinal motoneuron activity from nerve 6 (cut distally) of the prothoracic ganglion by use of a monopolar silver wire hook electrode (75 μm) with the indifferent electrode in saline nearby. There was no observable difference in the results obtained in the two situations.

Recordings were taken from the neuropile segments of interneurons and motoneurons by use of glass microelectrodes pulled to a resistance of 50 MΩ when filled with 1 M potassium acetate. For most experiments the tips of these electrodes were filled with Lucifer yellow CH (4% in distilled water) (55), and the shafts were filled with 0.5 M lithium chloride. After recording, Lucifer yellow was passed into the impaled neuron with hyperpolarizing current (5 nA for 5–10 min), and the ganglia were subsequently processed for fluorescence microscopy (30 min in 4% paraformaldehyde, pH 7.2; 20 min in two changes of absolute alcohol; 10 min in methyl salicylate). Filled neurons were viewed under a fluorescence microscope. Interneurons were numbered according to the scheme outlined in Ref. 47. We found that certain interneurons (e.g., 301, 501, 504) could be reliably identified on purely physiological grounds. In later experiments involving these neurons, the microelectrodes were backfilled with 1 M potassium acetate, and no Lucifer yellow was used. In this way problems associated with the high resistance of Lucifer yellow-filled electrodes (>100 MΩ) could be alleviated.

Picrotoxin treatment

Picrotoxin (Sigma), when used, was applied to the preparation in the following way: a stock solution of 3×10^{-3} M picrotoxin in saline was kept refrigerated for no longer than 5 days. For each experiment this was diluted 10 times to give a 3×10^{-4} M solution. This was dropped gently into the interior of the preparation until the volume of solution bathing the ganglia was approximately doubled to give a final picrotoxin concentration of $\sim 1.5 \times 10^{-4}$ M. This concentration had an effect in 3–5 min. Attempts were made to reverse the picrotoxin block of connections

by washing with fresh saline. However, on most occasions the fragility of double penetrations in the neuropile prevented this. On one occasion a stable preparation was washed for about 20 min without apparent reversal of the picrotoxin effect.

Phase-response curves

To obtain data for the phase-response curves shown in Figs. 1*B* and 2*B*, the relevant neuron was stimulated during flight sequences with 30-ms duration depolarizing current pulses (~10 nA) delivered at a constant frequency of 2 Hz. Because the frequency of the flight rhythm in deafferented preparations was about 10 Hz, the stimulus fell at a variable phase of approximately every fifth cycle. To construct the phase-response curve, the time of stimulation was taken as the start of the stimulus pulse, and cycle periods were measured from the beginning of successive bursts of dorsal longitudinal activity. The latency of the stimulus was defined as the interval from the start of the perturbed cycle to the start of the stimulus. Stimulus phase was measured as the ratio of the latency to the average period of the two preceding unperturbed cycles. Phase shift was the ratio of the difference between the period of the perturbed cycle and the average period to the average period. The only data points plotted were those for which the average period at the presentation of that stimulus was within 1 SD of the mean cycle period for all flight sequences throughout that experiment. This avoided biases introduced by irregular flight sequences and the tendency for the frequency to decrease during a single flight sequence. Stimuli occurring late in a cycle had a greater effect on the subsequent cycle than on the cycle in which they occurred because time of stimulation was taken at the start of the current pulse, and late stimuli overlapped the end of the cycle. Thus for stimulus phases greater than 0.8 the phase shift was measured for the subsequent cycle. These points are plotted to the left of stimulus phase 0 on the graphs.

Interneurons and synaptic connections

Most of these interneurons have been described previously, and the reader is referred elsewhere for detailed descriptions of their morphology (47). However, the location, structure, and numbers of particular interneurons may have considerable importance in interpreting the results and assessing the degree to which interneurons might interact with and without action potentials. Of equal importance is the possible physiological role of each interneuron, e.g., premotor function and/or ability to reset the rhythm. Therefore, Table I is a compilation of published and heretofore unpublished data. In all cases, reference to a single interneuron is intended to include its contralateral partner except where otherwise indicated. All the interneurons are paired. The physiology applies to all known homologues of an interneuron.

Due to the nature of the recording techniques and the preparation, double penetrations were held for short periods only. Thus the tests for monosynapticity used in other systems (2, 19) could not be performed. However, certain characteristics of the latency of a connection (1-for-1 following; invariant synaptic delay of ≤1 ms after subtracting the conduction delay) were taken as

TABLE 1. *Basic features of some flight interneurons*

Interneuron	Cell Body Location	No.*	Axon Pathway	Serial Homologues†	Phase of Firing	Premotor Function†	Reset†
201	meso	2	desc	pro	D	exc to D	
202	meso	2	desc		D	exc to D	
206	meso	2	desc		E		
301	meso	1	desc		E		yes
401	meta	1	asc	abd 1 & 2	E		yes
501	meta	1	asc	abd 1, 2, & 3	D	inh to E	yes
503	meta‡	1	asc	abd 1, 2, & 3	D		
504	meta	1	asc	abd 1, 2, & 3	E	exc to E	yes
511	meta	1	asc		E	inh to D	
520	meta	1	asc		D		
701	meso	1	asc, desc		D	exc to D	

*, 2 indicates that multiple fills have shown at least two identical neurons on each side. †, Lack of an entry in a column indicates a negative finding to date. ‡, Published description of this interneuron does not include a metathoracic representative. We have now identified this interneuron. Abbreviations: abd 1, 2, and 3, first, second, and third abdominal; asc, ascending; D, depressor; desc, descending; E, elevator; exc, excitatory; inh, inhibitory; meso, mesothoracic; meta, metathoracic; pro, prothoracic. Refs. 47, 49. Interneuron 206 is described in this paper. Interneurons 202 and 520 are undescribed.

indications of a probable monosynaptic connection. This argument holds for all constant short-latency connections described here, but due to the uncertainty involved they are hereinafter referred to as short-latency connections.

RESULTS

We first describe the circuit formed by the connections between interneurons 301 and 501. Then we consider the properties of the 301 to 501 connection in more detail. Finally, we consider the input to and the output of the 301-501 circuit.

A circuit of delayed excitation and feedback inhibition

The interneurons that have central importance in the circuit presented at the end of this paper are 301 and 501. They have been described and shown to be members of the pattern generator (47). We have since discovered the structure of 301 in the metathoracic ganglion (Fig. 1*A*). The axonal branching of 301 in the metathoracic ganglion was extensive, bilateral, and located just under the dorsal sheath of the ganglion. It overlapped the region of dendritic branching of all serial homologues of 501 (see Fig. 2*A* for the structure of 501 of the first abdominal neuromere). The axon subsequently exited in the abdominal connective. Rhythmic activity and ability of transient perturbations of this activity to reset the rhythm are generally accepted as criteria necessary to show that a neuron is a member of the rhythm generator (29, 47, 61). Resetting is shown as a change in duration only of the cycle period in which a brief stimulus falls. The ability of a stimulus to reset the rhythm can vary depending on the phase of the cycle at which it is presented, and this relationship can be plotted as a phase-response curve (e.g., Ref. 43). A systematic study of the effects of short-duration current pulses delivered to 301 and 501 has revealed the phase dependence of their resetting ability and has enabled phase-response curves to be constructed. Appropriately timed pulses to 301 could reliably either advance or delay the occurrence of the following cycle (Fig. 1*B*), whereas similar pulses to 501 could, with regularity, only delay the next cycle (Fig. 2*B*).

The interactions between these interneurons are particularly interesting (Fig. 3*A*). Every spike in 301 was followed by a depolarizing potential in 501 (Figs. 3,*B* and *C*).

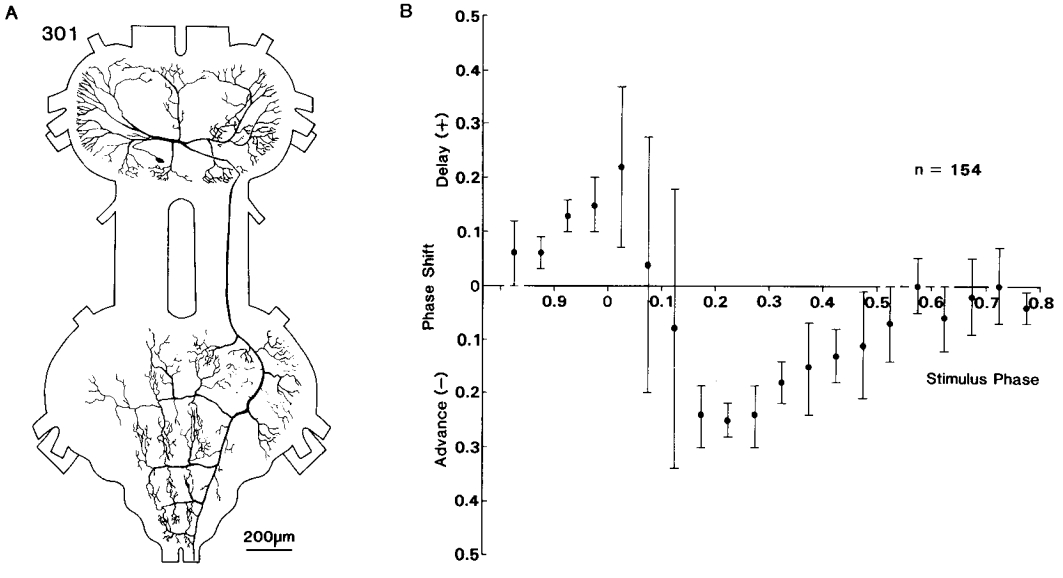

FIG. 1. Interneuron 301. *A*: drawing of structure of 301 in mesothoracic (*upper*) and metathoracic (*lower*) ganglia. *B*: phase-response curve for short depolarizing current pulses delivered to 301. Data are from a representative single experiment and plotted as the mean ± 1 SD for all stimuli falling in bins of 0.05 stimulus phase. Note that pulses to 301 can reliably delay and advance the occurrence of the subsequent cycle. See METHODS for details of construction of phase-response curve.

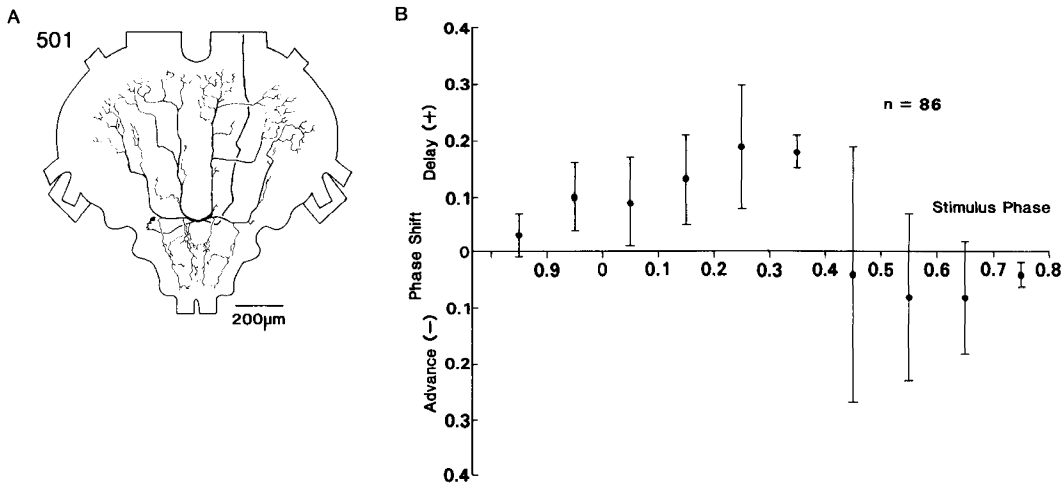

FIG. 2. Interneuron 501. *A*: drawing of structure of 501 in metathoracic ganglion. *B*: phase-response curve for short depolarizing current pulses delivered to 501. Data are from a representative single experiment and plotted as the mean ± 1 SD for all stimuli falling in bins of 0.1 stimulus phase. Note that pulses to 501 can reliably delay occurrence of subsequent cycle but that such advances as are seen are not significantly different from normal variation in period of rhythm. See METHODS for details of construction of phase-response curve.

The latency from spike to postsynaptic event was about 6 ms, which was longer than for short-latency connections (2–3 ms). The 301-501 connection is thus referred to as a delayed excitation. Every spike in 501 was followed by an inhibitory postsynaptic potential (IPSP) in 301 with a latency appropriate for a monosynaptic connection (Fig. 3*D*). Accurate measurements of the time courses of these potentials were possible in three experiments. Mean values were as follows for the delayed excitatory potential: latency, 6.2, time to peak, 16.6, and duration, 29.6 ms; and for the IPSP: latency, 3.0, time to peak, 5.1, and duration, 18.3 ms. Although 301 had delayed excitatory connections with 501 on both sides, each 501 had a short-latency inhibitory connection with only the contralateral 301. The connections between 301 and 501 form a simple circuit (Fig. 3*A*) that seems suited for burst generation. The 301 activity might drive 501 to fire after a fixed delay, and 501 firing would then feed back to 301, thus inhibiting it and consequently turning off 501's source of activation. When 301 is turned off 501 ceases firing and releases 301 from inhibition, thus enabling it to fire once more. If this is true, then tonic activation of 301 might cause rhythmical activity. This was confirmed. Depolarizing current pulses (~10 nA, 400–1,000 ms) were passed into the neuropile segment of 301 when the flight rhythm was not being expressed. In about 30% of the preparations this stimulation caused bursting in 501 and, significantly, in dorsal longitudinal motoneurons (Fig. 4). Frequency of bursting activity was close to the usual frequency of the rhythm expressed by our preparation (compare Fig. 4*B* with Fig. 4*A*), and the normal phase relationship between 501 activity and dorsal longitudinal activity was maintained (both fire at depressor phase). We are uncertain about why rhythmic activity was not evoked in all preparations, but it may be related to variability in the general level of excitability in different preparations. In one experiment, pulses of depolarizing current delivered to 501, similar to those used to stimulate 301, caused bursts of dorsal longitudinal activity. The evoked activity in 501 was rhythmically modulated, presumably via feedback from an unknown part of the flight circuitry. An important observation was that 301 was monitored simultaneously, the stimulus induced only a slight oscillation of its membrane potential, and at no time did it fire.

Properties of the delayed excitatory connection

Delayed excitatory connections were found from 301 to 501, 503, 520, and some de-

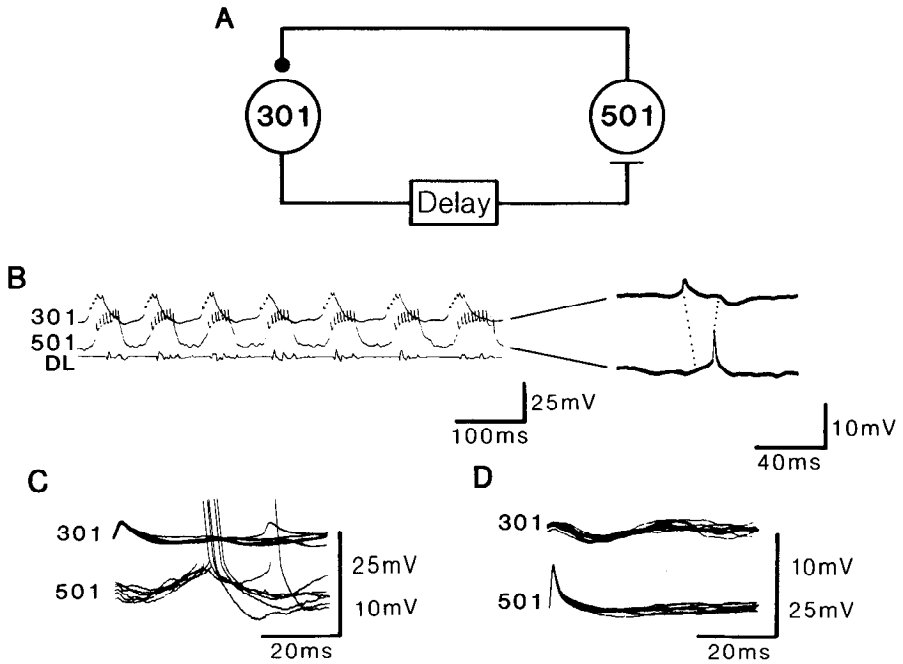

FIG. 3. Delayed excitation from 301 to 501 and feedback inhibition from 501 to 301. *A*: diagrammatic representation of connections between 301 and 501. *B*: Simultaneous recordings from 301 and 501 during a flight sequence. At the end a portion of traces has been enlarged to show a spike in 301 followed by a spike in 501 followed by an IPSP in 301 (sequence indicated by *dotted lines*). Note that this expanded record was taken when the preparation was not expressing flight rhythm. *C*: delayed excitatory con nection from 301 to 501. Note latency of 6 ms. This is about twice the value of short-latency connections. *D*: short-latency IPSP from 501 to 301. Note that this IPSP can be discerned in *C* although the gain of the 301 trace is not as great. *B*, *C*, and *D* were taken from same experiment. In this and all subsequent figures DL trace is a monitor of activity of dorsal longitudinal motoneurons recorded either electromyographically or *en passant* from the nerve root containing their axons (see METHODS); inhibitory connections are diagrammed as *filled circles* and excitatory connections are diagrammed as *T-bars;* method used to display neuronal interconnections is to overlay multiple sweeps of oscilloscope triggered off rising phase of spike of presynaptic neuron.

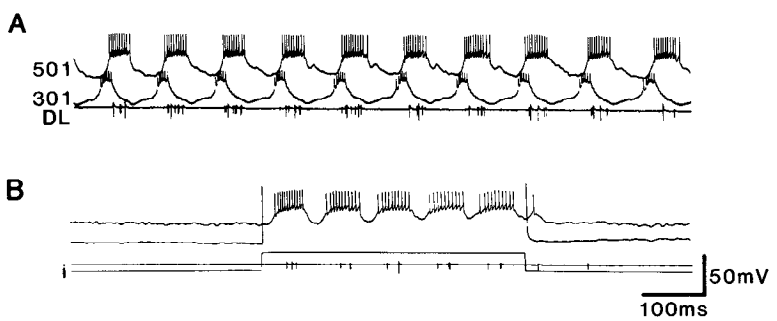

FIG. 4. Stimulation of 301 can produce rhythmic activity. *A*: simultaneous recordings of 501 and 301 during a normal flight sequence. *B*: a long-duration depolarizing pulse of current (about 10 nA) delivered to 301 when the flight rhythm was not being expressed induced rhythmical bursting activity in 501 and dorsal longitudinal motoneurons. Note that phasing and the frequency of this bursting activity is similar to normal flight activity (compare *B* with *A*). In this and all subsequent figures the trace labeled "i" is a current trace to monitor the duration of stimulus pulses.

pressor motoneurons. Latency of the delayed excitatory connection with each was similar and was found to be as invariant as those of presumed monosynaptic connections. The latency was about 6 ms and approximately double that of short-latency connections from 301 to interneurons in the same region of the ganglion as 501. This difference is clearly demonstrated in Fig. 5 where the delayed excitation from 301 to 501 (Fig. 5*A*) can be compared with a short-latency connection from 201 to the same 501 (Fig. 5*B*). The conduction pathways are the same length in each case, and the axon diameters of 201 and 301 are similar, thus suggesting similar conduction velocities for the spikes in each.

An interesting property of the delayed excitatory connection was that the injection of hyperpolarizing current into the postsynaptic neuron reversed the sign of the postsynaptic potential (PSP) (Fig. 6). This occurred with about −6 nA of injected current. Unfortunately, due to the problems of recording in the neuropile with high-resistance electrodes, the absolute reversal potential for this PSP could not be reliably measured. Rough estimates based on current and voltage thresholds for 501 placed the reversal potential for the delayed excitation about 10–15 mV hyperpolarized from the resting potential. The latter was within the range of 50–60 mV. It is important to note that spontaneous IPSPs in 501 reversed at the same membrane

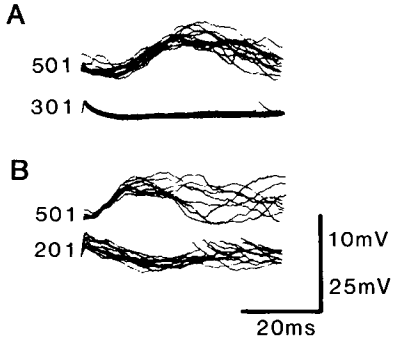

FIG. 5. Delayed and direct excitation of 501. *A*: spikes in 301 produce a delayed excitation of 501. *B*: 201 has a short-latency excitatory connection with same 501. Note that 301-501 connection has some monosynaptic characteristics (constant latency; one-for-one following), but that connection from 201 to 501 has a markedly shorter latency and shorter time course (compare *A* and *B*). Conduction delays from both neurons to 501 are assumed to be similar (see text).

potential as did the delayed excitatory potentials. This can be seen in the insets of Fig. 6 by comparing the smooth trace of 501 near the reversal potential for the delayed excitation with the ragged trace caused by IPSPs when no current is injected. Injection of chloride ions into 501 by use of a microelectrode containing 1 M KCl reduced the amplitude of the delayed excitatory potential recorded in 501 (from 5.3 mV to 3.5 mV after a 10-min injection with a 5-nA hyperpolarizing current; each value averaged from 125 successive potentials in a single representative experiment).

The fact that hyperpolarizing current reversed the delayed excitatory potential indicates that the potential was produced by a decreased conductance across the postsynaptic membrane. There are two ways that 301 might connect with 501 to produce a decreased conductance with the characteristics of a long-latency monosynaptic connection. The first is via a disynaptic disinhibitory pathway (e.g., Fig. 7*A*). We identified an interneuron (511) that receives a short-latency IPSP from 301 (Fig. 7, *B* and *D*), and that in turn causes a short-latency IPSP in 501 (Fig. 7, *C* and *E*). However, for such a pathway to produce a depolarization with the appropriate properties, there must be tonic and graded release of inhibitory transmitter at the 511-501 synapse, and the synapses must be close enough together to allow the 301-511 IPSP to have an effect on transmitter release at the 511-501 synapse. These characteristics have not yet been demonstrated for the pathway illustrated in Fig. 7*A*. The second way is via a monosynaptic decreased-conductance connection where the longer latency and time course are the direct result of the kinetics of the ion channels.

In an attempt to distinguish between these two possibilities we surmised that if IPSPs from 301 could be blocked without blocking the delayed excitation, then the possibility that it was a monosynaptic decreased-conductance connection would be more likely. Treatment with picrotoxin blocked the IPSP from 301 to 511 within about 4 min (Fig. 8). However, the same treatment also blocked the delayed excitation from 301 to 501 (Fig. 9), and this occurred after the same length of time. The effect of picrotoxin on 301 was to block spontaneous IPSPs, but there was

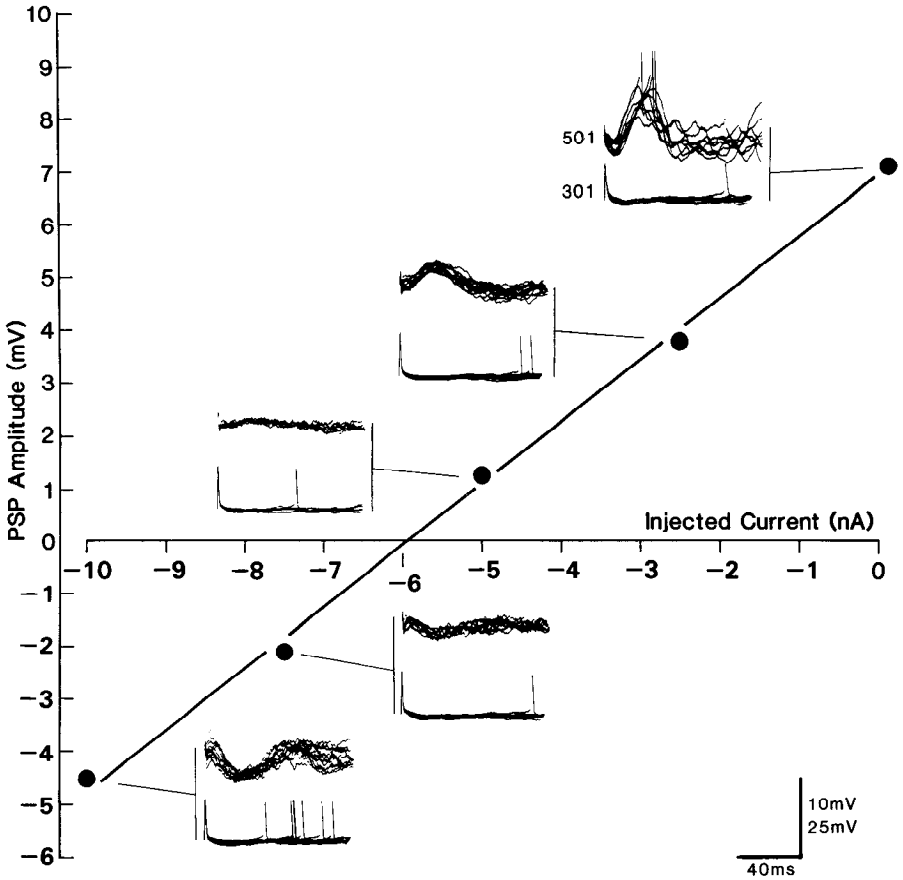

FIG. 6. Reversal of delayed excitatory postsynaptic potentials from 301 to 501. Increasing amounts of hyperpolarizing current passed into 501 gradually reduce amplitude of depolarizing potential until it is reversed with passage of −6 nA of current. *Insets* are examples of *traces* which provided the *data points*. Note that spontaneous IPSPs seen in 501 *traces* are reversed at same point as delayed excitation. This can be seen by relative smoothness of 501 *trace* when −5 nA are being injected compared with when no current is being passed.

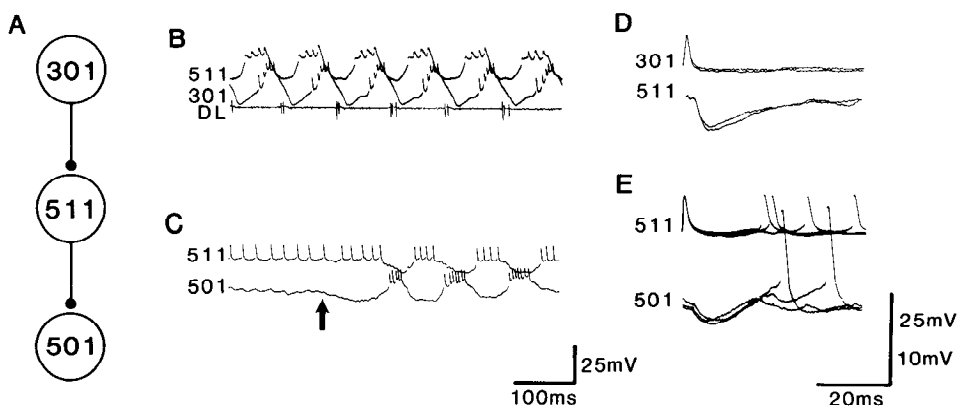

FIG. 7. A disynaptic pathway from 301 to 501 via 511. *A*: diagrammatic representation of pathway. *B*: simultaneous recordings of 511 and 301 during a flight sequence. *C*: simultaneous recordings of 511 and 501 during a flight sequence. *Arrow* under *trace* marks onset of hyperpolarization caused by wind stimulus that initiated flight. *D*: spikes in 301 produce an IPSP in 511. *E*: spikes in 511 produce an IPSP in 501. IPSPs can also be seen in *traces* of *C* before wind stimulus.

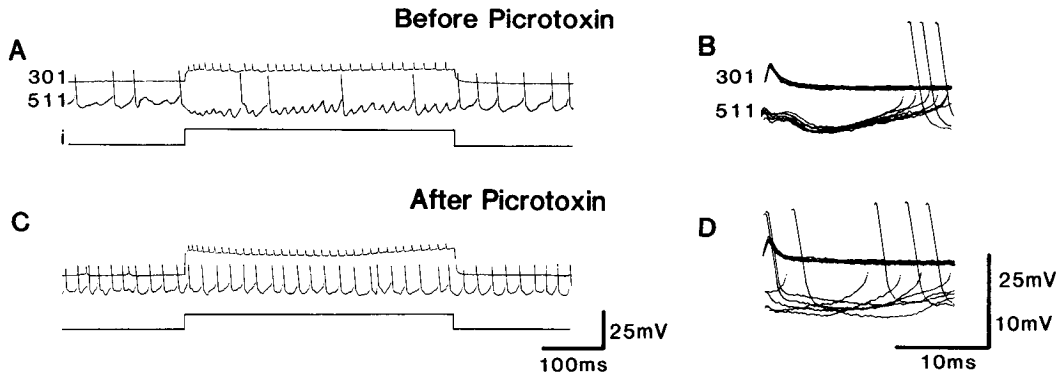

FIG. 8. Picrotoxin blocks IPSPs produced by 301. *A* and *B*: control. Simultaneous recordings of 301 and 511 show IPSPs produced in 511 after 301 is induced to fire with a depolarizing pulse of current (*A*). Multiple oscilloscope sweeps show the 301-511 IPSP (*B*). *C* and *D*: after bathing ganglia for about 4 min in $\sim 1.5 \times 10^{-4}$ M picrotoxin, similar recordings fail to reveal an IPSP. Note that in *A* and *C* the bridge for the 301 recording was not accurately balanced.

no effect on the general level of activity of 301 (Fig. 9, *A* and *D*). However, as well as blocking the delayed excitation from 301 to 501 and spontaneous IPSPs in 501, picrotoxin caused the membrane potential of 501 to be depolarized past threshold so that the neuron fired continuously (Fig. 9, *D* and *E*). This is consistent with the idea that picrotoxin acts to block a tonic inhibitory influence on 501. For this and other reasons that are outlined fully in the DISCUSSION we feel that the evidence suggests that the delayed excitation from 301 to 501 was caused by a disynaptic disinhibitory pathway with tonic and graded release of transmitter from the second synapse. Such connections were not restricted to 301, and similar delayed excitatory potentials were observed in 202, 301, 701, and some depressor motoneurons after each spike in 401 (an inhibitory interneuron that can

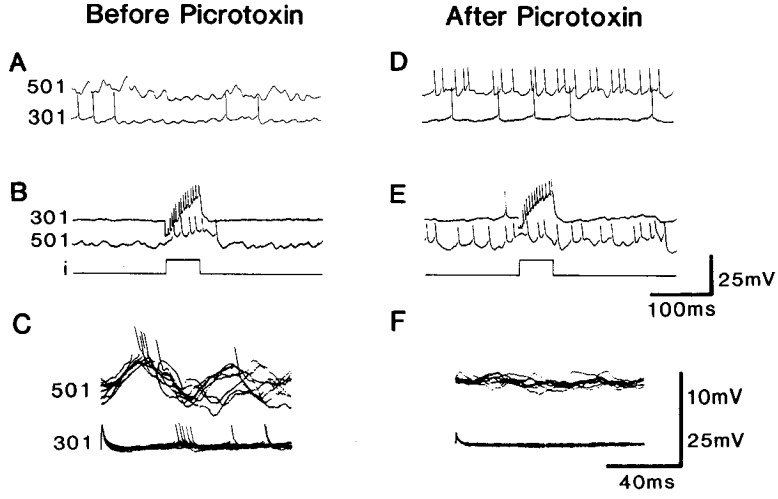

FIG. 9. Picrotoxin blocks delayed excitation produced by 301 in 501. *A–C*: control. A substantial depolarizing potential can be seen following each spike in 301 (*A*, *C*). A short pulse of depolarizing current delivered to 301 causes it to fire at high frequency and to drive 501 to burst (*B*). *D–F*: after bathing ganglia for about 4 min in $\sim 1.5 \times 10^{-4}$ M picrotoxin, spikes in 301 have no effect on membrane potential of 501 (*D*, *F*) even when 301 is driven to fire at high frequency (*E*). Note that picrotoxin treatment causes membrane potential of 501 to depolarize above threshold so that neuron fires continuously. This does not happen to 301 (compare *A* with *D*, and *B* with *E*). *A* and *D*, *B* and *E*, and *C* and *F* were taken from three different experiments. In *B* and *E*, bridge was not accurately balanced.

reset the rhythm). Interestingly, rhythmical activity could still be recorded after picrotoxin treatment. The frequency of the rhythm recorded under these conditions was substantially greater than under normal conditions (16–18 Hz compared with 10–12 Hz). It was also quite variable in its occurrence and regularity both between and within experiments, possibly due to the extent to which the picrotoxin was affecting other pathways. Also the structure of neuronal bursts was different, and the phase relationships were disrupted.

Input to the 301-501 circuit

The excitatory input for elevator motoneurons originates from a homologous set of interneurons 504 as well as other premotor interneurons (47). We found that each 504 also makes a short-latency connection with 301 (Fig. 10,*A* and *D*). In two experiments we established that the same 504 produced excitatory postsynaptic potentials (EPSPs) in an elevator motoneuron and in 301. This was done by first finding the connection from 504 to one of the neurons and then moving the second electrode to the other neuron without losing the initial penetration of 504. These connections ensure that activity in 504 will excite elevator motoneurons and 301 concurrently.

Short-duration current pulses delivered to 504 could reset the rhythm (Fig. 10*C*) possibly via its connection with 301. Long-duration current pulses, similar to those used to stimulate 301, caused rhythmical activity in the system (Fig. 11). In this case the rhythm was monitored by recording intracellularly from 301 and extracellularly from dorsal longitudinal motoneurons. The frequency of bursting was slightly less than the normal frequency (compare Fig. 11, *A* and *B*) and the phase relationship between 301 (elevator phase) and dorsal longitudinal (depressor phase) bursts was only slightly different from normal. Variability in the success of evoking the rhythm was similar to that found with 301.

Interneuron 504 received short-latency excitatory connections from a group of interneurons we have numbered 206 (Fig. 12). These are previously undescribed, and the morphology of a single 206 in the mesothoracic ganglion is shown in Fig. 12*A*. Multiple fills showed that there were at least two of this type of interneuron with the same morphology and physiology on each side of the mesothoracic ganglion. The course of the axon in the metathoracic ganglion is unknown at present.

Attempts were made to drive the rhythm by stimulation of 206. There were indications (not shown) that 206 had this capability in

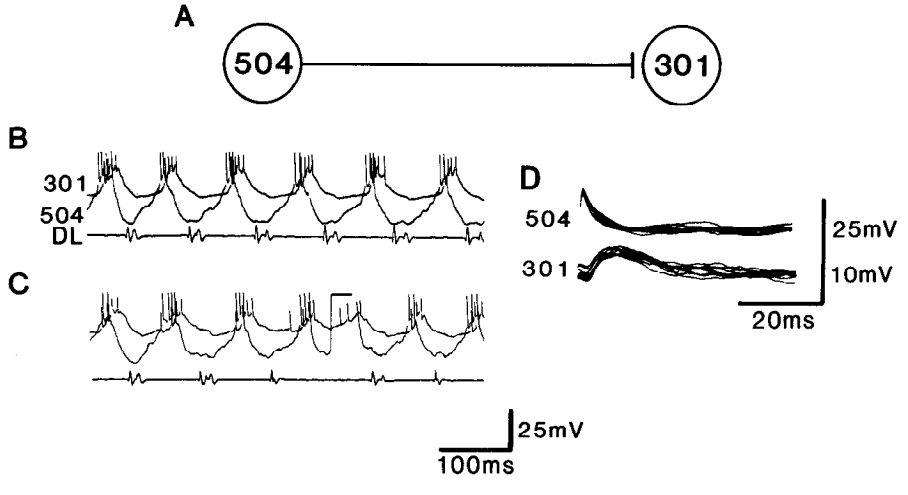

FIG. 10. Excitatory connection from 504 to 301. *A*: diagrammatic representation of connection. *B*: simultaneous recordings of 504 and 301 during the expression of flight rhythm. *C*: a 30-ms pulse of depolarizing current (~10 nA) delivered to 504 reset rhythm by increasing period of only cycle in which stimulus occurs. Compare with *B*, which is from same experiment. *D*: multiple oscilloscope *traces* demonstrate the short-latency EPSP from 504 to 301.

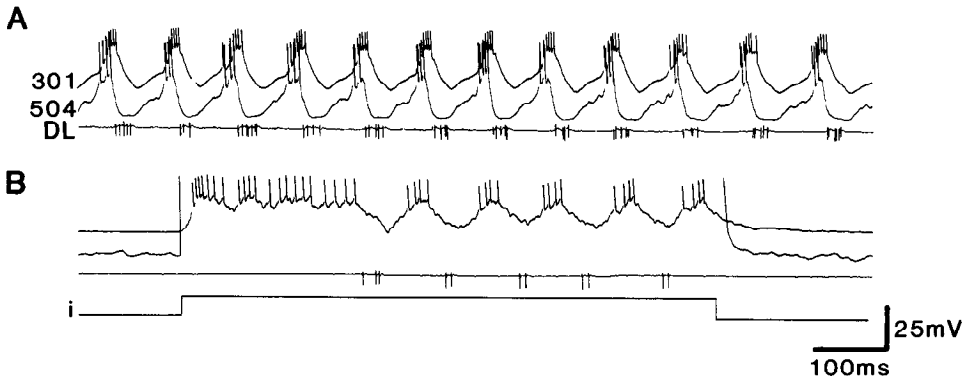

FIG. 11. Stimulation of 504 can produce rhythmic activity. *A*: simultaneous recordings of 301 and 504 during a normal flight sequence. *B*: a long-duration depolarizing pulse of current (~10 nA) delivered to 504 when flight rhythm was not being expressed could induce rhythmical bursting activity in 301 and dorsal longitudinal motoneurons. Note that once rhythm begins, phasing and frequency of bursting activity is similar to normal flight activity (compare *B* with *A*).

that stimulation of it occasionally caused one or two cycles of flightlike bursting. However this result was not consistent and needs further substantiation.

Output of the 301-501 circuit

It was mentioned earlier that 301 had a similar delayed excitatory connection with 503 as it had with 501. This is demonstrated in Fig. 13. The significance of this connection is that it is the initial step in a pathway from 301 to depressor motoneurons. Interneuron 503 is known to drive 201 in the prothoracic ganglion [201(T1)] (47). We have now found that 503 also has a strong connection with 201 in the mesothoracic ganglion [201(T2)] (Fig. 13, *E* and *F*). This connection was sufficiently strong for the 503 burst to have a direct influence on the number of spikes in each burst of 201 (Fig. 13*D*). Interneurons

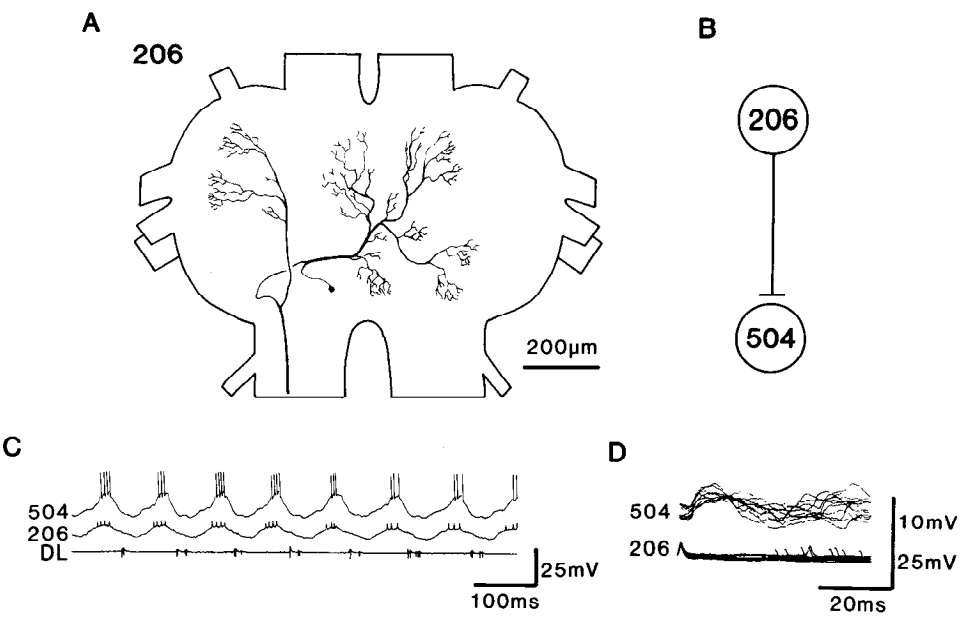

FIG. 12. Excitatory connection from interneuron 206 to 504. *A*: structure of 206 in mesothoracic ganglion. *B*: diagrammatic representation of the connection from 206 to 504. *C*: simultaneous recordings of 504 and 206 during a flight sequence. *D*: short-latency EPSP from 206 to 504 (from same experiment as *C*).

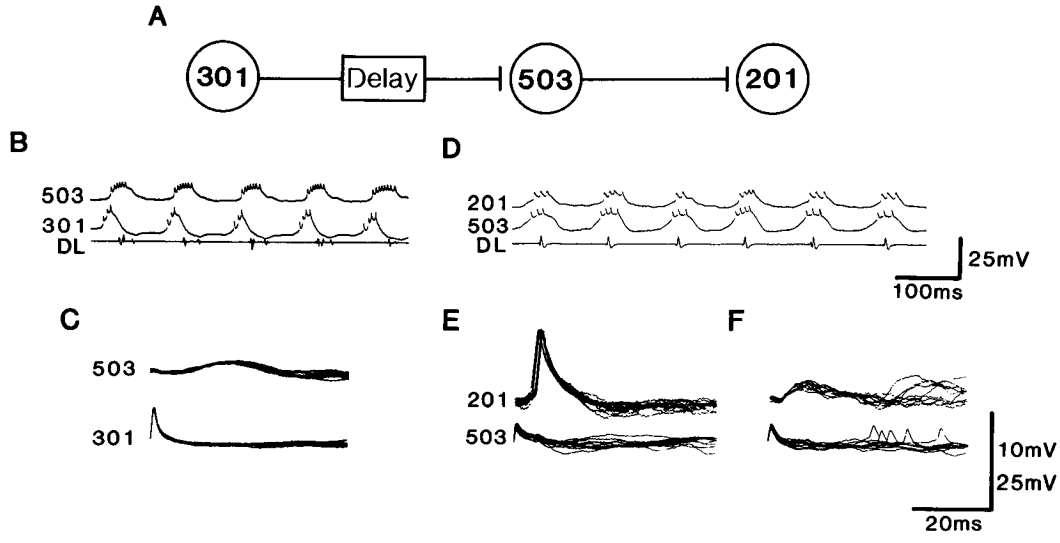

FIG. 13. Output of the 301-501 circuit to interneurons exciting depressor motoneurons. *A*: diagrammatic representation of the pathway from 301 through 503 to 201(T2). Interneuron 201(T2) makes short-latency excitatory connections to hindwing depressor motoneurons. *B*: simultaneous intracellular recordings of 301 and 503 during a flight sequence. *C*: delayed excitatory connection from 301 to 503. *D*: simultaneous recordings from 503 and 201. Note that, with the exception of last spike in third burst of 503, each spike in 503 is followed by a spike in 201. *E*: multiple oscilloscope sweeps show spike-for-spike following in 201 after 503. Note that this record, like connections demonstrated in other figures, was taken when preparation was not expressing flight rhythm, i.e., 503 exerts powerful control over 201 when the latter is at resting membrane potentials. *F*: slight hyperpolarization of 201 with about −2 nA of current reveals short-latency EPSP from 503 underlying spike in 201. Note that 301 is active during elevator phase, whereas 503 and 201 are active during depressor phase.

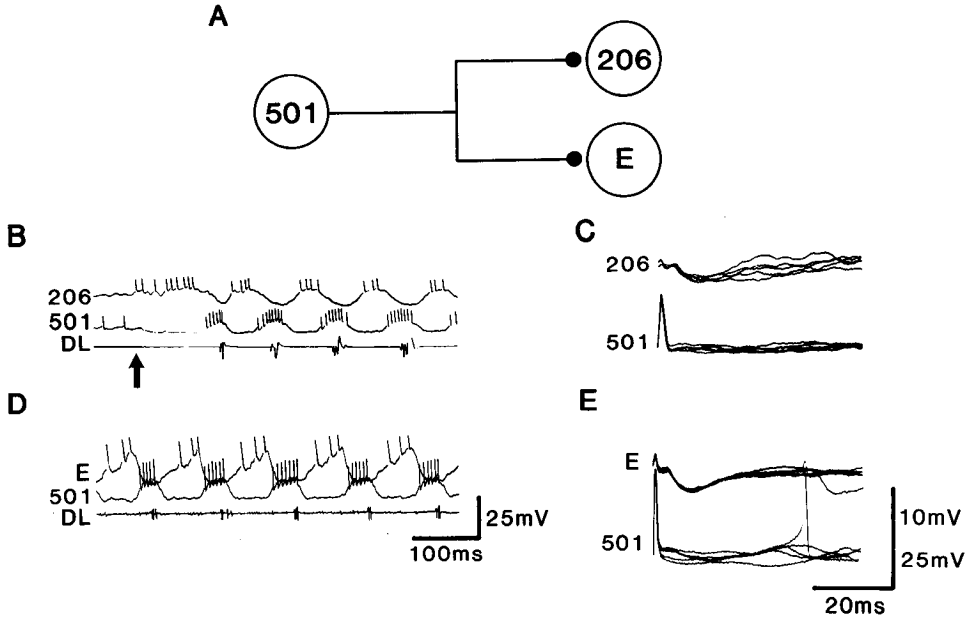

FIG. 14. Inhibitory connections of 501 to elevator motoneurons and to interneuron 206. *A*: diagrammatic representation of inhibitory connections from 501 to 206 and to elevator motoneurons. *B*: simultaneous recordings of 501 and 206 during a flight sequence. Note that 206 is depolarized by wind stimulus used to initiate the flight sequence (onset of response indicated by *arrow* under *trace*). *C*: short-latency IPSP from 501 to 206. *D*: simultaneous recordings from an elevator motoneuron (mesothoracic tergosternal) and 501 during a flight sequence. *E*: short-latency IPSP from 501 to a mesothoracic tergosternal motoneuron in a different experiment.

201(T1) and (T2) are premotor excitatory interneurons for depressor motoneurons of the forewing and the hindwing, respectively (47). The important point to note is that 301 fired at elevator phase, and all the follower neurons fired at depressor phase.

The inhibitory influence of 501 was quite extensive. We found a short-latency connection from 501 to 206 (Fig. 14, *A* and *C*). Interestingly 206 fired only a few spikes during the depolarized phase of its membrane-potential oscillation during flight sequences, and it appeared as if this burst resulted from periodic inhibition (primarily from 501) interrupting tonic activation due to the wind stimulus (Fig. 14*B*). This can be seen as a low tonic rate of firing except when inhibited or recovering from such inhibition (see 206 traces in Figs. 12*C* and 14*B*). In contrast to other flight interneurons there was no obvious indication of a phasic excitatory synaptic input underlying its burst.

We also found a strong short-latency IPSP from 501 to elevator motoneurons (Fig. 14, *A* and *E*). Finally, there is some indirect evidence (not shown) that 504 might also be inhibited by 501. Common IPSPs at the appropriate phase can often be seen in the traces of simultaneous recordings of 504 and other interneurons (e.g., 301) that are known to receive direct inhibition from 501.

DISCUSSION

Graded interactions in the flight system

The delayed excitatory connection between 301 and 501 plays a prominent part in the circuit presented below, and it is therefore important to determine as far as possible the mechanism underlying it. The connection exhibits two significant properties which theories of the mechanism should accomodate. First, latency is about twice as long as that seen for short-latency PSPs. Second, the PSP is reversed by the passage of hyperpolarizing current into the neuropile segment of the postsynaptic neuron and is thus caused by a decreased ionic conductance across the postsynaptic membrane. Two pathways could account for this: a direct decreased-conductance synapse or a disynaptic disinhibitory pathway with tonic and graded release of transmitter at least at the second synapse. We consider that the evidence to date is best accounted for by the second of these for the following reasons. *1*) The time courses of the decreased-conductance synapses which have been described are exceptionally long (latency about 1 s, duration greater than 30 s) (10, 13, 14, 62, 63), whereas the increased time course of the connection described here (latency 6 ms, duration 30 ms) was only about twice that of short-latency connections. Moreover, the role of the described decreased-conductance EPSPs is considered to be modulatory, increasing the effect of other inputs (4, 11, 52), rather than directly excitatory such as in the present case. Although it is impossible to determine the number of synapses which may be involved given only the value of the latency, it is possible that the longer latency of the connection described here could be produced by a disynaptic connection. The increase in latency relative to monosynaptic connections may be introduced by another synaptic delay and passive conduction time between the input and output sites on the intervening interneuron.

2) The delayed excitation was reversed at the same membrane potential as spontaneous IPSPs seen in 501, indicating that probably the same ionic conductances were involved in driving both potential changes. Also the rough value of the reversal potential is similar to that of a γ-aminobutyric acid (GABA)-mediated inhibitory response in the cockroach central nervous system (44). Injection of chloride ions into 501 reduced the amplitude of the delayed excitatory potential suggesting that these ions are involved in mediating the potential. Since GABA-mediated changes in chloride-ion conductances probably underlie much central inhibitory transmission in insects (12, 17, 27, 38, 44), it seems likely that inhibitory processes help to produce the delayed excitatory potentials. In contrast all described decreased conductance EPSPs are dependent on acetycholine- (4) or serotonin- (18, 51, 58) mediated changes in potassium ion conductances (10, 26, 63).

3) Approximately 1.5×10^{-4} M picrotoxin simultaneously blocked both IPSPs and the delayed excitatory potential following each spike of 301. Picrotoxin has been shown to block GABA, acetylcholine, and glutamate inhibitory connections produced by increases in conductance to both chloride and potassium ions (30, 42, 72). Only rarely has it

been proposed as a blocker of excitatory potentials (3, 23, 31, 32). In addition, picrotoxin at 10^{-4} M has no effect on a GABA-mediated depolarizing potential caused by a decreased conductance to potassium ions recorded in R15 in *Aplysia* (73).

4) Another effect of the picrotoxin treatment was to depolarize the membrane potential of 501 in a manner consistent with the removal of a tonic inhibition (a necessary condition for the disinhibitory pathway). Freeman (15) described a general excitatory effect of picrotoxin at concentrations greater than 5×10^{-5} M on crustacean axonal membrane. This effect was due to a block of potassium conductances, including the delayed rectifying current, with the result that spike widths were significantly increased with a concurrent depolarization of the membrane potential (15). Such an effect appears unlikely here because the depolarizing action was not seen on all neurons and because the spike width in 501 was unaffected by picrotoxin treatment.

5) Interneuron 301 is known to make short-latency inhibitory connections with other interneurons, and we have identified a disinhibitory pathway from 301 to 501 via 511. Unfortunately we have not yet demonstrated that there is tonic inhibition from 511 to 501 or that this connection operates in a graded (nonspike) fashion with changes in the membrane potential of 511. Given the size of the delayed excitatory potential (sometimes as great as 8–9 mV compared with 3–5 mV for conventional direct connections) it is conceivable that several intervening interneurons may be involved in transmission and inversion of the signal from 301. The delayed excitatory potential could be produced by disinhibition via only spike-mediated release of transmitter if the intervening neurons normally fired tonically at relatively high frequencies, and if their firing frequencies were smoothly modulated by synaptic input, i.e., if transmitter release at their endings appeared to be continuously graded. Such a possibility cannot be ruled out here, but is considered unlikely given the lack of precedent for such interactions compared with the mass of evidence for nonspiking release of transmitter in arthropods (9, 22, 36, 70).

Whereas none of the data are conclusive, we feel that they provide a convincing argument for treating the delayed excitatory connection as a disinhibitory pathway with graded transmitter release at the second synapse. There is now considerable anatomic evidence that there are numerous output synapses on the dendrites of flight motoneurons (59) and an intersegmental interneuron (60) in the locust. Also, graded transmission between neurons is well established in arthropods, and in many cases this acts at dendritic sites (9, 22, 36, 70). We propose that the disinhibitory pathway in this system is best modeled as acting at dendritic sites (Fig. 15). There are two other possibilities, neither of which can be definitely excluded with the present data. First, 301 may act through the axon terminals of interneurons which tonically inhibit 501. However, major fluctuations in axonal membrane potentials are very uncommon in this system, and we have never seen direct connections from 301 (or any other flight interneuron) to axons. Second, rather than acting through intersegmental interneurons, 301 could act through local interneurons in which the distinction

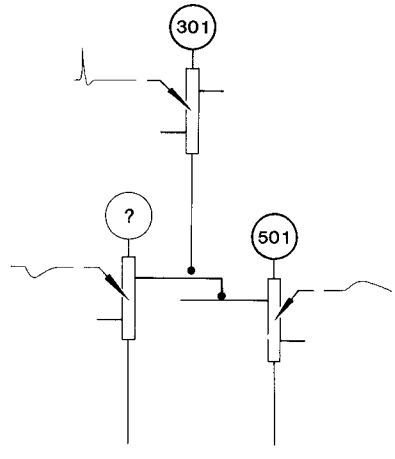

FIG. 15. Delayed excitatory connection from 301 to 501 modeled as disinhibition with graded and tonic release of transmitter via a neuron or neurons unknown (*?*). Diagrammatic *traces* represent proposed sequence of activity that would be recorded: a spike in 301, followed by a monosynaptic IPSP in *?*, followed by a delayed excitatory potential in 501. Note that disinhibition is proposed as occurring at a dendritic site (*long rectangles* represent the neuropile segments of each neuron with the lateral branches as dendritic branches and the *descending line* as axon). Elements of second synapse (*?* to 501) are shown contiguous to indicate necessity for tonic release of transmitter at this synapse. Further details in text.

between dendrite and axon is hazardous to make. This is a real possibility for this connection, considering the large number of local interneurons with graded interactions already discovered in locust thoracic ganglia (8, 53, 71). One reason we have modeled the disinhibition through the dendrites of an interneuron with a distinct axon in Fig. 15 is that the model is partly based on the identified disinhibitory pathway (301-511-501), and 511 is an intersegmental interneuron. Another is that, although we have identified some local interneurons in the flight system (unpublished observations), these are relatively few, and none have the characteristics suited for mediating the inhibition.

The delayed excitatory connection from 301 to 501 is not an isolated phenomenon. Interneuron 301 has similar connections with 503, 520, and some depressor motoneurons as well as with 501. Moreover the same type of delayed excitation can be seen from 401 (another resetting interneuron with direct inhibitory connections) to 202, 301, 701, and some depressor motoneurons (data not shown). Such connections therefore seem to be widespread in the flight system. This is taken as indicating that graded interactions have a significant part to play in setting up the membrane-potential oscillations seen in interneurons and motoneurons. Clearly the extent to which graded interactions are involved needs to be determined by a more thorough investigation. However, it is probable that they operate only within individual ganglia, the distance between the prothoracic and mesothoracic and between the mesothoracic and metathoracic ganglia precluding nonspike transmission of membrane potential changes between them. Thus, pattern generation in the flight system seems to rely on both spike and nonspike transmission. The former may be involved in structuring individual bursts, whereas the latter may help to set up and maintain the membrane-potential oscillations in neurons during flight sequences.

Can interneurons be separated into distinct functional categories?

The proposal that there may be a separation of function among interneurons (to facilitate sensory-induced changes in the motor pattern without affecting the wingbeat frequency) was based on negative evidence, and we suggested that this conclusion may need to be modified as more data on flight interneurons accumulated (47). There is now no doubt that interneurons originally described as premotor can reset the flight rhythm (e.g., 504, Fig. 10*C*) and that interneurons originally described as being involved in pattern generation can make strong direct connections with flight motoneurons (e.g., 501, Fig. 14*E*). Furthermore, other interneurons described as premotor have access to the rhythm generator via direct connections with pattern-generator interneurons [e.g., short-latency EPSPs can be recorded in 501 following spikes in 201(T2), Fig. 5*B*] and thus, theoretically, might be able to affect the rhythm. This data shows that no clear separation exists between premotor and pattern-generator interneurons.

Another way interneurons may be segregated is into two groups concerned either with setting up the basic pattern or with modulating this under the influence of the periphery. Note that we are dealing here with only ~20% of the interneurons we have identified as phasically active with the rhythm, and most of these were selected for study because they produce clean high-frequency bursts in deafferented preparations. Intuitively it might be expected that any premotor interneuron concerned with transmitting sensory information would produce a disrupted and weak burst in situations where the sensory input is unnatural and severely curtailed. We now know that deafferentation produces several important changes in the recorded flight motor pattern (24). Therefore, in addition to the bias introduced by selecting only those interneurons with seductive bursts, it seems that many controlling influences on the motoneurons are absent in the deafferented preparation. Moreover, Reichert and Rowell (45) have identified phasically active thoracic ocellar interneurons that project to flight motoneurons and form a different population of interneurons from those we have described. To date, these are not known to affect the timing of the rhythm (Reichert, personal communication), but obviously we must be wary of forming conclusions based on negative evidence. No definite conclusions can yet be drawn about a possible functional hierarchy of interneurons in the flight system.

However, it is conceivable that the interneurons which we have described are primarily responsible for patterning the basic activity in flight motoneurons (i.e., concerned with timing and structuring the rhythm and driving motoneurons). A different population may exist to modify motoneuronal activity under the influence of sensory feedback.

Neural circuits in the flight system

Figure 16 illustrates some of the neuronal connections that we have found in the flight system to date. This circuitry is only a small part of the total that is formed by flight interneurons and should not be regarded as the basis for rhythm generation in the system. However, in spite of our limited knowledge of the neuronal circuitry, the connections shown in Fig. 16 are consistent with two basic features of the deafferented flight rhythm. The fixed delay and the functional unity of the elevator-depressor sequence could be because 504 directly and concurrently drives elevator motoneurons and 301. Interneuron 301 then causes a delayed excitation of depressor motoneurons via intervening interneurons 503 and 201 (Fig. 13) and a delayed inhibition of elevator activity via 501. The second feature is the observation that depressor motoneuron bursts have a relatively constant duration (24). A property of circuits incorporating delayed excitation and feedback inhibition similar to the 301-501 circuit is that burst durations tend to be relatively independent of cycle period (20). This property is conferred to motoneurons because 301 indirectly drives depressor motoneurons and removes an inhibition to them from 511, thus creating a time window within which depressor motoneurons can fire. Concurrently, elevator motoneurons are silenced for the same duration by inhibition from 501.

The circuitry shown in Fig. 16 also suggests a scheme whereby rhythmic activity could be initiated by wind on the head, which is known to be capable of initiating and maintaining flight (64). The activation of 206 by wind (Fig. 14*B*) would excite 504, which in turn would start bursts in elevator motoneurons and 301. Interneuron 301 then directly inhibits 511 thus releasing depressor motoneurons from inhibition. At the same time, 301 indirectly excites depressor motoneurons

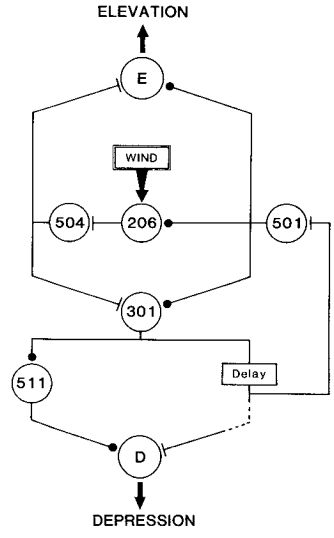

FIG. 16. Summary circuit diagram that can account for several features of flight motor rhythm recorded from deafferented preparations. Full explanation in text.

after a delay that is introduced via a delayed excitatory connection (illustrated in Fig. 16 as a delay box in an excitatory pathway) and intervening premotor interneurons (dashed line in Fig. 16). Via a similar (delayed excitatory) connection, which is probably mediated by a disinhibitory pathway, 301 also excites 501. Activity in 501 feeds back to inhibit elevator motoneurons, 206 and 301, directly. Inhibition of elevator motoneurons terminates the burst in these neurons. Inhibition of 206 removes a general source of activation of the system, and inhibition of 301 turns off the source of activation for depressor motoneurons and for 501 itself. As 501 is no longer driven, it ceases to fire, and its general inhibition of the system is removed, thus enabling another cycle to be produced.

From our current knowledge of the circuitry, 301 can be considered as an interneuron responsible for switching from elevator to depressor phase. It is active at the interface between elevator activity (e.g., 511 in Fig. 7*B*) and depressor activity (e.g., 501 in Fig. 4*A*), and the connections of 301 are what help ensure the appropriate phase relationships of 511 and 501 (Fig. 7*C*) and the transition from elevator to depressor phase. More significantly the 301-501 circuit seems an important functional element in the flight

system and appears at least partially responsible for generating depressor bursts and inhibiting elevators. This is consistent with the known asymmetrical nature of the system such that depressors are more liable to burst than elevators (57).

Comparison with other systems

With the current information on the locust flight system it is possible to make some limited comparisons with other described rhythm-generating systems. In general, there are several similarities. First, graded interactions seem important in this as in many other systems (22, 37, 40, 54, 71). The functional advantages of such interactions are considered to be to enable a finer control to be exerted between pre- and postsynaptic elements, even at subthreshold membrane potentials, and to allow neurons to have more than one functional role by virtue of the limited distance over which membrane potential changes can be conducted electrotonically (22, 39). The graded interactions in the locust flight system are particularly important in permitting inhibitory interneurons to have powerful excitatory effects. Second, it is becoming clear that rhythm-generating systems tend not to rely on a single mechanism to ensure rhythmicity. This is exemplified by the lobster stomatogastric system in which many parallel processes interact, and no single interneuron or mechanism can be said to have prime responsibility for generating the rhythm (33, 34). In the locust flight system the 301-501 circuit probably has oscillatory capabilities but is certainly not the only part of the system with this capability. Third, as in other systems (28) there is a preponderance of inhibitory connections. In fact the 301-501 circuit, if redrawn to reflect the probability that the delayed excitatory connection is mediated via a disinhibitory pathway, would resemble a cyclical inhibitory network (16). Fourth, the 301-501 connection is represented functionally in Fig. 16, and in this way the circuit resembles more the delayed excitation and reciprocal inhibition at the heart of the *Tritonia* escape swimming circuit (20). In this case, however, the delayed excitation is mediated by different processes such as multicomponent synapses dependent on only impulse-evoked transmission (19) and the operation of the potassium A current (21). It is important that graded interactions have not been reported in the *Tritonia* system, otherwise the multiple PSPs following a single spike could reflect circuitry involving graded transmitter release (as for the 301-501 connection described here) rather than multiple effects at a single synapse. So far there is no evidence for multicomponent synapses in the locust flight system.

The essence of the circuit we have described is delayed excitation through a graded disinhibitory pathway with feedback inhibition. We are not yet in a position to make definite statements concerning how the rhythm is generated in the locust flight system. However, no novel processes seem to be involved. The flight system is thus unique only in the way in which the components are assembled and in how they operate together.

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