Delayed Excitatory Connections in the Flight System of the Locust

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SUMMARY AND CONCLUSIONS

1. Synaptic interactions between identified neurons in the flight system of the locust were investigated by the use of standard intracellular recording and staining techniques. The intent was to determine the distribution and functional significance of delayed excitatory connections, which have been previously described.

2. For one inhibitory connection it was demonstrated that subthreshold depolarization of the presynaptic neuron was sufficient to cause release of transmitter at the synapse. This established the existence of graded interactions between spiking flight neurons.

3. Three inhibitory interneurons were found to cause delayed excitatory responses in several other neurons. Often these were coupled with direct inhibitory connections between the same pre- and postsynaptic neurons, resulting in an inhibitory/excitatory (I/E) postsynaptic potential (PSP). The two phases of this PSP were variable.

4. Delayed excitatory connections appeared powerful while the flight system was inactive. However, these connections were disabled during flight rhythms at the phase when the presynaptic neuron was depolarized and firing action potentials. This was likely due to the nature of the disynaptic disinhibitory interaction being via (an) intervening neuron(s) with oscillating membrane potentials and thresholds for release of transmitter.

5. Thus connections demonstrated when flight rhythms were not expressed changed their character during flight rhythms. The delayed excitatory connections in this system probably reflect complex circuits of inhibition mediated by graded interactions and have little functional significance as phenomena in their own right.

INTRODUCTION

Precise movements are dependent on precisely timed motor patterns, and the neural mechanisms underlying the timing of motor patterns are of considerable interest. Such mechanisms are important not only for coordinating individual movements but also in the generation of rhythmic neuronal activity. In several instances it has been demonstrated that proprioceptive input is important in timing the different phases of a sequence of movements (Pearson and Duysens 1976; Pearson and Ramirez 1990). In addition, the properties of the central neuronal circuits generating motor patterns confer an ability to introduce precise time delays between excitation at one phase and excitation at the following phase. The introduction of a delay between a neuronal action and antagonism of this action is recognized as being a necessary feature of rhythmically active systems (Friesen and Block 1984; Getting 1988). Numerous neurobiological mechanisms could be used to implement the requirement for delays. In the escape swimming system of Tritonia, both multicomponent synapses and activation of a transient potassium current are involved in generating the appropriate delays (Getting 1981, 1983). Other pattern generators use different combinations of cellular, synaptic, and network properties; in the locust flight system, conduction path lengths (Robertson and Pearson 1985b) and a delayed excitatory connection (Robertson and Pearson 1985a) have been implicated in timing the phases of the deafferented flight motor pattern. This paper is concerned with the distribution and functional significance of the delayed excitatory connection in the locust flight system.

Wingbeating during flight in the locust is a behavior that has received much attention in the past. The system controlling the wingbeat has served as a model system for several different general properties of motor control systems. Notably, research with the locust flight system has been influential in the continuing debate over the relative importance of central versus sensory mechanisms of motor pattern generation (Pearson 1985, 1987; Stevenson and Kutsch 1987). Notwithstanding the controversy, there is no doubt that central mechanisms generate the basic pattern of rhythmic alternating bursts of activity. Although the characteristics of the system make it particularly suitable for manipulation of the afferent input, and knowledge of the role of different phasically active sense organs is continually accumulating (Pearson et al. 1983, 1989; Pearson and Wolf 1988; Wolf and Pearson 1988), much less is known of how the central circuitry contributes to rhythm generation and coordination of the pattern (Robertson 1990; Robertson and Pearson 1985a; Robertson and Reye 1988). The current model of burst generation in the system (Robertson and Pearson 1985a) is only preliminary and cannot explicitly account for the observation that hemisection of the mesothoracic ganglion has little obvious effect on flight motor output (Ronacher et al. 1988; Wolf et al. 1988). More complete information on the central circuitry is clearly needed.

One of the main features of the model circuit is a delayed excitatory connection between two identified interneurons. This interaction has more than twice the latency of connections that are considered monosynaptic. The depolarization is driven by a decreased conductance across the postsynaptic membrane and it is eradicated by the application of picrotoxin. These and other observations led to the hypothesis that the delayed excitatory connection is mediated by disynaptic disinhibition with tonic release of transmitter, at least at the second synapse (Robertson and Pearson 1985a). A likely implication of tonic release is that it would not be mediated by spikes. Indirect evidence for non–spike-mediated release of transmitter from spiking flight interneurons has been reported (Robertson and Reye 1988); but there is so far no direct evidence. A similar disynaptic disinhibitory connection, which is mediated by a nonspiking neuron, has since been described in the proprioceptive con-
TABLE 1. Synaptic interactions of interneurons having delayed excitatory connections with other neurons

<table>
<thead>
<tr>
<th>Interneuron</th>
<th>Phase</th>
<th>Direct Connection</th>
<th>Direct Connection To</th>
<th>Delayed Excitation To</th>
</tr>
</thead>
<tbody>
<tr>
<td>301</td>
<td>Elevat</td>
<td>Inhibitory</td>
<td>DMn, 114, 308*, 501, 511*</td>
<td>DMn, 201, 202, 301*, 318, 501*, 503*, 507, 511, 520*</td>
</tr>
<tr>
<td>302</td>
<td>Depres</td>
<td>Inhibitory</td>
<td>EMn*</td>
<td>701</td>
</tr>
<tr>
<td>401</td>
<td>Elevat</td>
<td>Inhibitory</td>
<td>DMn*, 109, 201, 202*</td>
<td>DMn*, 201, 202*, 301*, 401, 701*</td>
</tr>
</tbody>
</table>

DMn, depressor motoneuron; EMn, elevator motoneuron. *Indicates synaptic interactions previously described in Robertson and Pearson (1985a,b); and Robertson and Reye (1988).

trol of leg movements in the locust (Burrows and Pflüger 1988). The connection in the flight system has an important role in the model of burst generation and of switching from elevator to depressor phase in the deafferented flight rhythm. The results presented here show that delayed excitatory connections are common but that they have limited functional significance in their own right during expression of the flight rhythm.

METHODS

Male and female Locusta migratoria were obtained from a crowded colony maintained at the Department of Biology at Queen’s University. Most of the experiments were performed using males; nevertheless, when females were used, no difference in the results obtained was observed. Experiments were performed at room temperature, which ranged between 22 and 26°C according to the season.

A deafferented preparation capable of expressing flight motor patterns was used (Robertson and Pearson 1982). The wings and legs of a locust were removed at their bases. The thoracic nervous system was exposed via a dorsal dissection, and the meso- and metathoracic ganglia were raised and stabilized on a rigid stainless steel plate. Nerves 3 and 4 of both ganglia were cut centrally to denervate the dorsoventral flight muscles. This functionally deafferented the preparation by preventing movements of the wing stumps. The thoracic cavity was bathed in saline containing (in mM) 147 NaCl, 10 KCl, 4 CaCl₂, 3 NaOH, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.2.

**FIG. 1.** Examples of delayed excitatory connections from 301 to several other flight interneurons. In this and subsequent figures, connections are displayed with multiple oscilloscope sweeps triggered off the rising phase of presynaptic spikes. **A:** spikes in 301 were followed by depolarizing PSPs in 201. **B:** dorsal longitudinal motoneurons (DL). **C:** 202. **D:** 507. Passage of hyperpolarizing current (-3 nA) into the postsynaptic neuron reversed the sign of the PSP (A). **E** and **F:** stimulation of the presynaptic neuron sufficient to cause it to fire a burst of spikes resulted in stronger depolarization and spiking in the postsynaptic neuron (E and F are taken from the same experiments as B and D, respectively).
Flight rhythms were initiated by blowing air on the head of the animal. The time of depressor activity in the rhythm was monitored with an electromyographic electrode inserted into one of the dorsal longitudinal muscles.

Intracellular recordings were made from selected flight neurons by impaling their neuropile segments with glass microelectrodes pulled such that their resistances when filled with 1 M potassium acetate were 20-50 MΩ. For most experiments, these electrodes were filled at the tip with 4% Lucifer yellow (Aldrich, Milwaukee, WI) in 0.5 M lithium chloride and their shafts were back-filled with 0.5 M lithium chloride. These electrodes had resistances of >100 MΩ. Electrical signals were amplified conventionally and stored on FM tape for subsequent analysis. After recording, Lucifer yellow was iontophoresed into the neuron using 2–10 nA of hyperpolarizing current for ~5 min. The ganglia were excised, fixed for 1 h in 4% paraformaldehyde, dehydrated in an ethanol series, and cleared in methyl salicylate. Neuronal structures were observed in whole mount under a Leitz Diaplan epifluorescence microscope. Neurons were identified by their physiology and structure and numbered according to Robertson and Pearson (1983). Occasionally neurons were identified during the experiment, after filling but before fixation, by the use of a Leitz epifluorescence attachment on the binocular dissection microscope. Some flight interneurons could be reliably identified on the basis of their physiology alone, and experiments could be performed using electrodes filled with 1 M potassium acetate. This had the advantage of avoiding the high resistances of dye-filled electrodes and allowing easier passage of current into the impaled neuron to manipulate its membrane potential.

Connections between flight neurons were judged to be monosynaptic if the branches of the two neurons overlapped in the neuropile, if the synaptic delay between the presynaptic spike and the postsynaptic potential (PSP) was short (~0.8 ms) and constant, and if PSPs followed the spikes in a 1:1 manner. Synaptic delay was estimated by measuring the latency from spike to PSP in paired recordings and subtracting a conduction time of 1 ms if the recording sites were in different ganglia (meso- or metathoracic) or zero conduction time if the recording sites were in the same ganglion. These approximate conduction times were derived from numerous paired recordings of activity of a single neuron impaled either twice in the same ganglion or once in each of the different ganglia specified above. Immunocytochemical studies have supported the use of these criteria to establish the monosynaptic nature of connections (Robertson and Wisniowski 1988).

Paired microelectrode recording from two neuropile sites in the thoracic ganglia was difficult enough to be less than routine. Thus it was not possible to record from all possible pairs of identified flight neurons, although this would have been highly desirable. Additionally, the ability to demonstrate disynaptic interactions was more subject to individual variation in the general state of the preparation than it was for direct connections. Finally, there was an obvious sampling bias such that interneurons that were considered to be the most interesting were preferentially selected for study and others were neglected. The data presented here were collected over the past several years from numerous penetrations of the different interneurons. In a general sense the phenomena have been confirmed many times. Specific connections have been demonstrated at least twice. Variability in the occurrence of particular phenomena is commented on in RESULTS.
RESULTS

Delayed excitatory connections were recorded after spikes in three different interneurons (301, 302, and 401; see Table 1). Each of these has been described as an interneuron with direct inhibitory connections to other neurons (Robertson and Pearson 1983, 1985a,b; Pearson and Robertson 1987). There was no consistent feature indicating that connections of this sort had a unique role in the operation of the circuit. Thus the phase of activity of the presynaptic neuron could be either elevator or depressor and, whereas direct interactions tended to be to neurons that were active in the opposite phase, the delayed excitatory interactions could be with neurons in either the same or the opposite phase. Nevertheless, the most effective connections were found after spikes in 301 (Fig. 1), and they appeared to promote the switch from elevator to depressor phase by directly inhibiting neurons active in the elevator phase and indirectly exciting neurons active in the depressor phase. The synaptic delays and time courses of the delayed excitatory connections were similar in each case (synaptic delay ~5 ms; duration 25–35 ms; Fig. 1). In most cases it was possible to test the nature of the conductance change during the PSP by passing current across the postsynaptic membrane. The delayed excitatory PSPs were reversed by hyperpolarizing the postsynaptic membrane (Fig. 1A), indicating that they were caused by a decreased conductance mechanism. In spite of this mechanism, which would limit the amplitude of the postsynaptic effect, the delayed PSPs were found to be effective in exciting the postsynaptic neuron. Unitary PSPs often generated spikes in the follower neuron (Fig. 1, A and D), and stimulation to cause a burst of spikes in the presynaptic neuron could cause a strong depolarization and spiking in the follower neuron after a pronounced delay (Fig. 1, E and F).

The decreased conductance underlying the delayed excitatory PSP is thought to be due to an interruption of tonic inhibition from an intervening neuron, and this implies graded release at the second synapse. The search for direct...
Evidence of non–spike-mediated release of transmitter at a synapse between flight interneurons was not fruitful. At only one of the direct connections is there evidence for such interaction (Fig. 2). Short pulses of current into 302 cause depolarization, which can result in a hyperpolarization of a known direct follower (mesothoracic tergo-sternal motoneuron) before the generation of a spike in 302 (Fig. 2C). It should be noted that it was not possible to demonstrate the non–spike interaction for all paired recordings of 302 and mesothoracic elevator motoneurons.

Several neurons received both a direct inhibitory connection from either 301 or 401 as well as the delayed excitatory connection (Table 1). Usually one of the interactions could be detected every time one of these neurons and a postsynaptic neuron were recorded together, whereas the other interaction occurred with more variability. In the case of the interactions between 301 and 511 (Fig. 3), it was the direct inhibitory connection that was always seen, and the occurrence of the indirect excitatory interaction depended on the preparation. For the interactions between 301 and 501 (Fig. 4), the delayed excitatory connection could always be detected, whereas the ability to detect the direct connection depended on the peculiarities of the preparation. This correlated with the phase of activity of the follower neuron and is consistent with the idea that activity in 301 tends to inhibit neurons active in elevator phase (e.g., 511) and excite neurons active in depressor phase (e.g., 501). As well as
being variable in occurrence in different preparations, the relative amplitudes of the two PSPs could change considerably within a very short time (Fig. 5). Successive spikes in the presynaptic neuron could be followed by predominantly excitatory potentials, joint inhibitory/excitatory (I/E) potentials, or predominantly inhibitory potentials. Some of this variation could be correlated with changes in the membrane potential of the postsynaptic neuron (Fig. 6). Slight spontaneous depolarization favored the detection of direct IPSPs, whereas spontaneous hyperpolarization favored the detection of the delayed EPSPs (Fig. 6B).

All of the above connections were demonstrated while flight rhythms were not being expressed by the preparation. In a disynaptic disinhibitory pathway it is probable that the intervening neuron would exhibit membrane potential oscillations in antiphase with the first neuron in the pathway. It is further to be expected that the intervening neuron would exhibit a membrane potential threshold for release of transmitter, even though there was tonic release at baseline, nonrhythmic, membrane potentials. A possible consequence of this would be that delayed excitatory connections of the sort described here could be disabled when the circuit was rhythmically active, because whenever the first neuron was generating a burst of spikes the intervening neuron would be driven beneath threshold for release. Some evidence that this occurs was found by stimulating a 301 with pulses of depolarizing current during expression of the flight rhythm. Such a pulse reset the flight rhythm and caused extra depressor motoneuron activity (Fig. 7). The described phase response curve for pulsed excitation of 301 (Robertson and Pearson 1985a) shows that pulses delivered to 301 cannot reset the rhythm if they occurred around the phase when 301 would normally be active, even though its firing rate and spikes per burst were increased by the stimulus. This is shown in Fig. 8, which also demonstrates that the delayed excitatory connection from one 301 to its contralateral partner could be detected only outside the time of its normal activity in the rhythm. Pulses of current that almost double the number of spikes per burst (from 6 to 11 spikes) and increase the intraburst spike frequency and duration of a burst of a 301 during the time when it would normally be active had no detectable effect on the activity of its contralateral partner (Fig. 8B).

**DISCUSSION**

The results presented in this paper demonstrate that delayed excitatory connections similar to the one that has already been described (Robertson and Pearson 1985a; Robertson and Reye 1988) are relatively common in the flight system. These connections were characterized as slowly developing depolarizing potentials that follow presynaptic spikes after a long latency and that have a comparatively long duration. They were reversed by passing hyperpolarizing current across the postsynaptic membrane and thus were caused by a decreased conductance across this membrane. Three identified interneurons have been directly shown to cause delayed excitatory connections, and it is likely that other inhibitory interneurons can also mediate indirect excitation.

The mechanism underlying these delayed depolarizations is thought to involve a cessation of tonic inhibition of the postsynaptic neuron via an interneuron or interneurons as yet unidentified. Yet it has proven difficult to demonstrate non-spike-mediated release of transmitter from flight interneurons. It is possible to account for this shortcoming by considering the limitations of stimulating and recording through the same glass microelectrode and the location of the site of impalement. It is notoriously difficult to stimulate neurons with high resistance electrodes, especially if they are filled with dye. Given the difficulty of balancing out the effect on the recorded potential of passing current across the electrode resistance, it is also difficult to be sure that the stimulus is indeed changing the membrane potential even at the recording site. To compound this, in most cases neurons were impaled in the larger diameter neuropile segment, which is closer to the spike initiation zone than to the sites of output synapses on the terminal branches (Watson and Burrows 1983). It is therefore possible that the small subthreshold stimuli were often inadequate to spread to the target synapses. Larger stimuli would have preferentially activated spikes that could easily mask any non-spike-mediated effects. Nevertheless there is now clear evidence for non-spike-mediated release of transmitter from 302 (Fig. 2) to add to the indirect evidence for the same from 301 (Robertson and Reye 1988).

Another problem for substantiating the proposed mechanism underlying the delayed excitatory connections is that the identities of the proposed intervening neuron(s) remain elusive. One possibility is that these are small local interneurons that so far have not been successfully penetrated.
An alternative possibility is that the neurons and connections have already been identified but that the contribution of each individual is too small for that neuron to have been considered as a serious candidate. There is a high degree of connectivity in the flight system, and each depolarization due to the delayed excitatory connections could be the result of summing the small effects of several intervening neurons. This would also account for some of the variability seen in the parameters of the PSP. It would be difficult to determine whether such a mechanism exists with two microelectrodes, but the general plausibility of the hypothesis could be tested by the use of computer simulations of cable models (e.g., Koch et al. 1990).

A feature of the connections described here was their variability both between preparations and during the course of a single experiment. This was evident for the direct as well as for the indirect connections. Flight interneurons are subject to a continuous barrage of inhibitory and excitatory input that would alter the conductance of their membranes. If the membrane is near reversal potential for the input, this could occur without causing alterations of their membrane potentials. The variability that was seen is likely due to a shifting balance in the magnitude of tonic driving forces and silent inhibitory conductances. The situation is further complicated by the disynaptic nature of the delayed excitation and the fact that the nature of the combined input to different flight neurons is different. For example, some interneurons were near reversal for inhibitory input at “resting” membrane potential levels, whereas others were not. This could be due to tonic inhibition, and, in such a situation, other direct IPSPs would be much reduced, although the delayed excitatory connection, being a decreased inhibitory conductance, would be prominent. On the other hand, at times when the tonic inhibition has been spontaneously reduced, the delayed excitation would be reduced, whereas direct IPSPs would become more prominent because of increased resistance of the membrane and because of depolarization increasing the driving force acting on the ions carrying the inhibitory current.

Whatever the basis for delayed excitatory connections, there is no doubt that they were common in recordings from flight neurons and effective in promoting spiking. Also, the demonstrated existence of I/E connections makes it attractive to implement them in models of burst generation and rhythmicity. However, there is reason for suggesting that delayed excitatory connections, as entities observed when flight rhythms were not being generated, have little functional significance in their own right during the generation of flight motor patterns and that the temptation to implement them in models should be resisted. First, they seem to have no unique role in the system. Neurons active at either the elevator or the depressor phase of the rhythm were observed to cause and to receive delayed excitatory connections. Although each example may have been similar in its characteristics and underlying mechanism, there was no clear unifying functional characteristic. Second, in spite of the fact that the temporal characteristics of all the delayed excitatory connections were similar, there was not a consistent relationship between the timing of the bursts of flight activity of the pre- and postsynaptic neurons. For example, 301 had I/E connections with both 511, active in the elevator phase, and with 501 and depressor motoneurons, which were active at different times in the depressor phase. This indicates that the timing of rhythm was not a direct consequence of the properties of these connections. Third, they could not be detected during the normal operation of the flight circuitry. It is difficult to discern unitary PSPs on the oscillating membrane potential of an active flight neuron. Another way to demonstrate connections is to stimulate the presynaptic neuron to generate a burst of spikes and to observe the result on the postsynaptic neuron. When this was done during the expression of the flight rhythm, the delayed excitatory connection had a phase-dependent occurrence. It was not detectable at times when the presynaptic neuron was normally active in the flight rhythm. For reasons presented in Results, this is not surprising, accepting that the intervening neuron has an oscillatory membrane potential and a threshold for transmitter release. Rather than considering delayed excitatory connections as phenomena important in their own right, it seems more profitable to interpret their existence as a reflection of the underlying circuitry in the system and as a consequence of nonspiking and subthreshold interactions between flight neurons. Thus what was observed as pronounced excitation when flight was not expressed is best considered as sequential inhibitions during generation of rhythms. The delayed excitatory connections retain importance for what they indicate about the operation of the system. Interactions between flight neurons, even when flight rhythms are not being generated, are complex and numerous. It remains to be determined precisely how activation of the system affects the described interactions and what particular benefits are conferred by the existence of subthreshold interactions in this system.

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REFERENCES


