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Roger Lyons Reep

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NEURONAL PATHWAYS INVOLVED IN TRANSFER OF INFORMATION
RELATED TO LEG POSITION LEARNING IN THE COCKROACH,
PERIPLANETA AMERICANA

By

Roger Lyons Reep

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ABSTRACT

NEURONAL PATHWAYS INVOLVED IN TRANSFER OF INFORMATION RELATED TO LEG POSITION LEARNING IN THE COCKROACH, PERIPLANETA AMERICANA

By

Roger Lyons Reep

During shock avoidance leg lift training using headless cockroaches (Periplaneta americana), "Positional" (P) animals receive shocks upon prothoracic leg extension and "Random" (R) animals receive shocks regardless of leg position. In a later testing period both animals receive shocks independently upon mesothoracic leg extension.

Information related to leg lift learning transfers from the prothoracic to the mesothoracic ganglion if one or both prothoracic-mesothoracic connectives are intact during training, but no transfer occurs if both connectives have been cut prior to training.

The present behavioral studies were done in order to verify that transfer is a real phenomenon and to characterize what kind of learning occurs, what kind of information transfers, and the time courses of these events. It was found that escape and avoidance learning occur but only the latter is important in transfer, and that transfer happens rapidly, such that a behavior change is seen in a P mesothoracic leg after ten minutes of prothoracic leg training. Facilitation of P behavior (i.e., the transfer of information about P learning) generates the P-R testing difference.

Anatomical experiments were undertaken to identify the neuronal pathways by which information related to learning might transfer from the prothoracic to the mesothoracic ganglion. Phase and electron microscopy were used to map the degeneration products, and therefore trace the course of, sensory fibers. Cobalt staining was used to map the projections and branching patterns of motoneurons and interneurons. All sensory and motor neurons had processes confined to the ipsilateral portion of the ganglion from which their axon exited peripherally. Interneurons with axons in the prothoracic-mesothoracic connectives had branches in the ipsilateral and contralateral portions of the prothoracic and mesothoracic ganglia. Giant fibers were found to ascend at least as far as the prothoracic ganglion and to have short branches in the prothoracic and mesothoracic ganglia.

Electrophysiological experiments were performed to determine what kinds of information travel in ascending and descending prothoracic-mesothoracic connective fibers. Suction electrodes were used to record action potentials from motoneurons and connective fibers. It was found that connective fibers could be classified as small, medium or large on the basis of spike amplitude. Some large fibers carry corollary discharges of motoneuron activity; a one-to-one copy of spikes in the case of corollary units of flexor motoneurons in nerve 6Br4 and an approximate copy of spikes in the case of corollary units of extensor motoneurons in nerve 5r1. Corollary units were found in ipsilateral and contralateral descending connective fibers for prothoracic flexor and extensor motoneurons, and in ipsilateral and contralateral ascending connective fibers for mesothoracic flexor and extensor motoneurons.

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A model was constructed based on the present findings. It hypothesizes that sensory information related to prothoracic leg shock and motor information related to prothoracic leg position are independently transferred down each prothoracic-mesothoracic connective to the mesothoracic ganglion, where they are associated and lead to a behavior change in P but not R mesothoracic legs.

This thesis is dedicated to my companion, Maxwell, without whose steadfast friendship and loyalty I could never have come this far.

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I deeply thank my parents for providing me with the opportunities that have culminated in the production of this thesis. Perhaps more importantly, I thank them for encouraging me to attain independence of spirit and freedom of thought.

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INTRODUCTION

Our understanding of the neuronal functions which mediate behavior and behavior modification (plasticity) has been aided greatly in recent years through the use of invertebrate preparations. Since many of the basic mechanisms of single neuron function were first investigated in invertebrates and later found to be applicable to vertebrates as well, it is reasonable to assume that studies on neuronal interactions in invertebrates will also yield general principles of operation.

Behavior is characterized by the concerted action of specific parts of the organism and is therefore an ideal starting point for the study of neuronal interactions. This approach, to begin with a behavioral phenomenon and seek to find the cellular basis by which it is mediated, has been aptly termed Neuroethology by Hoyle (1970). It has been most successful in experiments using invertebrates, many of which have extensive behavioral repertoires and relatively simple nervous systems compared to vertebrates. Whereas the brains of vertebrate animals contain on the order of 10^{10} nerve cells forming a mass of interconnections, invertebrate nervous systems are composed of chains of relatively autonomous ganglia, each of which contains on the order of 10^3 nerve cells. Furthermore, many invertebrate behaviors have been shown to occur in experimentally isolated portions

of the animal, thereby greatly reducing the number of cells which need to be considered. Invertebrates also possess many identifiable nerve cells; that is, cells which are the same in location, branching pattern, connections, and physiology from animal to animal within a species (and in some cases, in different species). This has led to many studies that combine behavioral, anatomical and electrophysiological experiments to produce a unified picture of the neural mechanisms underlying a given behavior.

The concept of Neuroethology may be extended so that it applies not only to behavior per se, but also to modification of behavior, or plasticity. Then the changed interactions between neurons become of greatest interest. The insect leg position learning preparation has a relatively long history of the combined approach mentioned above, and is beginning to yield information concerning the neural bases of learning.

LITERATURE REVIEW

Behavioral Experiments

Introduction

In 1962 Horridge (1962) reported on an insect (cockroach and locust) preparation which could be used for studies on instrumental conditioning. The procedure he used has been followed without significant alteration in most of the studies since then. A generalized version is given below.

Two animals are matched for size and activity, then decapitated and attached by their dorsal surfaces to glass rods. One leg of each animal is fitted with wire leads so that shocks may be delivered. The animals are then positioned over separate dishes containing saline.

During an initial training period, usually about 30 min duration, one animal of the pair completes a shock circuit upon extension of its leg lead into its saline dish. Then shocks (about 5 msec, 3/sec) are delivered to the same leg of this animal and the corresponding leg of the second animal. When the first animal flexes its leg, its lead is withdrawn from the saline and no shocks are received by either animal. The first animal is referred to as the Positional or P member of the pair, since all shocks it receives are contingent upon leg extension. The other member of the pair is designated the

Random or R animal, since its shocks are presumably randomly correlated with its leg position.

Following training there is a 10 or 15 min rest period during which no shocks are given. A testing period, usually about 30 min duration, follows and now the circuit is altered so that each animal receives shocks independently, when its own leg lead extends into its own saline dish.

From this description one can see that during training P receives shocks as a result of leg extension, whereas R may receive shocks when its leg is in a variety of positions. Since R receives the same temporal sequence and intensity of shocks, it is referred to as a yoked control animal. The logic of this procedure is that if any significant difference in number of shocks received during testing exists between P and R, such that P takes fewer shocks than R, it must be due to the association of leg position with training shocks, since the R animal controls for any non-specific effects of shock receipt (e.g., increased leg flexion to shock itself). Hence P-R testing differences are used as an index of learning. Large individual variability necessitates grouping the results of several P-R pairs and treating this combined data statistically.

Horridge (1962) found significant P-R testing differences and concluded that headless animals were capable of learning. He further showed that an association between leg position and shock receipt which was made at the prothoracic level during training could be manifested at the metathoracic level during testing (as judged by a P-R difference in these posterior legs).

Experiments in which the same leg is trained and tested will be referred to as same leg experiments. Those which use one leg for training and another for testing are hereafter termed transfer experiments. Table 1 is a summary of the behavioral experiments that have been done on leg position learning and transfer in insects. Intact, headless and isolated ganglion preparations have been used, as well as a variety of legs.

Same Leg Experiments

Headless, Isolated Ganglion and Intact Preparations.

Horridge's original experiments (Horridge, 1962) showed that in headless animals, shocks contingent upon leg extension may be used to train a prothoracic or metathoracic leg to assume a more flexed position. This finding was later confirmed (Disterhoft et al., 1971; Harris, 1971; Lovell and Eisenstein, 1977) and extended to include the mesothoracic legs of headless animals as well (Harris, 1971).

In 1965 Eisenstein and Cohen (1965) reported that an isolated prothoracic ganglion preparation (one which has had its ventral longitudinal nerve cord connectives severed anteriorly and posteriorly to the ganglion) could learn the leg lift task. Harris (1971) confirmed this and also showed that the isolated mesothoracic ganglion preparation could learn. Two studies on the isolated metathoracic ganglion have been made; in the first of these, Aranda and Luco (1969) claim to have obtained P-R differences in testing, but no statistics or group graphs are given. Pak and Harris (1975) report that P animals (isolated metathoracic preparation) did not take fewer shocks over time during a 30 min training session. These studies do not

Table 1.--A List of Leg Position Learning and Transfer Experiments.

Author(s)	Preparation	Single Leg Expt.	Transfer Expt.	Train	Rest	Test	Shocks
Aranda and Luco 1969	Blatta o. headless	isolated meta- thoracic		10	5-30	10	
Disterhoft, 1972	P. americana intact	right prothoracic (escape v. avoidance)		30	--	--	50 V, 5 msec, 3/sec
Disterhoft et al. 1971	P. americana headless	right prothoracic (extended, flexed, free)		35	10	35	50 V, 5 msec, 5/sec
Disterhoft et al. 1968	P. americana intact	left metathoracic		?	15 min, 24 hr, 48 hr	40	3/sec
Eisenstein and Cohen, 1965	P. americana isolated and ganglioness	left prothoracic		45	15	45	80 V, 3 msec, 4/sec
Eisenstein and Krasilovsky, 1967	P. americana isolated	P and R in same ganglion		30	--	--	3 msec, 4/sec
Harris, 1971	Leucophoraea headless	Prothoracic and Mesothoracic	Prothoracic to Mesothoracic	30	10-15	30	3 msec, 2/sec
Horridge, 1962	P. americana S. gregaria headless	right prothoracic right metathoracic	right pro to right meta	45	10	45	3-6 msec, 1-2/sec

Table 1.--continued.

Author(s)	Preparation	Single Leg Expt.	Transfer Expt.	Train	Rest	Test	Shocks
Lovell, 1975	P. americana intact, headless	right prothoracic		45	10	10	50 V, 4/sec
Nathanson, 1973	P. americana headless	right metathoracic	lift: extend:	30 12	10 1	20 20	30 V, 3 msec, 2/sec
Pak and Harris 1975	P. americana intact	isolated meta- thoracic	meso to meta	6 x 30	10	30	7 msec, 2/sec
Pritchatt, 1970	P. americana intact	metathoracic (changes in leg position after training)		78, 20, 6	--	--	300 msec, 2/sec
Pritchatt, 1968	P. americana intact	left and right metathoracic (flex and extend)		78	--	--	300 msec, 2/sec
Willner and Mellanby, 1974	P. americana headless	left metathoracic		45	--	--	30 V, 2 msec, 1/sec
Woodson et al. 1972	P. americana headless	right metathoracic		30	--	15	2 msec, 1.5 sec

provide us with convincing evidence for or against the learning ability of an isolated metathoracic ganglion. However, since metathoracic legs can learn in intact (Disterhoft et al., 1971; Pritchatt, 1968) and headless (Horridge, 1962; Nathanson, 1973; Willner and Mellanby, 1974; and Woodson et al., 1972) preparations it is reasonable to assume that the isolated metathoracic preparation can learn as well.

Intact animals have been used in several leg position learning studies. Prothoracic (Disterhoft, 1972; Lovell, 1975) and metathoracic (Disterhoft et al., 1968; Pritchatt, 1968) legs have been trained but thus far no mesothoracic legs have been used in intact animal experiments.

Characteristics of Same Leg Experiments. From the preceding discussion it is probable that any leg in an intact, headless or isolated ganglion preparation is capable of leg lift learning. In many respects the behavior of these three preparations is similar, but there have been reports that isolated ganglia learn more rapidly than headless otherwise intact preparations (Eisenstein, 1972; Kerkut et al., 1970), which in turn learn more rapidly than intact (Kerkut et al., 1970; Lovell, 1975). Comparisons among the prothoracic, mesothoracic and metathoracic legs with regard to learning ability are not as straightforward, since each leg has its own set of angles and planes in which it moves. Thus adequate control procedures must be applied for such comparisons.

An almost universal finding is that during training P assumes an increasingly flexed leg position and approaches an asymptote in number of shocks taken within 5-10 min. In contrast, R's leg

position is either extended or alternates between periods of flexion and periods of extension. The major exception to these findings is that of Pritchatt (1968), wherein P and R both assume an increasingly extended leg position during training (but R does so to a significantly greater extent). One major difference in Pritchatt's methodology that may explain this anomolous result is that he used shocks of 300 msec duration, whereas all other workers used shocks in the 1-10 msec range. As Pritchatt mentioned, it is quite possible that this longer duration introduced a fatigue component into his data.

In summary, the constant features of training may be said to be (1) a change in leg position by P, such that the leg is progressively more flexed than at rest, over the first 10 min of training, and (2) increased extension or alternating periods of flexion and extension by R's leg.

Testing is somewhat harder to characterize accurately since many workers used only training periods, and there is thus more training than testing data available.¹ In those experiments comprised of both training and testing, it is common to find that during testing P assumes a flexed leg position within the first few minutes but that R performs similarly to a naive leg (i.e., as a P leg during training), reaching an asymptote within about 10-15 min. Eisenstein

¹Disterhoft (1972) states that if one wishes to examine the learning process, it seems more "straightforward" to use differences in P and R training performance as the criterion of learning instead of the retention measure of P-R testing differences. However, Eisenstein (1968) has noted that it is too early to determine which behavioral aspects of training (e.g., P-R difference, rate of P decrease) best reflect the underlying changes that lead to the generation of a P-R testing difference. "Once this is known, however, a test period will not be necessary" (Eisenstein, 1968).

(1972, 1970b) and Eisenstein and Cohen (1965) have proposed the hypothesis that P-R testing differences may be due to R learning as well as P learning during training. That is, R learns competing responses to shock during training since it receives shocks while in a variety of leg positions. The incorrect responses must be extinguished during testing before R can assume a flexed leg position. One method of assessing whether R learning has occurred is to compare R test behavior to P train behavior. If during testing the R leg receives more shocks than the P leg initiated during training, then there has been some inhibitory effect (on learning) of the shocks R received during training. If R test equals P train in shocks received, the effect of R training shocks is apparently neutral. Finally, if one finds that R test is more flexed than P train, then there has been a facilitory effect of R train shocks. The results of several studies (Disterhoft et al., 1968; Eisenstein and Cohen, 1965; Harris, 1971; Horridge, 1962; and Pritchatt, 1968) indicate that there is a small inhibitory effect; R test animals receive more shocks than do P train animals. If this inhibitory effect is indeed the result of competing responses, one would predict that greater shock/flexion correlation in R is associated with greater inhibitory effect. Furthermore, such a line of analysis would characterize the threshold and linearity of the inhibitory effect. For instance there may be essentially no effect until 50% correlation between flexion and shock is reached, and from that point there may be a sigmoid dependence of the inhibitory effect on flexion/shock correlation.

A careful analysis of R learning should allow one to judge the merits of the "competing response hypothesis" and would thus

provide important information concerning the dynamics of shock/leg position association which may then be applied to P animals.

Disterhoft et al. (1971) used a similar approach in attempting to answer the question of whether R learning occurs during training. Identical training shocks (corresponding to a typical P training curve) were given to three groups of animals; the first group had the leg fixed in a flexed position, the second in an extended position, and the third had its leg free. These three groups then correspond to percent shock/extension correlation values of 0, 100, and 50, respectively (assuming that the third group is a random control). After 35 min of this "training," the legs were freed and tested in the usual manner (shocks delivered upon leg extension). It was found that the legs held in an extended position during training initiated the fewest number of shocks in testing and that the legs held in a flexed position initiated more than the extended group but fewer than the control (R) group. These results were explained by assuming that the facility with which learning occurred during "testing" depended on the number of competing responses that had to be eliminated. Thus the extended group had no such competing responses, the flexed group had to reverse its responses, and the control group had many competing responses. These findings of Disterhoft et al. support the notion that R testing behavior is a function of the number of competing responses learned during training.

In most experiments P reaches an asymptote (takes fewer shocks) by the fifth to tenth minute of training and it is therefore of interest to know if a 10 min training period would be sufficient to generate a P-R test difference. Since the majority of shocks

occur during the first 10 min (the first third of a normal 30 min training session), learning may be complete by the end of this "critical" period. Alternatively, the relatively maintained flexion of the P leg for the remaining 20 min of training and the few shocks which are taken may be important as well, with regard to testing performance.

Aranda and Luco (1969) used training times of 10 min (whereas all other reported training times are 30 min or more) and although they report P-R differences during testing, lack of statistics or group data makes it impossible to evaluate whether these differences are real.

Pritchatt (1970) dealt with the problem in a slightly different manner. He trained animals for either 6 or 20 min, then compared their leg behavior during a non-shock rest period to see if the persistence of learned leg flexion depended upon training time. It was found that following 6 min of training (during which P usually reaches an asymptote in number of shocks received) there was no maintained P-R difference in leg position, but rather P returned to its pre-training (spontaneous) relatively extended position. If 20 min of training were used the changes in leg position persisted for at least 78 min (the duration of the experiments). These results imply that the minimal training period needed to generate a P-R testing difference lies somewhere between 6 and 20 min. Thus it could be that the minimal period is the 10 minutes usually needed for P to reach an asymptote during training.

It is important to know the time course of retention of a learned behavior when postulating mechanisms of learning and memory.

This becomes of particular importance in pharmacological studies which attempt to relate the biochemical determinants of retention to underlying molecular mechanisms of learning and the formation of memory.

In leg position learning studies using the normal 30-45 min training period and variable rest periods, significant P-R testing differences are obtained using rest times of 10 min to 30 min (Eisenstein, 1970a). If a 1 hr rest time is used there is no P-R test difference (Aranda and Luco, 1969). Significant differences return with rest times of 2 hrs or 12 hrs (Eisenstein, 1970a). Disterhoft et al. (1968) reported significant differences at 24 hrs but a reversed P-R difference at 48 hrs, such that R took fewer shocks than P. Eisenstein (1970a) noted that a similar non-linear relation between retention and rest time also exists in the rat and is known as the Kamin effect. The closeness of time course between the rat and cockroach retention curves may reflect fundamental mechanisms of the memory process that are common to all animals.

Leg Extension Learning. The previous discussion has dealt exclusively with leg lift training, where shocks are given upon leg extension and hence the leg is trained to flex. In 1968 Pritchatt (1968) reported that the metathoracic leg of an intact animal could be trained to extend to avoid shocks contingent upon leg flexion rather than extension. There was a significant difference in leg position between P and R during a 78 min training period, but no test period was used. A more recent study on leg extension learning was done by Nathanson (1973). Using the metathoracic legs of headless animals, he found significant P-R testing differences after a 12 min

training period and 1 min rest period. In other experiments where legs were trained to a given criterion (less than 25 shocks/3 min for leg extension learning, less than 4 shocks/3 min for leg lift learning), he found that the basic difference in extension and flexion learning was that in the former, fewer flexion-extension (f/e) movements were made per min but more shocks were taken per f/e.² In other words dip duration was longer in extension training than in flexion training.

Escape and Avoidance Learning. Disterhoft (1972) pointed out that two types of change in leg behavior could result in an animal taking fewer shocks over time. In the first (type I) fewer dips into the saline are made over time but the duration of each dip is constant. In the second (type II) the leg withdraws more rapidly on subsequent dips but the number of dips over time does not change. A third possibility (type III) is that fewer shocks result from a combination of the above two behavior changes. If this were true one should find a decrease in dips over time and a decrease in dip duration as well.

Type I above is an example of avoidance learning, wherein the animal alters its behavior so as to decrease the probability of occurrence of a noxious event (in this case through decreased frequency of leg extension). Classically, the distinction is made

²A flexion/extension, or f/e, is defined as one up-down or down-up movement cycle of the leg. Nathanson (1973) found 3 f/e per minute for extension learning, 9 f/e per minute for flexion learning; 30 shocks per f/e for extension learning, 4 shocks per f/e for flexion learning.

between active and passive avoidance. In the former there is execution of new behavior which results in avoidance and in the latter there is suppression of behavior with which the punishment is associated. In the present case such a distinction becomes arbitrary since leg flexion and extension are coupled and mutually exclusive behaviors. Thus we cannot speak of increased execution of leg flexion independent of suppression of leg extension.³

Type II above is an example of escape learning (which may be a special case of avoidance learning) wherein the animal alters its behavior so as to decrease the duration of a noxious stimulus upon successive encounters. Again, by the same argument as in the above paragraph, no distinction can be made between active and passive responses in escape learning.

These behavioral experiments all suffer from the fact that "flexed" and "extended" are the only labels used for leg position; all the exploratory movements made when the leg lead is held above or in the saline are bypassed in the data taking and subsequent analysis.

Disterhoft (1972) found that in the prothoracic legs of intact animals the decrease in number of shocks over time is due to fewer dips over time and not to a decrease in dip length. In contrast, Lovell and Eisenstein (1977) reported a decrease in dip duration as well as a decrease in number of dips for the same preparation. Thus there is disagreement on type II (escape) learning and full agreement on type I (avoidance) learning in intact animals.

³They could be distinguished in electrophysiology experiments, where the relative contribution of flexor and extensor muscles to the behavior can be examined.

In headless animals it has been found that there is no escape learning (type II) but rather that the decrease in number of shocks over time is due entirely to fewer dips over time (type I) (Lovell and Eisenstein, 1977). If intact animals do indeed have an escape component, it may involve tonic descending inhibitory input from the brain. Headless animals are released from this inhibition, and are known to have greater activity in their thoracic motoneurons than intact animals (Weiant, 1958). If such inhibition operated selectively it could be a factor contributing to the behavioral differences seen in intact and headless animals.

P and R in the Same Ganglion. Eisenstein and Krasilovsky (1967) performed experiments where one prothoracic leg was treated as P, the other as R. Training was typical, with the P leg becoming more flexed over time and the R leg maintaining a relatively extended position. During testing there was no P-R difference but rather, R performed as well as P, starting at a low level of shocks and asymptoting rapidly. It can be concluded that whereas R was unable to control its receipt of training shocks initiated by P, it was able to "tap into" and utilize P's training experience during the testing period. R therefore performed as well as P during testing. Thus by virtue of the neural connections between P and R there was facilitation of R test behavior.

Classical Conditioning Experiments. The leg position learning experiments described above are all examples of instrumental conditioning, where reinforcement depends on the animal's spontaneous behavior (leg extension and flexion) and there is a temporal

association between the behavior and reinforcement. Classical conditioning involves a temporal association between one stimulus (the conditioned stimulus or CS) and another (the unconditioned stimulus or UCS) which follows it. The UCS elicits a given response when presented alone and the temporal pairing of CS to UCS eventually leads to the response being elicited by CS presentation alone.

Chen et al. (1970) used the metathoracic legs of intact, headless and isolated ganglion preparations in such a paradigm. The CS consisted of two pulses (4 v, 1.5 msec, separated by 24 msec) that were initiated upon extension of the leg lead into the saline. These pulses were sub-threshold for the avoidance response (leg flexion) seen during training or testing. The UCS consisted of a train of shocks (10-35 v, 500 msec duration) that followed the CS by 900 msec. Animals were trained to a criterion of 100% avoidance. An avoidance response consisted of lifting the leg during the 900 msec interval following CS and preceding UCS; this would result in no shocks being given.⁴ A later testing period examined the response to CS presentation alone. Controls consisted of CS-UCS presentations with variable CS-UCS interval (6-30 sec), and showed no response to later CS presentation alone.

It was found that the intact group reached criterion within 100 trials and that retention lasted several days without decrement. Headless animals could also be trained to reach criterion, but retention times were less (1-2 days) than intact animals. In

⁴These experiments have elements of instrumental as well as classical conditioning, since leg position determines CS presentation and the UCS can be avoided.

two-ganglion (meso- and metathoracic) and one-ganglion (metathoracic) preparations there was a failure to reach criterion, but this may have been due to the fact that fewer trials were given per day (20 instead of 140).

The most interesting finding by this group of workers was that if an intact animal was trained and its head removed after reaching criterion, retention was 100% up to 3 days later. If the metathoracic ganglion was isolated by cutting connectives after the intact animal reached criterion, there was 90-100% retention up to 4 days later.

These experiments indicate that intact animals learn more readily than headless or isolated ganglion preparations, but that once training to 100% criterion is achieved, retention does not require the presence of any ganglion but the one being tested.

Transfer Experiments

In his original experiments on leg position learning Horridge (1962) found that, if the right prothoracic legs of P and R animals were trained, there was a significant P-R difference when their left metathoracic legs were tested. This phenomenon was termed "transfer" since the results of training in one ganglion were manifested in another ganglion. Harris (1971) later showed that transfer could occur from either prothoracic leg to either mesothoracic leg and was mediated by the paired ventral nerve connectives. Furthermore, either connective could mediate transfer from either prothoracic leg to either mesothoracic leg.

Harris (1971) investigated the time course of transfer and found that if the prothoracic-mesothoracic connectives were cut

following prothoracic training, there was still a significant P-R testing difference. This was true even when one connective alone (either one) was intact during training and then cut prior to testing. These results (similar to those of Chen et al. (1970) with single ganglion classical conditioning experiments) indicate that transfer is complete by the end of training whether it occurs ipsilaterally (right prothoracic leg to right mesothoracic leg) via one or both connectives, or contralaterally (right prothoracic to left mesothoracic) via one connective. Furthermore, the P-R difference seen in mesothoracic legs during testing must be a property of the P and R mesothoracic ganglia since all connections with the prothoracic ganglia have been severed.

To determine when transfer occurs during training, Harris (1971) used a reversible cold block (which prevented electrical conduction) on the prothoracic-mesothoracic connective(s). If the cold block was used during the entire training period no transfer occurred, although training was normal. If it was applied after training, transfer was normal. When it was used only in the first 10 min of training, no transfer occurred. These results show that cold block did not impair learning ability (i.e., there was no injury effect) and imply that the first 10 min of training are the most critical for transfer. To fully demonstrate the latter point it would be necessary to show that transfer occurred when the cold block was applied after the first 10 min of training (or more simply, that 10 min of prothoracic training is sufficient to generate a P-R testing difference at the mesothoracic level). If 10 min of training is indeed sufficient to generate a P-R testing difference in transfer

experiments, then this fact together with results in single ganglion experiments (see section on Headless, Isolated Ganglion and Intact Preparations above) implies that 10 min of training may be sufficient for the retention of leg position learning whether retention is measured in the same leg that was trained or in a leg to which training information has transferred.

A recent study on transfer from mesothoracic to metathoracic legs has potential significance for understanding the mechanism(s) of transfer. Pak and Harris (1975) report confirmation (although no data is shown) of Harris' (1971) finding that cutting both (mesothoracic to metathoracic) connectives prior to training results in no P-R testing difference, but if cutting occurs after training there is a P-R difference. Most importantly, these workers found that if both connectives were cut prior to mesothoracic leg training but six 30 min training sessions were used instead of one, there was a significant metathoracic P-R testing difference. This can be interpreted as evidence for a humoral component in transfer (at least transfer resulting from long training times). Pak and Harris found that cutting connectives impaired the ability of the now isolated metathoracic ganglion to learn (in contrast to results on prothoracic and mesothoracic ganglia). Therefore they suggest that the P-R testing difference found in metathoracic legs following mesothoracic leg training (both connectives cut) represents differences that are entirely generated during mesothoracic training, and then transferred to the metathoracic ganglion.

Is It Learning?

Eisenstein (1967) has argued that the critical factor which distinguishes classical and instrumental conditioning from other forms of behavioral plasticity such as habituation or sensitization is the encoding of a temporal sequence of events. According to this view learning involves an association among the originally ineffective stimulus (the conditioned stimulus, or CS) the so-called reinforcing stimulus (the unconditioned stimulus, or UCS), and the response to be made by the organism. The temporal order in which these three events occur determines the probability that the response will occur to the originally ineffective stimulus (the CS). Furthermore the nature of the reinforcing stimulus (the UCS) determines whether it will be a positive reinforcer (e.g., food) leading to an increase in the probability of the response occurring, or a negative reinforcer (e.g., shock) leading to a decreased probability of occurrence of the response.

"Non-associative" forms of learning also exist, wherein spatial or language relationships are incorporated into the behavioral repertoire without apparent or imposed reinforcement (Davis, 1976). These may therefore represent more advanced forms of learning, but as Davis (1976) points out, it is not possible to distinguish experimentally between non-associative learning and "simpler" forms of plasticity such as habituation. As in the previously mentioned case of distinguishing active from passive avoidance learning, a finer-grained understanding is needed. If one could specify cellular mechanisms corresponding to what is now called non-associative

learning and compare them to the cellular mechanisms underlying what is now called habituation, there would be no confusion.

The shock avoidance leg position learning experiments described above are generally considered to be examples of avoidance conditioning (characterized by the use of a negative reinforcer, e.g., shock), and come under the larger heading of instrumental conditioning. Both instrumental and classical conditioning involve the temporal association of events, but in the former the reinforcing event "is not inevitable as in classical conditioning, but is instead contingent upon the animal's 'voluntary' or 'spontaneous' behavior" (Eisenstein, 1967).

It has been common to consider proprioceptive feedback as the neural correlate of the CS in leg position learning experiments (see Eisenstein, 1972). According to this view proprioceptive feedback supplies the information on leg position that is necessary for an association between leg position and shock (or lack of shock) to be made. Behavioral support for this idea comes from the observation that during training, after a few shocks have been received by P upon leg extension, the P leg often descends slowly toward the saline and then rapidly raises before any contact is made (Eisenstein and Cohen, 1965; Horridge, 1962; Pritchatt, 1968). When experiments (in deafferented rats (McLoon and Buerger, 1974) as well as deafferented insects (Hoyle, 1965; Murphy, 1969)) suggested that proprioceptive feedback may not be necessary for leg position learning nor for learning to modify the firing rate of a motoneuron, the suggestion was made by some that leg position learning in the cockroach is not avoidance learning "because of the absence of any association between

the shock stimulus and another stimulus" (Alloway, 1973). Davis (1976) argues against this idea, noting that proprioceptors other than the ones extirpated may be operative and thus constitute the CS. More importantly, he states that "even if proprioception is shown to have no role in leg position learning, other mechanisms by which the nervous system can be informed of motor activity are known, including efference copy" (Davis, 1976). (Efference copy is treated in greater detail in the section ELECTROPHYSIOLOGY EXPERIMENTS.) It is significant in this regard that Horridge, in his original paper (1962), stated that "association has not yet been shown to depend upon sensory information derived from position-sense organs. The leg position could feasibly be inferred by a central mechanism taking note only of the frequency of impulses to the main muscles . . ." (Horridge, 1962).

Another fact which supports the notion that leg position learning is associative and not simply a reflex effect of shocks is that legs can be trained either to extend or to flex to avoid shocks. Similarly, in analogue electrophysiology experiments to be discussed below (Hoyle, 1975; Woolacott and Hoyle, 1977), the firing rate of a motoneuron can be trained to rise or fall in response to shocks given upon downward or upward changes in the spontaneous rate, respectively. These electrophysiology experiments have also shown that reversal occurs; a motoneuron trained to increase its rate can, after a short rest, be trained to decrease its rate and vice versa.

Biochemical Studies

Once leg position learning in insects was accepted as a valid phenomenon, it became of interest to understand its molecular basis.

As shown by Eisenstein (1970a) and others (Aranda and Luco, 1969; Disterhoft et al., 1968), there were short and long term memory components judging by retention data. Was the situation here similar to vertebrate learning and memory, where long term memory depended upon protein synthesis but acquisition and short term retention did not? Either way, an answer to this question would yield valuable information concerning the generality of the concept that short term memory is independent of, and long term memory is dependent on, protein synthesis.

Cyclohexamide

Early experiments (Brown and Noble, 1967, 1968; Glassman et al., 1970) showed that application of cyclohexamide (CXM), a protein synthesis inhibitor, to the prothoracic ganglion in headless or isolated ganglion preparations led to slower acquisition in CXM treated P animals compared to saline P animals.⁵ The CXM group was also more active, making more dips per minute (and therefore receiving more shocks) than the saline P group. Both groups showed a decrease in number of shocks over time during training, and savings in their testing performances. It may be (Eisenstein, 1968) that learning and memory occurred in both groups but that the CXM group was simply more active and took longer to reach the arbitrary criterion of learning.

⁵Brown and Noble (1968, 1967) used 375 µg/ml CXM in a 25 µl solution, or 9375 µg CXM. They found 90% inhibition of protein synthesis at this dose. Glassman et al. (1970) used 940 µg/ml CXM in 10 µl, or 9400 µg CXM. Whether these doses are comparable depends on the injection volume, which differed by a factor of two, as well as where the sites of CXM action which affect learning are located in the ganglion.

Eisenstein (1968) has argued convincingly that in order to properly assess the effects of drugs on insect leg position learning, one must use P and R animals, both of which have been given the drug. Then any differences in performance between drug treated P and R relative to saline control P and R animals may be attributed to the effect of the drug on the relationship between leg position and shock receipt, since the R animal controls for any non-specific effects of the drug such as increases in leg activity. In this way the action of drugs like CXM on the association process itself may be examined.

Lovell (1975) performed experiments using P and R animals. She found that CXM given one hour prior to training impaired acquisition in headless, but not intact animals. That is (similar to the above studies), headless CXM P animals took more shocks at the beginning of training and took longer to reach an asymptote than did saline P animals. The impairment seen during training in headless animals was apparently not due to altered sensitivity of the leg to shock, since there was no change in the twitching threshold of the leg to shock after CXM administration, nor was there a difference in average dip duration (a measure of the rapidity of leg withdrawal to shock) between saline and CXM groups within a P and R category.

The impairment in headless animals could be due to increased activity, since the CXM P animals made more dips than their saline controls. However CXM R animals did not differ from their saline controls in overall leg position or activity level. This argues in favor of a more specific effect of CXM.

Lovell (1975) found that CXM had no effect on testing performance in either intact or headless animals, using a 10 min rest

period between training and testing. Judging by the retention data of Eisenstein (1970a), this constitutes a test of the effect of CXM on short term rather than long term memory since rest times of more than one hour are needed to examine effects on the long term component.

The mechanism of the CXM impairment on acquisition in headless animals is still unclear. Its primary action could be to affect acquisition through changes in activity or through changes in the associative process itself. Aspects of Lovell's data that support the former hypothesis are:

1. Activity of CXM treated P animals is higher than saline control P animals during the first 10 min of training.
2. Since the slopes of the learning curves for CXM treated P and saline control P animals are similar, CXM may affect only the starting levels and not the rate of learning.

Evidence in favor of the latter hypothesis is:

1. CXM injected P animals take longer to reach asymptote than do saline control P's.
2. There is no difference in leg activity or position between CXM and saline injected R animals.
3. There is no difference between saline and CXM groups with respect to twitching threshold or dip duration, both of which are measures of the sensitivity of the leg to shock.

Measurements of Protein and RNA Synthesis

Kerkut et al. (1970) investigated directly the role of protein synthesis in the learning of leg position by headless animals by the use of an isotope double-labelling technique. ^3H -leucine was injected

into one animal and ^{14}C -leucine into the other. The animals were trained, one as P, the other as R, and then the metathoracic ganglia were excised and pooled. Differences in incorporation were detected using the $^3\text{H}/^{14}\text{C}$ ratio. It was found that incorporation of leucine into newly synthesized proteins was 70% greater in P than in R animals. In addition, P animals showed increased incorporation of three specific protein fractions.

A double labelling technique like the one above, but using uridine instead of leucine, was used to assess the role of RNA synthesis in leg position learning (Kerkut et al., 1972). It was found that P animals incorporated 44% more uridine into newly synthesized RNA than R animals, and 69% more than resting animals. This incorporation was greatest in the posterior region of the metathoracic ganglion, where some flexor motor neuron cell bodies are known to be located.

Although these results on protein and RNA synthesis show P-R differences in incorporation, there is the possibility that since P is more flexed overall than R, the greater incorporation by P reflects a higher metabolic rate in P flexor motor neurons rather than a more specific association aspect of the learning.

A recent report presents evidence that leg lift training in the grasshopper also leads to increased RNA production in P animals, compared to R animals or resting controls (Sukumar, 1975). This difference, as well as those of Kerkut et al. (1970), was found after a one hour training period. It would be quite interesting to compare the time courses of incorporation and retention to see if the rate of incorporation parallels the return of P-R testing differences after a rest period of one hour (Eisenstein, 1970a).

Facilitators and Inhibitors

Kerkut et al. (1970) have investigated the effects of several drugs on acquisition in the metathoracic legs of headless animals. They claim that RNA or protein synthesis inhibitors (CXM, actinomycin D, acridine orange, congo red, chloramphenicol) slow the rate of learning; i.e., time to a criterion of less than 4 shocks in a 3 min period. Other agents (prostigmine, physostigmine, amphetamine, edrophonium, magnesium pemolate) with a diversity of known actions were said to facilitate leg position learning. In all cases dose-response relationships were found such that larger doses magnified these inhibitory and facilitory effects (within a range of 50-100 μ g).

These findings, while intriguing, cannot be accepted at face value for several reasons. Primarily, there are no learning curves presented. The data that are discussed deals either with number of shocks or number of minutes to reach criterion, and as Davis (1976) has pointed out, neither of these measures can distinguish an effect on activity level from an effect on learning rate.

Cholinesterase Activity Changes

Kerkut et al. (1970) found there was a 50% reduction in cholinesterase (ChE) activity in the metathoracic ganglion of P animals relative to resting controls after one hour of metathoracic leg training. R animals showed a 25% reduction compared to resting controls. A later study by the same group (Kerkut et al., 1972) demonstrated that the 25% net reduction in ChE activity of P relative to R was due to a decrease in activity of the major ChE iso-enzyme,

and that the time course of ChE activity reduction followed that of the P-R difference in leg position which develops during training.

Davis (1976)⁶ notes that there is a "serious inconsistency" in the data from the Kerkut group. Since they found no change in V_{max} for ChE due to training, any determination of ChE activity made at much higher substrate concentrations than K_m could not reveal activity differences between experimental and control animals. The earlier study of Kerkut's group used concentrations approximately ten times higher than K_m and resulted in changed ChE activity; thus part of their data must be erroneous. Either V_{max} changes and there is changed ChE activity, or V_{max} does not change and there is actually no change in ChE activity.

In two independent studies, no decrease in ChE activity was found to accompany training. Willner and Mellanby (1974) detected no difference in ChE activity in P and R metathoracic legs during training of headless animals. There was also found to be no difference in P and resting controls. These workers examined leg activity and reported no difference between P and R, so this should not be a factor in the ChE results. In the second study, by Woodson et al. (1972), no difference was found between metathoracic ganglion ChE levels in P trained versus resting animals. No data are reported for R animals.

Willner and Mellanby (1974) reported that the time of homogenization drastically affects ChE activity; if ganglia were placed

⁶This argument was developed by Dr. Jeffrey Ram, who at one time worked in Davis' laboratory.

in cold Ringer solution and then homogenized at different times afterward, there was no loss of activity for times up to 24 hours. But if homogenization was done first, and tests for activity made at different times later, a 40% drop in activity was seen after 1 hour. Since Kerkut et al. (1972, 1970) do not state the interval they used between homogenization and assay (or indeed if this was constant), there is no way of knowing if this was a factor in their results.

There is obviously a large amount of conflicting data with respect to possible changes in ChE activity that accompany leg position learning. But if indeed ChE activity is reduced in P trained animals, and K_m changes but V_{max} does not, it is likely that this represents the action of a competitive enzyme inhibitor that "raises the efficacy of synaptic transmission in the specific neuronal circuits that mediate the learned task" (Davis, 1976).

Findings related to putative neurotransmitters are that P training leads to lower GABA levels and lower GAD activity (Oliver et al., 1971). In other experiments the Kerkut group found that AChE, GABA and GAD are all decreased in the head after training such that intact P animals have lower levels than intact resting animals (Oliver et al., 1971). These changes may be components of the behavioral differences seen by Lovell and Eisenstein (1977) in intact versus headless animals.

Cyclic AMP and Leg Position Learning

Nathanson (1973) demonstrated that cyclic AMP (cAMP) and all its associated enzymes are present in cockroach thoracic ganglia in concentrations comparable to those in vertebrate brains. The putative

central transmitters dopamine, norepinephrine and serotonin all caused increased cAMP levels when applied to the ganglia. Octopamine, which activates phosphorylation in the thoracic nerve cord (Robertson and Steele, 1973, 1972), caused a much larger rise in ganglionic cAMP than any of these agents.⁷

Nathanson (1972) found a cAMP-dependent protein kinase which catalyzes phosphorylation of three specific ganglionic proteins in the presence of cAMP. It is of interest that Kerkut et al. (1972) found that three protein fractions show increased incorporation of leucine following training. Although these three fractions have the same relative mobilities as Nathanson's three proteins, one cannot directly compare the findings of the two groups. Kerkut's group used standard gels whereas Nathanson used SDS gels. The former separate different proteins on the basis of charge, molecular weight and three-dimensional structure; the latter separate only on the basis of charge.

In both leg lift and leg extension experiments, Nathanson found that Theophylline and SQ 20006 (both phosphodiesterase inhibitors that prolong and intensify the action of cAMP by preventing its metabolic breakdown) reduce the average learning time and number of trials to reach a given criterion. These effects were specific, since there were significant differences between P and R performances. Exogenously applied cAMP gave the same results.

⁷250 μ M octopamine led to a 500% increase in cAMP levels whereas norepinephrine and serotonin at the same concentration led to 150% increases. 250 μ M dopamine produced a 300% increase. Thus octopamine was by far the most potent of any compound tested.

Nathanson's findings are consistent with the Greengard hypothesis of cAMP action in neural transmission (Beam and Greengard, 1976): transmitter release → increased cAMP → increased protein kinase activity → phosphorylation (in this case, of three specific proteins) → changes in ion permeability of post-synaptic membrane → change in nerve activity, perhaps long-lasting. This sequence of events could be operative in leg position learning if it occurred in specific neurons. As mentioned above, there may be inhibition of ChE activity by a competitive enzyme inhibitor, a control point in this sequence serving to increase the post-synaptic effect of a given quantity of transmitter.

Anatomy Related to Leg Position Learning and Transfer

Flexor and Extensor Muscles, Motor-neurons, and Interneurons

The leg movements which predominate in cockroach and locust leg position learning experiments are levation and depression of the femur about the coxal-trochanteral joint. Pearson and co-workers have found that leg movements of this kind are also important in walking (Pearson, 1972; Pearson and Iles, 1970). Their studies have used cockroaches; intact walking animals, deafferented and isolated ganglion preparations. In all three cases the neuronal circuitry described below was found to mediate these movements or their neuromuscular correlates (Pearson, 1972; Pearson and Bergman, 1969; Pearson and Fournier, 1975; Pearson and Iles, 1973, 1971, 1970).

Nerve 6Br4 (branch B, ramus 4) innervates the posterior coxal levator muscles, via twelve axons, six of which are spontaneously

active in all but the most rapid leg movements. During these rapid movements (e.g., escape behavior) the other six axons are also active. The axons are labelled 1-12 in order of increasing diameter. The spontaneously active axons are numbers 1-6, those associated with rapid movements are numbers 7-12. No junctional potentials (jp's) have been found to be associated with axons 1 & 2, so their functions remain unknown. Axon 4 is a steadily firing slow excitatory fiber whose effect is strongly inhibited by inhibitory junctional potentials (ijp's) from axon 3. Axons 3 and 4 have been shown to function as a postural control system. Axons 5 and 6 are excitatory and are spontaneously active in bursts that are correlated with the flexion movements seen in walking. Axon 3 has little effect on their action.

Coxal depressor (D) muscles are innervated by nerve 5r1 (ramus 1), which contains five axons labelled D_{1-3} , D_s and D_f . D_{1-3} are branches of three different common inhibitory neurons (defined as motoneurons whose branches innervate functionally different muscles). D_3 is a branch of the same neuron that forms axon 3 in N6Br4. D_s is a slow excitatory fiber and D_f is active only in fast leg movements (hence the subscripts s and f). D_s fires in bursts antiphasic to the levator bursts in axons 5 and 6, and its action is strongly inhibited by D_3 .

Pearson and Bergman (1969) reported that common inhibitors⁸ (CI, one branch of which is axon 3 of N5r1 and another of which is D_3 of N5r1) were located in the mesothoracic and metathoracic ganglia.

⁸There are three common inhibitors under consideration, as stated; however the one with branch D_3 in N5r1 and axon 3 in N6Br4 is the most well known and is hereafter referred to as the common inhibitor (CI).

Iles (1977) later showed that there were homologous CI's in the prothoracic ganglion as well. The metathoracic CI's (symmetrically located on either side of the ganglion) distribute out ipsilateral nerve trunks 2-6 and send a branch into the ipsilateral posterior connective as far as the first abdominal ganglion. Another branch in the ipsilateral anterior connective ascends to the suboesophageal ganglion without distributing side branches to the peripheral trunks of the mesothoracic or prothoracic ganglia (Crossman et al., 1972).

A system of non-spiking interneurons has been found to underlie the alternating burst pattern seen in levator axons 5 and 6 and depressor axon D_s (Pearson and Fourtner, 1975).

Interneuron I has an oscillating membrane potential whose depolarization is in phase with bursts in axons 5 and 6. When Interneuron I is depolarized it excites axons 5 and 6 and inhibits D_s . When hyperpolarized it inhibits axons 5 and 6 but does not excite D_s (in contrast to a symmetrical non-spiking interneuron system in the crab (Mendelson, 1971); rather D_s is released from the inhibition of the depolarizing phase.

Interneuron II specifically inhibits axons 5 and 6 when depolarized and has no effect on D_s .

Interneuron III specifically inhibits D_s when depolarized but has no effect on axons 5 and 6. It is therefore reciprocal in action to Interneuron II.

Interneuron IV excites D_s when depolarized but has no effect on axons 5 and 6.

It is known that Interneuron I is contained entirely within the ganglion and that it is primarily responsible for generating the

oscillating burst pattern seen in walking. This is due to the fact that depolarizing current injected into Interneuron I on its hyperpolarizing phase resets the burst rhythm, whereas current injected into any of the other interneurons does not reset the rhythm (Pearson and Fourtner, 1975). A group of oscillatory non-spiking interneurons with similar properties has been found in the locust by Burrows and Siegler (1976).

Coordinating Fibers Between Ganglia

Pearson and Iles (1973) found that there were axons in the dorsolateral part of the ipsilateral meso-metathoracic connective which discharged in phase with flexor bursts (in axons 5 and 6) in either the mesothoracic or metathoracic ganglion. Since the flexor motorneurons are wholly contained within the ganglia, these connective fibers are probably interneurons driven by the burst generating system. Pearson and Iles (1973) found evidence of probable monosynaptic connections between the connective fibers and Interneuron I (as well as the known monosynaptic connections between Interneuron I and the flexors).

Stein (1971) found similar fibers in the swimmeret system of the crayfish. These "coordinating" fibers connect oscillators (presumably interneurons like Interneurons I-IV) of one segment with those of another. Thus motorneurons driven by these oscillators are coordinated in these actions by virtue of this central representation of efferent (motorneuron) discharge.

Davis et al. (1973) found fibers in the cerebrobuccal connectives of the pleurobranch mollusk which coordinate feeding rhythms

generated independently by systems in the brain and buccal ganglion. Some of these neurons carry an exact one-to-one replica of motoneuron impulses ("efference copy") while others simply carry bursts that are of similar duration and occur at about the same time as motoneuron bursts ("corollary discharge"). By these criteria the connective fibers described by Pearson and Iles (1973) should be considered corollary discharge coordinating fibers.

Other fibers that discharge upon stimulation of various leg parts were found by Pearson and Iles (1973) in the ventral part of the ipsilateral connective. This implies that they are either afferent collaterals or interneurons strongly excited by primary afferents.

Dorsal Unpaired Median (DUM) Neurons

In addition to the connective fibers described by Pearson and Iles (1973) and the CI neuron, which projects between ganglia, there is another class of neurons which shares this feature and may be of importance in transfer. These are the eight dorsal unpaired median (DUM) cells. They were first examined in the metathoracic ganglion of the cockroach and locust by Crossman et al. (1972, 1971) and later in the prothoracic ganglion of the cockroach by Iles (1977). The cells are clustered in a group near the dorsal midline of the ganglion. Their axons distribute bilaterally to nerve trunks 3-6 and to the anterior and posterior connectives in the cockroach, and to trunks 3-5 and the anterior connective in the locust. In contrast to most insect neurons, their cell bodies are electrically excitable; however, the spike activity which propagates into the peripheral trunks is of very low amplitude (Crossman et al., 1972).

Hoyle (Hoyle, 1974; Hoyle et al., 1974) has found that one DUM neuron, DUMETi, innervates the extensor tibiae muscle in the locust and causes inhibition of the tonic contraction rhythm in this muscle when it fires. DUMETi synthesizes octopamine in its 70 μm soma and axon (Hoyle and Barker, 1975) (but neither norepinephrine nor dopamine). The inhibitory action of DUMETi on the extensor tibiae muscle is mimicked by infusion of norepinephrine or dopamine at concentrations of 10^{-6} M or by infusion of octopamine at concentrations of 10^{-9} M (Hoyle, 1975). This thousand-fold sensitivity to octopamine, the findings above regarding synthesis, and the presence of dense core vesicles⁹ (DCV's) (Hoyle et al., 1974) all indicate that DUMETi is an octopaminergic neuron.

Nathanson (1973) found that the degree of cAMP increase was very sensitive to octopamine, and that cAMP played an important role in leg position learning. This, together with Hoyle's findings on DUMETi, indicates that all the DUM neurons should be fully investigated to see if they are all octopaminergic, and to determine their exact innervation patterns. Since they have bilaterally symmetrical axon distributions in the ganglion and connectives, it is possible that they may play a role in transfer of learning information and in single ganglion learning.

⁹These are found in the soma, scattered in the axon, and abundantly packed in the terminals. They are 600-2000 Å in diameter.

Electrophysiology Experiments Related to Leg Position Learning

Investigations of electrical activity patterns that accompany leg position learning have been few. In the first one, Aranda and Luco (1969) found higher spontaneous firing rates in metathoracic nerve 5 of the isolated ganglion of P trained animals compared to R animals. This difference was found only on the trained side of P; on the contralateral side there was no difference between P and R. The threshold for evoked responses (due to stimulation of the anterior connectives) was also lower on the trained P side than the untrained P side, and lower than either side of R. These changes were evident up to 30 min after training but then disappeared. This supports the two-stage memory hypothesis mentioned earlier in connection with retention studies (see Eisenstein, 1968).

Hoyle and his colleagues (Hoyle, 1965; Tosney and Hoyle, 1977; Woolacott and Hoyle, 1977) have developed what may be considered an electrophysiological analogue of leg position learning. In the locust a single excitatory motoneuron innervates the anterior coxal adductor (AAdC) muscle, which is a major flexor muscle. This excitatory fiber and a smaller inhibitory fiber emanate from nerve 4. The ijp's generated by the inhibitory fiber do not cause any significant reduction in the tension produced by the excitatory fiber. Therefore by recording ijp's from fibers of the AAdC muscle one can monitor the firing rate and relate it to muscle tension. In the free leg an increased firing rate would lead to increased muscle tension and thus leg flexion. Shock leads are used and, as in the behavioral experiments, are tied around the tibia.

Hoyle (1965) originally observed that following behavioral training of headless locusts to lift a metathoracic leg to avoid shocks, there was a high frequency maintained discharge in the coxal adductor muscles. No other muscles (flexors or extensors) likely to be involved in leg position learning were found to exhibit such changes. Thus he reasoned that perhaps one could observe changes in the AAdC firing rate (in either the muscle or motoneuron) as they occurred in response to selectively timed shocks.

Animals were "trained" by delivering shocks every time the AAdC firing rate fell below a chosen demand level (generally 10-15% above the average spontaneous rate) over a 10 sec interval. In this way, it was found (Tosney and Hoyle, 1977) that one could drive the firing rate from its average spontaneous frequency of 14/sec up to 30/sec in 28 min. Animals given shocks not correlated with changes in their AAdC firing rate showed little or no change in rate. Since the frequency/tension curve for AAdC is sigmoid and is steepest in the range 15-30 Hz, these learned changes in firing rate are likely to be behaviorally significant. Following up-learning there is retention of the increased firing rate for up to 2 hrs. Usually one sees a 40% decline in the rate after an hour following training.

Animals can also be trained to decrease their spontaneous firing rate. AAdC's with relatively high spontaneous rates (30/sec avg; AAdC spontaneous frequency range is 2-43 Hz) could be trained to reduce the rate from 30 Hz to 10 Hz after 20 min in response to shocks given following increases in the spontaneous rate. As before, randomly applied shocks generated no such changes.

Evidence was found for reversibility; an animal trained to decrease its firing rate could, after a 20 min rest, be trained to increase its rate. Whether this is true of the opposite case, that an animal can be trained to decrease its rate 20 min after being trained to increase it, remains to be seen.

Once it was established that changes in AAdC firing rate could result from training shocks (Tosney and Hoyle, 1977) it became of interest to determine the neuronal locus of these changes (Woolacott and Hoyle, 1977, 1976). The spontaneous rate of AAdC is partly an intrinsic property of the AAdC motoneuron. This pacemaker function is evidenced by the fact that infusion of low Ca^{++} , high Mg^{++} saline (which inhibits synaptic transmission) produces a steadying effect on the spontaneous rate; there is a marked narrowing of the standard deviation in the interval histogram (Woolacott and Hoyle, 1977). Following infusion, the firing rate may undergo a rise or fall, or stay the same. It is suggested that these effects result from the removal of different relative amounts of excitatory and inhibitory inputs to the AAdC motoneuron.¹⁰

The training procedure in the infusion experiments was to determine the intrinsic pacemaker rate after low Ca^{++} , high Mg^{++} infusion, then to return the saline to determine the saline rate. Training (up or down) was then carried out and, afterward, the intrinsic pacemaker rate was again determined. In this way, one could

¹⁰If these inputs were balanced prior to infusion, no net change in rate would occur. If they were unbalanced, a downward change would occur if excitation predominated, and a change in the upward direction would result if inhibitory input was dominant.

separate the overall frequency change (i.e., the change in the saline rate over the training period) from pacemaker changes (the pacemaker rate after training compared to that before training).

Up-training resulted in an average total frequency increase from the resting (saline) value of 8.6 Hz to the trained (saline) value of 21.4 Hz. The pacemaker rate increased from 9.8 Hz before training to 15.0 Hz after, therefore comprising about 40% of the total frequency increase. Similarly, in down-learning the total frequency decreased from 26 Hz to 15 Hz and pacemaker decreases accounted for 35% of this change. In no experiment did pacemaker changes account for the entire effect. Thus the trained changes in AAdC firing rate (up or down) represent both pacemaker changes (about 40%) and synaptic input changes (about 60%).

In the above infusion experiments no R animals were used and thus one has no direct measure of the influence of random shocks (i.e., shock itself) on the pacemaker rate. Presumably, the fact that up-learning and down-learning can occur indicates that shocks do not have an invariant effect. Furthermore in most experiments shocks were followed by a brief excitatory burst and then a longer period of inhibition. In up-learning this inhibitory period was followed by a sharp increase in frequency then a return to baseline; the inhibitory period is reduced in length as training progresses. During down-learning the inhibitory period gradually lengthens (Woolacott and Hoyle, 1977).

Preliminary findings indicate that down-learning is accompanied by an increase in the frequency of ipsp's recorded in the AAdC

motoneuron soma, whereas up-learning is correlated with increased epsp frequency. No psp amplitude changes were seen in either case (Tosney and Hoyle, 1977).

Current-voltage measurements revealed that resistance shifts occur in the soma during training (Woolacott and Hoyle, 1970). Up-learning results in an increase from 4 M Ω to 7 M Ω and down-learning leads to a decrease from 9 M Ω to 4 M Ω .

The shape of the soma-recorded action potential is dependent upon frequency (whether spontaneous or trained) (Tosney and Hoyle, 1977). At lower frequencies there is a large undershoot, possibly representing a potential-dependent K⁺ conductance that is reduced at higher frequencies. TEA (tetra-ethyl ammonium, a specific blocker of K⁺ channels), injected into the cell blocks this undershoot and leads to a rise in frequency. Thus the changes in frequency following training may represent (in part) changes in potassium conductance, g_K . Up-learning would lead to a decrease in g_K while down-learning would lead to an increase. These conductance changes may causally precede the observed frequency shifts.

In summary, the trained changes in AAdC firing rate are mediated by pacemaker changes and by synaptic input changes. It is possible that non-spiking interneurons play a role in learning by producing DC shifts in the AAdC motoneuron membrane potential. Since soma recordings cannot resolve such changes in potential, it is likely that recordings from the principal neurite of the AAdC motor-neuron will be needed in order to evaluate this possibility.

RATIONALE FOR EXPERIMENTS

The cockroach leg position learning paradigm offers a unique opportunity for the investigation of cellular mechanisms by which information related to learning transfers from one location to another and also provides us with the means by which to characterize the kinds of information that transfer. Each pair of legs is supplied with a thoracic ganglion and the ganglia are interconnected by paired ventral nerve connectives. These connectives are the only neuronal pathways by which interganglionic communication can occur.

This animal represents one of the few invertebrate systems in which associative learning has been shown to occur that is also large enough in size to permit the use of conventional electrophysiological (and other) techniques.

The main purpose of the present experiments was to begin the cellular analysis of the pathways and mechanisms by which transfer occurs, using anatomical and electrophysiological techniques. First, since there were only two reliable reports that transfer of information related to learning occurred in the cockroach (Harris, 1971; Horridge, 1962), behavioral experiments were carried out to verify that training at the prothoracic ganglion level resulted in changes in behavior at the mesothoracic level, and that one connective alone

could mediate the transfer of information related to this behavioral effect.

The time course of transfer is important with respect to any proposed mechanism by which transfer occurs. Therefore a determination was made of the rapidity with which a behavioral change can be seen in the leg to which training information is being transferred.

If one is to examine the transfer of information related to learning, one must first decide what types of learning are occurring. To this end the prothoracic and mesothoracic legs were both characterized in terms of escape and avoidance learning components. Furthermore the P and R treatments were assessed to see if both kinds of information showed evidence of transfer.

All of the behavioral experiments and analyses mentioned above were carried out not only to verify and extend the nature of the transfer phenomenon, but also to lay a foundation for anatomical and electrophysiological experiments. Some of these have been done and are mentioned below.

Previous anatomical work on the cockroach was not done for the purpose of discovering the pathways important to leg lift learning and transfer. The only neuronal elements of this system that have been examined structurally in any detail are the motoneurons to leg flexor and extensor muscles. Therefore anatomical studies were done to determine the central projections of sensory fibers in the leg which carry shock input to the central nervous system. Did these fibers carry shock input directly to all the thoracic ganglia via the connectives? Were their projections ipsilateral and/or contralateral? Could

their branching patterns be correlated with the location of known neuronal elements?

Other anatomical experiments focused on the characterization of nerve fibers in the connectives. Did the connectives contain direct sensory and/or motor projections that could mediate transfer or did they consist wholly of interneurons? Did the connective fibers make ipsilateral and contralateral contacts once they reached a ganglion, thus explaining the finding by Harris (1971) that either connective alone could mediate transfer?

The previous electrophysiological analyses of leg lift learning (or more accurately in this case, leg position learning) have dealt almost exclusively with single ganglion learning and not with transfer. Thus in the present experiments recordings were made from the connectives and motoneurons at both the prothoracic and mesothoracic levels in order to answer several questions. First, what classes of units are there in the connectives and which classes respond most when the leg is shocked? Second, apropos of the finding by Harris (1971) that either connective alone can mediate transfer, how do responses in the ipsilateral connective compare with those in the contralateral connective? Third, does shock information travel anteriorly and posteriorly, and if so, do the same units or classes of units carry out the function in both instances? Finally, when a motoneuron that is known to be important in leg lift learning fires action potentials is there any correlated discharge in any of the connectives? Answers to these kinds of questions would allow one to make significant advances in deciding among the possible means by which transfer could occur. For instance, sensory and motor events

at one ganglion may be represented independently in the connective discharges to other ganglia. Some connective firing might be coupled in a strict one-to-one fashion with such events, whereas other information is only represented by a discharge of similar duration occurring at about the same time. Alternatively, sensory and motor events could first be associated at their ganglion of origin and then integrated information transferred.

The use of several techniques is helpful since it allows one to compare data arrived at by different means but dealing with the same problem, and thus to confirm or deny the plausibility of hypotheses developed through the use of one technique alone.

BEHAVIORAL EXPERIMENTS

Methods and Materials

Preparation of Animals

Adult male cockroaches, Periplaneta americana, were used in all behavioral experiments. They were housed together in a large bin and given water and dog food for nourishment. The two animals to be used for an experiment (one experimental animal, one control) were taken from the bin and anesthetized using pure carbon dioxide gas bubbled through water. They were then decapitated (thus removing the head parts and suboesophageal and supraesophageal ganglia) and the neck filled with vaseline to prevent loss of hemolymph. Each animal was attached by its dorsal surface to a glass rod with melted wax.

For animals in which connectives were cut, the ventral cuticular flap between the prothoracic and mesothoracic ganglia was lifted after cutting around its margin. Connectives were always cut between the prothoracic and mesothoracic ganglia, close to the prothoracic ganglion and anterior to the junction of mesothoracic nerve 2 with the connective (Pipa and Cook, 1959). The mesothoracic nerve 2 could not, therefore, serve as a conduction pathway between the ganglia on the cut side(s). After the connectives had been cut, the ventral cuticular flap was replaced and waxed into position.

Three groups of animals were used in the experiments: Intact animals, in which no connectives were cut; Left-Cut animals, which had their left prothoracic-mesothoracic connective cut prior to training; and Both-Cut animals, which had both their prothoracic-mesothoracic connectives cut prior to training. There were ten experimental and ten control animals in each of the three groups.

The right prothoracic leg of each animal was used for training and the right mesothoracic leg for testing. All other legs were covered by a strip of gauze to prevent their interference with these two legs. Pieces of 0.001" silver wire were tied around the femur and tibia of the right prothoracic and mesothoracic legs. These were for the delivery of shocks. A piece of 0.002" wire was waxed to each tarsus, the wax serving to insulate the leg from the wire. This lead extended from the tarsus and could make contact with a saline bath (1M KCl). Each leg of each animal had its own bath.

All animals were allowed to rest undisturbed for one hour following these procedures.

Experimental Procedure

During the 30 min training period one animal of a pair (chosen by flipping a coin), called the Positional or P animal, received shocks whenever it extended its right prothoracic leg lead into its saline bath, thereby completing a shock circuit. The second animal, designated the Random or R animal, was connected in series with P so that both animals received shocks to their right prothoracic legs until P lifted its leg lead out of the saline. Thus during training

R could receive shocks when its leg was in a variety of positions whereas P only received shocks when its leg was extended.

During training, when P's leg lead contacted its saline bath, the pulses initiated (50 V, 6 msec, 4 Hz) were not only delivered to both P and R as shocks, but were also recorded on a polygraph as a record of P's leg position and number of shocks received by P and R. When R's leg lead contacted its saline bath, no shocks were received but the pulses generated (5 V, 6 msec, 4 Hz) were recorded on the polygraph as a record of R's leg position.

After training there was a rest period which ranged from 5 to 25 min during which no shocks were given. Then the testing period began and lasted for 30 min. During testing the right mesothoracic legs of P and R were connected in parallel to the shock circuitry so that each animal received shocks independently, when its right mesothoracic leg lead was extended into its saline bath. The pulses initiated by each animal were recorded on the polygraph and represented the number of shocks received as well as leg position for each animal.

Data Analysis

Two response measures based on the pulses initiated by P and R leg behavior were used for most of the behavioral analysis. First, leg position was measured by the number of pulses initiated per minute, an index of how long a leg is extended and its lead is in contact with the saline. Secondly, leg activity was measured by counting the number of times per minute the leg lead made (extension) and broke

(flexion) contact with the saline. One such cycle is termed a flexion/extension (f/e).

The Wilcoxon matched pairs test (Siegel, 1956) was used to test for significance among matched animals. Where non-matched animals were compared, the Mann-Whitney U test (Siegel, 1956) was used. Two-tailed tests have been employed unless otherwise noted.

Results

Transfer as Measured During Testing

During training (Figure 1) P animals of all three groups (Intact, Left-Cut, Both-Cut) hold their right prothoracic leg in a progressively more flexed position, thus receiving fewer shocks over time. As found in past work in this and other laboratories, the asymptote is rapidly approached within 5 to 10 min (Eisenstein, 1972). R animals in all three groups demonstrate no such tendency toward maintained leg flexion. Rather they show fluctuations in leg position and a tendency to gradually maintain their legs in a more extended position.

During training the correspondence between leg extension and shock occurrence is 100% for the P animals in each group. For the R animals it is found that Intact R animals have their leg in an extended position 36% of the times that training shocks are initiated by P. This correspondence value is 30% for the Left-Cut groups and 45% for the Both-Cut group. Thus R animals receive shocks during the training period somewhat more often when their legs are flexed than when they are extended.

Figure 1. Right Prothoracic Leg Training and Right Mesothoracic Leg Testing. Only 20 of the 30 min used in training and testing are shown. In every case the remaining 10 min are consistent extensions of the patterns seen in the first 20 min.

A: Intact group

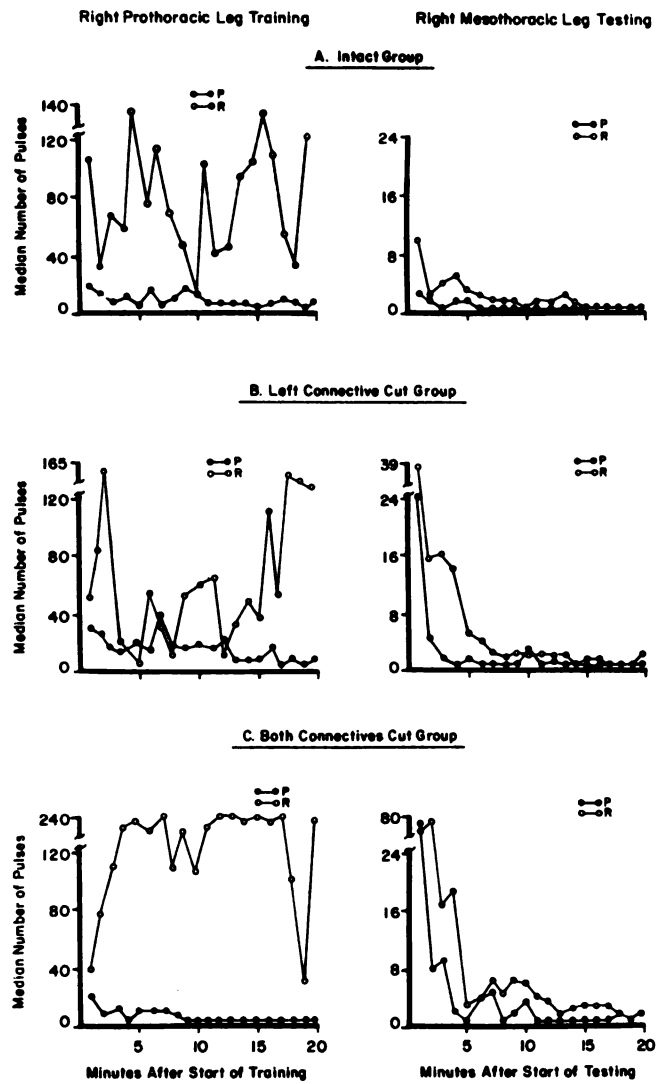
During training, P animals assume a progressively more flexed leg position and, therefore, fewer shocks are received by their right prothoracic legs. R animals show marked fluctuations in leg position with no tendency toward increasing leg flexion over time. During the first 5 min of testing there is a significant difference between the number of shocks initiated by the right mesothoracic legs of P and R ($p < .01$, one-tailed; min 1-5. $N = 10$ pairs).

B: Left-Cut group

During training, P animals initiate fewer shocks over time. R animals show marked fluctuations in leg position with a tendency toward increased leg extension over time. The first 5 min of testing reveals a significant P-R difference ($p < .04$, one-tailed; min 1-5. $N = 10$ pairs).

C: Both-Cut group

Training is similar to the above groups for P animals. R animals show a progressive tendency toward increased leg extension. Testing shows no significant P-R difference ($p > .16$, one-tailed; min 1-5. $N = 10$ pairs).



During testing both P and R of the Intact group (Figure 1A) initiate progressively fewer shocks, but R starts at a relatively higher level and reaches an asymptote more slowly. The P-R testing difference, which is a measure of transfer, is significant ($p < .01$, one-tailed; min 1-5). After ten min the P and R curves merge at a maintained low level of shock initiation. The Left-Cut group testing curves (Figure 1B) also show a significant P-R difference ($p < .04$, one-tailed; min 1-5) and converge at a low level by the tenth min. In the Both-Cut group (Figure 1C) both P and R initiate progressively fewer shocks, but in contrast to the other two groups, there is not a significant P-R difference ($p > .16$, one-tailed; min 1-5).

The resting time between the end of training and the start of testing is a critical factor in retention of leg lift learning when training and testing occur in the same ganglion (Disterhoft et al., 1968; Eisenstein, 1972). It has been shown that there is a progressive decrease in retention, as testing is conducted at increasing intervals up to one hour (Eisenstein, 1970a). In the present experiments, varying the resting time interval from to to 25 min had no effect on the transfer of a P-R difference in the Intact and Left-Cut groups.

Presence or absence of transfer is also reflected in the activity patterns of the legs. R test activity (Figure 3) is greater than P test activity (Figure 2) in the two groups showing transfer ($p < .01$, min 1-10, for the Intact and Left-Cut groups) but there is no such difference in activity in the Both-Cut group ($p > .13$, min 1-10). Furthermore, P train activity is greater than P test activity in the Intact and Left-Cut groups ($p < .01$, min 1-10, in both cases) but not in the Both-Cut group ($p > .29$, min 1-10). There were no

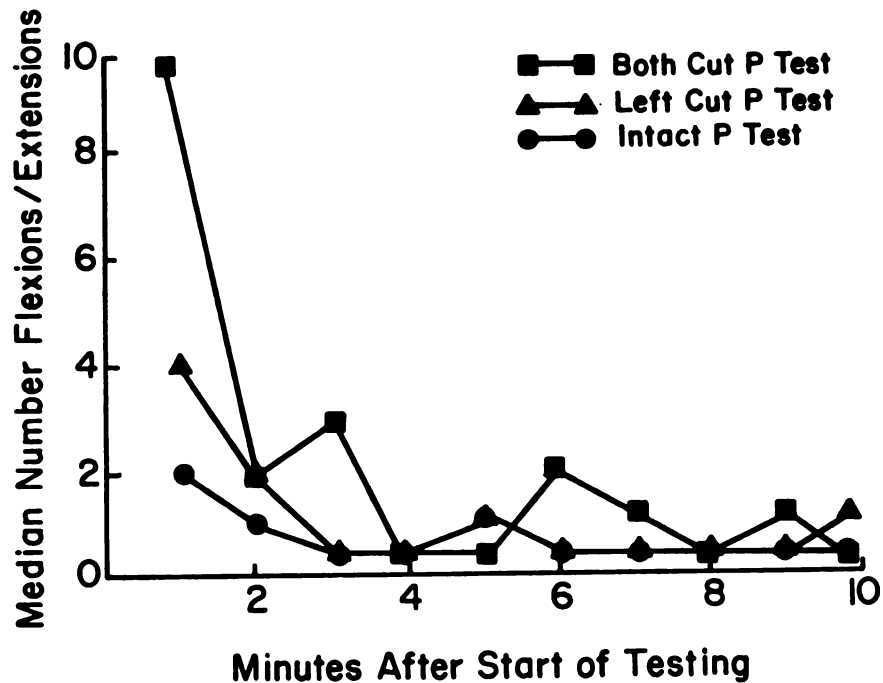


Figure 2. Mesothoracic Leg Activity in P Test Groups Increases When Connectives Have Been Cut Prior to Training. The flexion/extension activity of the Left-Cut group is significantly greater than that of the Intact group ($p < .01$, min 1; Mann-Whitney U Test. $N = 20$ animals). The Both-Cut group also has significantly higher activity than the Intact group ($p < .02$, min 1; Mann-Whitney U Test. $N = 20$ animals) but there is no difference between the Left-Cut and Both-Cut groups ($p > .40$, min 1; Mann-Whitney U Test. $N = 20$ animals).

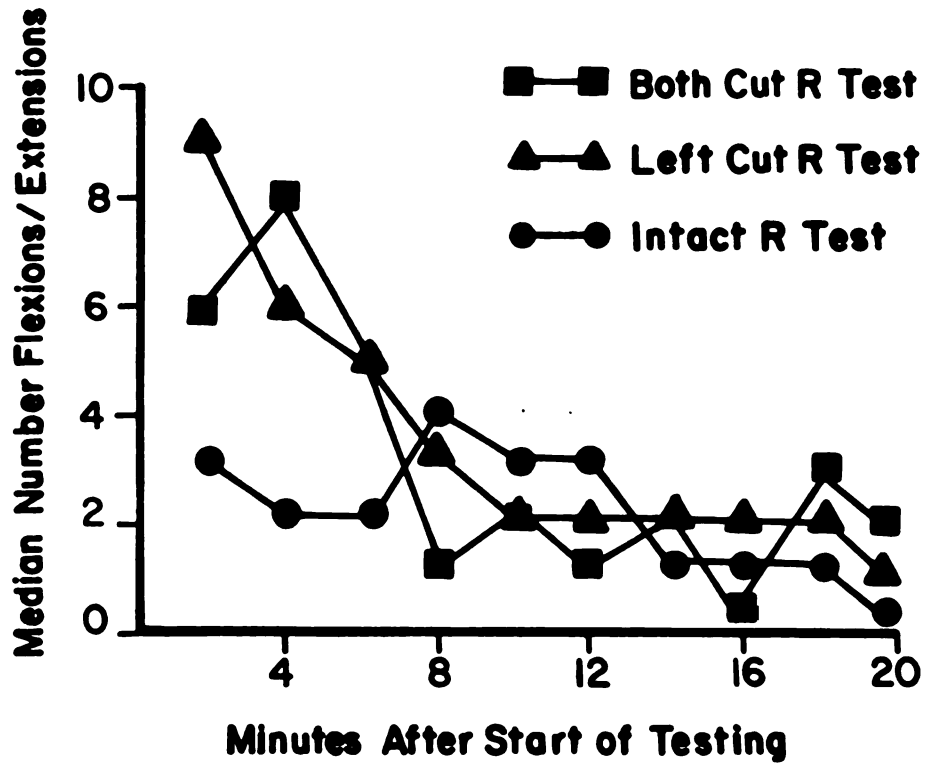


Figure 3. Leg Activity in R Test Groups. Unlike P test activity (Figure 2) there is no significant difference in R test flexion/extension activity between any pair of the three groups (Mann-Whitney U Test). However, the trend is similar to that of the P test groups; in that the Both-Cut and Left-Cut groups show more activity than the Intact group.

intrinsic differences between the training behavior of prothoracic and mesothoracic legs (to be discussed in detail in the section entitled Escape Learning); therefore such factors do not contribute to the above mentioned difference between P train (prothoracic) and P test (mesothoracic) activity.

These leg activity results reflect the fact that more activity is correlated with more shocks taken (note the similar time courses of the activity curves of Figure 2 and the shock curves of Figure 1). Thus only transfer of P information leads to decreased leg activity and decreased number of shocks taken, while transfer of R information or transfer of no information (as in the Both-Cut group) results in no decrease in activity or shocks.

The Influence of CNS Lesions on Initial Shock Levels and Leg Activity

The initial number of shocks received during testing by P animals (Figure 1A, B, C) is greater for the Left-Cut group than for the Intact ($p < .02$, min 1) and is also greater for the Both-Cut group than for the Intact ($p < .02$, min 1). There is no significant difference between the Left-Cut and Both-Cut groups ($p > .23$, min 1).

There is a similar trend among R test animals. The initial shock level is greater for the Left-Cut and Both-Cut groups than for the Intact ($p < .05$, min 1, in both cases). Again, there is no significant difference between the Left-Cut and Both-Cut group starting levels ($p > .70$, min 1).

Thus for both P mesothoracic test and R mesothoracic test animals, there is a higher initial shock level in the two groups that

have had connective(s) cut prior to prothoracic training, compared to the Intact group. Interestingly, neither the Left-Cut nor Both-Cut group differs significantly from the Intact group with regard to initial shock level during training ($p > .10$, min 1, in both comparisons). Therefore, the differences seen in initial shock level during testing probably represent changes specific to the mesothoracic level.

In order to assess whether the CNS lesions produced by cutting connective(s) in the Left-Cut and Both-Cut groups altered the activity of mesothoracic legs, thereby affecting the initial number of shocks taken during testing, P test flexion/extension activity was compared among the Intact, Left-Cut and Both-Cut groups (Figure 2). It was found that the Left-Cut group was more active than the Intact group ($p < .01$, min 1) and that the Both-Cut group was also more active than the Intact group ($p < .02$, min 1). The Left-Cut and Both-Cut groups did not significantly differ ($p > .40$, min 1). When activity analysis was carried out on the P prothoracic training behavior of each group it was found that just as in the case of initial shock levels, there was no significant effect of lesions on the activity of either the Left-Cut or Both-Cut group relative to the Intact group ($p > .10$, min 1, in the former comparison; $p > .30$, min 1, in the latter).

The flexion/extension analysis to this point has shown that for P animals, sectioning one or both of the prothoracic-mesothoracic connectives prior to training specifically increases the activity of mesothoracic legs.¹¹ This may account for the higher number of shocks

¹¹ Whether this increase is an effect of the lesion per se or of shock on lesioned animals remains to be shown.

taken by Left-Cut or Both-Cut P mesothoracic legs (relative to the Intact group) at the start of testing (Figure 1A, B, C). R animals of the three groups showed similar trends with regard to initial activity levels during testing (Figure 3). The Both-Cut R group did not differ significantly from the Intact but the Left-Cut group did ($p < .02$, min 1), indicating that lesioned R animals are more active than Intact R animals.

Transfer of a P-R Difference in the
Intact Group Is Due to the Transfer
of P Information

When transfer occurs (as in the Intact and Left-Cut groups), there is a P-R testing difference such that a P mesothoracic leg takes significantly fewer shocks than an R mesothoracic leg. However, due to the fact that a different leg is tested than that which was trained, it is not immediately apparent whether the observed P-R difference is due to facilitation of P mesothoracic testing performance (as a result of P prothoracic training in the same animal), inhibition of R mesothoracic testing performance (due to R prothoracic training in the same animal), or a combination of the two.

In order to determine the basis of the P-R difference, testing performances of right mesothoracic legs of the following three groups of intact animals were compared. The first group had its right prothoracic leg trained as P, the second group had its right prothoracic leg trained as R, and the third group had no training shocks. These groups were thus designated P, R and Naive, respectively.¹²

¹²The P and R animals are the same as those of the Intact group of Figure 1A.

Figure 4 shows that there is no difference in the testing performances of mesothoracic legs of the R-trained group and the Naive group ($p > .20$, min 1-5). However, the mesothoracic legs of the P-trained group took significantly fewer shocks than either the Naive ($p < .01$, min 1-5) or the R-trained group ($p < .02$, min 1-5). Thus, although R prothoracic leg training does not appear to influence the testing performance of a mesothoracic leg, P prothoracic leg training has a facilitating influence. These results are in contrast to the data of Harris (1971) which indicate that R prothoracic training interferes with the ability of a mesothoracic leg to perform as a P leg during testing.

Escape Learning

The training procedure used in these experiments allows for the measurement of both escape and avoidance components in any learning and transfer which occurs. Avoidance learning can be measured as a decrease over time in number of extensions of the animal's leg lead into the saline, whereas escape learning is measured by the change over time in the rapidity with which the leg lead is withdrawn from the saline once shocks are initiated. The escape component will thus be represented as a decrease in the number of pulses initiated per flexion/extension during successive extensions of the leg.

An examination of the number of shocks per flexion/extension taken by the prothoracic or mesothoracic legs of intact (i.e., headless, no connectives cut) P animals (Figure 5) shows a significant escape component for the prothoracic leg from the first to the second flexion/extension ($p < .05$, $N = 9$) but not so for animals in

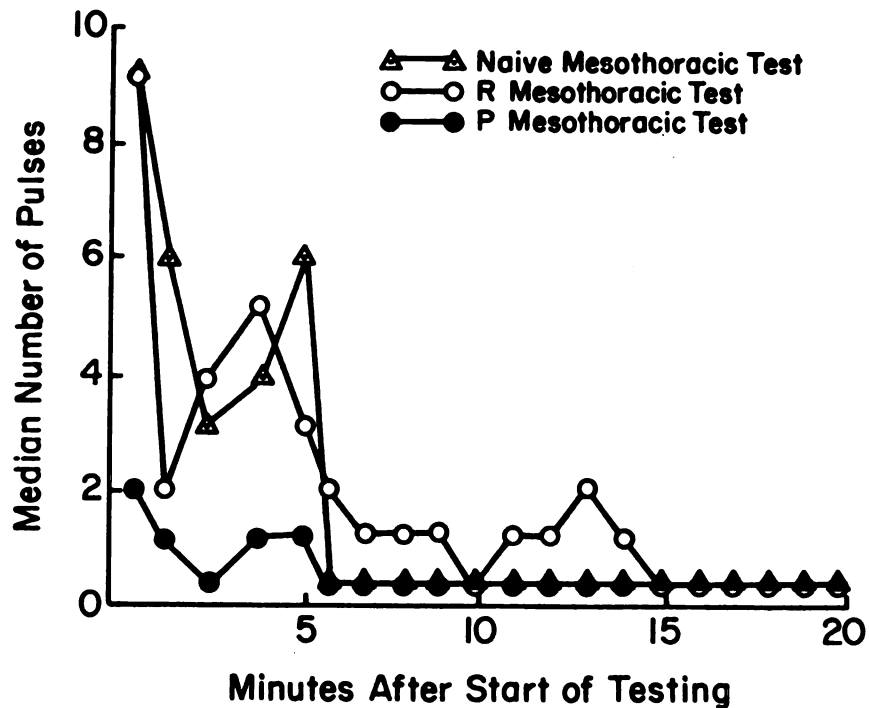


Figure 4. Transfer of a P-R Difference in the Intact Group Is Due to the Transfer of P Information. There is a significant difference between testing performance of the mesothoracic legs of animals with previous P prothoracic leg training and the testing performance of Naive animals (animals with no prior training) ($p < .01$, min 1-5; Mann-Whitney U test. $N = 20$ animals). There is also a difference between P-trained and R-trained animals ($p < .02$; min 1-5. $N = 10$ pairs). However, no difference was found between the Naive and R-trained groups ($p > .20$, min 1-5; Mann-Whitney U Test. $N = 20$ animals).

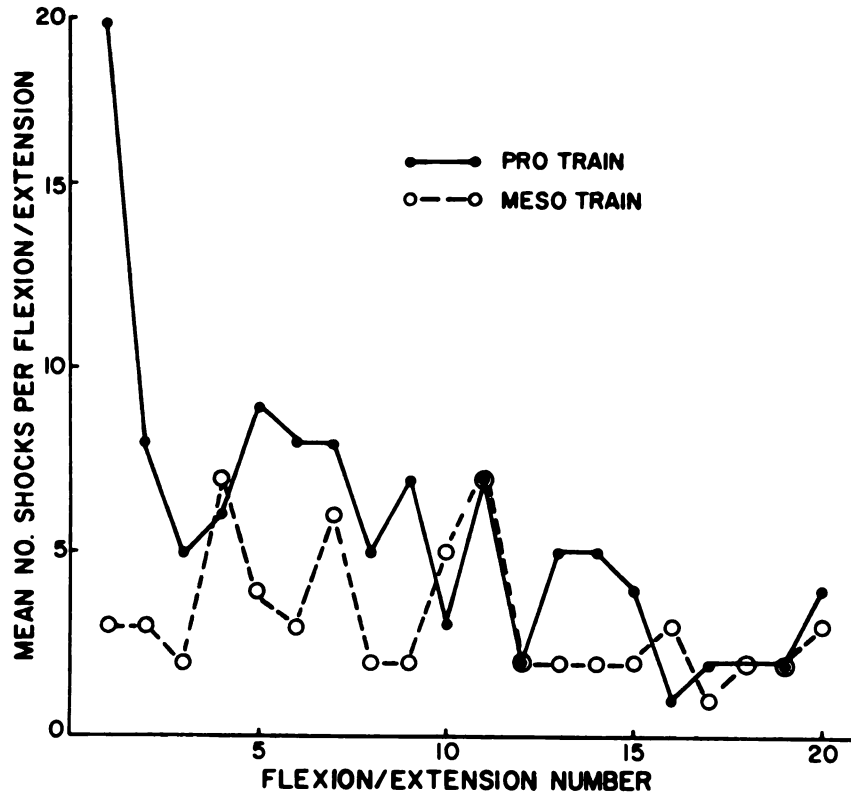


Figure 5. Escape Learning Is Seen in Prothoracic But Not Mesothoracic Leg Training. During prothoracic training, progressively fewer shocks are taken per flexion/extension, with the greatest decrease occurring between the first and second flexion/extension ($p < .05$, $N = 9$). Mesothoracic leg training shows a relatively constant and low number of shocks per extension. There is a significant difference in the number of shocks per flexion/extension between the prothoracic and mesothoracic legs for the first ten flexion/extensions ($p < .02$, Mann-Whitney U test. $N = 18$). The twentieth flexion/extension occurs on the average by the fifth minute of training.

which the mesothoracic leg was trained. There was a significant difference between the number of shocks per flexion/extension for the prothoracic and mesothoracic legs over the first ten flexion/ extensions, representing approximately the first 2 min ($p < .02$, $N = 18$). When the testing behavior of P and R mesothoracic legs were examined, no evidence of escape learning was found in any group; Intact, Left-Cut or Both-Cut ($p > .20$ in all cases, $N = 60$; six groups of ten animals each).

Disterhoft (1972) found no evidence of escape learning in the prothoracic leg training of non-decapitated cockroaches, however he compared the average number of shocks for the first five flexion/ extensions to that for the last five within the first 10 min of a 30 min training period. Since our results (Figure 5) indicate that most of the escape learning has occurred by the second flexion/extension, it is possible that Disterhoft's analysis obscured the escape effect through averaging over the first five flexion/extensions. Lovell and Eisenstein (1977) reported the presence of an escape component in the prothoracic training of non-decapitated animals. Similarly, Eisenstein (unpublished, personal communication) found that in four separate experiments with headless animals in which a prothoracic leg was used for both training and testing, escape learning was seen from min 1 to 2 in all P groups during training and in all P and R groups during testing. These results, together with those of the present experiments, indicate that prothoracic training includes an escape component in headless and non-decapitated cockroaches.

The original purpose in distinguishing avoidance and escape learning components was to see if both showed evidence of transfer. Since no escape learning has been seen in mesothoracic legs with or without previous prothoracic leg training, it is not possible to distinguish lack of transfer of an escape component from inability of the mesothoracic ganglion to support escape learning.

To further assess whether the inability of the mesothoracic leg to demonstrate escape learning represents an intrinsic property of the ganglion or is an artifact of either recording technique or the different mechanics of movement for prothoracic and mesothoracic legs, the legs were compared with respect to number of shocks per min and flexion/extensions per min during training. The prothoracic and mesothoracic legs behaved similarly in training; there was no significant difference between them over the first min or the first five min, for either shocks initiated ($p > .58$, min 1; $p > .46$, min 1-5) or number of flexion/extensions ($p > .28$, min 1; $p > .72$, min 1-5). Therefore, the difference shown in Figure 4 must reflect the fact that the prothoracic ganglion can support escape learning whereas the mesothoracic ganglion cannot.

In view of the complexity of the behaviors mediated by the prothoracic ganglion (grooming, feeding), it is quite possible that this ganglion may be capable of supporting a greater variety of learning and more complex learning than more posterior ganglia.

Transfer as Measured During Training

In order to determine if evidence of transfer could be observed during the training period itself rather than measuring it as

a P-R difference during the testing period, the following procedure was used: the right prothoracic leg of an intact animal was trained as P. During the training period the position of the same animal's right mesothoracic leg was recorded but no shocks were given to it. Figure 6A shows that prothoracic training proceeds normally, with progressively more leg flexion and thus a decline in number of shocks received. In addition, the mesothoracic leg shows increasing and maintained flexion after about a 10 min lag ($p < .03$, comparing min 1-10 with min 11-20). This change in mesothoracic leg behavior was found to be entirely an avoidance phenomenon and contained no escape component.

The reverse of the above experiment also was done. The right mesothoracic leg was trained as P and the position of the right prothoracic leg recorded during this training. In this case (Figure 6B) the mesothoracic leg generated a training curve indicating increased leg flexion but the prothoracic leg showed no evidence of increasing flexion and instead, maintained a completely extended position throughout the entire training period.

It thus appeared that transfer could be measured during training in the anterior to posterior direction but not vice versa. We have called this "behavioral rectification" of transfer.

Discussion

The results of the present experiments on interganglionic transfer confirm the previous finding of Harris (1971) that information related to prothoracic leg training is transferred and manifested as a significant P-R difference at the mesothoracic level during

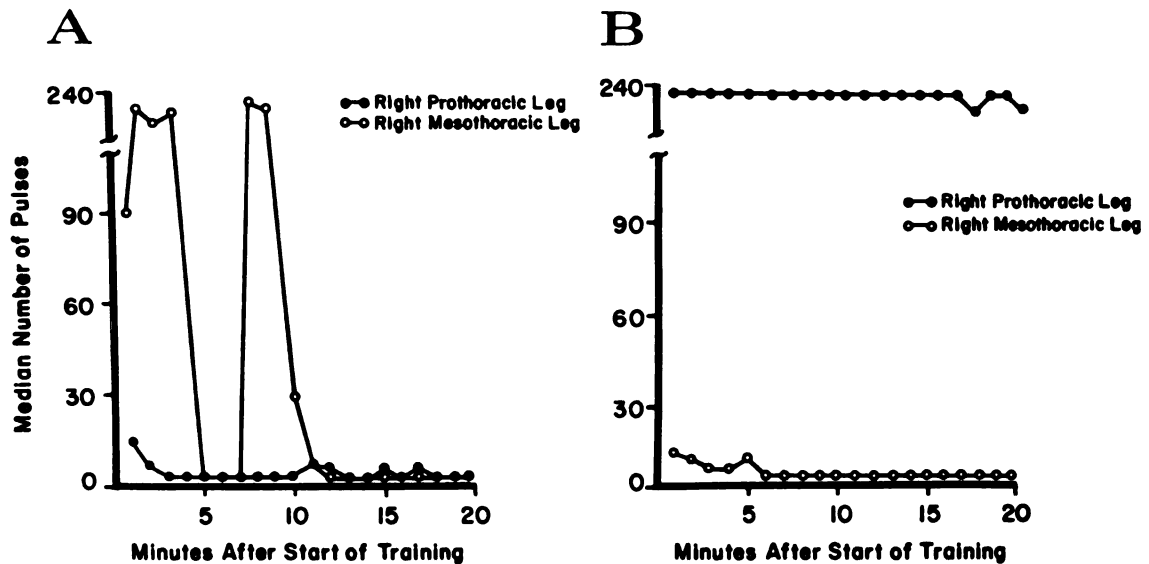


Figure 6. Transfer as Measured During Training.

A--When a right P prothoracic leg is trained it becomes progressively more flexed and, therefore, takes fewer shocks over time. There is a corresponding behavior change seen in the right P mesothoracic leg, such that it is significantly more flexed during minutes 11-20 than minutes 1-10 ($p < .03$, $N = 9$ pairs).

B--In contrast to the above, when a right P mesothoracic leg is trained there is no such change in the right P prothoracic leg behavior.

testing (in the Intact and Left-Cut groups). Lack of transfer in the Both-Cut group indicates that transfer is mediated by the ventral interganglionic nerve connectives.

* The presence of only one connective is sufficient for transfer to occur, as shown by the significant P-R testing difference in the Left-Cut group. This confirms a previous finding by Harris (1971) that either the left or right connective alone could mediate right prothoracic to right mesothoracic transfer. Thus, whatever information is represented in each connective, it is redundant with respect to ability to produce a significant P-R testing difference.

By cutting both connectives following prothoracic leg training and still finding a significant P-R difference during mesothoracic leg testing, Harris (1971) concluded that information necessary for the generation of a P-R testing difference is transferred during the training period. The present finding that during prothoracic leg training a behavioral change (increased flexion) occurs in the mesothoracic leg after 10 min (Figure 6A) suggests, together with previous cold-block studies (Harris, 1971), that much of the transfer occurs early in training. As stated in the section LITERATURE REVIEW, Behavioral Experiments, Pritchatt (1970) found that 6 min of training, which is sufficient to produce an asymptote in number of shocks taken, resulted in no maintained change in position of that leg after training shocks were terminated. However a training period of 20 min did result in a maintained change in position. This finding may apply to the time course of transfer as well: although the external leg behavior may have changed all it is going to (both in the prothoracic leg undergoing training and in the mesothoracic leg that shows

flexion after 10 min), the few shocks that are taken after the first 10 min of training may be of great importance in reinforcing and maintaining the behavioral change that has already occurred.

From the above discussion it is evident that until the minimum training time needed for significant transfer is determined with greater certainty, one must take 30 min, the full extent of the training period, as a conservative estimate.

The time course of transfer has been dealt with at some length because of its important relationship to a consideration of the mechanism(s) of transfer. Specifically, one can ask if the information that transfers down the connectives from the prothoracic to mesothoracic ganglion is electrical or molecular in nature.

Since transfer is complete by the end of a 30 min training period (at the latest), any molecular mechanism must be able to express itself within this time. Axoplasmic flow is the usual means by which molecular components of nerve cells (e.g., proteins, neurotransmitters, etc.) are transported both anterogradely and retrogradely in nerve fibers. Since the length of the prothoracic-mesothoracic connectives is about 5 mm, the minimum flow rate necessary for a molecule to express itself by the end of training is 5 mm/30 min, or 240 mm/day. Smith (1971) found that the fastest reliably observed flow rate of proteins in cockroach connectives is only 72 mm/day. At this rate a minimum of 1.7 hr would be needed for the expression of a molecular component. Thus present evidence suggests that axoplasmic flow is not capable of mediating transfer. Smith's study is the only one that has been made of axoplasmic flow in connective fibers, and until further studies confirm or deny 72 mm/day as the highest flow rate, and

whether other molecules besides proteins travel at this rate, the negative conclusion above should be made tentatively.

Another molecular mechanism which logically could mediate transfer is the diffusion of a molecule from the prothoracic to mesothoracic ganglion via the hemolymph. Conceivably, P animals would produce such molecules whereas R animals would not. The immediate problem with this humoral hypothesis is one of time course: in the only study presenting evidence that a diffusible substance influences transfer, Pak and Harris (1976) found that very long training times (six 30-min sessions) were needed to obtain a significant P-R difference.

Eisenstein has conceived of the possibility that a humoral mechanism might reveal itself in parabiotic experiments, as Bodenstein (1955) showed for the case of limb regeneration in cockroaches. Thus if two roaches were joined during training such that their hemolymph was in contact, would the training of one cockroach transfer to the parabiotic partner and show up in a later testing period? Another similar means of testing the humoral hypothesis would be to inject the hemolymph of a trained animal into an untrained one and observe the testing behavior. If an effect were found, the active substance in the hemolymph could be determined by successive fractionations and injections. For the present, as in the case of axoplasmic flow, the existence of a humoral component in transfer seems unlikely.

Electrical activity appears to be the most probable (or at least the dominant) means by which transfer occurs. Due to the 5 mm distance between the prothoracic and mesothoracic ganglia, passive conduction of graded potentials would be of little if any importance,

as such signals would undergo severe attenuation. Therefore action potentials would seem to be the means by which information is transferred in the connectives.

As Eisenstein (1972) has noted, there are three immediately obvious types of electrical activity which can be viewed as candidates for a transfer mechanism: (1) A corollary discharge of the primary sensory (shock) input to the prothoracic ganglion is transferred posteriorly via the interganglionic connective(s); (2) There is independent transfer of sensory and motor information from the prothoracic to the mesothoracic ganglion, where they are associated; (3) There is transfer of a pre-formed input-output association from the prothoracic to mesothoracic level.

The major difference in the above alternatives is that, in the first two, any integration or association would occur independently at the prothoracic and mesothoracic levels, whereas, in the last alternative, integration would occur at the prothoracic level and then be transferred posteriorly. On the basis of behavioral data alone, it is not possible to judge the relative merits of the above three possibilities.

Whether transfer occurs is independent of the absolute level of activity shown or shock received. For example, the Left-Cut P test group is not significantly different in either shocks received (Figure 1) or activity level (Figure 2) from the Both-Cut P test group, yet the former shows significant transfer and the latter does not. Conversely, the Left-Cut P test group does significantly differ from the Intact P test group in shock level and activity, yet both groups show significant transfer. Interestingly, in the R test

groups, although there is no transfer, the lesioned (Left-Cut and Both-Cut) groups show higher initial shock level (Figure 1) and activity (Figure 3) than the Intact group. Thus cutting one or both connectives causes increases in mesothoracic leg shock level and activity which are independent of whether or not transfer also occurs.

The increase in leg activity is only seen posterior to the cut connective. Whereas the mesothoracic legs showed greater activity, there was no increase in shock level or activity level in prothoracic legs. This unidirectionality strongly suggests that the increased activity is not caused by a widespread injury response, as such a response would be expected to propagate in both directions, affecting the prothoracic as well as the mesothoracic ganglion. Furthermore, it is unlikely that there are localized injury responses in specific connective fibers that normally carry impulses posteriorly from the prothoracic to the mesothoracic ganglion, as such responses are seen immediately after a connective is severed and disappear within one or two minutes (Weiant, 1958); the activity changes reported here were seen approximately 90 min after connectives were severed.

A plausible explanation for the unidirectional increase in mesothoracic leg activity is that there has been an interruption of descending inhibitory input to the mesothoracic ganglion. Weiant (1958) has shown that severing the cervical (suboesophageal to prothoracic) connective(s) leads to higher discharge rates in metathoracic motor neurons due to release from inhibition and that motor neurons on one side of the ganglion are affected similarly by severance of either connective. Rowell (1964) has similarly demonstrated a descending inhibitory influence of the suboesophageal

ganglion on a grooming response involving the prothoracic legs of the locust. Thus, previous work indicates that there is descending inhibitory input to thoracic leg motor neurons and that the influence of this input can be detected behaviorally. Our results showing increased leg activity with severance of connectives are entirely consistent with this explanation.

Descending input may be of importance in another respect; the present results (Figure 6) indicate that there is behavioral rectification such that prothoracic leg training leads to a behavior change (greater flexion) in the mesothoracic leg after 10 min, but that mesothoracic leg training results in no such corresponding change in prothoracic leg behavior. The lack of an effect in the posterior to anterior direction may either be due to ascending information about training events at the mesothoracic level not being received by the prothoracic ganglion or not being utilized. This question is further considered in the section ELECTROPHYSIOLOGY EXPERIMENTS below.

Extrapolating from these training results showing rectification to a testing situation, one might predict that P-R testing differences would occur less readily or not at all in an experiment where mesothoracic leg training is followed by prothoracic leg testing.

The present case of rectification of information transfer may represent an invertebrate form of cerebral dominance in which anterior regions of the CNS exert control or influence over more posterior regions.

ANATOMY EXPERIMENTS

Introduction

The interganglionic connectives mediate transfer of information related to learning, as found by myself (see BEHAVIORAL EXPERIMENTS section) and by Harris (1971). The presence of either connective alone is sufficient for transfer to occur and it makes no difference whether it is the ipsilateral or contralateral one (Harris, 1971).

Although behavioral data alone can tell us the gross location of the pathways by which transfer occurs, it cannot (as stated previously) distinguish between information transmitted by direct sensory and motor pathways (collaterals of sensory and motor neurons) and information transmitted by interneuron pathways. For this reason, several anatomical questions arise. First, are there direct projections from prothoracic sensory and motor neurons to the mesothoracic ganglion? Secondly, if there are no such direct projections, do connective fibers exist which have branches in the prothoracic and mesothoracic ganglia, thereby providing a possible pathway for interganglionic communication? In either case there must be anatomical redundancy since either connective can mediate transfer. Thus in the first case we would expect to see direct projections in both connectives, and in the second case we would expect to see connective fibers having ipsilateral and contralateral branches.

In order to find out if direct and indirect pathways exist, and to determine the general structural features of the pathways of transfer, sensory, motor and connective nerve fibers were stained by the cobalt method (Pitman et al., 1972).

Nerve 5 is the only nerve which innervates leg regions distal to the trochanter (Pipa and Cook, 1959) and therefore it must be the means by which shock input to the tibia-femur reaches the ganglion. Thus nerve 5 was stained in the femur to determine the course of the sensory fibers of interest. Nerve 5 also contains the extensor motor neurons known to be active in leg movements of the kind seen in leg lift learning (Pearson, 1972; Pearson and Iles, 1973). Thus it was also stained to determine the central projections of the extensor motor fibers of interest.

Nerve 6Br4 contains the flexor motor neurons known to be most active in leg movements of the kind associated with leg lift learning (Pearson, 1972; Pearson and Iles, 1973). Therefore it was stained to determine the central projections of the relevant flexor motor neurons.

The connectives were stained to see if nerve fibers in them have branches in the ganglia, and to determine the ipsilateral/contralateral extent of any such branching.

Because cobalt staining of nerve 5 in the femur could not stain sensory fibers without also staining some motor fibers, an alternative method was sought by which the central projections of sensory fibers in nerve 5 could be identified. Fortunately, the cell bodies of most sensory neurons in invertebrates are located peripherally, near the structures which they innervate. The cell

bodies of motor neurons are located centrally, in the ganglion. Thus if a leg is sectioned, Wallerian degeneration (degeneration distal to the cell body) will affect only those sensory fibers whose cell bodies are distal to the cut. Lamparter et al. (1969) showed that this situation could be used to trace the degeneration products of sensory fibers with phase and electron microscopy. Hence, I decided to adopt such methods in the present case, to selectively trace the course of sensory fibers.

Methods and Materials

Cobalt Staining

Fifty cockroaches (male and female) were used. Late stage nymphs tended to show better uptake of cobalt than adults, though both could be used with success. In vitro staining of excised portions of the nervous system was tried but gave much less satisfactory results than in vivo staining. The best method proved to be in vivo staining at about 4° C (in a refrigerator). Bacon and Altman's (1977) silver intensification procedure was routinely used, as it greatly enhanced the intensity of stained profiles.

An animal to be stained was first anesthetized using CO₂ gas bubbled through water, then mounted ventral side up on a clay block. The legs were restrained using pieces of clay. For staining of connectives, the soft ventral tissue was penetrated and the connective to be filled was cut close to the ganglion from which fibers were to be traced. The cut end was held in a pair of forceps and vaseline was injected beneath and around it, forming a well through which only the cut tip of the connective protruded. This well was filled with a

drop of distilled water to dilate the nerve fibers in the connective and to check for leakage. After a few minutes the distilled water was withdrawn and replaced with a drop of 3-7% cobalt chloride (containing 0.013 g bovine serum albumin and 2 ml dimethyl sulfoxide per 100 ml cobalt chloride). The top of the well was sealed over with vaseline to prevent drying and the preparation placed in the refrigerator for 24-72 hrs.

At the end of the staining period the preparation was taken from the refrigerator and the well uncovered and remaining cobalt solution withdrawn. No CO₂ anesthesia was necessary since the animals had been exposed to prolonged low temperature (they were usually still alive). The vaseline was picked away with a probe and the general area flooded with saline. The ganglia and connective of interest were removed, care being taken to remove as much fat and trachea as possible and to cut unwanted nerves close to the ganglia.

The tissue was next placed in a beaker containing 20 ml saline and two drops of concentrated ammonium sulfide. After 15 min, during which a black cobalt sulfide precipitate forms in filled processes and cell bodies, the tissue was rinsed in saline and put into Carnoy's fixative (60 ml of 100% ethanol, 30 ml chloroform, and 10 ml acetic acid) for 15-60 min.

After fixation the tissue was hydrated (in preparation for silver intensification) in successive 15 min changes of 95, 90, 70, 50, 30, and 15% ethanol. Then came a change of distilled water followed by a change of warm distilled water (60° C in an oven). The tissue was then pre-soaked for 1 hr in 10 ml of "solution A," which consisted of: 10 g sucrose, 3 g gum arabic, 0.8 g citric acid, and

0.17 g hydroquinone, all added to 100 ml of hot buffer. The buffer was made up so as to be pH 2.6 at 60° C and consisted of: 17.76 ml 0.1 M citric acid and 1.24 ml of 0.2 M disodium phosphate.

Next, 1 ml of 1% silver nitrate (freshly made) was added to the pre-soak solution and the preparation kept in the oven in darkness. The duration of this step (which involves the reduction of silver nitrate to silver through a reaction with hydroquinone that is catalyzed by heavy metals such as cobalt; Bacon and Altman, 1977) depended on several factors including age and pH of solution A, and density of cobalt filling. Generally, for a solution A of pH 2.5-2.7 and less than four days old, times of 20-35 min were used. Intensification is optimum when the cobalt-filled profiles appear jet black against a background of light golden color.

Preparations were checked by taking them from the silver nitrate solution into warm water and observing through a dissecting microscope. If more intensification was needed, they were returned to a fresh solution A/silver nitrate mixture. After the final intensification the tissue was placed in distilled water and then dehydrated in an ascending alcohol series, with two changes of 100% ethanol at the end. Next came a change in a one-to-one mixture of 100% ethanol and oil of wintergreen followed by a change in 100% oil of wintergreen. This was found to be the best clearing agent among several, including cedarwood oil, methyl benzoate, toluene and xylene.

Preparations were mounted in the following manner: a thick coating of Pro-Texx mounting medium (Scientific Products) was put around the perimeter of a slide, leaving a space in the middle. After 5 min (for curing) a few drops of oil of wintergreen were put

in the space, along with the tissue, and the slide was gently cover-slipped. Slides must be stored in a flat position.

For staining of peripheral nerves the soft cuticle was penetrated and the nerve to be filled was cut, a well built, and the procedure above followed. For staining of nerve 5 in the femur all other nerves were cut close to the ganglion and the leg was sectioned. Nerve 5 was not drawn into a well, but rather the leg was bathed in cobalt solution inside a wall of vaseline.

Degeneration as Viewed with Phase and Electron Microscopy

Adult male animals were used in all cases. A total of ten animals was studied. The right prothoracic or right mesothoracic leg was cut at the femur-tibia junction and the animal housed alone with food and water for 3-7 days. Then the animal was anesthetized using CO² bubbled through water, and the ganglia and connectives of interest removed. This tissue was placed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (also containing 0.5% dimethyl sulfoxide), pH 7.2-7.4. After 3 hrs the tissue was rinsed in 0.15 M buffer (two changes, 15 min each) and then placed in 2% osmium tetroxide in 0.2 M buffer for 2-3 hrs. At this stage the tissue was transferred to new vials and, after three rinses with distilled water (15 min each), was soaked 5-18 hrs in 4% uranyl acetate (in Triton X, pH 4.5). Next came three rinses in distilled water and dehydration in 70, 90, 95, 100, 100 and 100% ethanol (15 min each change, except the final 100% change, which was one hour. This was found to be a critical step). The tissue was then placed in a two-to-one mixture of 100% ethanol and Spurr's embedding medium (10 g ERL,

6 g DER and 26 g NSA mixed together, then 0.4 g DMA or S1 added) and rotated gently for 3-13 hrs. This was followed by one-to-one and one-to-two mixtures of ethanol and Spurr's, also rotated for 3-12 hrs. Finally the tissue was spun in two changes of 100% Spurr's medium (2 hrs each change). The tissue was then oriented and embedded in 100% Spurr's and baked at 65° C for 1-2 days in an oven.

From some blocks thick sections (4 μ m) were cut and observed with phase microscopy to determine the location of degeneration profiles. The area of interest could then be trimmed and thin sections cut on a Sorvall ultramicrotome. These were mounted on copper grids.

For other blocks, serial thick sections were cut and mounted on glass slides. These could then be viewed with phase microscopy, and areas of degeneration traced through a drawing tube.

Results

Projections of Nerve 5

Figure 7 gives a dorsal view of a prothoracic ganglion in which the right N5 was filled in the femur for 48 hours (mesothoracic N5 fills gave similar results). Some of the stained fibers are attached to cell bodies in the ganglion, indicating that they are motoneurons. But a large number of fibers were found to have no apparent attachments and must therefore be branches of sensory neurons, whose cell bodies are in the periphery.

A cluster of large cell bodies (~50 μ m) is located on the ventro-lateral surface near the ascending ipsilateral connective. Among the identified cells known to be positioned here is D_f, the fast excitatory extensor motoneuron (Pearson and Fournier, 1973).

Two other ventrally located cell bodies, medium ($\sim 30 \mu\text{m}$) in size, are seen near the midline; these appear to correspond to the common inhibitors D_1 and D_2 of Iles (1976). Three dorsally located cell bodies were found. One is large and located halfway between the midline and the exit of N5; the other is a smaller cell body (barely visible) just lateral to the anterior cluster of large cells. The slow excitatory extensor motoneuron, D_5 , has its cell body situated on the dorsal surface near the lateral edge of the ganglion between N5 and the ipsilateral descending connective, as found by Iles (1976).

Just after the stained fibers enter the ganglion, they separate into dorsal and ventral bundles which tend to form planar sheets in the corresponding neuropile regions. The middle of the ganglion (in terms of its dorso-ventral extent) is left relatively free of branches. This segregation of branches into dorsal and ventral sheets corresponds to the long-standing observations that branches of motor fibers lie dorsally whereas those of sensory fibers lie ventrally (see Gregory, 1974 and Bullock and Horridge, 1965 for more complete discussions). Both groups are confined to the ipsilateral half of the ganglion (Figure 7).

Degeneration studies which focused specifically on the sensory projections of N5 from the femur confirmed the suggestion made above that these projections are ipsilateral and ventral. Figure 8A shows an example of ipsilateral degeneration products in a mesothoracic ganglion after a survival time of 4 days. This picture is of the posterior ventral region of the ganglion at the ventral-most level of the attachment of N5 to the ganglion, and corresponds to section F of Figure 10. A section through the same location contralaterally

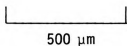
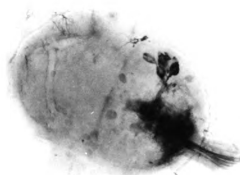


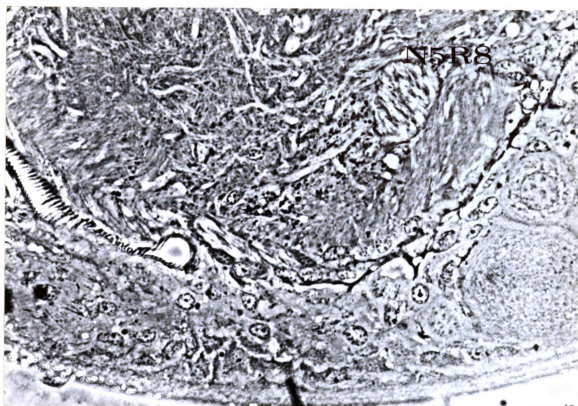
Figure 7. Cobalt Staining of Nerve 5. Dorsal view of a prothoracic ganglion; anterior toward the top. Nerve 5 was stained in the femur. Several cell bodies are visible as well as extensive branching of incoming nerve fibers. All stained components of nerve 5 are contained within the ipsilateral half of the ganglion and no processes are seen to leave the ganglion through the connectives.

Figure 8. Degeneration in the Ganglion Following Leg Amputation Is All Ipsilateral.

A--Horizontal section through the right half of a mesothoracic ganglion at the level of the ventral-most attachment of N5 to the ganglion. The right mesothoracic leg had been amputated at the femur 4 days previously. The posterior margin of the ganglion is visible at the bottom. A border of trachea and glial processes separates the outer cell body layer from the inner neuropile. The midline of the ganglion is represented by the right edge of the figure.

A fairly continuous series of round black profiles extends postero-laterally from N5R8 (seen as the large oval bundle of fibers cut in cross-section at the uppermost extent of the black profiles), thus appearing to correspond to degeneration in N5R11.

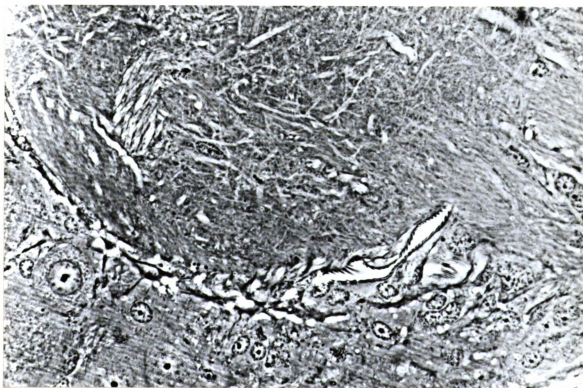
B--A horizontal section through the left half of the same ganglion as above. No degeneration profiles are seen. Midline toward the left edge.



A

100 μm

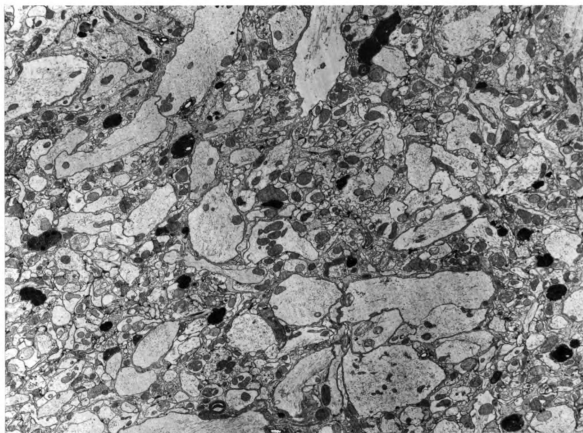
B



(Figure 8B) reveals a lack of degeneration, and therefore, lack of any crossing fibers (likewise, no degeneration was seen in normal tissue). The topographic map of degeneration to be presented (Figure 10) makes this clearer.

The degeneration products seen in the horizontal section of Figure 8A represents neurites in cross-section, but for sections in the same plane as N5 one can see degeneration along the length of fibers. Figure 9 shows the appearance of degeneration products as viewed through the electron microscope at low power. At a survival time of 4 days, as in the present case, degeneration profiles are dense black in appearance. However at shorter survival times such as 2 days, one can occasionally observe dark profiles that contain vesicles and have close apposition to other neurites, indicating that they are degenerating presynaptic terminals. Therefore at least some of the dark black profiles seen at four days are probably presynaptic terminals undergoing later stages of degeneration.

The topographic extent of degeneration was mapped in prothoracic and mesothoracic ganglia using serial horizontal thick ($4\text{ }\mu\text{m}$) sections, and was found to be essentially the same in both cases. Figure 10B-J illustrates the results for a mesothoracic ganglion. All the degeneration is ipsilateral and the bulk of it is at the ventral-most level of N5, extending posteriorly along the cell body-neuropile border from the lateral edge of the ganglion, where N5 exits, toward the midline. This corresponds very well to Gregory's (1974) N5R11, a bundle of 700 small and very small sensory fibers (see his Figure 28). The present results are also consistent with those of Lamparter et al. (1979) in the ant, in that sensory fiber



20 μ m

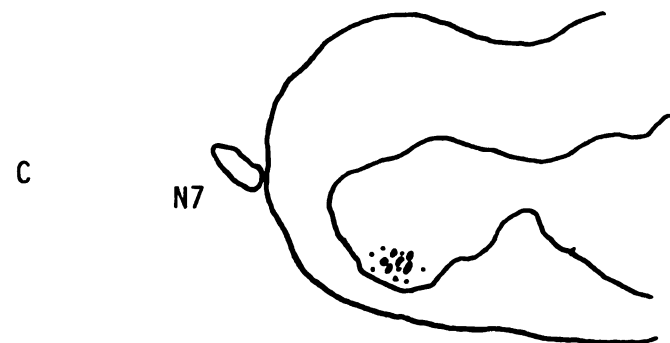
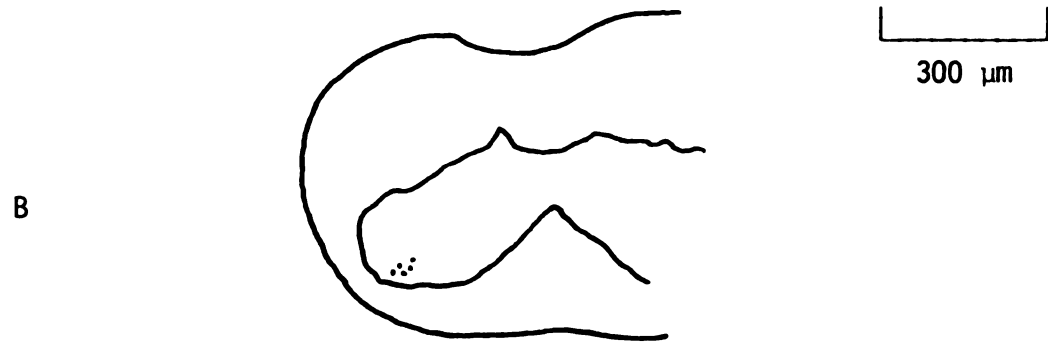
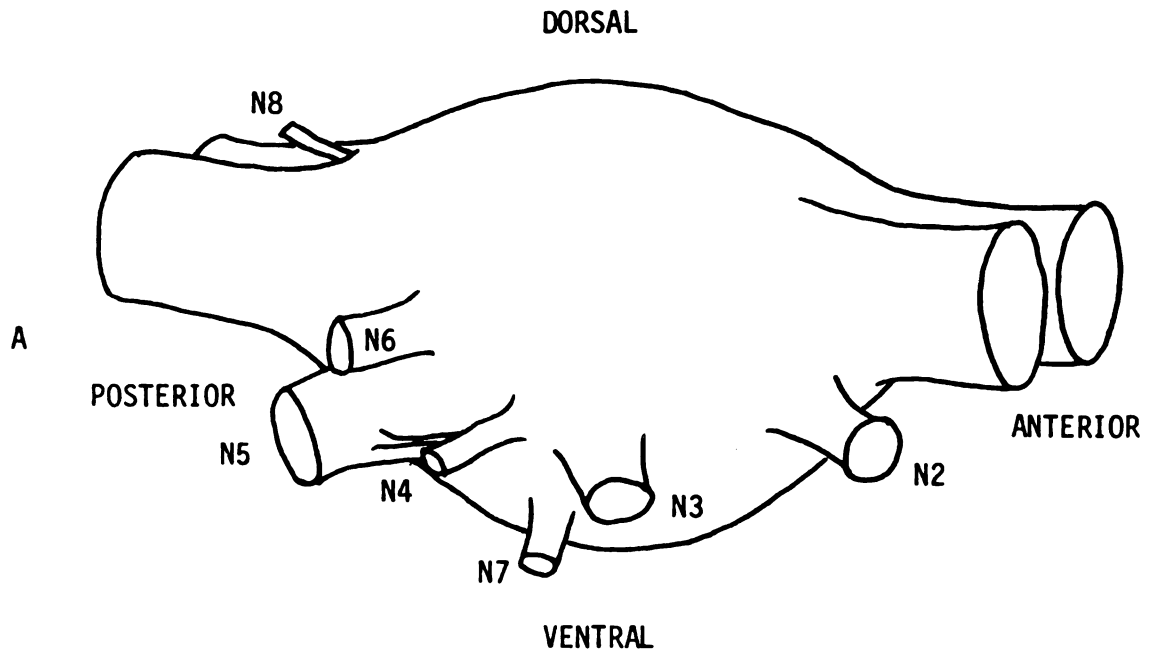
Figure 9. An Electron Micrograph Showing an Area of Degeneration in a Mesothoracic Ganglion. 4 1/2 day survival time.

Figure 10. Topographic Distribution of Degeneration in the Mesothoracic Ganglion Following Mesothoracic Leg Amputation.

A--Mesothoracic ganglion as viewed from the right side. Dorsal is toward the top, anterior to the right. The bases of the peripheral nerve roots and interganglionic connectives are shown. After Gregory (1974).

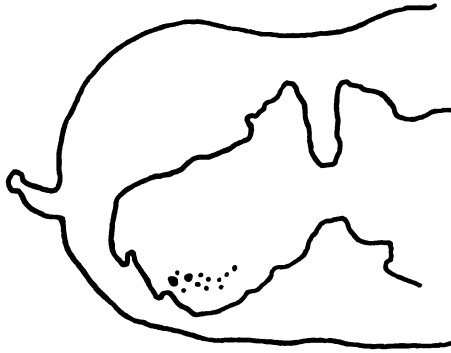
B-J--Topographic distribution of degeneration profiles in a mesothoracic ganglion. The right mesothoracic leg had been amputated 4 days earlier. Tracings were made from camera lucida projections of serial horizontal 4 μ m sections. Every fourth section in the region of degeneration is shown here. The sections proceed from ventral to more dorsal levels.

Degeneration is confined to the ipsilateral posterior ventral portion of the ganglion, at a ventro-dorsal level between N7 and N5.



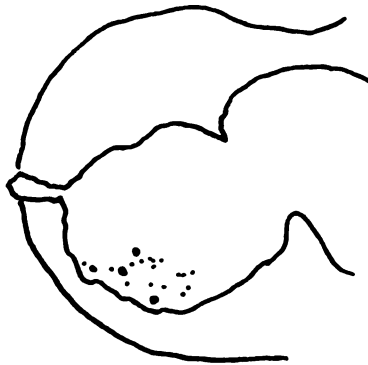
D

N7

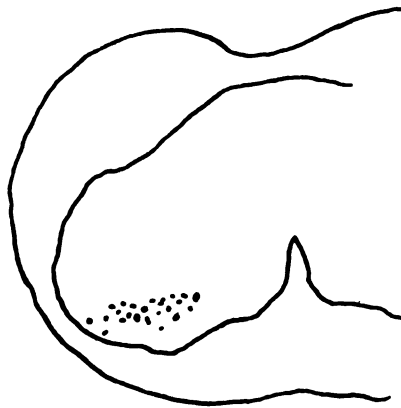


E

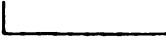
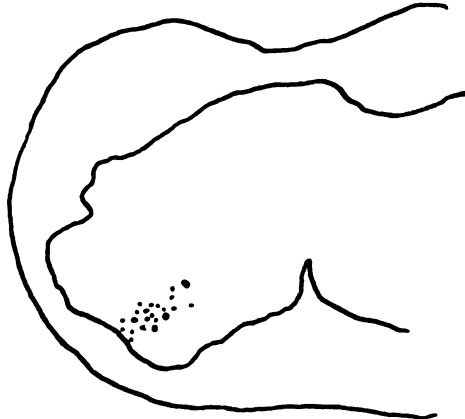
N7



F

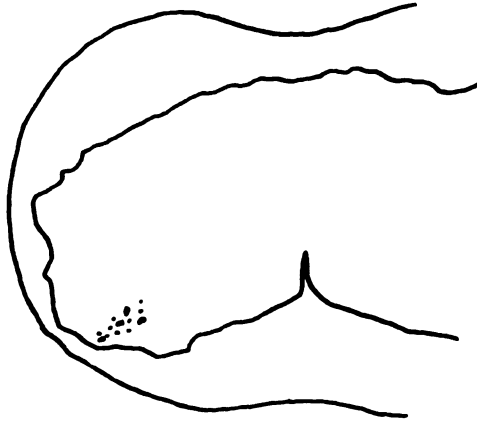


G

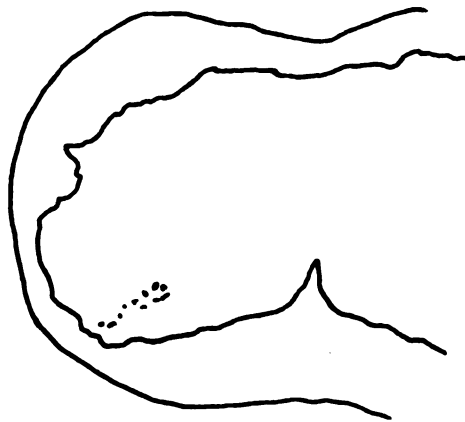


300 μm

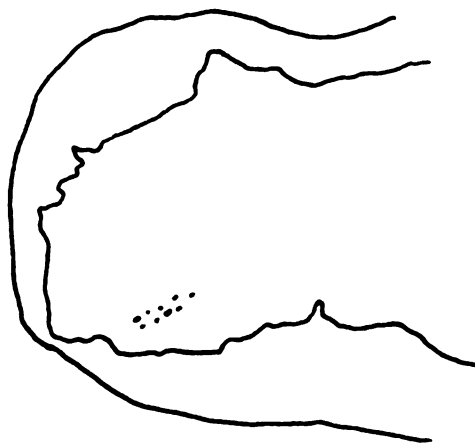
H



I


300 μm

J


N5

degeneration is confined to the ipsilateral side of the ganglion. Furthermore, Gregory (1974) found that all sensory fibers in the mesothoracic ganglion have ipsilateral projections.

Staining of Nerve 6

Figure 11 gives a ventral view of a prothoracic ganglion in which the right N6B was filled for 48 hrs (mesothoracic ganglion N6B fills gave similar results). Four or five large cell bodies are seen to be located anteriorly on the ventral side, clustered near the edge of the ganglion at the level of N2 and N3. One large cell body is found posteriorly between N6 and the ipsilateral descending connective. Two smaller cell bodies (not visible here) are located just anterior to the exit of the ipsilateral descending connective. This distribution of cell bodies is similar to that found by Pearson and Fournier (1973) in the metathoracic ganglion and to Gregory's (1974) results in the mesothoracic ganglion.

The branches of the flexor motoneurons of N6B are found dorsally and are wholly contained in the ipsilateral half of the ganglion. The two main branches seen in Figure 11, just after N6B enters the ganglion, appear to correspond to dorsal roots N6DR1 and N6DR3 of Gregory (1974) for the mesothoracic ganglion. Similarly to the present results, Gregory (1974) reported no contralateral branches of N6B.

Staining of Prothoracic-Mesothoracic Connective Fibers

When the right connective is stained just anterior or posterior to the mesothoracic ganglion and the prothoracic ganglion is examined,



Figure 11. Cobalt Staining of Nerve 6. Ventral view of a prothoracic ganglion; anterior toward the top. Nerve 6B was stained. Cell bodies and branches of N6B are visible in the ganglion. All of these components are confined to the ipsilateral half of the ganglion and no processes leave via the connectives.

one always finds several cell bodies in characteristic locations and a similar pattern of branching. As illustrated in Figure 12A, three large cell bodies are found on the contralateral dorso-lateral surface just lateral to the descending connective. Also found on the contralateral side is an anterior cluster of cells. The most lateral of these is located on the dorsal surface but all the others are found on the ventral surface.

Several smaller cell bodies were found on the posterior ventral surface, stretching from the lateral edge of the ipsilateral connective to the midline. Two such cells are visible in Figure 12A (arrow) just lateral to the stained ipsilateral connective.

The bulk of the branching of connective fibers is in the dorsal half of the ganglion, corresponding to the finding that most of the stained fibers are in dorsal tracts as they pass through the ganglion. As shown in the denser fill of Figure 12B, there is a considerable amount of contralateral as well as ipsilateral branching. Whereas ipsilateral branching extends throughout the entire medio-lateral extent of the neuropile, the lateral extent of contralateral branching is confined to the medio-lateral extent of the contralateral connective.

When a connective is stained just anterior or posterior to the prothoracic ganglion and the mesothoracic ganglion is examined (Figure 13A), one sees that, as in the prothoracic ganglion, there are ipsilateral and contralateral branches. However, branching is less extensive in the mesothoracic ganglion (compare Figures 12B and 13A). This point is further illustrated in the bilateral fill of Figure 13B. There is separation of right and left branching fields

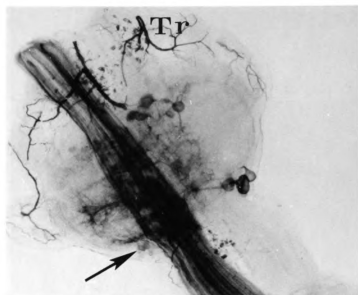
Figure 12. Branching of Connective Fibers in the Prothoracic Ganglion.

A--Ventral view of a prothoracic ganglion, anterior toward the top left. The right connective was stained at the mesothoracic level and is seen to branch ipsilaterally and contralaterally upon entering the ganglion. Several large (~50 μ m) cell bodies are also visible.

All stained connective fibers leaving the ganglion anteriorly do so on the same side through which they entered. Stained branches of trachea can be seen, especially at the anterior margin of the connective.

B--Dorsal view of a prothoracic ganglion, anterior toward the top. Stained as above but intensified to a greater extent. The ipsilateral branches fill the entire medio-lateral and anterior-posterior extent of the ipsilateral neuropile but the contralateral branches extend laterally only as far as the width of the contralateral connective.

A



500 μm

B



Figure 13. Branching of Connective Fibers in the Mesothoracic Ganglion.

A--Dorsal view of a mesothoracic ganglion, anterior toward the top left. The right connective was stained at the prothoracic level and is seen to have ipsilateral and contralateral branches in the ganglion. Four small cell bodies are visible, two in the posterior midline region, one contralaterally between N6 and the contralateral connective, and one contralaterally just below N2 (arrows).

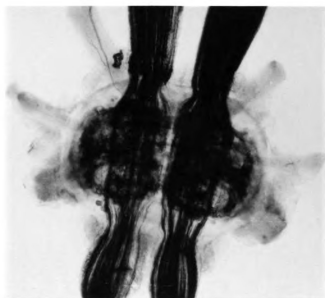
B--Dorsal view of a mesothoracic ganglion, anterior toward the top. Both connectives were stained as above. The midline is free of stained fibers except at discrete points of crossing.

A



500 μm

B



except at discrete points of crossover at the midline. These transverse projections correspond to the commissures noted by Pipa et al. (1959) and by Gregory (1974).

Four cell bodies were seen in the mesothoracic ganglion when prothoracic-mesothoracic connectives were stained. One was small and located contralaterally on the dorso-lateral surface between N6 and the descending connective (Figures 13A and B). A second cell was also small and found on the contralateral side, anteriorly on the lateral surface near N2 (Figure 13A). Two ventrally located small cells were seen just contralateral to the midline at the level of N5. One is visible in Figure 13A.

Staining of Giant Fibers

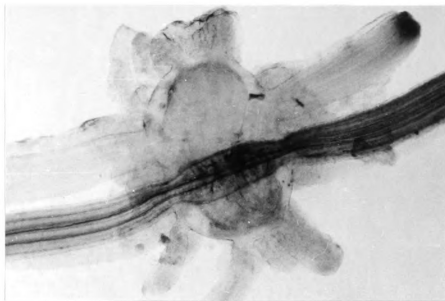
In order to determine if any of the giant fibers (which originate in the last abdominal ganglion) ascend as far as the prothoracic or mesothoracic ganglion, and might therefore be involved in intra-thoracic communication via branches in these ganglia, the connectives were stained in the abdominal A2-A3 region. Figure 14A shows that giant fibers course through the mesothoracic ganglion and give off a few lateral branches which are confined to the ipsilateral side. There is a similar pattern in the prothoracic ganglion (Figure 14B). Thus, in addition to reaching the metathoracic ganglion (Harris and Smyth, 1971), at least some of the giant fibers ascend to the prothoracic and mesothoracic ganglia. Evidently some fibers pass to even more anterior regions, since the ipsilateral ascending prothoracic-suboesophageal connective is seen to contain stained fibers in Figure 14B.

Figure 14. Giant Fibers Have Branches in the Prothoracic and Mesothoracic Ganglia.

A--Ventral view of a mesothoracic ganglion, anterior toward the left. The right connective was stained in the abdomen, at the A_2 - A_3 level. Giant fibers are seen to pass through the ganglion and give off short branches.

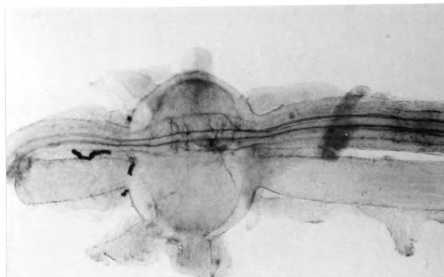
B--Dorsal view of a prothoracic ganglion, anterior toward the left. The right connective was stained as above. Giant fibers pass through the ganglion and give off a few short branches before continuing anteriorly through the ascending connective.

A



500 μm

B



Discussion

The branches of motor fibers in N6B and N5 are wholly contained in the ipsilateral portion of the ganglion and there are no direct projections to other ganglia. This latter point has been noted previously for other motoneurons, in studies utilizing methylene blue (see Bullock and Horridge, 1965) and cobalt staining (see Burrows, 1975). Therefore, if information about motor events in one ganglion is sent to other ganglia, it must be via interneurons.

Sensory fibers in N5 which innervate the femur (and points more distal) have projections confined to the ipsilateral portion of the ganglion and no branches in any of the connectives, just as for motoneurons. This arrangement appears to be uniform for all nerves in the cockroach (Gregory, 1974) but Tyrer and Altman (1974) found that certain sensory fibers in the locust which are important in flight send branches to other ganglia via the ipsilateral connective. Such organization is not unique to the locust; Bullock and Horridge (1965), in their extensive review of the earlier literature, reported that in many invertebrates sensory fibers send branches up or down the ventral cord to other ganglia. In the cockroach then, if information about sensory events is sent to other ganglia, it is apparently done via interneurons.

The longitudinal fibers of the connectives have extensive ipsilateral and contralateral branching, providing pathways for the interganglionic flow of information via interneurons. Since most of the branching is dorsal, as is the branching of motoneurons in N5 and N6, it may be that much of the interganglionic information is motor in nature. This would be consistent with findings in other invertebrate

systems (e.g., Davis et al., 1973), wherein coordinating fibers are used to carry out behaviors that involve more than one ganglion. The coordinating fibers are motor in nature and since many such behaviors have been shown to occur in deafferented preparations, sensory information can be viewed as a fine tuning mechanism local to each ganglion.

Bullock and Horridge (1965) reported that connective fibers which branch in the ganglia have either ipsilateral or contralateral branches but that fibers with both are rare. Cobalt staining has revealed at least one connective fiber in the cockroach that has ipsilateral and contralateral branches (O'Shea et al., 1974), and until selective cobalt staining of one or a few connective fibers at a time is done, the percentage of fibers having both ipsilateral and contralateral branches will remain unknown.

The presence of contralateral branches in connective fibers is important because it provides a possible means by which transfer of learning-related information could occur when only the contralateral connective remains intact. Since sensory and motor fibers branch only on the ipsilateral side, the information they carry must reach the contralateral connective by either local interneurons (confined to one ganglion) which cross to the contralateral side, or directly, by contacts between the sensory and/or motor fibers and branches of the contralateral connective fibers. The present results indicate that the latter possibility is quite real. If, as was alluded to in the preceding paragraph, single connective fibers have both ipsilateral and contralateral branches, then they could conceivably transmit information from ipsilateral and contralateral sensory and/or motor inputs.

Nunnemacher et al. (1974) found that there was a greater number of connective fibers in the thoracic connectives than anteriorly, in the prothoracic-suboesophageal connectives, or posteriorly, in the abdominal connectives. This is indicative of a high degree of local intra-thoracic communication. The same group of workers also found that of the 9,700 fibers in the prothoracic-mesothoracic connective, 80% are less than 1 μm in diameter, 19% are 1-6 μm , and 1% are over 6 μm . Furthermore, the very small fibers that constitute the bulk of fibers in the connectives are located in the ventral part of the connective. In the present results the dorsal parts of the connectives were heavily stained and the dorsal portion of the ganglion was where most of the branching occurred. This plus the finding of only a few cell bodies indicates that many small fibers (and their corresponding cell bodies) were not being stained. Consequently, in the present material more is revealed concerning the larger dorsal fibers, which are probably concerned with motor information, than for the smaller ventral fibers, which are likely to be concerned with sensory information.

It is possible that giant fibers mediate some degree of intra-thoracic communication since they have branches in the prothoracic and mesothoracic ganglia (as well as in the metathoracic; Harris and Smyth, 1971). Farley and Milburn (1969) and Spira et al. (1969) reported that giant fibers ascend beyond the prothoracic ganglion, but their reports presented no information on branching of giants within the thoracic ganglia.

In summary, the questions posed in the introduction have been answered. There are no direct sensory or motor projections, either

to the contralateral side of the ganglion or to other ganglia, but rather connective fibers branch ipsilaterally and contralaterally, thereby providing the means for information transfer and the redundancy necessary such that either connective alone can mediate transfer.

ELECTROPHYSIOLOGY EXPERIMENTS

Introduction

The present electrophysiological studies were undertaken for the purpose of determining which connective fibers are likely to be the most involved in transfer. Since my anatomical studies (see the previous section, ANATOMY EXPERIMENTS) showed that all the fibers in the prothoracic-mesothoracic connectives are interneurons, the responses of connective fibers to leg shock were examined to see if any could be reliably characterized as "sensory" or "motor" in nature, a distinction which is of importance if one is to determine what kinds of information are transferred.

Pearson (1972) and Pearson and Iles (1973) showed that nerve 6Br4, which supplies flexor muscles, and nerves 5r1a and b, which supply extensor muscles, are the motor nerves necessary and sufficient for leg movements of the kind seen in behavioral leg lift learning. Therefore simultaneous recordings were made from these motor nerves and connectives for the purpose mentioned in the preceding paragraph.

Since either the ipsilateral or contralateral connective alone can mediate transfer (see the section, BEHAVIORAL EXPERIMENTS), comparisons were made between the responses of these connectives to leg shock. Thus if the same kind of information transfers down either connective, it should be discernible as a common feature in their responses to leg shock.

Finally, in light of the behavioral finding that prothoracic leg training results in a rapid behavioral change in the mesothoracic leg but not vice versa, ascending and descending prothoracic-mesothoracic connective fibers were compared regarding their responses to leg shocks.

Methods and Materials

Twenty-three animals (mostly males) were used in these experiments, in which suction electrodes were used to record electrical activity from the cut ends of connective and motor nerves. These were made by heating polyethylene tubing (I.D. = .023", O.D. = .038"; Scientific Products) over a soldering iron and then pulling it to obtain a tapered tip. The tip was cut to a diameter appropriate for the nerve from which recordings were to be made. Next a pin was used to make a hole in the tubing shaft, about 2 cm from the tip. A piece of .002" silver wire was inserted into this hold and threaded to within 0.5 cm of the tip. The hole in the shaft was sealed with melted wax and the silver wire protruding from the hole was soldered to a larger insulated wire which led to the positive input side of a Tektronix 122 preamplifier. One end of a second piece of silver wire was wrapped around the outside of the tip of the electrode and the other end was soldered to a larger insulated wire which led to the negative input side of the preamplifier.

The tubing length of an electrode was about 40 cm. About the middle 15 cm was placed inside a glass tube which provided stability and thus allowed the electrode to be mounted on a micromanipulator. The shaft end of the tubing was connected to a syringe, which allowed

suction to be applied at the tip. Thus a cut nerve could be sucked into the electrode.

The stimulating electrode consisted of two fine insect pins embedded in plastic so that their tips protruded and were separated by about 2 mm. The pin shafts were soldered to larger insulated wires that led to a stimulator consisting of a Tektronix 162 waveform generator and Tektronix 161 pulse generator. Shocks were square wave pulses (5-30 V, 6 msec), and as in the behavioral experiments, shock amplitude was adjusted to be twice the threshold for tarsal twitching.

All recordings were done inside a three-sided screened cage to reduce transient electrical signals. The output of the pre-amplifier was led to a junction box and then split to a Tektronix 502A two-channel oscilloscope, Sony TC-654-4 four-channel magnetic tape recorder, and audio monitors. Data was played back from the tape recorder into a Gould two-channel brush recorder for analysis.

An animal to be used for recording was anesthetized using CO₂ gas bubbled through water, decapitated, then mounted ventral side up on a clay block and its legs restrained with pieces of clay. For recordings from connectives, the soft cuticle between the ganglia was removed and the connectives of interest were cut close to the prothoracic ganglion (in the case of ascending fiber recordings) or the mesothoracic ganglion (in the case of descending fiber recordings).

For recordings from flexor motor nerve 6Br4 the leg was rotated anteriorly to expose the soft cuticle just central to the coxal rim. This tissue was peeled away, thus exposing nerve 6B, which was cut as close as possible to the coxal rim. This provided a long

length of nerve with which to work and eliminated recording from nerves 6Br1, 2, and 3, which are more central branches of nerve 6B.

For recordings from extensor motor nerve 5r1a or b (each of which contains the same motor neurons), the coxal cuticle was cut away and superficial muscles removed, thus exposing nerve 5. Then either nerve 5r1a or b was cut as distal as possible before branching, thereby providing a long length of nerve with which to work.

Some animals were decapitated and the connectives cut posterior to the mesothoracic ganglion, producing an isolated prothoracic-mesothoracic preparation.

The clay block with the animal was then placed on a platform inside the cage and the shock pins inserted into the femur of the leg to be shocked. Next the recording electrodes were brought into position and the appropriate nerves sucked into their tips. Throughout the course of the dissection and recording procedures, cockroach saline (Pearson and Fournier, 1975) was frequently applied to exposed areas to prevent drying.

Animals were allowed to rest at least 30 min before any shocks were given to the leg. The intensity, duration and rate of shocks could be controlled automatically by settings on the pulse and waveform generators.

For statistical treatment of electrophysiological data, two-tailed Wilcoxon matched pairs tests (Siegel, 1956) were used unless otherwise noted.

Results

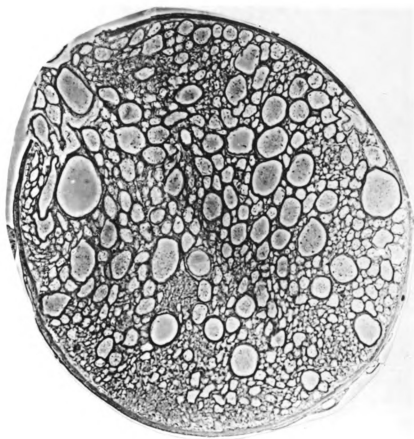
Fiber Classes in the Connectives

A prothoracic-mesothoracic connective viewed in cross-section (Figure 15) is seen to have hundreds of nerve fibers, ranging in size from very small (less than 1 μm) to very large (over 10 μm). Very large fibers are the fewest in number. Suction electrode recordings from the cut ends of prothoracic-mesothoracic connectives (whether from the portion attached to the prothoracic ganglion or that attached to the mesothoracic ganglion) show a similar range of spike heights that can be broadly grouped into small, medium and large classes (Figure 16). Since suction electrodes (unlike hook electrodes or other en passant extracellular recording techniques) record concentrically from all the fibers in a nerve, there is faithful representation of signals such that larger spikes correspond to larger diameter fibers.

The spontaneous connective discharge is typified by a predominance of small spike activity and little large spike activity (Figure 16A). A shock to the leg increases the firing rate of all fiber classes but most dramatically for the large class (Figure 16B). Amplitude histograms constructed from the evoked response to leg shock (Figure 17) verify that there are small, medium and large spike classes in both ascending and descending connective fibers.

General Features of Connective Discharges

Having shown that there are discrete classes of nerve fibers in the connectives, the firing pattern of each class in response to



50 μ m

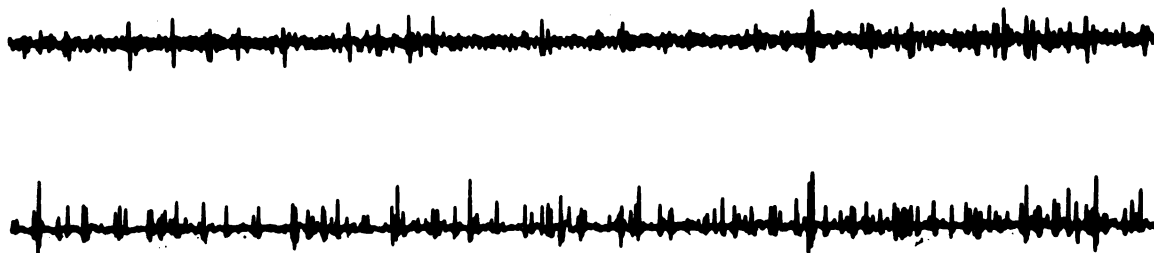
Figure 15. Phase Micrograph of a Prothoracic-Mesothoracic Connective Viewed in Cross-section. Dorsal toward the top. Most large fibers are dorsal to the horizontal midline whereas the bulk of small fibers lie ventrally.

Figure 16. Spike Activity in Connective Fibers. Upper trace in A and B is activity in ipsilateral ascending fibers from the mesothoracic ganglion, lower trace is activity in ipsilateral descending fibers from the mesothoracic ganglion.

A--Spontaneous activity. There is a moderate degree of activity, with smaller units predominating.

B--Response to mesothoracic leg shock. There is a sharp increase in spike activity, most notably in the larger fibers.

A



500 msec

B

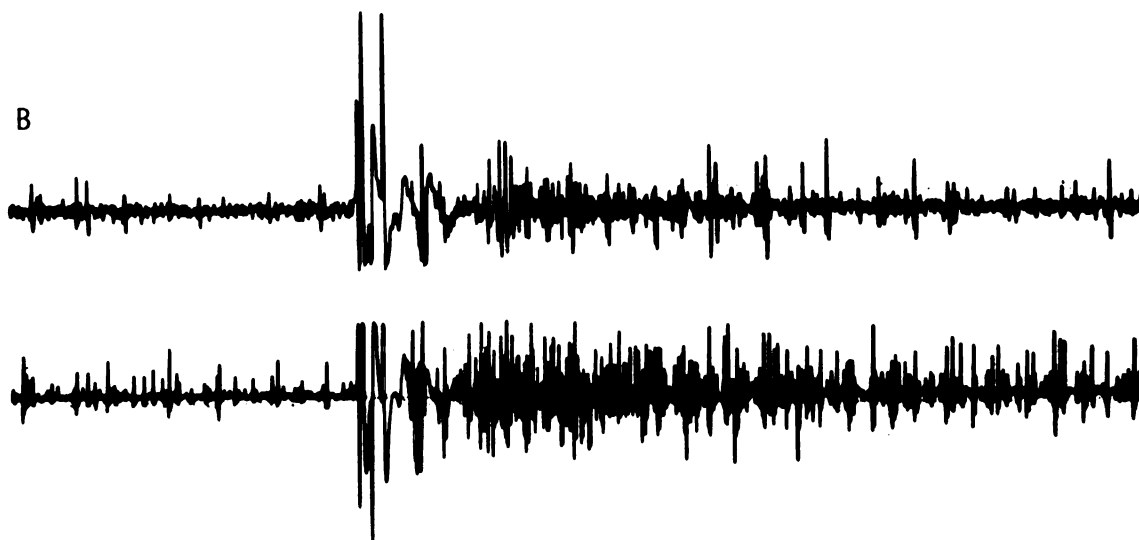
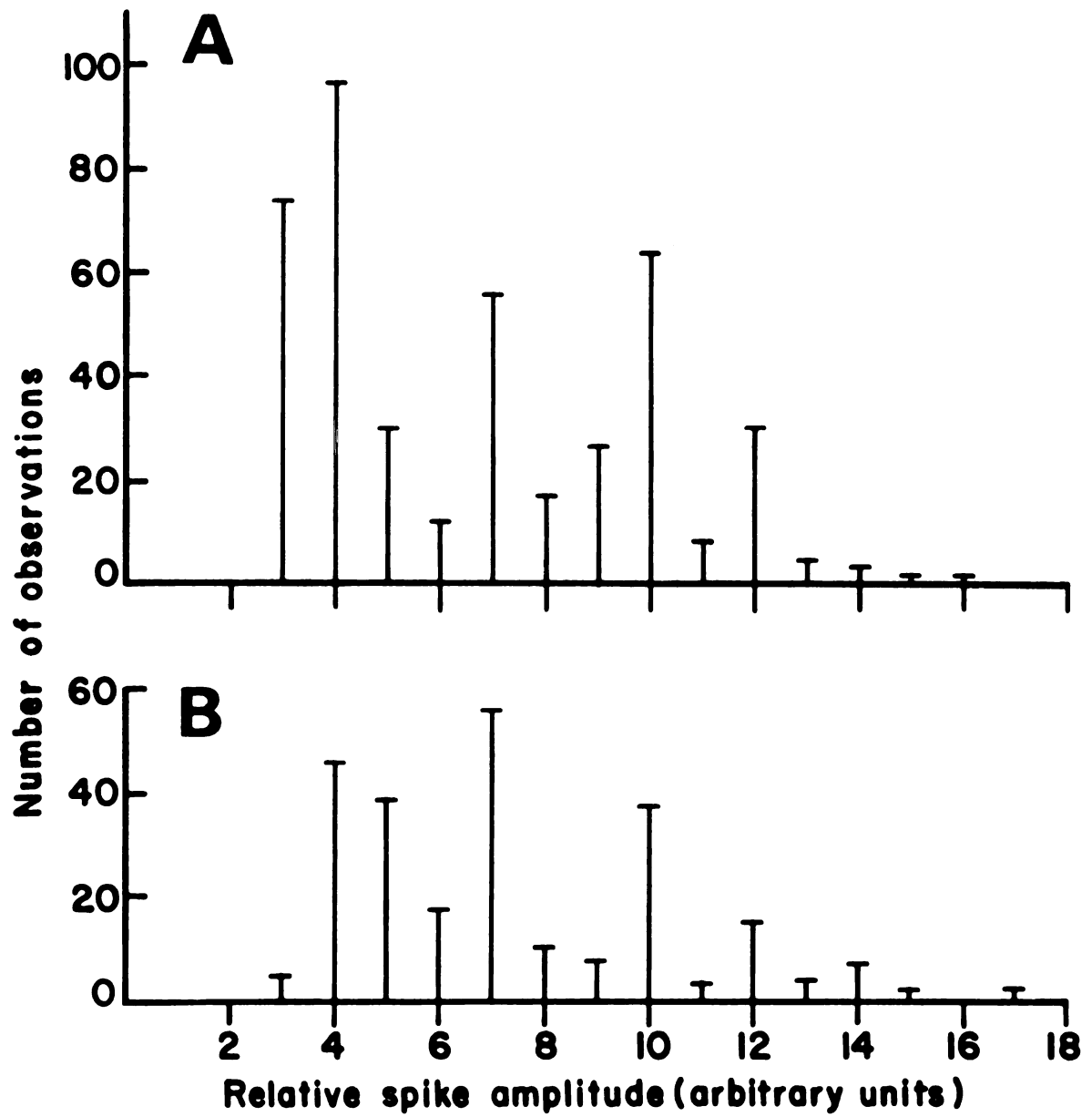


Figure 17. Amplitude Histograms of Prothoracic-Mesothoracic Connective Fiber Action Potentials.

A--Descending fibers from the prothoracic ganglion distribute into three main peaks, at 3-4,7 and 10.

B--Ascending fibers from the mesothoracic ganglion also distribute into three peaks, at 4-5,7 and 10.

The similarity between the histogram profiles of ascending and descending fibers indicates that small, medium and large classes of fibers can be distinguished in each case.



leg shock could then be examined. A series of ten shocks, one every 2 seconds or every 5 seconds, was given to either a prothoracic or mesothoracic leg and responses recorded from either descending or ascending connective fibers, respectively. Since the maximum response occurred in the first half-second following stimulation (Figures 16B, 19C, 20C), this interval was chosen for analysis of post-stimulus firing rates. Figure 18 gives the combined results from thirteen such experiments.

In every case (Figure 18A, B, C, D) the spontaneous activity is highest in small fibers and lowest in large fibers, with medium fibers in between. The slightly higher spontaneous rate of small and medium fibers ipsilaterally (i.e., on the same side as the leg being shocked) versus contralaterally may be a result of putting the shock pins in the ipsilateral femur (compare A to B and C to D).

When the first shock in a series of ten is given, there is a rise in firing rate for all fiber classes in all cases (Figure 18A, B, C, D). This rise is significant ($p < .02$) for all but the small ipsilateral descending class.

Descending Connective Fibers

Comparing the response patterns of ipsilateral and contralateral descending connective fibers (Figure 18A, B), one finds that the most outstanding difference is in the large fibers. Ipsilaterally the large fiber class shows a greater initial increase in firing rate than either the small or medium fiber classes ($p < .05$ in both comparisons) and its response habituates upon repeated stimulation (the response to stimulus 1 is significantly greater than the response

Figure 18. Responses of Small, Medium and Large Ascending and Descending Prothoracic-Mesothoracic Connective Fibers to Leg Shocks. In A and B the responses of descending fibers to prothoracic leg shocks are plotted. In C and D the responses of ascending fibers to mesothoracic leg shocks are plotted.

A--The large fiber class shows a greater initial increase in firing rate than either the small or medium class ($p < .05$, comparing the response to stimulus 1 minus the spontaneous rate, in both cases), and its response habituates upon repeated stimulation ($p < .05$, comparing the response to stimulus 1 to that of stimulus 10). The habituated response is greater than the spontaneous rate ($p < .05$). The medium class shows a significant initial increase in rate ($p < .05$, comparing the response to stimulus 1 with the spontaneous rate), and there is no habituation. As a class, small fibers do not change their spontaneous firing pattern following shocks.

B--There is an initial increase in firing rate upon leg shock for all fiber classes ($p < .05$) but the increase in the large class is no greater than that of the small or medium class.

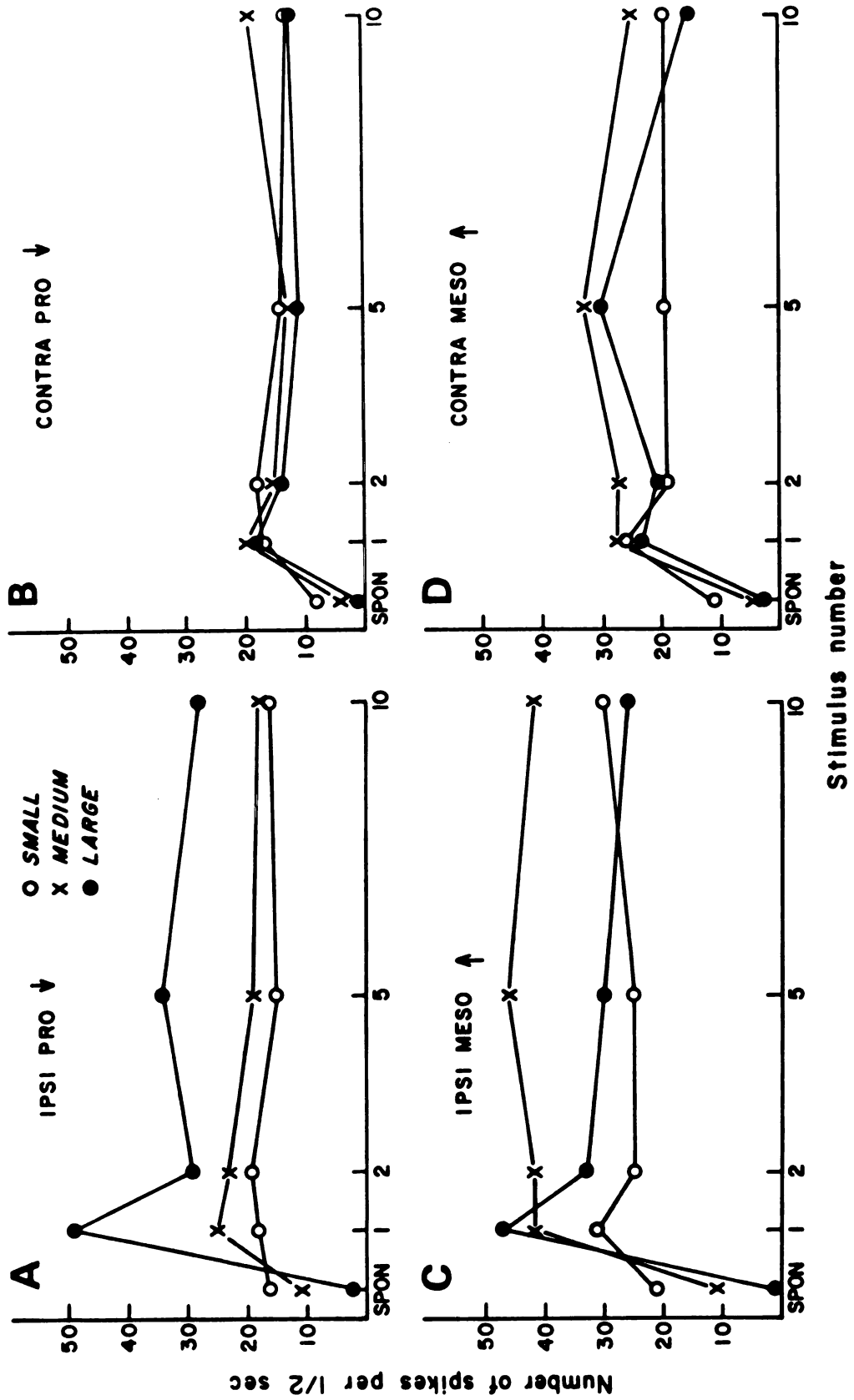
The ipsilateral descending large fiber response (A) is significantly greater than the contralateral (B) for stimuli 1, 2 and 5 ($p < .05$).

C--All fiber classes have an increased firing rate initially ($p < .05$), with the large class having the greatest increase and showing significant habituation ($p < .05$). The habituated large fiber response is higher than the spontaneous rate ($p < .05$, comparing the responses to stimuli 1, 2, 5 and 10 to the spontaneous level).

D--All fiber classes show an initial increase in activity upon leg shock ($p < .05$). The medium class response to stimuli 1, 2, 5 and 10 is greater than its spontaneous level ($p < .05$ in all cases), but not so for the small and large classes.

The ipsilateral ascending large class fiber response (C) is significantly greater than the contralateral (D) for stimulus 1 ($p < .05$). The medium fiber response ipsilaterally (C) is greater than that contralaterally (D) for all stimuli ($p < .05$ in all cases).

Ipsilateral ascending medium fibers (C) have significantly greater maintained activity than ipsilateral descending medium fibers (A) ($p < .02$, comparing stimuli 2, 5 and 10. Two-tailed Mann-Whitney U Test).



to stimulus 10; $p < .05$). However, the habituated response remains well above the spontaneous baseline level ($p < .05$, comparing the response to stimulus 10 with the spontaneous level).

Contralaterally the initial increase in firing rate of large fibers is no larger than for the small or medium fibers, and there is no significant habituation.

The ipsilateral large fiber response is greater than the contralateral for stimuli 1, 2 and 5 ($p < .05$).

The medium fiber class shows an initial rise in firing rate on both sides. This increase is maintained to a significant extent ipsilaterally and contralaterally ($p < .05$, comparing the response to stimulus 10 with the spontaneous rate).

Small descending fibers in both connectives show a slight initial rise followed by a gradual fall back to baseline.

Ascending Connective Fibers

As in the case of descending fibers, a major feature in the response of ascending connective fibers to leg shock is the difference between large fibers ipsilaterally and contralaterally (Figure 18C, D). Ipsilaterally there is a sharp initial increase followed by significant habituation ($p < .04$). As in the large ipsilateral descending class, the habituated response is significantly greater than the spontaneous level ($p < .05$). Although the initial rise is greater for large fibers than for small or medium fibers, it is not overwhelmingly or significantly so, in contrast to the ipsilateral descending connective large fibers.

The initial rise in firing rate of large ascending ipsilateral fibers is significantly greater than the rise contralaterally ($p < .05$). But for stimuli 2, 5 and 10 the responses do not significantly differ.

The medium ascending fibers show a maintained increase in firing rate ipsilaterally and contralaterally ($p < .05$, comparing the response to stimulus 10 with the spontaneous level in both cases). The increase is much more pronounced ipsilaterally ($p < .05$, comparing the ipsilateral versus contralateral rates for all stimuli).

The small ascending fiber response is similar to that of small descending fibers; there is an initial increase followed by a decline to a flat response intermediate between the spontaneous rate and the initial stimulated rate.

Summary

Of all the fiber class responses, only the large ascending and descending fibers of the ipsilateral connective show habituation. In both cases the habituated response is significantly greater than the spontaneous firing level.

The ascending and descending medium fibers of both sides show an increase in firing rate upon stimulation that is maintained at a level significantly greater than the spontaneous rate in every case. The ipsilateral ascending medium fiber response is greater than the contralateral.

Small fibers in all instances have the least pronounced changes and do not differ ipsilaterally versus contralaterally in contrast to the large and medium classes.

The major distinction between the ascending and descending fiber responses seems to be ipsilaterally, in the medium fiber class (Figure 18A, C). Whereas all medium fibers have the same spontaneous firing rate, those that ascend from the mesothoracic ganglion exhibit a markedly greater response to leg shock than those that descend from the prothoracic ganglion ($p < .02$, comparing stimuli 2, 5 and 10; $p < .06$ for stimulus 1. Two-tailed Mann-Whitney U Test).

Motor Corollary Discharge Units

When simultaneous recordings of electrical activity are made in the prothoracic leg flexor motor nerve 6Br4 and descending connective fibers (ipsilateral or contralateral), or in mesothoracic N6Br4 and ascending connective fibers (ipsilateral or contralateral), in both headless and isolated thoracic preparations, one finds that during a spontaneous burst of activity in N6Br4 or during one that is the result of mechanical stimulation of the leg, there is a similar burst of activity in the connective fibers (Figure 19A, B). Furthermore, as shown in Figure 19C, bursts of activity in N6Br4 following electrical stimulation of the leg are also accompanied by connective bursts. Thus for spontaneous activity and mechanical and electrical stimulation, activity in N6Br4 is represented by a corollary discharge in both ascending and descending connective fibers.

At a fast sweep speed (Figure 19D) it is seen that there is a one-to-one representation of activity such that for each spike in N6Br4 there is a spike in the connective. (Hence, by the criteria of Davis et al. (1973), these connective units are efference copy units.) This was found to be true for the ipsilateral and contralateral

Figure 19. Corollary Discharge in N6Br4 Motorneurons and Connective Fibers.

A--Spontaneous activity. Top trace is activity in ascending right connective fibers from the mesothoracic ganglion, bottom trace is activity in right mesothoracic N6Br4.

B--Responses to mechanical stimulation of the right mesothoracic femur. Top trace is activity in ascending right connective fibers from the mesothoracic ganglion, bottom trace is activity in right mesothoracic N6Br4.

C--Responses to electrical stimulation of the right mesothoracic leg. Top trace is activity in ascending right connective fibers of the mesothoracic ganglion, lower trace is activity in right mesothoracic N6Br4.

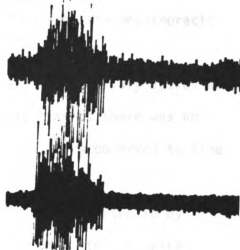
D--Two examples of spontaneous activity. Top trace is activity in descending right connective fibers of the prothoracic ganglion, bottom trace is activity in right prothoracic N6Br4.

Bar = 1 sec in A, B and C; 0.25 sec in D.

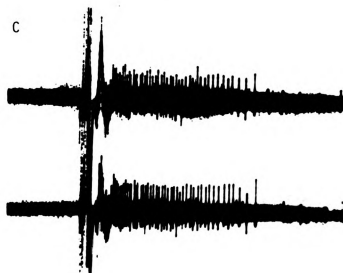
A



B



C



D



connective fibers descending from the prothoracic ganglion and for the ipsilateral and contralateral fibers ascending from the mesothoracic ganglion.

Spikes in N6Br4 occur ~ 2 msec later than corollary spikes in the connective in Figure 19D, but in many experiments there was no measurable latency. In no case were spikes in N6Br4 observed to fire before corollary spikes in the connective.

Several of the motor neurons in N6Br4 are represented by corollary discharge, as shown by the presence of different spike amplitudes which follow one-to-one in Figure 19D. The connective fibers that fire in synchrony with fibers in N6Br4 are of the large and medium classes, larger connective fibers being associated with larger motor fibers.

When the leg is given a strong shock (20-30 V instead of the usual 5-10 V) or strong mechanical stimulation, very large fibers in N6Br4 fire spikes and very large connective fibers are seen to fire in synchrony with these motor units (Figure 19B). The very large units of N6Br4 and of the connective are not spontaneously active, nor do they fire when the normal shock stimulus (5-10 V) is given to the leg.

When simultaneous recordings of electrical activity are made in prothoracic leg extensor motor nerve 5r1 and descending connective fibers (ipsilateral or contralateral), or in mesothoracic N5r1 and ascending connective fibers (ipsilateral or contralateral), in either headless or isolated thoracic preparations, one finds that during a spontaneous burst of activity in N5r1 or during a burst that is the result of mechanical stimulation of the leg, there is a similar burst

Figure 20. Corollary Discharge in N5r1 Motorneurons and Connective Fibers.

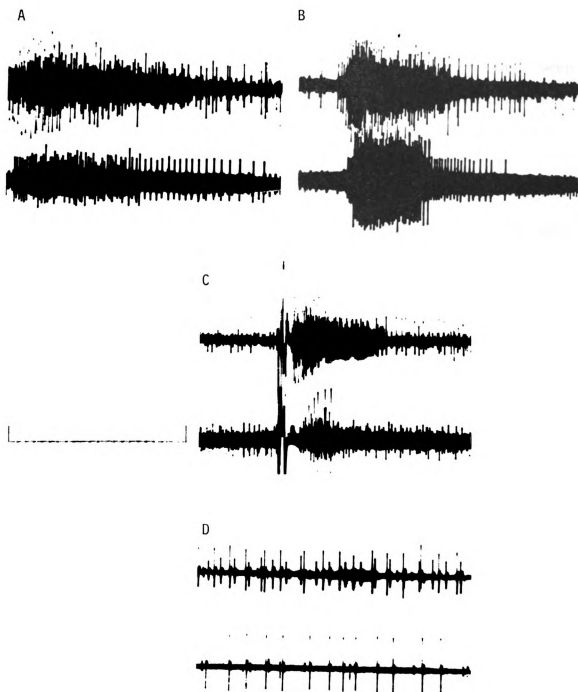
A--Spontaneous activity. Top trace is activity in descending right prothoracic-mesothoracic connective fibers, bottom trace is activity in right prothoracic N5r1.

B--Responses to mechanical stimulation of the right prothoracic femur. Top trace is activity in descending left prothoracic-mesothoracic connective fibers, bottom trace is activity in right prothoracic N5r1.

C--Responses to electrical stimulation of the right prothoracic leg. Top trace is activity in descending right prothoracic-mesothoracic connective fibers, lower trace is activity in right prothoracic N5r1.

D--Spontaneous activity. Top trace is activity in ascending right prothoracic-mesothoracic connective fibers, lower trace is activity in right mesothoracic N5r1.

Bar = 1 sec in A and C; 0.5 sec in B; 0.25 sec in D.



of activity in the connective (Figure 20A, B). Furthermore, as Figure 20C illustrates, bursts of activity in N5r1 following electrical stimulation are also accompanied by connective bursts. The fast sweep of Figure 20D shows that there is not a one-to-one representation of N5r1 spikes in the connective. This lack of correspondence can also be seen for the large N5r1 spikes in Figures 20A and B.

The patterns noted above for the corollary discharge interneurons of flexor and extensor motoneurons were found even after many shocks had been given to a leg. For example, if the usual series of ten shocks (one every 2 or 5 sec) was given to a leg, the corollary units of N6Br4 continued to follow one-to-one at all times, even though by the tenth shock there was often significant habituation of the large fiber response.

Discussion

The findings that there are discrete classes of connective fibers as judged by spike amplitudes, and that each class has characteristic response properties to leg shock, make it desirable to correlate spikes of a given amplitude with nerve fibers of a specific diameter. Pearson et al. (1970) found that for cockroach motoneurons there was a uniform relationship between fiber diameter and (extracellularly recorded) spike amplitude, such that

$$d = 5.7 \sqrt{V} , \quad (1)$$

where d is the diameter of the fiber and V is the peak-to-peak amplitude of the action potential. Giant fibers (which fire rarely but were recorded from occasionally upon cercal stimulation) have an

average diameter of 11 μm in the prothoracic-mesothoracic connectives (Spira et al., 1969). Therefore their relative spike amplitudes were measured directly off the (uncalibrated) chart record and used to obtain a scaling factor for V. Once this scaling factor was determined, the fiber diameters of small amplitude spikes could be calculated using equation 1. According to this method the smallest connective fibers recorded from were about 3 μm in diameter. If this is in fact true then the present recordings represent a sample of only those fibers greater than or equal to 3 μm in diameter, or less than 20% of the total number of fibers in the connective (see Nunnemacher et al., 1974). On the other hand, the relation between fiber diameter and spike amplitude in equation 1 may be valid only for motoneurons or large diameter fibers in general, in which case the small spikes seen in the present recordings could correspond to activity in the small (i.e., <1 μm) fibers that constitute the bulk of connective fibers (Nunnemacher et al., 1974). This question of the relationship between spike amplitude and fiber diameter can best be resolved by: (1) extracellular recording from teased out connective fibers followed by cobalt staining via axonal back-filling, (2) intracellular recording from intact connective fibers followed by dye injection, or (3) intracellular recording from an interneuron cell body and simultaneous extracellular recording from its connective fiber, followed by dye injection into the cell body.

The finding that there are connective fibers which fire in phase with flexor motor nerve 6Br4 or extensor motor nerve 5r1 indicates that corollary motor information is sent between the prothoracic and mesothoracic ganglia. That these corollary responses are motor

rather than sensory in nature is shown by the fact that they occur during spontaneous motor nerve activity as well as upon mechanical and electrical stimulation. Similar corollary discharge units have been found in a variety of invertebrate and vertebrate systems (for a review see Kennedy and Davis, 1976).

Pearson and Iles (1973) reported finding motor corollary discharge units of N6Br4 in the ipsilateral mesothoracic-metathoracic connective having somewhat different properties than the ones I have investigated. Their corollary units (see their Figure 6) fire bursts that begin ~100 msec before bursts lasting ~200 msec in N6Br4, and there is no one-to-one relation between spikes in N6Br4 and those in the connective. This is in contrast to the simultaneous occurrence of bursts in N6Br4 and the connective, and the presence of one-to-one coupling between N6Br4 and the corollary units, in the present recordings.

Since there is often no delay between spike activity in N6Br4 and its corollary units, one might suspect that collateral branches of N6Br4 are responsible for corollary discharge activity. However three facts argue strongly against this possibility: (1) when there is a delay it can be as long as 2 msec, which is much too long to be accounted for by collateral branch conduction time (for the known conduction velocities of ~3 m/sec); (2) when there is a delay it is always the connective that fires first, before N6Br4; (3) it was shown in the ANATOMY EXPERIMENTS section that N6Br4 has no branches in the connectives, as also found by Gregory (1974). Since corollary discharge spikes never occur later than N6Br4 spikes, it is unlikely that

flexor motoneurons of N6Br4 synapse with corollary discharge interneurons.

It is known that in the metathoracic ganglion, flexor motoneurons whose axons are in N6Br4 are driven by monosynaptic input from non-spiking interneurons which have oscillating membrane potentials (Pearson and Fourtner, 1975), particularly by Interneuron I. Similar non-spiking interneurons have been found by Burrows and Siegler (1976) to drive motoneurons in the metathoracic ganglion of the locust and are suspected to exist in all the thoracic ganglia of both the cockroach and locust. As the magnitude of depolarization increases in Interneuron I there is orderly recruitment of motor axons in N6Br4 from smallest diameter to largest (Pearson and Fourtner, 1975), a general feature of many invertebrate motor systems driven by oscillating inputs (Kennedy and Davis, 1976).

Thus the simplest explanation of the present findings, assuming that there are interneurons similar to Interneuron I in the prothoracic and mesothoracic ganglia, seems to be that Interneuron I (whose branches are wholly contained in the ganglion; Pearson and Fourtner, 1975) makes monosynaptic contacts with spiking interneurons that function as corollary discharge units (in addition to its contacts with motoneurons of N6Br4). This would explain the one-to-one firing pattern seen for all motoneurons of N6Br4 and their corollary units. It would also explain the firing of very large motoneurons in N6Br4 and their corollary units when stronger than normal intensity shocks are given (assuming that sensory input directly or indirectly drives Interneuron I). The short but variable latency between corollary discharge spikes and motoneuron spikes is indicative of a

synaptic process and could be explained as being due to greater and more variable delay at the Interneuron I-motoneuron synapse(s) than at the Interneuron I-corollary unit synapse(s).

The main branches of Interneuron I are located laterally and dorsally, in the same area as the main branches of motoneurons in N6Br4 (Pearson and Fournier, 1975) and the branches of ipsilateral connective fibers. A smaller tuft of short branches is located more medially, directly in the path of ipsilateral dorsal tract connective fibers (see Figure 11 of Pearson and Fournier, 1975). The branches of some contralateral connective fibers extend far enough across the midline to contact these short branches of Interneuron I (see ANATOMY EXPERIMENTS section). These suggested anatomical connections are plausible means by which ipsilateral and contralateral corollary discharge units could fire one-to-one with motoneurons in ipsilateral N6Br4.

Since there is not a one-to-one representation of spikes in the corollary discharge units of N5r1, the presence of motoneuron collaterals functioning as corollary discharge units in the connectives is unlikely. Furthermore cobalt staining showed that the branches of N5r1 are confined to the ganglion, and this has been shown to be true for three of the five N5r1 motoneurons individually (Iles, 1972; Pearson and Fournier, 1975; Pitman et al., 1973). Thus, as in the case of N6Br4, corollary discharge units of N5r1 must be interneurons.

There are known to exist non-spiking interneurons (like Interneuron I) that upon depolarization excite extensor motoneurons in N5r1 (Pearson and Fournier, 1975). The structure of these

interneurons has not been determined, but assuming that they are similar to Interneuron I the same basic line of reasoning as above could be applied to corollary discharge mechanisms for N5r1, with one important exception. There is looser coupling between N5r1 motor-neurons and their corollary discharge units than between N6Br4 motor-neurons and their corollary discharge units, and this must eventually be explained. It may be that N5r1 corollary units receive convergent synaptic input from a variety of sources other than those representing activity in N5r1.

At first glance it may appear that the most parsimonious mechanism would be one whereby Interneuron I excites N6Br4 and its corollary discharge units upon depolarization and N5r1 and its corollary units upon hyperpolarization. However Pearson and Fournier (1975) showed that while hyperpolarization of Interneuron I releases motoneurons of N5r1 from inhibition it does not cause them to become particularly active. Thus a second interneuron which specifically excites motoneurons of N5r1 upon depolarization is needed, and exists (see the previous paragraph).

Since my cobalt studies showed that giant fibers traverse the length of the thoracic region, it is possible that at least some of the corollary discharge units are giant fibers. The largest amplitude spikes in connective fibers were seen only rarely, usually upon cercal stimulation, and thus probably represent discharge in giant fibers (Delcomyn, 1977). Similarly the very large amplitude corollary discharge units of the larger motoneurons in N6Br4 fire infrequently and have spike amplitudes approximately the same as those of giant fibers. These similarities are offset by three facts which argue

against the identification of giant fibers as the corollary discharge units of larger motoneurons: (1) Delcomyn (1977) found that giant fiber activity always travels in the posterior to anterior direction, whereas the present corollary discharges travel in both longitudinal directions; (2) Spira et al. (1969) found that giant fibers taper as they ascend through the thoracic region of the ventral nerve cord, hence their diameters and spike amplitudes do not stand out as dramatically as in the abdominal region of the cord where they are indeed giants; (3) if the previous suggestion is correct, that (ipsilateral and contralateral) corollary discharge units receive monosynaptic input from non-spiking interneurons which also drive motoneurons, then corollary discharge units in the opposite connective relative to a non-spiking interneuron must have contralateral branches which contact the non-spiking interneuron. As shown in the ANATOMY EXPERIMENTS section, and by Harris and Smyth (1971), giant fibers have only short branches in the thoracic ganglia, and these branches are confined to the ipsilateral sides of the ganglia.

On the basis of the above discussion it is concluded that corollary discharge units of large motoneurons are among the largest fibers in the thoracic connectives, but are not the "giant" fibers.

It is interesting that there is a one-to-one corollary discharge of activity in flexor motoneurons of N6Br4 but that activity in extensor motoneurons of N5r1 is represented by a "rough copy." This may be related to the fact that in walking, the durations of flexor bursts are relatively constant compared to extensor bursts (Pearson and Iles, 1970). These asymmetries show that, for some

reason, corollary flexor information is more detailed and possibly more important than corollary extensor information.

The large fiber class, of which the corollary discharge units are members, shows more dramatic responses to leg shock than the small and medium fiber classes, in the ipsilateral ascending and descending connective fibers. From the evidence that ipsilateral and contralateral ascending and descending connective fibers all fire corollary discharges to motoneuron activity, it can be deduced that the larger response in ipsilateral versus contralateral large fibers must represent response differences in large fibers other than corollary discharge units of N6Br4 and N5r1. It may be that corollary discharge units of other less important motoneurons have asymmetric activity ipsilaterally versus contralaterally, or these remaining large spikes may represent activity other than corollary motor discharge, for example, corollary sensory discharge.

Although the large fiber class was unique in that it showed habituation ipsilaterally (in ascending and descending fibers), it is unclear to what degree this applies to behavioral experiments on leg lift learning. In the first minute of training the usual number of shocks taken is ~20, yielding an average rate of ~1 shock per 3 sec. In the present experiments leg shocks were given once per 2 sec, nearly the same rate. Since the bulk of habituation occurs rapidly, from the first to the second shock, and behavioral escape learning also occurs rapidly, from the first to the second flexion/extension, habituation may be a component of escape learning. For example, in leg lift learning there may be greater habituation in the response of

extensor motoneurons to leg shock than in flexor motoneurons, thus contributing to rapid leg flexion.

Medium class fibers had properties similar to large fibers except they did not habituate upon repeated stimulation. Some medium class fibers are corollary discharge units but the majority are functionally unidentified. They, like the unidentified large fibers, could be corollary discharge units of motoneurons other than those in N6Br4 and N5r1, could be corollary sensory units, or could serve some as yet unspecified function.

Small fibers exhibited the least responses of all to leg shock and remain very much of an enigma. It may be that since small fibers are much more numerous than medium or large fibers (Nunnemacher et al., 1974), individual small fibers undergo significant changes in activity that are lost by recording from the whole connective.

The above discussion again illustrates the need for identified neuron studies now that particular classes of connective fibers have been shown to have characteristic properties. It is only by recording from and staining single fibers that the functions of these interneurons can begin to be understood, particularly with regard to the transfer of information related to learning.

DISCUSSION AND SUMMARY

Results from the anatomical and electrophysiological experiments now allow some specific statements to be made concerning the mechanisms of the transfer of training information in the behavioral experiments. The major results can be summarized as follows: (1) There are no direct interganglionic projections of sensory and motor neurons, at least in the pathways of interest here. Thus interganglionic communication must be mediated by interneurons in the connectives. (2) All relevant sensory and motor neurons have their branches confined to the ipsilateral side of the ganglion from which their peripherally directed axon originates. (3) Either connective can mediate transfer. Thus, with 2 above, if monosynaptic connections exist between sensory or motor neurons in the prothoracic ganglion and their connective interneurons to the mesothoracic ganglion (or vice versa), connective fibers with contralateral branches are likely to be involved. Such fibers have been shown to exist. (4) Motor corollary discharge interneurons were found in ipsilateral ascending and descending connective fibers between the prothoracic and mesothoracic ganglia.

Since P and R animals both have transfer of motor information via corollary discharge interneurons, but only P animals show positive transfer of learning information as measured behaviorally, there must be separate transfer of sensory information related to leg shock.

Conceivably, in P animals the interneuronal discharge representative of prothoracic leg shock (which, as stated in the ELECTROPHYSIOLOGICAL EXPERIMENTS section, may be via large or medium connective fibers that are corollary sensory units) arrives at the mesothoracic ganglion in close temporal relation to corollary extensor motoneuron discharge, since leg extension initiates shocks in P. Absence of corollary shock discharge is then always associated with corollary flexor motoneuron discharge. However in R animals there is no fixed relationship between corollary sensory discharge and corollary motor discharge since the R animal's leg is shocked in a variety of positions.

If sensory and motor events are represented independently in the connectives, association between leg position and shock must be made separately in the prothoracic and mesothoracic ganglia.¹³ At the present time there is virtually no information concerning which local interneurons might be involved in such association functions, except to say that since direct sensory and motor neuron projections are ipsilateral in the ganglion, association neurons are also likely to be located ipsilaterally.

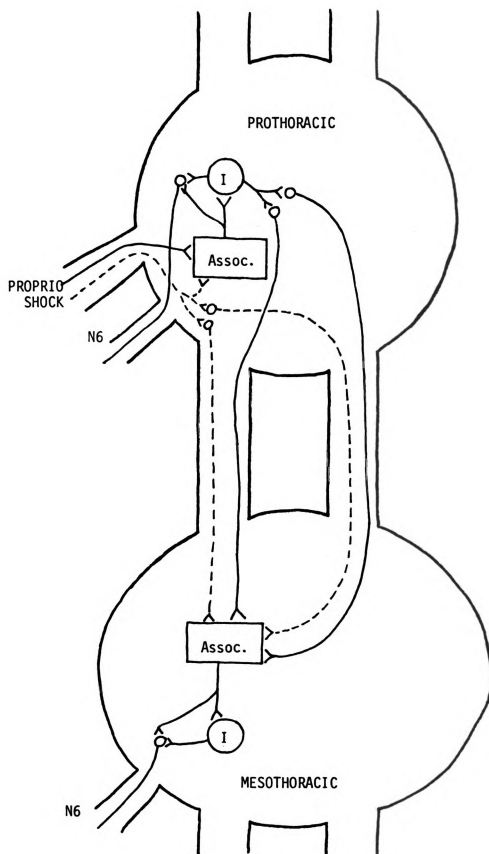
A model which incorporates the present ideas is shown in Figure 21. Descending ipsilateral and contralateral corollary interneurons of N6Br4 motoneurons are contacted monosynaptically by branches of Interneuron I, which also drives the motoneurons. A

¹³This corresponds to possibility 2 of Eisenstein (1972) and is in contrast to his possibility 3, wherein there is transfer of a pre-formed input-output association between leg position and shock from the prothoracic to mesothoracic level.

Figure 21. Schematic Model of the Probable Pathways by Which Information Related to Prothoracic Training Events Is Transferred to the Mesothoracic Ganglion. For the motor pathways only those for N6Br4 flexor motoneurons have been shown, but they are meant to be representative in principle of the N5r1 extensor motoneuron pathways also.

Leg shock input is represented by the dashed line and is seen to contact a prothoracic association center and ipsilateral and contralateral corollary discharge interneurons in the connectives. Prothoracic proprioceptive information also reaches the association center. The two inputs cause an increase in prothoracic leg flexion by making contact with N6Br4 flexor motoneurons directly or via Interneuron I.

Motor information, which can serve as an indication of leg position, transfers via ipsilateral and contralateral corollary discharge units (solid lines in the connectives) that are driven by Interneuron I. At the mesothoracic ganglion, independent representations of sensory and motor information are integrated at association centers and leg flexion is affected by direct or indirect contact with flexor motoneurons of N6.



similar arrangement is envisaged for the corollary units of N5r1 motoneurons.

The corollary motor discharge units for flexor and extensor motoneurons thus provide bilateral pathways for the interganglionic transfer of motor information. As Davis (1976) has discussed, corollary motor discharge provides a means other than proprioceptive input by which information on leg position (represented by the combined corollary discharges of all leg motoneurons having such corollary units) could be expressed. It seems plausible that proprioceptive input concerning leg position is normally utilized for associations between leg position and shock at the prothoracic ganglion during training, and that corollary discharge is used for the transfer of leg position information to the mesothoracic ganglion.

This model also illustrates bilateral transfer of corollary sensory information related to leg shock information, and independent association of sensory (leg shock) and motor (leg position) information in the prothoracic and mesothoracic ganglia. Since Harris (1971) showed that transfer occurred via a single connective when the right prothoracic leg was trained and the left mesothoracic leg tested, there must be bilateral representation of each connective's sensory and motor information in the mesothoracic ganglion, either directly via contralateral branches of the corollary units (as shown in Figure 21), or indirectly via local interneurons.

The next major steps for this research as I see them are:

- (1) identify specific motor corollary discharge interneurons;
- (2) identify the pathways by which sensory information related to leg shock travels between ganglia; and (3) identify those neurons or

groups of neurons which associate leg shock and leg position. I have previously discussed strategies for 1 above and similar strategies will undoubtedly yield results for 2 as well. Step 3 above relates not to the mechanisms of transfer, but rather what is done with the transferred information once it reaches the mesothoracic ganglion. It can also be viewed as an approach toward the analysis of single ganglion learning (e.g., prothoracic training) independent of transfer.

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