THE WITHDRAWAL RESPONSE OF A FRESHWATER
SNAIL (*LYMNAEA STAGNALIS* L.),

By ANTHONY COOK

*School of Biological and Environmental Studies, The New University of Ulster,
Coleraine, Northern Ireland*

SUMMARY

1. Electrical stimulation of a variety of nerves towards the brain results in movements of the neck of the snail similar to those associated with the withdrawal response of the intact animal.

2. The columellar and cervical nerves mediate most of the movements being measured.

3. Repetition of the stimuli results in a decline in response amplitude which is complicated by a superimposed incremental process which is itself subject to a decremental process as stimuli are repeated.

4. As stimuli are repeated the response latency increases.

5. Consecutive stimulation of pairs of nerves indicates that the response decrement is specific to the nerve being stimulated.

6. The involvement of the pleuro-pedal connectives in the response has been demonstrated both in a semi-intact preparation stimulated electrically and in a free-roaming animal treated surgically and stimulated visually.

7. Visual stimuli associated with the withdrawal response are detected by photoreceptors on the head and in the mantle.

INTRODUCTION

It has long been known that snails make body withdrawal responses to sudden stimuli (Pieron, 1911; Dawson, 1911), and that upon repetition of the stimuli the responses decline (Humphrey, 1930). A decline in response as a result of a repeated stimulus that is not followed by any form of reinforcement is known as habituation (Thorpe, 1963). In the opisthobranch *Aplysia californica*, Kandel and his co-workers have demonstrated, cell by cell, a nervous pathway, involved in the habituation of the gill withdrawal response (Castellucci *et al.* 1970; Kupfermann *et al.* 1970; Pinsker *et al.* 1970). This response is not ideally suited for extensive behavioural investigation since the animal must be restrained for the response to be elicited and observed. The body withdrawal response of a shelled gastropod provides a useful subject for behavioural investigation of habituation (Humphrey, 1930; Cook, 1970) but its usefulness as a subject for physiological investigation has yet to be demonstrated. The habituation of neuronal responses has been demonstrated in both *Lymnaea* and *Helix* spp. (Pakula & Sokolov, 1973) but its relationship to behavioural responses is not clear. The work in this paper is an attempt to identify the ganglia involved in the body withdrawal

response to visual and other stimuli, of the pulmonate snail *Lymnaea stagnalis* L.¶ and an investigation into the habituation of the electrically stimulated response. In conducting these investigations it is hoped to provide a link between the behaviour of a free-roaming and, where possible, intact animal, and that of a semi-intact preparation.

The withdrawal response is a snail's normal reaction to sudden stimuli and presumably serves to protect the head and foot from small predators. The shell is brought forward and down over the head, the tentacles are brought rapidly down, locomotion ceases and air may be expelled from the lung. In the present experiments it is the movement of the shell alone which is being investigated.

MATERIALS AND METHODS

Animals

Lymnaea stagnalis were obtained from Haig's Farm, Newdigate, Surrey, various drainage ditches around Cambridge, and from Traad Point on Lough Neagh, Co. Londonderry. Snails were kept in pond water at about 20 °C and fed on lettuce.

Anatomy

Animals were relaxed in 0·1 % nembutal for 1 h, the shell was removed and the body fixed in Bouins fluid for 24 h. The head was split open in larger specimens to assist the passage of fixative to the interior. After fixation the snail was dissected so that the anterior portion of the head remained as a frame holding the brain *in situ*. Preparations were dehydrated in ethanol, embedded in 52 °C wax and sectioned at 10 μm. Sections were stained in Delafields haematoxylin and eosin, and mounted in Balsam.

Electrical stimulation

A preparation of the animal was made which allowed the recording of the movement of the insertion of the columellar muscle onto the shell in response to the electrical stimulation of various nerves. The shell was removed and the attachment of the columellar muscle to the shell carefully severed. The animal was then placed in a dish with one pin through its buccal mass and one through the posterior region of its foot. The dish was flooded with *Lymnaea* Ringer (de Vlieger, 1968). An incision was made in the head of the animal extending from the pin in the buccal mass to the neck region. The skin was then pulled back to expose the brain. A small crocodile clip was attached to the point at which the columellar muscle inserted into the shell. The clip was connected to a mechano-electrical transducer with thread and the output of the transducer recorded on a pen recorder. Electrical stimuli from a Farnell pulse generating system were delivered via glass suction electrodes to the cut ends of nerves at 100 Hz for 1 sec. The amplitude of the stimuli was double threshold value; it varied from 0·2 V to 2 V. In general the better the fit between the nerve and the electrode, the lower the threshold. The tips of the electrodes could not be made too small since they became easily blocked with mucus.

In many of the experiments, connectives were cut. In some cases nerves are closely associated with these connectives and in no cases were these nerves separated from the ganglion into which they insert. After its nerves or connectives had been cut the animal was allowed 10 minutes to settle down.

Lesions

The anaesthetic procedure employed was modified from that of Lever, Jager & Westerveld (1964). The animal was placed in a 0·1% solution of nembutal at 30 °C and nitrogen was bubbled through the solution for 5 min to encourage the animal to extend out of its shell. This was followed by 8 min during which CO_2 was bubbled through the nembutal solution. The animal was then transferred to a 0·2% solution of MS 222 (Sandoz) for 5 min.

Operations were conducted using a tissue-covered plasticene block as a support. The block was constructed so that the head of the animal was higher than the rest of the body. This allowed body fluids to drain away from the head and prevented excessive loss of haemolymph. The procedure for operations was as described by Joosse & Lever (1959). The anaesthesia achieved in the present experiments did not appear to be as deep as that reported by Lever and his co-workers. When connectives were cut, for instance, the animal responded with a withdrawal, and therefore for these operations the head was restrained with a pin through the buccal mass and the shell was clamped steady.

After operation, the animals were left to recover in running water for 7 days. Their responsiveness to 20 'light off' stimuli of 1 sec duration delivered every 10 sec was then tested. Procedures for the delivery of such stimuli are described elsewhere (Cook, 1970). Following this test the animals were given a tactile stimulus to the head to test whether they were capable of giving a withdrawal response. All lesions were verified by dissection.

RESULTS

Anatomy

A diagram of the brain was reconstructed from serial sections (Fig. 1). This diagram represents the relaxed brain that one might reasonably expect to see in the anaesthetised or freshly dissected animal. Fixation without relaxation causes the connectives to contract and the brain to appear as a small knot of ganglia.

In Fig. 1 names and numbers have been assigned to each nerve. The names are similar to those in de Vlieger (1968) and the numbers are arbitrary. The nerves with which this work is primarily concerned are the optic (8), the tentacular (9) the pallial (13, 14, 18, 19), columellar (22) and the anterior and posterior cervical nerves (21 and 20 respectively).

Electrical stimulation

In accord with the philosophy of using a system as close as possible to the behavioural one, the optic nerve (8) was dissected free from the tentacular nerve (9) and stimulated. This, however, gives no response even when a stimulus of 20 V is applied. Withdrawal responses are, however, elicited from stimuli applied to a complex of three nerves, the optic (8), tentacular (9) and the minor labial nerve (a branch of the inferior labial nerve (7)), all of which run together after leaving the CNS. Further experiments have been conducted in which these three nerves (hereafter called the 'optic complex') were stimulated together. It is therefore uncertain which sensory modalities are being stimulated. In, addition the motor nerves might be antidromically excited.
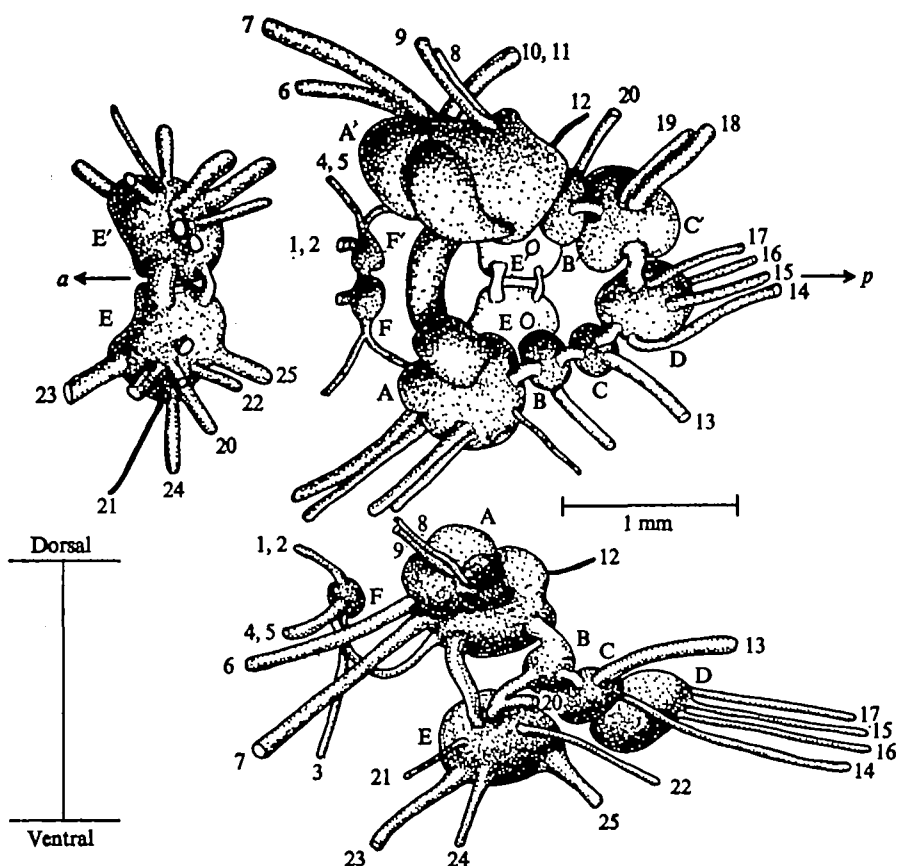
Fig. 1. Diagrams of the Central Nervous System of *Lymnaea stagnalis* reconstructed from sections, showing a dorsal view (above) and a lateral view (below). A and A', cerebral ganglia; B and B', pleural ganglia; C and C', parietal ganglia; D, visceral ganglion; E and E', pedal ganglia; and F and F', buccal ganglia. Nerves from the buccal ganglia: (1) anterior gastric; (2) posterior gastric; (3), (4), (5) primary, secondary and tertiary pharyngeal nerves. Nerves from the cerebral ganglia; (6) superior fronto-labial; (7) inferior labial; (8) optic; (9) tentacular and minor labial; (10), (11) penis nerves; and (12) nuchal nerve. Nerves from the visceral ring (parietal/visceral ganglia): (13) left pallial; (14) cutaneous pallial; (15) anal; (16) intestinal; (17) genital; (18) internal right pallial; and (19) external right pallial nerve. Nerves from the pedal ganglia; (20) posterior cervical; (21) anterior cervical; (22) columellar; (23) anterior pedal; and (24) posterior pedal nerve.

Motor innervation

The optic complex was stimulated whilst motor nerves that may potentially mediate the contractions were cut a pair at a time. Fig. 2 shows the result of one such experiment. This experiment was repeated, cutting nerves in the reverse order, with a similar result. After the columellar nerves (22) are cut, a small reduction in the size of the response occurs. When the posterior cervical nerves are cut, the amplitude of the response is drastically reduced. Further diminution of the response is caused by cutting the anterior cervical nerves (21). The residue of the response is mediated via the pedal nerves to the rest of the foot (23, 24, 25).
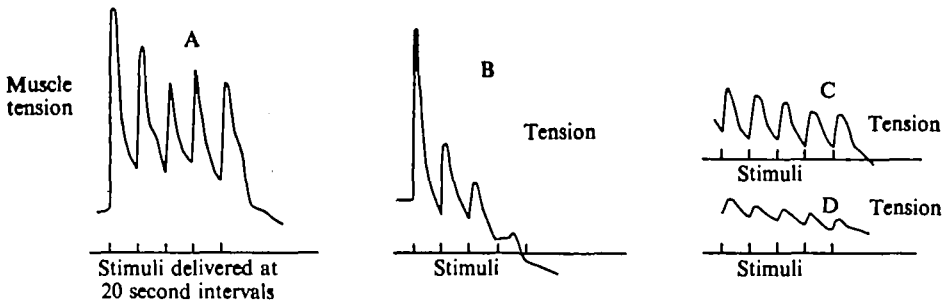
Fig. 2. The effects of progressively cutting motor nerves that potentially mediate the movements of the insertion of the columellar muscle. (A) muscular contractions in response to repeated stimulation with the nervous system intact except for one severed 'optic' complex, which was being stimulated. (B) same as (A), after cutting the columellar nerves (22). (C) the same as (B), after cutting the posterior cervical nerves (20). (D) the same as (C), after cutting the anterior cervical nerves. An upward excursion of the trace represents a muscular contraction. The areas innervated by nerves 20, 21 and 22 are shown in Fig. 8.
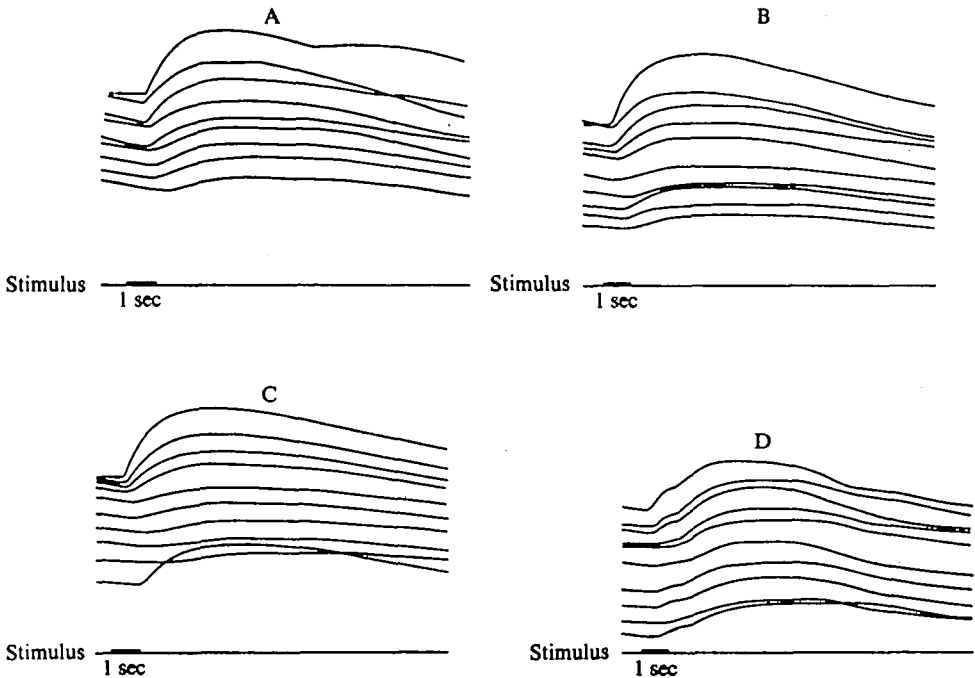

Fig. 3. Records to show the latency of response to electrical stimulation. (A) Internal right pallial nerve (18), (B) external right pallial nerve (19), and (C) the optic complex (8+9). These three records were produced in response to stimuli directed towards the brain. (D) Responses to the stimulation of the columellar nerve towards the muscle. In all cases stimuli were of one second duration and were repeated at ten second intervals. The first response is at the top and subsequent ones are arranged in register below at convenient spacings. An upward excursion of the trace represents a contraction.
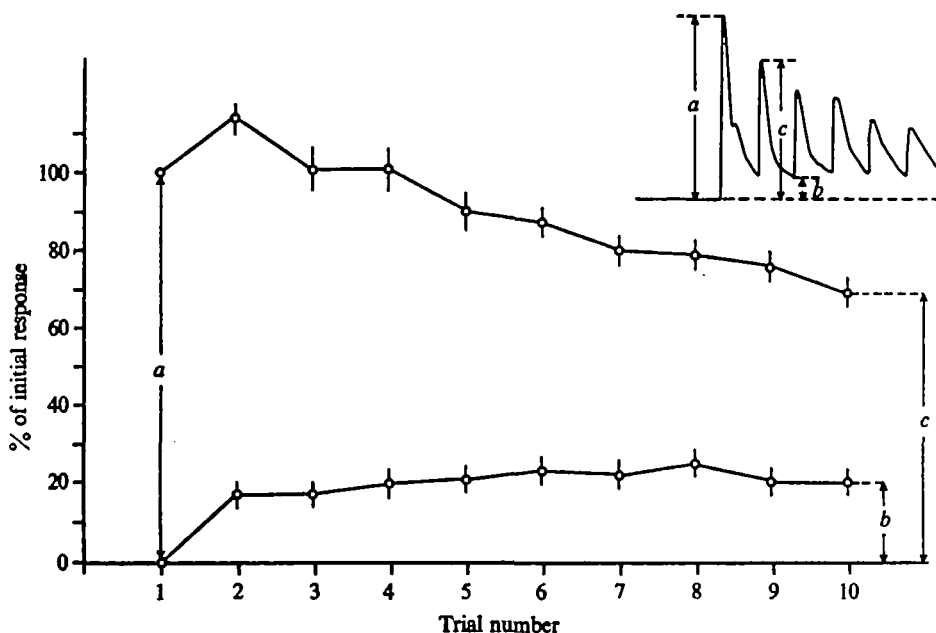
Fig. 4. The amplitude of responses to repeated electrical stimulation of the optic complex (8+9). The basal height (b) and the total height (c) are expressed as a percentage of the initial response height (a). The means and standard errors from thirty experiments are shown. The inset illustrates the source of the measurements, a, b, and c.

Effects of a repeated stimulus

When electrically stimulated, all nerves entering the CNS are capable of mediating the withdrawal response, with the exception of the optic nerves (8) and the nerves entering the buccal ganglia. Where some responses have been elicited, some decrease in the amplitude of the responses (habituation) takes place when the stimuli are repeated. In the case of the optic complex (8, 9) and the nuchal nerve (12) the responses generally disappear completely. When the left pallial nerve (13) is stimulated, however, the response amplitude, whilst declining initially, reaches a steady level at which responses persist.

Analysis of a number of records allows some quantitative statements to be made:

(i) *Latency*. The latency of response varies a great deal from snail to snail, but individuals are consistent, even when different nerves are stimulated (Fig. 3A, B, C). Stimulation of the columellar nerve towards the muscle produces a response with a latency similar to that observed from the stimulation of other nerves towards the CNS, and it appears that the greatest delay in the pathway occurs between the stimulation of the motor nerve and the development of tension in the muscle. When stimuli are repeated, there is a gradual increase in latency in all cases. This corresponds with an increase in response latency seen in the behaviour of the intact animal (Cook, 1970).

(ii) *Amplitude*. Only the responses to the stimulation of the optic complex have been studied sufficiently intensively to allow generalizations to be made about the change in amplitude observed on the repetition of the stimulation.
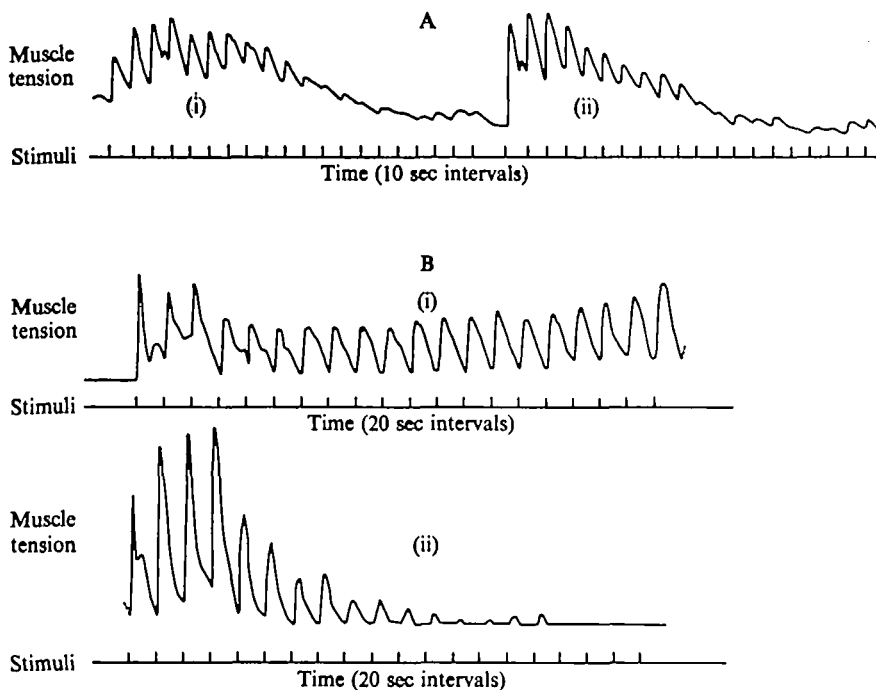
Fig. 5. The effects of consecutively stimulating pairs of nerves. (A)(i) The left optic complex (8+9) followed by (ii) the right optic complex (8+9). Stimuli were delivered at ten second intervals. (B)(i) The left pallial nerve (13) followed by (ii) the left optic complex (8+9). Stimuli were delivered at 20 sec intervals. There is no interaction between the responses given by these pairs of nerves.

The amplitude of the first ten responses was measured from the records of thirty experiments in which the optic complex was stimulated. Taking the level before the initial response as a base, two measurements were made: first from this base to the peak of the response (total height) and secondly from the base to the level at which the response started (basal level). Both these measurements have been expressed as a percentage of the initial response height. Fig. 4 shows the means and standard errors for these 30 experiments. It will be noted that the total height declines with stimulus repetition, that there is an increase in amplitude affecting the first few responses, and that there is an increase in the basal level of contraction of the muscle between responses. Of the thirty experiments used in this analysis, in only five cases was the second response smaller than the first. Of the remaining 25 the increase in the basal level was sufficient to account for the increase in total height in only 9 cases. In the rest (16 records) the actual contraction (i.e. the difference between the total height and the basal level) was larger in response to the second stimulus than to the first. Furthermore, whilst the responses themselves may return to their initial amplitude with a rest following stimulation, only rarely does the initial rise in response height return. The incremental process, therefore, is subject to a decline with repeated stimulation, and the recovery of this second decremental process has a different time course from that of the decremental process involved in the response decrement self.
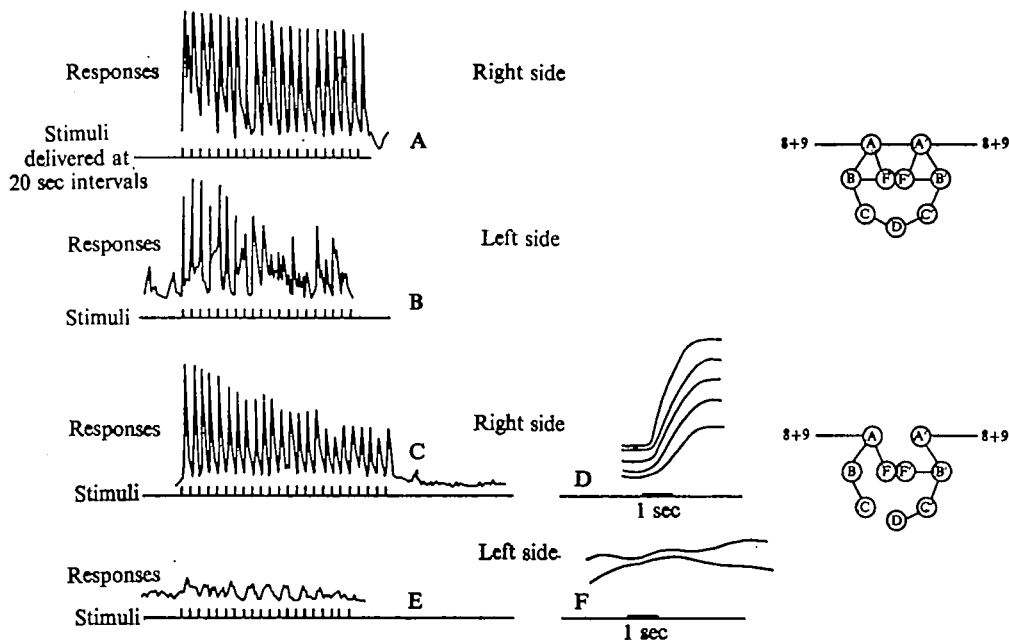
Fig. 6. Typical results of an experiment using one side of the brain as a control for the other. Stimuli were delivered to the left and right optic complexes (8+9) at 20 second intervals and were of one second duration. (A) and (B) normal sequences of responses from the right and left sides respectively. The cerebro-cerebral, left viscero-parietal, left pleuro-pedal and the right cerebro-pedal connectives were then cut (see insets labelled as in Fig. 1). Subsequent stimulation to the right optic complex showed little change in responses (compare A with C). The responses were of normal latency and amplitude (D). Stimulation to the left optic complex, however, produces abnormal responses to a sequence of stimuli (compare B with E). The responses are of low amplitude and long, variable latency (F).

(iii) *Consecutive stimulation of pairs of nerves.* Experiments have been conducted in which a series of stimuli were delivered to one nerve followed immediately by a series delivered to another. If a habituation process is shared either partially or wholly by these two nerves it would be expected that the responses to the stimulation of the second would be of lower amplitude and habituate faster than those to the stimulation of the first. Fig. 5B shows the effect of stimulating the left pallial (13) and the left optic complex consecutively. There appears to be no interaction between these two nerves.

In 15 experiments in which the left and right optic complexes were successively stimulated no interaction was observed between the two. A sample record is shown in Fig. 5A. It may be concluded, therefore, that habituation to a stimulus is at least specific to the nerve along which it arrives.

Investigation of the pathway

The roles of various connectives in the brain have been investigated in a series of 26 experiments involving the cutting of these connectives.

The experiments have been of two types. First, stimuli have been delivered via one nerve and the effects of progressively cutting connectives has been observed. Secondly, one side of the CNS may be used as a control for the other. This is achieved by cutting
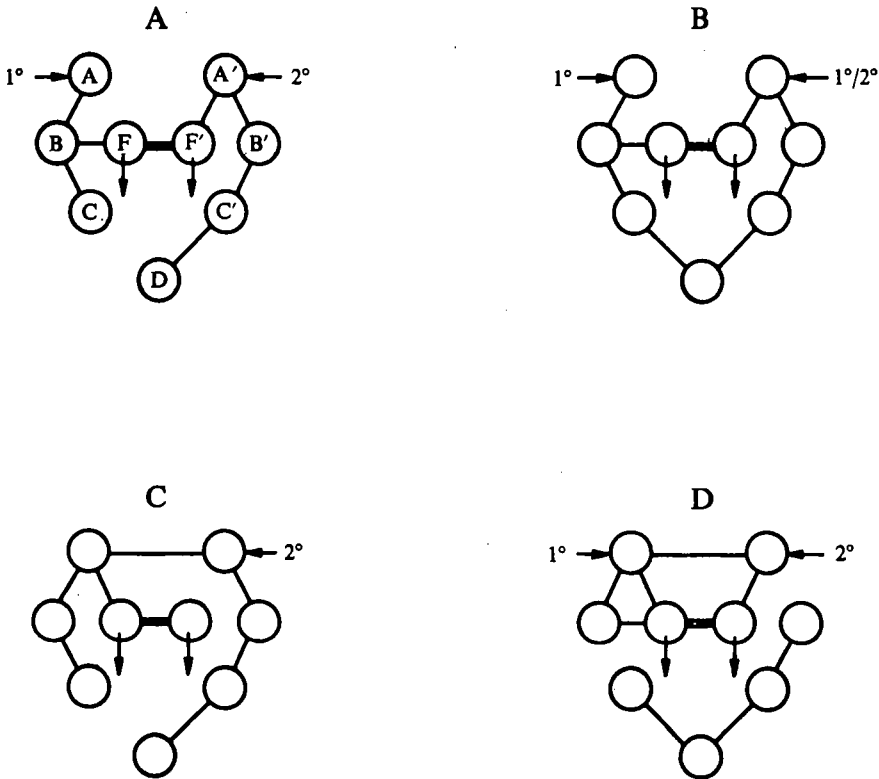
Fig. 7. The type of contractions produced in response to electrical stimuli to the optic complexes. 1° responses are normal. 2° responses are abnormal. (A) The ipsilateral pleuro-pedal connective will mediate primary responses; the ipsilateral cerebro-pedal connective will mediate only secondary responses. (B) The contralateral pleuro-pedal connective will sometimes mediate primary responses via the visceral ring of ganglia. In six experiments, 3 gave primary and 3 gave secondary responses. (C) The contralateral cerebro-pedal connective will mediate secondary responses via the cerebro-cerebral commissure. (D) The contralateral pleuro-pedal will not mediate primary responses via the cerebro-cerebral commissure.

the cerebro-cerebral commissure and viscero-parietal connective. Stimuli delivered to the left-hand side cannot now enter the right-hand side of the brain without passing through the pedal ganglion. Fig. 6 shows the sort of result obtained in this type of experiment. In these experiments, responses are only rarely abolished, but those that are made can be classified into one of two types. Primary responses (Fig. 6A, B, C, D) are similar to those normally produced before any connectives are cut. They have a short latency and are, initially at least, of large amplitude. Secondary responses (Fig. 6E, F) are elicited from animals in which the pleuro-pedal connectives have been cut. They are of long, variable latency and of low amplitude when compared to the primary responses of the same animal. Similar results have been obtained stimulating the left pallial nerve (13). The results of this series of experiments are summarized in Fig. 7, and the major conclusions to be drawn are, first, that the pleuropedal connectives are of paramount importance in the mediation of normal (primary) responses, and, secondly, that the pathway is response specific, i.e. the same pedal connective is concerned, irrespective of the nerve stimulated, provided that it did not enter the pedal ganglion directly.

Lesions

The experiments involving electrical stimulation of nerves give simple and clear-cut results but unfortunately do not give information about what is happening in a free-roaming, unstressed animal. The applicability of lesions is limited by the necessity both of performing the operation and of keeping the animal alive long enough for it to recover from the shock of the operation.

Location of the photoreceptors

Various nerves and combinations of nerves have been cut and the animals subsequently tested for their responsiveness to a visual stimulus. Table 1 gives a catalogue of these experiments. In only one experiment was the response to light changed. This was experiment 6 in which the optic (8), tentacular (9), left pallial (13), internal right pallial (18) and the external right pallial (19) nerves were cut. Responses were not abolished in all individuals but the number of responses made by the group of 19 as a whole to 20 stimuli was significantly lower than that made by a control group that had had their brains manipulated but no nerves cut (Mann–Whitney U Test, $P < 0.01$).

Pathway

To ascertain whether the pathway appropriate for the mediation of the withdrawal response to electrical stimuli was also appropriate in response to visual stimuli, the following experiment was performed. Ninety-three animals were used in all; 31 controls, which merely had their brains manipulated, 32 in which the left and right pleuro-pedal connectives were cut, and 31 in which the left and right cerebro-pedal connectives were cut. All those animals in which the cerebro-pedal connectives were cut died within one week of the operation and it must be assumed that either there is something particularly vital about this part of the nervous system or that the manipulations required to cut this connective are unacceptably severe. Of the 32 animals with the pleuro-pedal connective cut 9 survived for one week. Animals with this connective cut had their withdrawal response severely impaired. All hung out of their shells. The typical response to light was a rapid downward movement of the tentacles but no body withdrawal was ever observed. The response to tactile stimulation to the head of the animal was a local withdrawal of the labial and buccal areas but no body withdrawal. The 11 survivors from the control experiment responded normally.

Observations made on the third day after the operations (i.e. before the expiry of the normal recovery period) on those animals with their cerebro-pedal connectives cut indicated that they were capable of total withdrawal responses to tactile stimuli.

DISCUSSION

The responsiveness of *Lymnaea stagnalis* to a 'light off' stimulus has been shown to be reduced by denervation of the eyes, tentacles and most of the mantle. This result is unlikely to be a non-specific consequence of the extent of the denervation since more extensive denervation operations have been conducted without impairing the respon-

Table 1. *A summary of operations conducted to discover photoreceptive areas associated with the withdrawal response to 'light off' stimuli*

Experiment no.	Nerves cut	Number of survivors	Effect on the number of responses to 20 'light off' stimuli
1	8 and 9 (optic complex)	15	None
2	6 and 7 (labials)	2	None
3	6, 7, 8, 9 (optic complex and labials)	7	None
4	7, 8, 9, 10, 11, 12 (all cerebral nerves except inferior labials)	2	None
5	13, 18, 19 (parietal pallials)	15	None
6	8, 9, 13, 18, 19 (optic complex and parietal pallials)	19	Reduced significantly
7	None	15	None

siveness (Table 1, experiment 4). Medioni (1959) and Stoll (1972) using the same species found that the ablation of the eyes was not sufficient to prevent the withdrawal response to a moving shadow. Photoreceptors associated with the withdrawal response must be located in the tentacles and/or eyes, and in the mantle. Crisp (1972) has found structures interpreted as photoreceptors in the general body surface of *Nassarius reticulata*. *Lymnaea* has a shell which is often translucent and light could be transmitted through the shell to the mantle. Other specimens, however, have highly encrusted shells and light would not pass through. The edge of the mantle has a lighter pigmentation than the rest of the mantle and can be seen immediately below the shell edge. In this position it would be well situated to receive light stimuli. Denervation of the eyes and the major part of the mantle is not suffiecnt to prevent all responsiveness to light in all snails. The residual responsiveness could arise in various ways. First, other areas of the body might be light sensitive. This is a possibility which is difficult to eliminate experimentally since more extensive denervation of the body surface might cause behavioural changes unrelated to the animal's responsiveness to light. A second possibility is that the brain itself might be sensitive to light. Light sensitive neurones have been described in the CNS of *Aplysia* (Arvanitaki & Chalazonitis, 1961) and in the peripheral nervous system of *Mactra*, a bivalve (Kennedy, 1958). There are, however, indications that such cells are not involved in the mediation of the withdrawal response to light stimuli. First, the surface pigmentation of *Lymnaea* varies greatly: some are almost black whilst others are a light grey-brown. If the brain were acting as a photoreceptor it might be expected that the heavily pigmented animals would be less responsive than the lighter ones. Such a correlation has not been noted (Cook, 1974, unpublished data). Secondly, the action spectrum of the withdrawal response ranges from 400 to 650 μm with a peak at 520 μm (Medioni, 1959). Benjamin & Walker (1972) have separated a variety of carotoproteins and haemoproteins from the brain of *Lymnaea* and none of their absorption peaks correspond to the action spectrum of the response. There appears, therefore, to be little correlation between the response and the pigments.

The motor nerves involved in the response serve the muscles of the 'neck 'of the animal. The cervical nerves (20, 21) enter and presumably innervate longitudinal
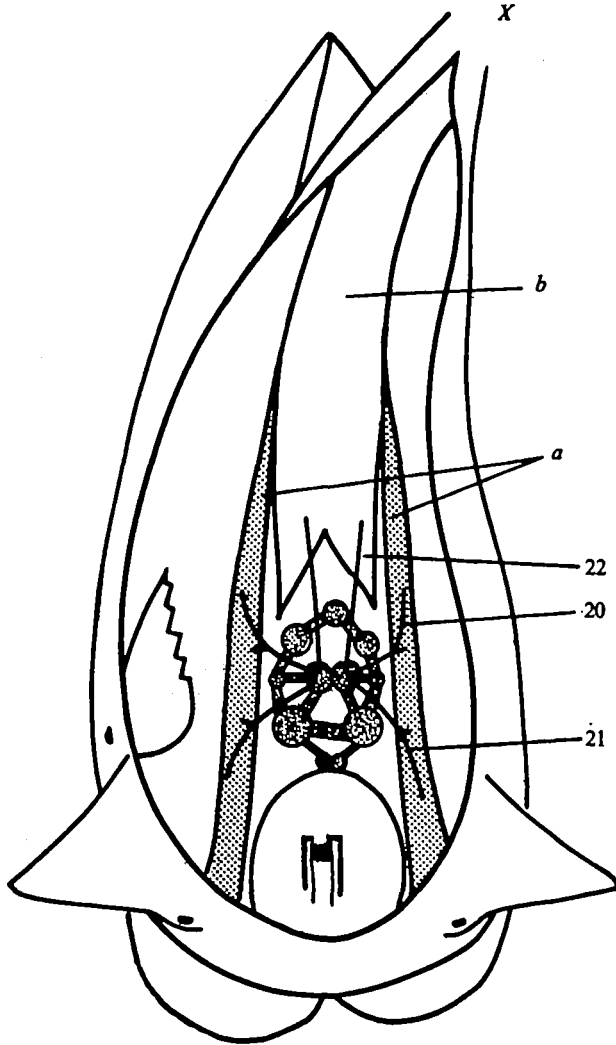
Fig. 8. A diagram of the muscles involved in the movement of the point of insertion of the columellar muscle onto the shell (*X*). (*a*) ' Cervical strips' of muscle innervated by the anterior (21) and posterior (20) cervical nerves. (*b*) Columellar muscle innervated by the columellar nerve (22). Most of the movement comes from the cervical strips and this is probably because the transducer is situated about 45° from the horizontal. The columellar muscle has a large vertical (out of the plane of the page) component to its contraction.

strips of muscle overlying the general foot musculature (Fig. 8). Their contraction tends to bring the head of the animal nearer the neck. The contraction of the columellar muscle tends to move the shell of the animal downwards and forwards. Given that the transducer used was attached to the columellar muscle at an angle of about 45° from vertical it is reasonable to expect that forward movement brought about both by the cervical longitudinal strips of muscle and the columellar muscle itself would be major contributors to the total movement of the point of attachment of the transducer.

A major objective of this work has been to learn something about the nature of the pathways and processes concerned in habituation seen at a behavioural level. On the

behavioural evidence it has been argued that habituation in this snail need not be a highly complex process (Cook, 1970). If we are prepared to make the assumption that the experiments concerned with the electrical stimulation of nerves described above are relevant to the behavioural situation, then the following generalisations may be added to our knowlege of habituation of *Lymnaea*. First, habituation is specific to the nerve along which the stimulus arrives (Fig. 5); i.e. it is not only modality-specific (Cook, 1970) but also spatially specific. Spatial specificity is well known from the habituation of other invertebrates, for instance *Notonecta glauca* (Wolda, 1961). Secondly, certain repeated forms of stimulation (viz. electrical stimulation of the optic complex) involve both decremental and incremental processes and that the incremental process itself is subject to a separate decremental process (Fig. 4). These processes may correspond to habituation and sensitization (= dishabituation) at the behavioural level (Cook, 1970). Thirdly, the pathway concerned in the response at the gross level of the ganglia and the connectives is response specific, i.e. the pleuro-pedal connective mediates the response no matter which peripheral nerve is stimulated. Verification of this pathway by a technique of anaesthetics and lesions proved only partially successful. The pleuro-pedal connective was shown to be essential for the mediation of the response in animals which were as near undamaged as possible. All those animals in which the alternative route (via the cerebro-pedal connective) was cut, died within 1 week. Consequently, one cannot be certain that the withdrawal response to light is mediated solely by the pleuro-pedal connective; it may, for instance, require the integrity of all pedal connectives. Observations made on animals with cut cerebro-pedal connectives during the recovery period when they are normally considered to be 'ill' suggested that they were capable of normal responses to tactile stimulation. Since the pathway for electrical stimuli has been shown to be response rather than stimulus specific it is unlikely that the difference in stimuli is significant. On balance therefore, the evidence suggests that the pathway worked out for electrical stimulation is relevant to the behavioural response to light.

de Vlieger (1968) investigated the pathway involved in an electrical response from the left pallial nerve (13) to a tactile stimulus delivered to the lips. He found that 'the majority of the connections between ganglia of the C.N.S. (commissures and connectives) are unspecific as far as the capacity to transmit tactile signals from the lips is concerned'. This may well be the case for the components of the withdrawal response mediated via nerves serving such areas as the mantle. The present experiments show it not to be the case for the major muscular movements themselves.

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