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THE NEURAL CONTROL OF EGG-LAYING BEHAVIOUR IN THE POND SNAIL *LYMNAEA STAGNALIS*: MOTOR CONTROL OF SHELL TURNING

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Summary

Behavioural and neurophysiological techniques were used to study the neuronal control of shell turning during egg-laying in the pond snail *Lymnaea stagnalis*. Egg-laying consists of three phases: resting, turning and oviposition, and is triggered by an electrical discharge in a group of neuroendocrine cells, the caudodorsal cells. During the discharge, several peptides encoded on two *CDCH* genes are known to be released. Behavioural experiments in which different combinations of nerves were lesioned indicated that the inferior cervical nerves are necessary for turning behaviour to occur. The right inferior cervical nerve innervates the right dorsal longitudinal muscle and contains axons of neurones that are active just prior to, and during, shell movements in freely behaving animals. These axons are probably the axons of motor neurones. The motor neurones of the dorsal longitudinal muscle were identified in the cerebral A and pedal N clusters. We have demonstrated that there is a correlation between the state of excitability of the caudodorsal cells and the electrical activity of the pedal N motor neurones. Our results indicate that the pedal N motor neurones are involved in executing the turning phase during egg-laying.

Introduction

A recent goal of behavioural neuroscience is to determine the role that peptidergic neurones play in producing and modulating behaviour. Particularly interesting is the question of why some peptidergic neurones release more than one peptide or hormone. The egg-laying behaviours of the gastropod molluscs *Aplysia* and *Lymnaea* have provided convenient model systems for such studies because the discharge of a large number of identifiable neurosecretory neurones initiates stereotyped patterns of behaviour (for a review, see Geraerts *et al.* 1988). In both cases, the peptidergic neurones controlling egg-laying produce multiple peptides (Kupferman, 1967; Rothman *et al.*

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1983; Geraerts *et al.* 1985; Sigvardt *et al.* 1986), which are cleaved from a common precursor (Scheller *et al.* 1983; Vreugdenhil *et al.* 1988; Li *et al.* 1992). Before egg-laying, these cells produce a discharge of action potentials (Pinsker and Dudek, 1977; Ter Maat *et al.* 1986) during which the peptides are released and egg-laying behaviour is initiated.

In neither *Aplysia* nor *Lymnaea*, however, have the motor neurones that drive the egg-laying behaviour been identified. The effects of the peptides on these motor neurones are therefore unclear.

In the pond snail *Lymnaea stagnalis* the neuroendocrine cells controlling egg-laying are the caudodorsal cells (CDCs). This is a group of about 100 electrotonically coupled neurones, located in the cerebral ganglia. These cells have axons that form a neurohaemal area in the cerebral commissure (Wendelaar Bonga, 1970; De Vlieger *et al.* 1980). From this area, CDC peptides are released into the blood during the electrical CDC discharge (Kits, 1981; Buma and Roubos, 1983).

The CDCs exhibit three states of excitability, resting, active and inhibited, each with distinct electrophysiological characteristics. Transitions between these states can occur spontaneously or can be induced experimentally. When the CDCs are in the resting state, a train of depolarising current pulses elicits an afterdischarge, the active state. From this active state, the cells enter the inhibited state (Kits, 1980). The ability to generate discharges is positively correlated with the amplitude of the depolarising afterpotential that is induced by a short train of depolarising current pulses (Brussaard *et al.* 1988).

In the isolated central nervous system, a CDC discharge can be elicited by repetitive suprathreshold depolarisation of resting-state CDCs (De Vlieger *et al.* 1980). In the intact animals, the electrical CDC discharge can be initiated by stimulating the animals with clean water (Kits, 1980; Ter Maat *et al.* 1983). CDC discharges also occur spontaneously.

Within minutes of the start of the electrical discharge in the CDCs, animals show egg-laying behaviour (Ter Maat *et al.* 1986). This egg-laying behaviour is a sequence of stereotyped movements in which three phases can be distinguished: resting, turning and oviposition (see also Ter Maat *et al.* 1989). During turning, the animals make long-lasting turns of their shell through more than 60 ˚ relative to the head-foot. These turns only occur in the second phase of egg-laying and are not part of the animal's other behaviour patterns. It is to be expected, therefore, that the motor neurones involved in these shell movements will be inhibited during the resting phase and excited during the turning phase of egg-laying behavior.

The present study attempts to determine which muscles, nerves and motor neurones are responsible for the stereotyped shell movements observed during egg-laying and what effect a CDC discharge has on these motor neurones.

Materials and methods

Animals

Adult specimens of *Lymnaea stagnalis* (L.) (age 4–6 months, shell length 25–35 mm), bred under standard laboratory conditions, were used in all experiments (Van der Steen *et al.* 1969). The animals were housed in perforated jars placed in a large tank with

running fresh water (20 °C), and were kept under a 12 h:12 h light:dark cycle and fed daily on lettuce.

Lesion experiments

The experimental animals were anaesthetised by injection of 1.5 ml of $MgCl_2$ (50 mmol l^{-1}) into the foot. Within 2 min, all responses to tactile stimulation had disappeared. The head-foot was then opened dorsally over a length of 3 mm. The slit was held open with surgical hooks, and nerves were lesioned by cutting a specific nerve either unilaterally or bilaterally. Unoperated and sham-operated animals were included as controls. The latter animals were operated on in the manner described above, but no nerve lesion was performed (N=6 for each group). After the operation, animals were housed individually for 7 days in closed jars. The water in these jars was not replaced during this period. Egg-laying was then induced by transferring the animals to a jar with clean water (i.e. a clean water stimulus; Ter Maat *et al.* 1983). From the moment of placing the animals into the jar until 1 h after oviposition the animals were continuously recorded on video tape.

All lesions were checked after the experiments.

Analysis of shell turning

Shell movements were analysed starting approximately 1 h prior to the end of oviposition. Shell movements were analysed using two different methods. In the first method, shell movements were determined using a PC Vision Plus (Imaging Technology) video digitizer and a PC/AT personal computer to capture and process a video frame every 15 s. Shell position relative to the head-foot complex axis was measured by determining the coordinates of the left and right tentacles, the anterior end of the shell and the apex (i.e. posterior end) of the shell. These coordinates were rotated to a standard position, as shown in Fig. 1A. The angle between the line connecting the tentacle coordinates and the line connecting the shell coordinates was then calculated (Fig. 1A). Each point in this figure corresponds to a position of the apex of the shell sampled at 15 s intervals over approximately 1 h prior to oviposition. The normal position of the shell relative to the head-foot was between 175 ° and 180 °.

In order to obtain a dynamic description of shell movements, a second method of analysis was used. In this method, measurements were made of (i) the normal positions of the shell, (ii) the anterior shell positions (retracted) and (iii) positions of the shell greater than 10 ° to the left or right of the normal position of the shell (i.e. 180 °). Movements of the shell in the posterior or anterior directions while it remained turned to the left or right were indicated as left′ and right′. Only one position could occur in a given period (Fig. 1B).

Whole-nerve recordings in freely behaving animals

Permanently implanted electrodes were used to monitor the electrical activity of nerves in freely behaving animals (Parsons *et al.* 1983). Stainless-steel fine wire electrodes (25 μm in diameter, California Fine Wire Company) were implanted to record electrical spiking activity in the inferior cervical nerves (N=10). In order to implant the electrodes,

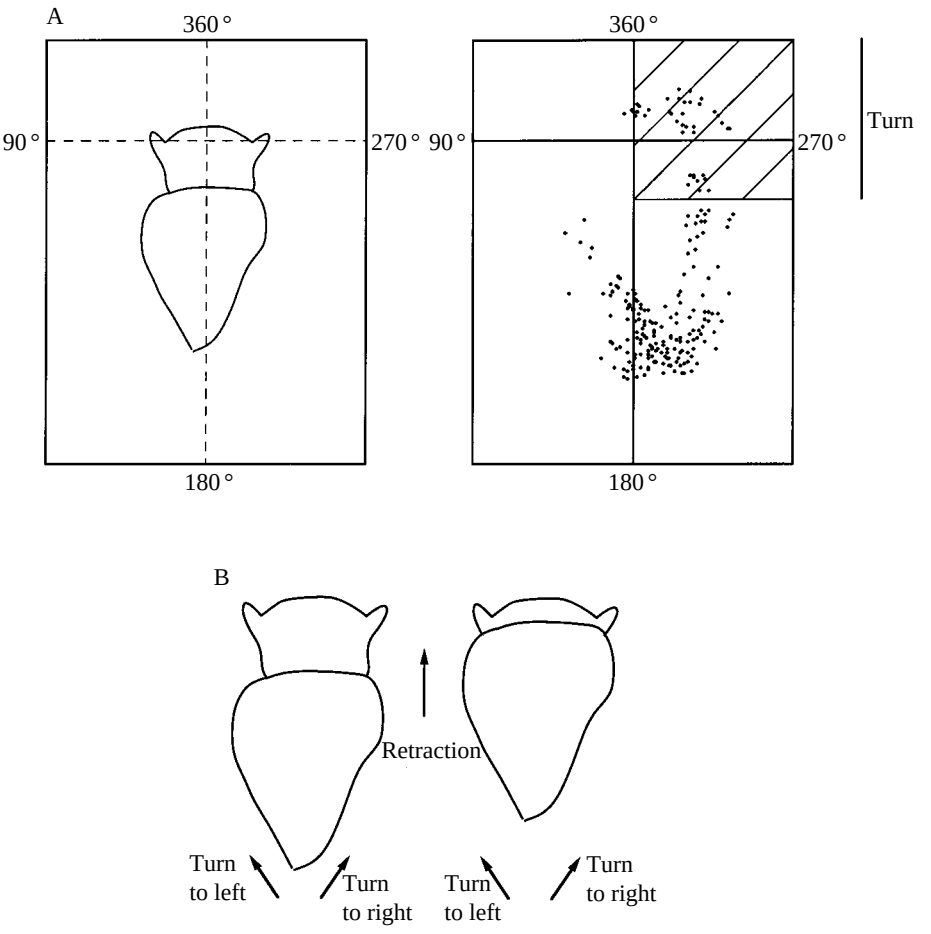

Fig. 1. Analysis of turning behaviour in freely behaving animals. (A) The position of the shell was measured by determining the coordinates of the left and right tentacles and the anterior end and apex of the shell. The normal position of the shell relative to the head-foot was between 175 ˚ and 180 ˚. A movement of the shell was considered to be a turn when the position of the shell was more than 250 ˚ relative to the head-foot (i.e. 70 ˚ relative to the normal position of the shell; shaded area on right). Each point corresponds to a position of the apex of the shell sampled at 15 s intervals. (B) In a second method, measurements were made of the extent of shell retraction and the position of the shell when it was more than 10 ˚ to the left or right of its normal position (i.e. 180 ˚).

animals were anaesthetised and the head-foot was opened as described above. Electrodes were inserted into the body cavity through the body wall and positioned around the right inferior cervical nerve. The nerve was dried with a jet of air and the wire was secured in position with tissue adhesive (Pattex supergel, Henkel) or with dental impression material (Reflect, Kerr). A loop of the wire was left in the body cavity and the electrode lead was secured to the shell with tissue glue. The entire procedure took about 20 min. Animals were then transferred to an experimental chamber provided with a continuous supply of

water and left overnight to recover from the anaesthesia. The day after electrode implantation, the animals were placed in an observation jar and the electrodes were connected to a WPI DAM 80 differential amplifier (10 Hz high-pass filter, 1 kHz low-pass filter). Electrical activity in the nerve as well as the behaviour of the animal were recorded simultaneously and stored on video tape. A time code generator (TC 30, Alpermann and Velte) was used to provide every video frame with a time code in order to be able to synchronise the behaviour stored on video tape with the digitised electrical activity of the nerve.

Analysis of nerve activity

A spike train analysis program (a modified version of the program described by Jansen and Ter Maat, 1992) was used to reconstruct the firing patterns of individual neurones from wave forms in the *in vivo* whole-nerve recordings. This program enabled us to detect the occurrence of matching wave forms, requiring no prior knowledge other than the approximate duration of the wave form. The wave forms were digitised using a Cambridge Electronic Design, model 1401, 12-bit analog-to-digital converter. An instantaneous frequency plot of each individual wave form was made.

Identification and morphology of neurones

Neurones that project into the right inferior cervical nerve were identified by backfilling this nerve with nickel–lysine (1.7 g of $NiCl_2{\cdot}6H_2O$ plus 3.5 g of L-lysine free base in 20 ml of water; BDH Chemicals and Sigma) for 24 h at 20 °C. The preparation was stained with rubeanic acid (Janssen Chimica; Quicke *et al.* 1980), dehydrated in a graded series of alcohols and cleared in methyl salicylate (Sigma). The preparations were embedded in Entellan (Merck), examined under the microscope and photographed.

The morphology of motor neurones was revealed by intracellular anterograde tracing with Lucifer Yellow (Sigma). Neurones were impaled with electrodes containing a 5 % aqueous solution of Lucifer Yellow. The dye was injected into the cells using a pressure injector. After injection, the brain was fixed in 4 % formalin, dehydrated in alcohol and cleared in methyl salicylate. The cleared preparations were viewed under a fluorescence microscope and photographed.

Preparations and recordings

Reduced preparations consisted of the central nervous system (CNS), the right inferior cervical nerve and the right half of the head-foot complex. An incision was made along the dorsal midline of the head. The preparation was pinned out flat in a recording chamber lined with a silicon elastomer (Xantropen-Blue, Bayer) with the internal surface of the right head-foot complex uppermost. All nerves other than the right inferior cervical nerve were cut. The CNS was pinned down on a Xantropen-covered platform suspended above the muscular part of the preparation. The outer layer of connective tissue of the CNS was carefully removed in order to expose the neurones to be impaled.

The preparations were maintained in Hepes-buffered saline of the following composition (in $mmol\,l^{-1}$): NaCl, 30.0; KCl, 1.7; $MgCl_2$, 1.5; $CaCl_2$, 4.0; $NaHCO_3$, 5.0; sodium methylsulphate, 10.0; Hepes, 10.0; adjusted to pH 7.8 with NaOH. Except for

sodium methylsulphate (Merck), the chemicals used in the saline were obtained from J. T. Baker. The neurones were impaled with glass microelectrodes (GC150F-10, Clark Electromedical; impedance 50–60 MΩ), filled with 0.5 mol l^{-1} KCl. An *en passant* extracellular electrode was used for recording electrical activity of the nerve. Extracellular recordings of the electrical activity of the muscle were made using glass microelectrodes ground to an appropriate diameter. The electrical activity of the neurones, nerve and muscle were recorded on FM tape (TEAC XR-310; bandwidth d.c. to 1250 Hz). The experiments were carried out at room temperature.

Effect of caudodorsal cell afterdischarges on the electrical activity of motor neurones

The effects of CDC afterdischarges on the electrical activity of identified motor neurones were studied as follows. At the start of each experiment, the state of excitability of the CDCs was determined with brief electrical stimulation (20–40 suprathreshold 1 nA pulses of 100 ms at 3 Hz) that could induce depolarising afterpotentials (DAPs). DAPs only occur when the CDCs are in the resting state (Brussaard *et al.* 1988; Kits, 1980).

Recordings were made from preparations in which the CDCs were in the resting state (in which no afterdischarge was induced) as well as from preparations in which the CDCs were in the inhibited state (where no afterdischarge could be induced). In a third group, afterdischarges were induced by electrical stimulation (N=7 for each group) using repetitive suprathreshold depolarising stimulation at 2 Hz for periods of 1–4 min. Simultaneous recordings were made of the electrical activity of the motor neurones, the CDCs, the right inferior cervical nerve and the right dorsal longitudinal muscle. Preparations and recordings were as described above.

The electrical activity of the neurones, nerves and muscles were recorded from 15 min after the neurones had been impaled with an electrode until at least 135 min after impalation. An afterdischarge was induced 30 min after the start of the recording.

Statistical techniques

Throughout this paper, the minimum criterion of statistical significance was $P<0.05$. Group means are always presented with their S.E.M.

A factorial analysis of variance (Bonferroni adjustment) was used to evaluate differences in the total duration of shell turns during the 1 h prior to oviposition between animals with different nerve lesions and controls. Prior to this test, data were tested for normality.

The Wilcoxon signed-rank test was used to evaluate differences between the mean spiking frequency of units in the right inferior cervical nerve, 4 s before and 4 s after a transition of the shell from one position to another position.

A factorial analysis of variance (ANOVA) was performed to evaluate effects of the CDC afterdischarge on the spiking frequency of motor neurones (preparations with the CDCs in an active state *versus* a resting state) and to evaluate differences in the spiking frequency of the motor neurones in preparations with the CDCs in a resting state *versus* an inhibited state. Prior to ANOVA, firing rates were tested for normality and transformed to their $\log_{10}$ values.

Results

Lesion experiments

To investigate which muscles are involved in shell movements and turning behaviour during egg-laying, normal turning during egg-laying was recorded and analysed (N=6). Egg-laying was induced with clean water and shell position determined every 15 s. Each dot in Fig. 1A represents one position of the apex of the shell sampled during turning until oviposition.

The number and duration of turning movements in the turning phase showed considerable variability between animals. However, in all animals, the normal position of the shell relative to the head-foot was between 175 ° and 180 ° (i.e. with the apex of the shell pointing backward). During a period of approximately 1 h prior to the end of oviposition, all the animals turned their shells counter-clockwise more than 60 ° relative to the normal position 2–4 times, each time maintaining this position for several minutes. Fig. 2A gives an example of the shell movements of a control animal for 1 h prior to oviposition.

Muscles involved in the whole-body withdrawal response are the columellar muscles (CM) and the dorsal longitudinal muscles (DLM; Cook, 1975; Ferguson and Benjamin, 1985, 1991*a*). These muscles are also involved in shell movements during locomotion (Winlow and Haydon, 1986). Therefore, it is possible that the CM and the DLM are involved in shell movements and turning behaviour. The nerves that innervate these muscles or that have projections into the mantle or column areas are the columellar nerves, the superior and inferior cervical nerves, the cutaneous pallialis nerve and the parietal nerves (Elo, 1938; Janse, 1974; Ferguson and Benjamin, 1991*a*).

To determine which of these nerves are necessary for shell movements, lesions were made of the cutaneous pallialis nerve, the left parietal nerve, the columellar nerve or the inferior cervical nerve. The latter nerve was lesioned either unilaterally or bilaterally (N=6 for each lesion). In both lesioned and sham-operated animals, egg-laying was induced with clean water. Shell movements were analysed for 1 h prior to oviposition using the method described above (see also Fig. 1A).

Lesion of the left parietal nerve, the cutaneous pallialis nerve or a bilateral lesion of the columellar nerves had no apparent effect on shell movements during egg-laying (Fig. 2C–E). Animals with a lesion of one of these nerves made long-lasting counter-clockwise rotations of more than 60 ° relative to the normal position of the shell. Their shell movements were comparable with those of unoperated animals (Fig. 2A) or sham-operated animals (Fig. 2B). The mean total duration of shell turns for 1 h prior to oviposition did not differ from that of the controls (Fig. 3; Bonferonni adjustment). In contrast, after a bilateral lesion of the inferior cervical nerves, the animals were unable to turn or move their shells. The position of the shell relative to the head-foot remained between 175 ° and 180 ° (Fig. 2F), which is the normal position with the apex pointing backwards. No rotations of the shell occurred during the 1 h before oviposition (P<0.001 compared with the controls; Fig. 3). Unilateral lesion of the right inferior cervical nerve also resulted in a strong reduction of shell movements. Rotations that occurred in these animals never exceeded 20 ° (not shown).

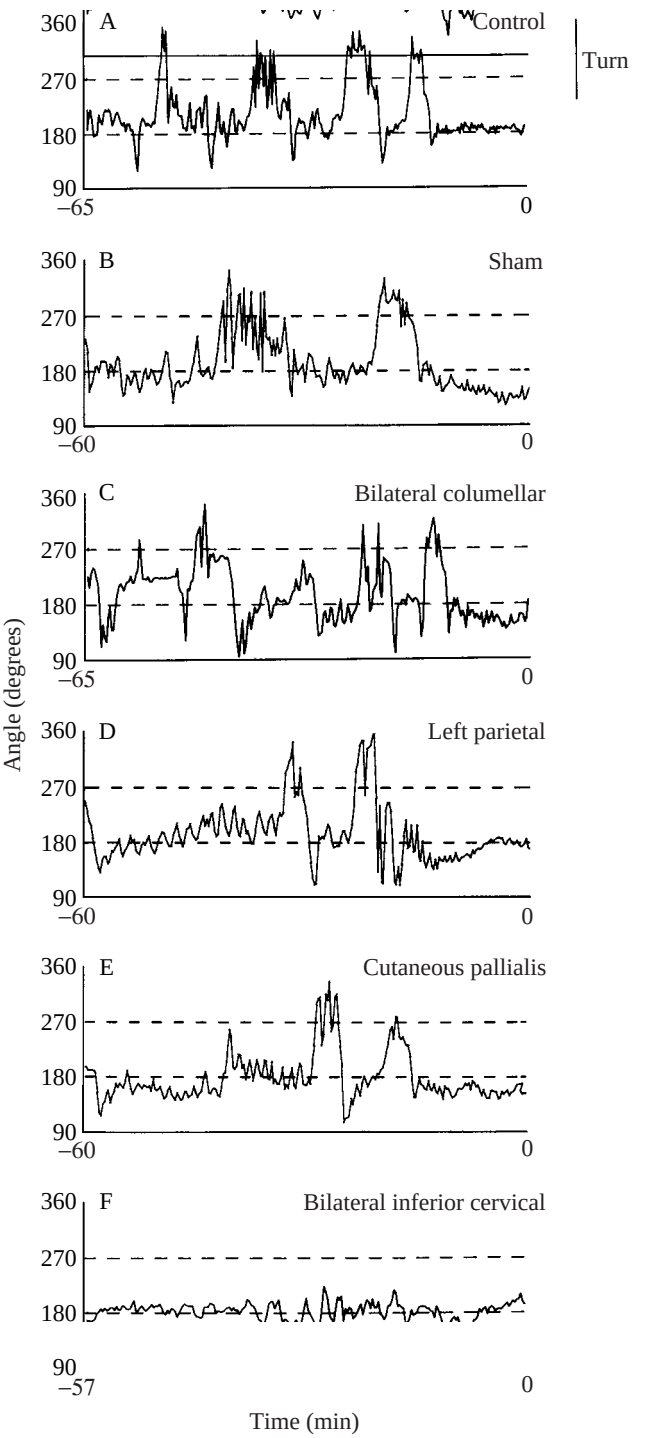
A
Control
Turn
B
Sham
C
Bilateral columellar
D
Left parietal
E
Cutaneous pallialis
F
Bilateral inferior cervical
360
270
180
90
−65
−60
−57
0
Angle (degrees)
Time (min)

Fig. 2

Fig. 2. Turning behaviour during egg-laying recorded for approximately 1 h prior to oviposition (time=0). (A) Example of normal turning behaviour (unoperated). (B) Example of turning behaviour after a sham operation. (C–F) Examples of turning behaviour after successive bilateral lesions of the columellar nerves (C), lesion of the left parietal nerve (D), lesion of the cutaneous pallialis nerve (E) or bilateral lesions of the inferior cervical nerves (F). Note the complete abolition of turns after bilateral lesions of the inferior cervical nerves.

It was not possible to lesion selectively the superior cervical nerves because this procedure damaged the surrounding blood vessels, and nerves coming from the cerebral ganglia and the animals did not survive. It cannot, therefore, be excluded that the superior cervical nerves are also involved in turning behaviour. However, the total abolition of shell movements after a lesion of the inferior cervical nerves indicates that these latter nerves contain most of the elements necessary for shell movements.

Our conclusion is that the inferior cervical nerves are necessary for turning behaviour to occur.

Whole-nerve recordings during turning in freely behaving animals

In order to demonstrate that the inferior cervical nerve contains axons of motor neurones that are involved in turning behaviour, it is necessary to show that there is appropriate electrical activity in this nerve before or during shell movements in freely behaving animals.

Whole-nerve recordings were made with fine wire electrodes implanted into freely

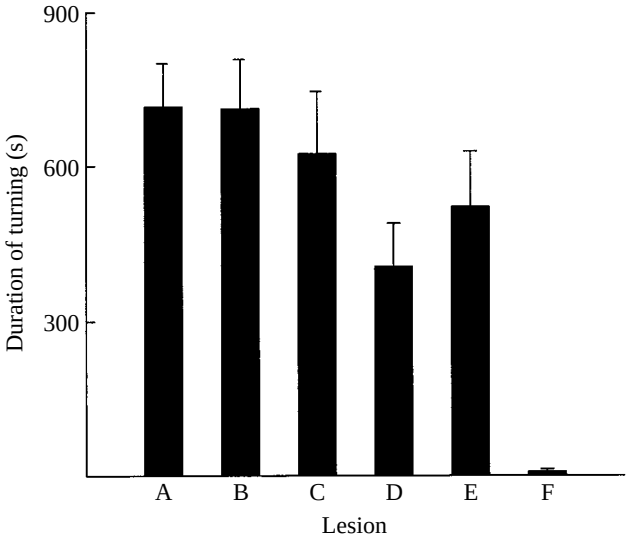

Fig. 3. Turning behaviour during egg-laying over a period of approximately 1 h prior to oviposition. Mean of the total duration of turns for 1 h prior to oviposition (+ S.E.M., N=6) after lesion of a nerve, and in controls. (A) unoperated; (B) sham; (C) bilateral columellar nerves; (D) left parietal nerve; (E) cutaneous pallialis nerve; (F) bilateral cervical nerves. The total duration of shell turns after lesion of the inferior cervical nerves is significantly different from that of the controls (P<0.001; Bonferroni adjustment).

behaving animals (*N*=8). Most of the recordings were made from the right inferior cervical nerve, because the animal turns the shell counter-clockwise, i.e. to its right. The electrical spiking activity was recorded onto the same video tape that contained images of the animal.

To estimate the effect of electrode implantation on turning behaviour, the position of the shell relative to the head-foot was analysed for 1 h prior to oviposition. Fig. 4A illustrates shell movements of an animal with an implanted electrode. The animal was able to make long-lasting counter-clockwise rotations of its shell, comparable to the rotations seen in control animals (c.f. Fig. 2A,B). It seems that the electrode did not damage the nerve or hinder shell movements.

In order to describe changes in the electrical activity in the right inferior cervical nerve during turning behaviour, a dynamic description of shell movements was made and spiking activity was analysed (*N*=4). The extent of shell retraction and the extent of shell rotations greater than 10 ° relative to the normal position of the shell (i.e. 180 °) were quantified (see Fig. 1B). The transition from one position of the shell (i.e. the shell pointing backwards or retracted) to another (i.e. forward or rotation movements) was then determined.

The electrical activity of particular units (i.e. wave forms) was reconstructed from the whole-nerve recording of the right inferior cervical nerve. Instantaneous spiking frequencies of each unit were correlated with shell movements.

The mean spiking frequencies were determined from 4 s before until 4 s after a shell transition. All the animals analysed had units in the right inferior cervical nerve whose spiking frequency changed significantly just before a forward movement of the shell (retraction; backward–forward). Transitions to the right (backward–right or forward–right) were also preceded by significant changes in the spiking frequency of some units. Most of these units (20 %) increased their spiking frequency just before the transition. Only 5 % of the units showed an increase following a transition. We never found a change in the spiking frequency of units in the right inferior cervical nerve before or following a transition of the shell to the left (forward–left).

Fig. 4B illustrates a short period of electrical activity in the right inferior cervical nerve (top panel) during turning, together with the positions of the shell in that period determined by the dynamic analysis method (bottom panel). Fig. 4C shows changes in the mean spiking frequency (spikes s^{-1}) of selected units (insets) in the right inferior cervical nerve (obtained from one animal). During retraction of the shell from a backward-pointing position to a more forward position, there was a significant increase in the spiking frequency, starting 1 s before the transition and lasting for up to 2 s after the transition. A transition from forward to right was also preceded by a significant increase in the spiking frequency, which slowly decreased after the transition. There was also a significant increase in the spiking frequency 1 s before a transition from backward to right. The spiking frequency decreased to the normal level almost immediately after that period.

Animals retracted their shell before they turned it to the right by more than 80 °, or to the left.

Although the activity of not all units in the right inferior cervical nerve was correlated

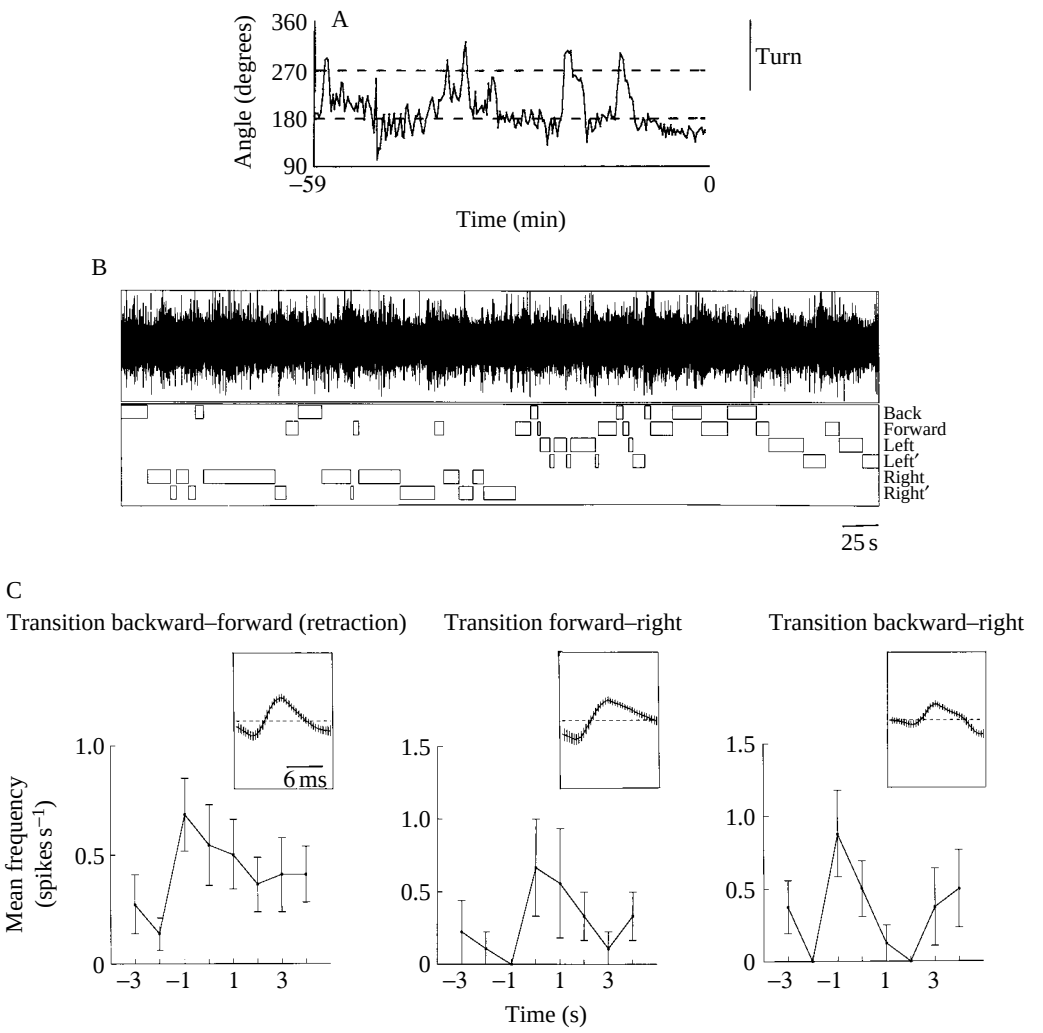

Fig. 4. Electrical activity in the right inferior cervical nerve and turning behaviour in freely behaving animals implanted with fine wire electrodes. (A) Example of the turning behaviour over a period of approximately 1 h prior to oviposition. (B) Example of the electrical activity of the right inferior cervical nerve (top panel) and the positions of the shell in that period scored with the dynamic analysis method (bottom panel). Movements of the shell in the posterior or anterior direction while the position of the shell remained in the left or right position, are indicated as left′ and right′. (C) The mean spiking frequency (spikes s^{-1}; ± S.E.M.) of three wave forms in the right inferior cervical nerve, for a period beginning 4 s before and ending 4 s after a transition from one shell position to another. The insets show the wave forms. The electrical activity in the nerve and the shell movements were analysed for 1 h prior to oviposition. The transition backward–forward (retraction) showed a significant increase (P<0.05, N=22 transitions, one animal; Wilcoxon signed-rank test) for 1 s before, and for up to 2 s after the transition. The transition forward–right showed an increase in spiking frequency at the moment of the transition (P<0.05, N=9 transitions). The spiking frequency of a third unit increased 1 s before the transition backward-right (P<0.05, N=8 transitions) and decreases almost immediately.

with shell movements, we conclude that the right inferior cervical nerve is not only necessary for turning behavior to occur, but also contains axons of neurones that are electrically active at the appropriate moments. Because the increase in the spiking frequency generally occurred before the transition began, it is likely that these units are axons of motor neurones innervating the right dorsal longitudinal muscle.

We focused on units that were involved in shell movements, and did not attempt to analyse units that were tonically active at specific shell positions. Therefore, it is unclear whether a tonic increase in activity is required to maintain each shell position.

Identification and morphology of motor neurones

Nickel–lysine retrograde staining was used to identify neurones with an axon in the inferior cervical nerves (N=8). These experiments confirmed the findings of Ferguson and Benjamin (1991*a*). Neurones in the cerebral A, pedal N, E, F, G and A clusters (Slade *et al.* 1981) and neurones in the parietal, pleural and visceral ganglia have an axon in the inferior cervical nerves. These projections, except those of the pedal A cluster and that of one neurone in the pedal G cluster, are all ipsilateral. These exceptions project ipsilaterally as well as contralaterally.

To investigate which of the neurones revealed by the nickel–lysine backfills were motor neurones of the dorsal longitudinal muscle, we used a reduced preparation consisting of the CNS, the right inferior cervical nerve and the right half of the head-foot complex. Intracellular recordings of the electrical activity of neurones were made simultaneously with extracellular recordings of the electrical activity of the right inferior cervical nerve and of the right dorsal longitudinal muscle. A neurone was considered to be a motor neurone if its soma spikes were followed 1:1 at all spiking rates by (1) spikes in the nerve and (2) muscle potentials, both with constant latencies.

The results of this study confirmed the findings of Ferguson and Benjamin (1991*a*,*b*) that neurones in the cerebral A and the pedal N clusters are motor neurones of the dorsal longitudinal muscle (Fig. 5).

The right pedal N cluster contains 4–6 neurones that have an axon in the right inferior cervical nerve and somata that are 20–30 μm in diameter. All neurones in the right pedal N cluster with an axon in the right inferior cervical nerve induced contractions in the right dorsal longitudinal muscle (Fig. 5C,D).

Staining the right pedal N neurones with Lucifer Yellow (N=8) showed that some had axons in the right inferior cervical nerve and the right columellar nerve and/or in the right inferior pedal nerve (Fig. 6A). Two neurones in the right pedal N cluster had an axon in both the right superior cervical nerve and the right inferior cervical nerve. Other neurones of the right pedal N cluster had an axon that passed through the dorsal region of the pedal commisure and into the left columellar nerve. These neurones had no axon in the right inferior cervical nerve (Fig. 6B). All stained neurones had an axon through the right pedal–pleural connective and had neuropilar branches near the ventral surface of the right pedal ganglion.

The right cerebral A cluster contains 8–12 neurones that have an axon in the right inferior cervical nerve and somata that are 30–60 μm in diameter. They had resting membrane potentials between -60 and -65 mV and showed no spontaneous activity in a

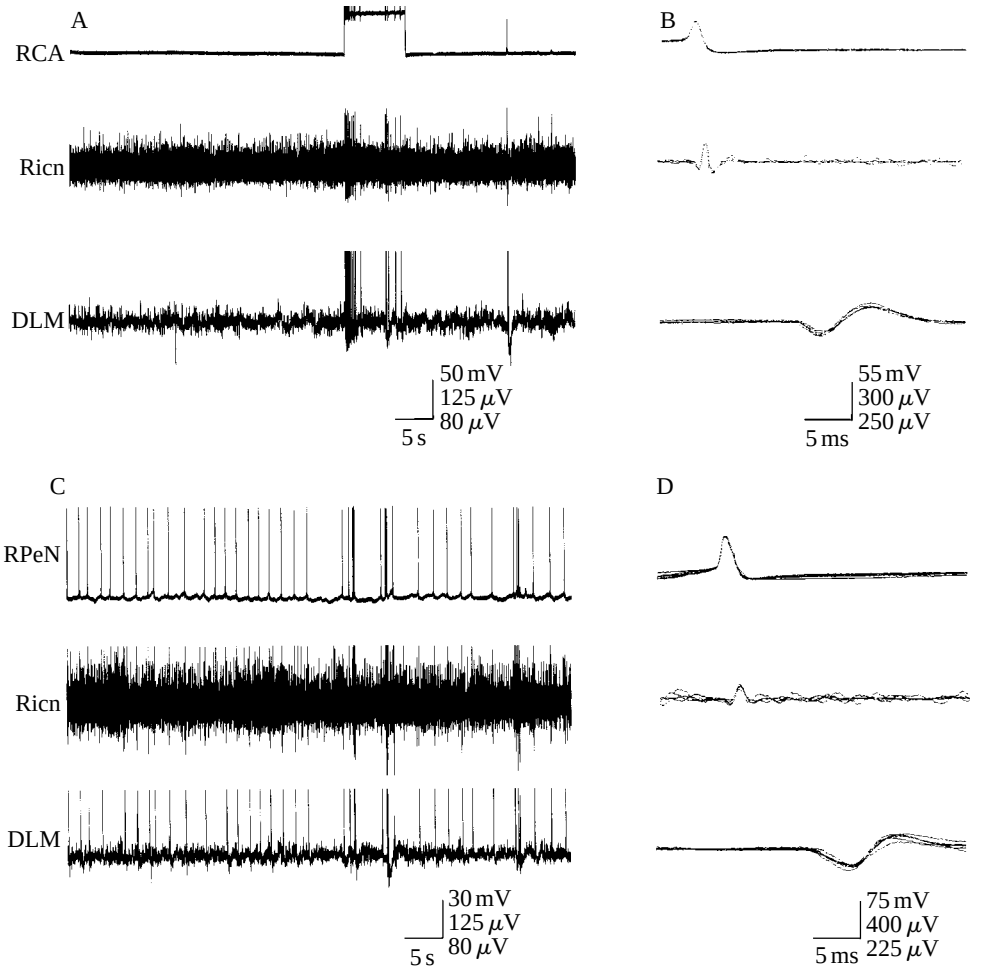

Fig. 5. Identification of the motor neurones of the dorsal longitudinal muscle. Simultaneous recordings were made of the electrical activity of the neurones (intracellular; upper traces), the right inferior cervical nerve (middle traces; extracellular) and the right dorsal longitudinal muscle (lower traces; extracellular). (A,B) Identification of one right cerebral A motor neurone. Action potentials in the neurone were induced by suprathreshold stimulation. (C,D) Identification of one right pedal N motor neurone. Sweeps in B and D were triggered from six soma spikes. The muscle recording is clipped at the top in A and C. RCA, right cerebral A neurone; RPeN, right pedal N neurone; Ricn, right inferior cervical nerve; DLM, dorsal longitudinal muscle.

reduced preparation. Suprathreshold depolarisation of cerebral A neurones with an axon in the right inferior cervical nerve always induced biphasic muscle potentials in the dorsal longitudinal muscle (Fig. 5A,B; see also Ferguson and Benjamin, 1991*b*).

The function of the other neurones with an axon in the inferior cervical nerves is not clear. They did not induce muscle contractions and we therefore concluded that they are not motor neurones of the dorsal longitudinal muscle.

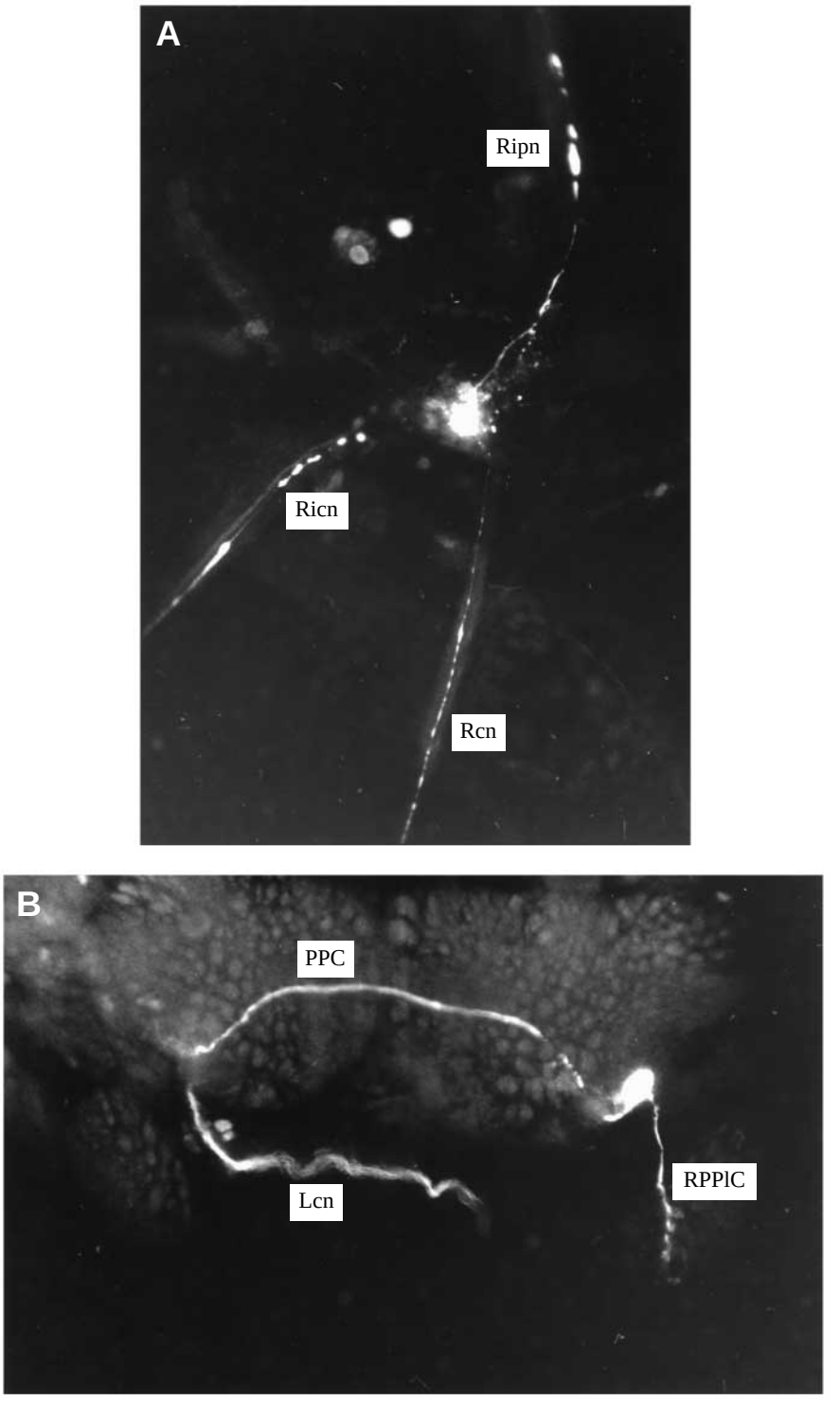

Fig. 6

Fig. 6. Lucifer Yellow staining of right pedal N neurones. (A) A right pedal N motor neurone innervating the dorsal longitudinal muscle. (B) A right pedal N neurone with no axon in the right inferior cervical nerve, which is therefore not a motor neurone of the dorsal longitudinal muscle. Ripn, right inferior pedal nerve; Ricn, right inferior cervical nerve; Rcn, right columellar nerve; Lcn, left columellar nerve; RPPlC, right pedal–pleural connective; PPC, pedal–pedal commissure.

Effect of caudodorsal cell discharges on the identified motor neurones

To investigate whether a CDC discharge has a modulating effect on the identified motor neurones described above, we used a reduced preparation consisting of the CNS, the right inferior cervical nerve and the right half of the head-foot complex. Intracellular recordings of the electrical activity of the right pedal N or right cerebral A motor neurones and the CDCs were made simultaneously with extracellular recordings of the electrical activity of the right inferior cervical nerve and the right dorsal longitudinal muscle.

The state of excitability of the CDCs was determined by inducing depolarising afterpotentials. An afterdischarge was induced in half of the preparations in which the CDCs were in the resting state (see Materials and methods).

The spontaneous spiking activity of the right pedal N motor neurones in preparations in which the CDCs were in the inhibited state differed from that in preparations in which the CDCs were in the resting state. Fig. 7A shows the mean of cumulative number of spikes of the right pedal N motor neurones in these preparations (N=7 for each group; P<0.05, ANOVA). It was also found that an afterdischarge of the CDCs had an inhibitory effect on the right pedal N motor neurones. This inhibition of the pedal N motor neurones lasted for hours after the induction of an afterdischarge in the CDCs. Fig. 7B shows the mean

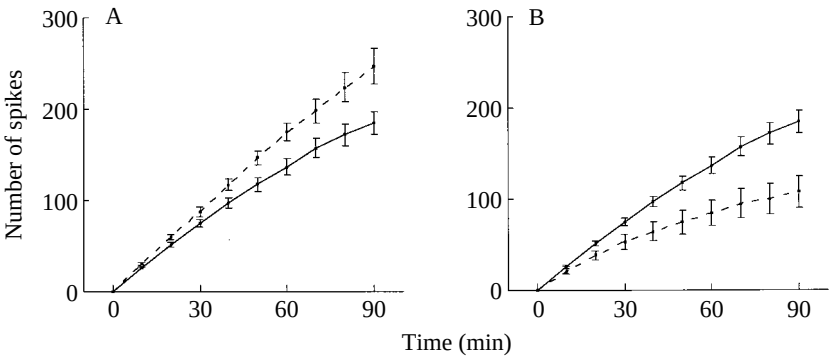

Fig. 7. Effect of the three different states of excitability of the CDCs on the electrical activity of the pedal N motor neurones. (A) Mean of the cumulative number of spikes ± S.E.M. (spikes min^{-1}; log_{10}-transformed data) of the pedal N motor neurones in preparations with the CDCs in the inhibited state (dashed line) and in the resting state (solid line). For both groups, N=7; P<0.05 (ANOVA). (B) Mean of the cumulative number of spikes ± S.E.M. (spikes min^{-1}; log_{10}-transformed data) of the pedal N motor neurones in preparations with the CDCs in an active state (dashed line) and in the resting state (solid line). At time zero, an afterdischarge was induced in the CDCs (active state) by electrical stimulation using repetitive suprathreshold stimulation at 2 Hz. For both groups, N=7; P<0.05 (ANOVA).

cumulative number of spikes of the right pedal N motor neurones in preparations with the CDCs in the resting and in the active state. There was a significant difference between these two groups (N=7 for each group; P<0.05).

The three different states of excitability of the CDCs did not affect the electrical activity or membrane potentials of the right cerebral A motor neurones (N=4 for each state in the CDCs; not shown).

We conclude that the electrical spiking activity of the right pedal N motor neurones correlates with the state of excitability of the CDCs.

Discussion

In this study, we have identified muscles, nerves and motor neurones involved in turning behaviour during egg-laying in the pond snail *Lymnaea stagnalis*. Furthermore, we have demonstrated that an afterdischarge in the caudodorsal cells affects the electrical activity of the identified pedal N motor neurones and that the state of excitability of the CDCs correlates with the electrical activity of the pedal N motor neurones. These conclusions are based on the results of (1) lesion experiments, (2) *in vivo* whole-nerve recordings and (3) induction of an afterdischarge in the CDCs in reduced preparations.

The egg-laying behaviour of the gastropods *Aplysia* and *Lymnaea* have provided good model systems for determining the role that peptidergic neurones play in producing and modulating behaviour.

In *Aplysia*, it has been demonstrated that peptides released by the bag cells during an afterdischarge have a number of effects on the electrical activity of central neurones *in vitro* (Mayeri *et al.* 1979*a*,*b*, 1985; Stuart and Strumwasser, 1980; Rothman *et al.* 1983; Sigvardt *et al.* 1986; Schaeffer and Brownell, 1986; Goldsmith and Byrne, 1993; for a review, see Mayeri and Rothman, 1985). These peptides also affect gill and siphon contractions (Schaeffer and Brownell, 1986; Goldsmith and Byrne, 1993) and the arterial system (Ligman and Brownell, 1985). Injections of these peptides into intact animals also induce behavioural changes (Stuart and Strumwasser, 1980).

An injection of CDCH (the ovulation hormone) induces egg-laying in *Lymnaea* although, following such injections, the animals do not show the first phase of egg-laying behaviour (Ter Maat *et al.* 1989). Alpha CDCP and CDCH have an auto-excitatory function in *Lymnaea* (Brussaard *et al.* 1990).

Until now, it has not been possible to study the role of the CDC peptides in regulating the neurones involved in overt egg-laying behaviour. With the identification of the nerves and motor neurones involved in turning behaviour during egg-laying in *Lymnaea*, we now have a neuronal model that may allow us to determine the effects of the peptides released by the caudodorsal cells during egg-laying.

Lesion experiments

Lesioning the columellar nerves had no apparent effect on turning behaviour. Similarly, lesions of the right parietal nerve, the analis nerve or the genitalis nerve (Ferguson *et al.* 1993) and lesions of the cutaneous pallialis nerve or the left parietal nerve (this paper) that innervated the column and the mantle had no effect on turning behaviour.

After a bilateral lesion of the inferior cervical nerves, however, the animals did not turn or move their shell. Unilateral lesion of the right inferior cervical nerve caused a strong reduction of shell movements.

Cook (1975) demonstrated in *in vitro* experiments in *Lymnaea* that lesions of the inferior cervical nerves caused a strong reduction of the withdrawal response. Lesions of the superior cervical nerves or the columellar nerves had almost no effect on the withdrawal response.

It was not possible to lesion all the nerves in the CNS, so we cannot conclude that the inferior cervical nerves are sufficient to produce turning behaviour. However, we can conclude that the inferior cervical nerves are necessary for executing turning behaviour during egg-laying.

In vivo *whole-nerve recordings*

The conclusions drawn from the lesion experiments concur with the results of whole-nerve recordings. Recordings of the right inferior cervical nerve together with the recordings of the behaviour in freely behaving animals clearly indicated that elements in this nerve are active just prior to and during shell movements. Most of these elements showed an increase in their electrical spiking activity just before anteriorly directed shell movements or shell movements to the right. This indicates that these elements represented the electrical activity of axons of motor neurones. The delay between the change in firing frequency of elements in the right inferior cervical nerve and the observed change in position of the shell is about 1 s. A similar delay was also found by McPherson and Blankenship (1991*a*), who demonstrated in *Aplysia* that the latency of contraction of a muscle, from first motor neurone spike to initial change of tension in the muscle, was between 800 and 533 ms in a reduced preparation.

We never found a change in the electrical activity of elements in the right inferior cervical nerve during spontaneous movements of the shell to the left. The motor neurones involved in this movement probably have their axons in the left inferior cervical nerve, which innervates the left part of the dorsal longitudinal muscle. This would be consistent with the observations of McPherson and Blankenship (1991*b*), who found that most motor neurones controlling movements of the parapodia and body in *Aplysia* have only ipsilateral effects.

Although not all elements in the right inferior cervical nerve can be correlated with shell movements, we conclude that this nerve contains axons of neurones that innervate the right dorsal longitudinal muscle and are active during turning behaviour. It is not possible to correlate the shape and form of elements recorded in an *in vivo* preparation with axon spikes of the identified motor neurones in the right inferior cervical nerve *in vitro*, because the shape and form change in a reduced preparation.

The inferior cervical nerves innervate the posterior parts of the dorsal longitudinal muscles (Ferguson and Benjamin, 1991*a*; Janse, 1974). The dorsal longitudinal muscle consists of two sets of three bands of bilaterally symmetrical muscles, which lie to the left and right sides of the midline of the dorsal surface of the body. When the dorsal longitudinal muscle contracts, the dorsal part of the head-foot is shortened along the anterior–posterior axis (Ferguson and Benjamin, 1991*a*). Several studies have

demonstrated that the columellar muscles are involved in the withdrawal response and locomotion of *Lymnaea* (Cook, 1975; Ferguson and Benjamin, 1991*a*,*b*; Winlow and Haydon, 1986). Our results indicate that the columellar muscles are less important for shell movements during egg-laying. Instead, the dorsal longitudinal muscles are essential for turning movements of the shell. This implies that shortening of the head-foot is necessary for rotations of the shell. This is supported by our finding that turns of the shell to the right by more than 80 °, as well as movements to the left, are preceded by a forward (retraction) movement of the shell.

Effect of caudodorsal cell afterdischarges

Induction of an afterdischarge in the CDCs resulted in a strong decrease in the spontaneous electrical spiking activity of the identified right pedal N motor neurones. These motor neurones have an axon in the right inferior cervical nerve, which innervates the right dorsal longitudinal muscle. Although we have not provided direct evidence, it seems reasonable to assume that peptides released by the CDCs mediate the inhibitory effect. In terms of behaviour, this effect would occur immediately after the onset of the CDC discharge and would thus correspond with the first phase of egg-laying behaviour, resting. In contrast with the resting phase *in vivo*, this inhibition *in vitro* of the spiking activity of the right pedal N motor neurones lasted for hours after the induction of a discharge in the CDCs. This indicates that another excitatory input onto the right pedal N motor neurones is necessary to express the electrical spiking activity corresponding to the specific shell turns seen in the second phase of egg-laying.

In *Aplysia californica*, the discharge of the neurosecretory bag cells is sufficient to initiate the appetitive phase of egg-laying behaviour, but sensory input from the ovulated eggs is necessary for the occurrence of consummatory egg-laying behavioural patterns (Cobbs and Pinsker, 1982; Ferguson *et al.* 1986). Ter Maat *et al.* (1989) showed that there is a positive correlation between the number of eggs in the egg mass and the duration of the turning phase in *Lymnaea*. This suggests that input from the reproductive tract is necessary for full expression and coordination of egg-laying behaviour. Ferguson *et al.* (1993) showed that lesioning the intestinal nerve also abolished the shell turns during egg-laying. However, the animals were still able to move their shell after this lesion. Sensory input from the reproductive tract, passing *via* the intestinal nerve, may be necessary for turning behaviour to occur, and the inferior cervical nerves may be the motor pathway underlying the behavioural patterns of the turning phase. However, it is not known whether the input from the reproductive tract is, in itself, sufficient to cause turning phase behaviour and whether this input has modulating effects on the pedal N motor neurones. The function of the turning movements is still unclear. It might be that shell movements are involved in the transport of the eggs.

Induction of an afterdischarge in the CDCs had no effect on the spiking activity or membrane potential of the right cerebral A motor neurones. These neurones are not spontaneously active in a reduced preparation. Cutting all nerves except the right inferior cervical nerve (and the right superior cervical nerve and columellar nerve in the study of Ferguson and Benjamin, 1991*b*) may have abolished the input pathway (neuronal or hormonal) needed to generate action potentials in the cerebral A motor neurones and thus

prevented muscle contractions. Suprathreshold depolarisation of these cells, however, was able to induce a contraction in the dorsal longitudinal muscle.

The correlation of electrical spiking activity of the right pedal N motor neurones and the state of excitability of the CDCs (resting *versus* inhibited; Kits, 1980) suggests that there is a common input to both groups of cells. Jansen and Bos (1984) and Jansen and Ter Maat (1985) have demonstrated that an identified unpaired neurone, the ring neurone, inhibits the CDCs and modulates the motor neurones of the columellar muscles. Therefore, it is possible that this ring neurone simultaneously excites the pedal N motor neurones and inhibits the CDCs. This has, however, not yet been proved.

In summary, we can conclude that it is likely that the CDC peptides have a modulatory effect on the right pedal N motor neurones. There is also a correlation between the state of excitability of the CDCs and the electrical spiking activity of the pedal N motor neurones. These motor neurones have an axon in the right inferior cervical nerve, which innervates the dorsal longitudinal muscle. The right inferior cervical nerve is necessary to execute turning behaviour and contains axons of neurones that are active during shell movements recorded in freely behaving animals, probably the axons of the pedal N motor neurones. Our results, therefore, indicate that the pedal N motor neurones are involved in executing the turning phase during egg-laying. Anticlockwise rotations of the shell to an angle of more than 260 ˚ relative to the head-foot, and movements to the left, are preceded by a forward movement of the shell, implying that shortening of the dorsal longitudinal muscle is necessary for rotations of the shell.

Further experiments are necessary (1) to demonstrate the modulatory effects of the individual CDC peptides on the electrical spiking activity of the pedal N motor neurones, (2) to determine the contributions of the reproductive tract and the intestinal nerve to the electrical spiking activity of these motor neurones and (3) to identify the common input onto the pedal N motor neurones and the CDCs.

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