SHORT COMMUNICATION

NEURONAL FEEDBACK IN EGG-LAYING BEHAVIOUR OF THE POND SNAIL *LYMNAEA STAGNALIS*

GRAHAM P. FERGUSON*, ANTON W. PIENEMAN, RENE F. JANSEN and
ANDRIES TER MAAT

Department of Biology, Vrije Universiteit, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

The egg-laying behaviour of gastropod molluscs is controlled by peptidergic neuroendocrine cells and has provided an important experimental system for behavioural neurobiology. The genes that code for multiple peptides have been sequenced and the peptides themselves have been identified, thus enabling us to investigate how they act on the nervous system to produce the overt behavioural pattern (reviewed by Geraerts *et al.* 1988). The two animals that have been studied most extensively are the opisthobranch *Aplysia californica* and the pulmonate *Lymnaea stagnalis*. In both cases, the peptidergic neurones controlling egg laying are normally electrically silent (both *in vivo* and *in vitro*; Kupfermann, 1967; Pinsker and Dudek, 1977; Kits, 1980; Ter Maat *et al.* 1986) and produce multiple peptides (Rothman *et al.* 1983; Geraerts *et al.* 1985; Sigvardt *et al.* 1986), which are cleaved from a common protein precursor (Scheller *et al.* 1983; Vreugdenhil *et al.* 1988). Before egg laying, the cells produce a long-lasting discharge of action potentials (Pinsker and Dudek, 1977; Ter Maat *et al.* 1986). This electrical discharge initiates egg-laying behaviour, and during it the peptides (one of which initiates ovulation) are released into the blood. The demonstration, in *Aplysia californica*, that these peptides could have various effects on the activity of central neurones (reviewed by Mayeri and Rothman, 1985) led to the hypothesis that egg-laying behaviour is a neuroendocrine fixed action pattern controlled and coordinated by the concerted actions of the released peptides (Scheller and Axel, 1984). This hypothesis is also thought to apply to *Lymnaea stagnalis* (Vreugdenhil *et al.* 1988) because of the structural similarities between precursors of *Aplysia californica* and *Lymnaea stagnalis* egg-laying hormones. In this paper we investigate how the sequence of the various components of the egg-laying behaviour pattern is achieved.

In *Lymnaea stagnalis*, the neuroendocrine cells controlling egg laying are the caudodorsal cells (CDCs), a group of about 100 electrotonically coupled neurones located in the cerebral ganglia and with axons forming a neurohaemal area in the intercerebral commissure (Wendelaar Bonga, 1970; de Vlieger *et al.* 1980). In the intact

animal, CDC activity can be initiated by giving animals a clean water stimulus (CWS; consisting of transfer from dirty to clean water, Ter Maat *et al.* 1983) or can occur spontaneously. In both cases, CDC activity lasts about 60min and is always followed by egg-laying behaviour (Ter Maat *et al.* 1986, 1989). This begins within minutes of the CDCs starting to fire (Ter Maat *et al.* 1986) and consists of three phases: resting, turning and oviposition (Ter Maat *et al.* 1989).

Egg laying can also be induced by injecting the ovulatory hormone (caudodorsal cell hormone, CDCH) directly into the blood. Following such injections, animals do not, however, show a resting phase. Instead, they continue moving and then enter directly into the turning phase (about 60min after the injection; Ter Maat *et al.* 1989). Animals that are given injections but do not respond by laying eggs show no egg-laying behaviour. These results demonstrate that the presence of CDCH in the blood is alone insufficient to produce the fully coordinated pattern of egg-laying behaviour but, as the other CDC peptides that are normally released synchronously with the CDCH were absent, this does not contradict the hypothesis of Scheller and Axel (1984).

The same previous study (Ter Maat *et al.* 1989) also revealed a positive correlation between the number of eggs in the egg mass and the duration of the turning phase; the more eggs there were, the longer it lasted. This observation seemed to challenge the hypothesis that the actions of the released CDC peptides can account for the fully coordinated pattern of egg-laying behaviour. Instead, it suggested that input from the ovulated eggs is necessary for full expression and coordination of the egg-laying behavioural pattern of *Lymnaea stagnalis*. This idea is investigated in the present study, where lesions were made to disrupt the pathways likely to be involved in the transmission of sensory input from the reproductive tract to the neurones in the central nervous system (CNS) responsible for producing egg-laying behaviour. The turning-phase behaviour of animals, including, for the first time, measurements of shell rotation, was then analysed.

Adult laboratory-bred snails were used. Prior to experiments these were housed individually in perforated jars placed within an aquarium tank supplied with continuously running fresh water (20°C) and kept under a 12h:12h light:dark cycle. Animals were fed a daily ration of lettuce.

Fig. 1 shows the normal egg-laying behaviour of a single *Lymnaea stagnalis* following clean water stimulation (CWS, Ter Maat *et al.* 1983). Rasping activity, locomotion and shell position were quantified by replaying episodes of egg laying from video recordings. Rasps were counted. Shell turning was determined using a PC Vision Plus (Imaging Technology) videodigitizer and a PC/AT personal computer to capture and process a video frame every 15s. The angle between the line connecting the two tentacles and the line connecting the anterior and posterior ends of the shell was used to measure shell position relative to the head-foot. Locomotion was determined by using the video digitiser/computer system (described above) to measure the distance travelled (using the midpoint between the tentacles of the animal as a landmark) by the animal in each 15 s interval.

Approximately 15min after the CWS the animal entered the resting phase. During resting, there were few changes of shell position, no rasps and little locomotory activity. After remaining in the resting phase for approximately 40min, the animal entered the
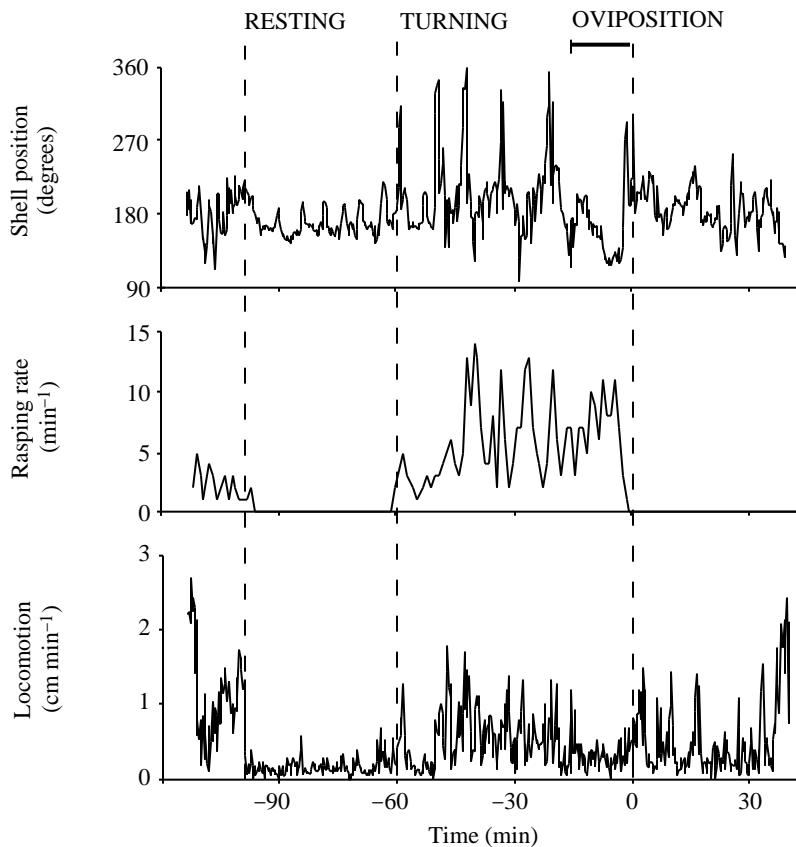
Fig. 1. The normal egg-laying behaviour of *Lymnaea stagnalis* after a CWS. Egg-laying behaviour was quantified by measuring the shell position, rasping and locomotory activity of the animal. During resting, there were few turning movements of the shell, no rasping and very little locomotion. During turning, shell position changed frequently, rasping activity was high and there was little locomotion. The animal remained in a localised area of the tank, where the eggs were deposited during the oviposition phase (indicated by the horizontal bar at the top of the figure).

turning phase and started typical turning-phase behaviour. There were frequent rotations of the shell, the animal started to use its buccal mass to make rasping movements against the substratum and moved slowly within a localised area of the tank. Turning-phase behaviour occurred for approximately 60min and was followed by the oviposition phase (lasting about 10min), when the eggs were deposited on the substratum. During oviposition, the shell was held almost parallel to the head-foot, the level of rasping declined and the animal moved slowly forward. In the 30min period following the completion of oviposition, shell movements resumed and the rate of locomotion increased, but no rasping movements were made.

    To determine whether neuronal input from the reproductive tract is important for the full expression of turning-phase behaviour, lesions were made (under $MgCl_2$ anaesthesia)

to disrupt the neuronal pathways between the reproductive tract and the CNS (see Fig. 2A). Previous anatomical studies (Elo, 1938) suggested that nerves from the parietal or visceral ganglia were most likely to innervate the reproductive tract. Lesions ($N=5$ for each group) were therefore made of both pleuroparietal connectives, the two nerves (the internal and external right mantle nerves) of the right parietal ganglion and all four nerves (the genital, anal, cutaneous mantle and intestinal nerves) of the visceral ganglion. These latter nerves were first all lesioned simultaneously and then, in separate groups of animals ($N=5$ for each), lesioned individually. Sham-operated animals ($N=10$) served as controls. Egg laying was then induced (on postoperative day 3) either by injection of CDCH [20pmol of synthetic CDCH (Eurosequence, Groningen) in 10 µl of dibutylamine and
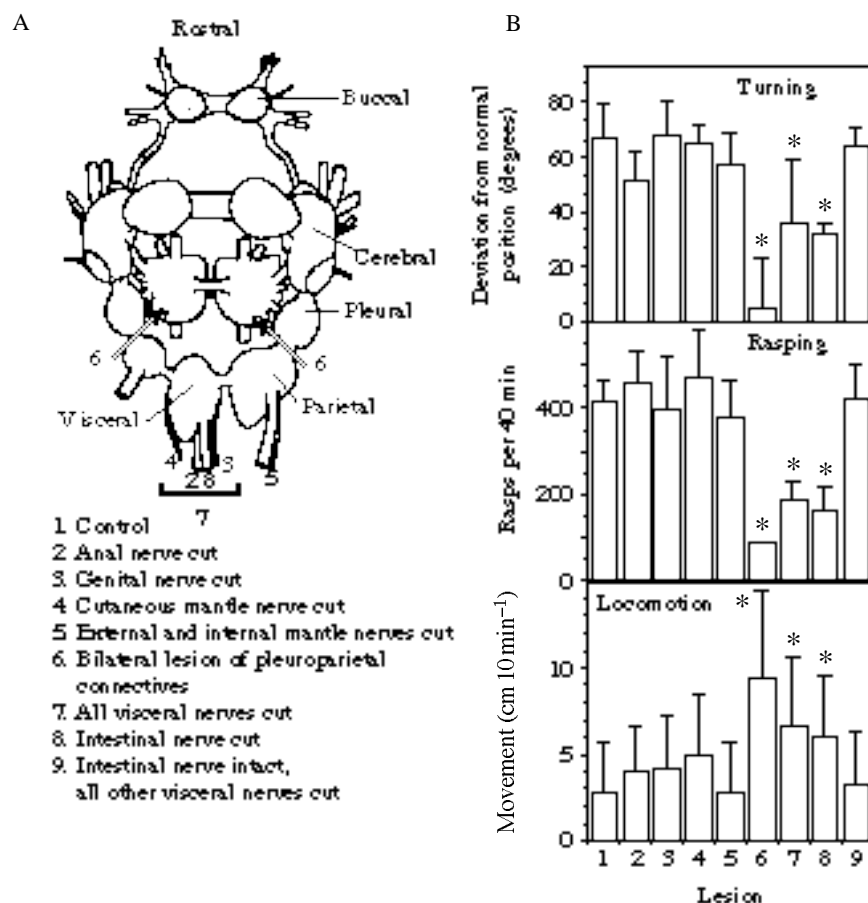

Fig. 2. Identification of the neuronal pathways necessary and sufficient for normal turning-phase behaviour. In A, the CNS of *Lymnaea stagnalis* is shown schematically. The buccal, cerebral, pleural and parietal ganglia on the right side of the CNS are labelled as is the unpaired visceral ganglion. The connectives and nerves lesioned are numbered. (B) Lesions of the pleuroparietal connectives (6), all visceral nerves (7) and the intestinal nerve (8) caused significant changes in all three turning-phase behavioural patterns (shell turning, rasping and locomotion; *$P<0.016$). When the intestinal nerve was left intact and the other visceral nerves were lesioned (9), normal turning behaviour occurred.

40 μl of Hepes saline] into the foot sinus, or by giving animals a CWS. The first of these methods was used for the group of animals with bilateral lesions of the pleuroparietal connectives, for the group of animals where all four visceral nerves were cut together and for five of the controls. The CWS was used for all other groups of animals and for the remaining five controls). The reason that CDCH injections were used to induce egg laying in the groups of animals with lesions of the pleuroparietal connectives or all visceral nerves was that previous work (Geraerts *et al.* 1984) suggested that they would not respond to the CWS. The turning-phase behaviour of the animals during the 40 min immediately prior to the start of oviposition was analyzed. Total rasps, average deviation from normal shell position and average speed were determined over this period.

Rasps, shell position and locomotion all showed considerable variability between animals. Therefore, in animals receiving the CWS, the three measures of these activities were also determined during 15 min following CWS, which is before the start of the CDC discharge (Ter Maat *et al.* 1989). In injected animals, the 30 min period prior to the injection was used. The rates of rasping, shell position and locomotion during these baseline periods correlated strongly with each of the rates exhibited during turning-phase behaviour. They were consequently used to correct rasping, shell position and locomotion of individuals during the turning phase (analysis of covariance, using the pre-egg-laying score as the covariate). The measures of all experimental groups, corrected for the covariate, were then compared with controls. Because this involves eight comparisons for each measure, the Bonferroni protection level ($P<0.016$; see Fig. 2) is given, instead of the P-value of the individual tests ($P<0.002$ at worst).

As shown in Fig. 2B, some of these lesions had a dramatic effect on turning-phase behaviour. Animals with lesions of the pleuroparietal connectives, all visceral nerves, or the intestinal nerve showed significantly less shell turning and fewer rasping movements, and a significantly higher rate of locomotion, than both groups of control animals. The higher locomotory level was because animals moved around the whole tank until the eggs started to leave the vagina. The other lesions did not significantly affect any of the three turning-phase behavioural patterns. The almost complete abolition of turning-phase behaviour after bilateral lesions of the pleuroparietal connectives, all visceral nerves or the intestinal nerve suggests that sensory input is important for the expression of turning behaviour. The results suggest that the information from the reproductive tract enters the CNS mainly *via* the intestinal nerve and is then transferred to the anterior ganglia of the CNS *via* the pleuroparietal connectives.

To investigate whether the intestinal nerve was sufficient for the production of turning-phase behaviour, it was left intact while the three other visceral nerves were cut ($N=5$). After egg laying had been induced, by injection of CDCH, these animals showed normal levels of all three turning-phase behavioural patterns in the last 40 min of the turning phase. This demonstrates that the intestinal nerve is, in addition to being necessary for normal turning, also sufficient for the production of normal turning behaviour when left intact.

In Fig. 3 the time course of the egg-laying behaviour of individual control and experimental animals is shown. Rasping and locomotion were quantified for the full egg-laying episode and shell position was scored for the hour prior to the end of oviposition.
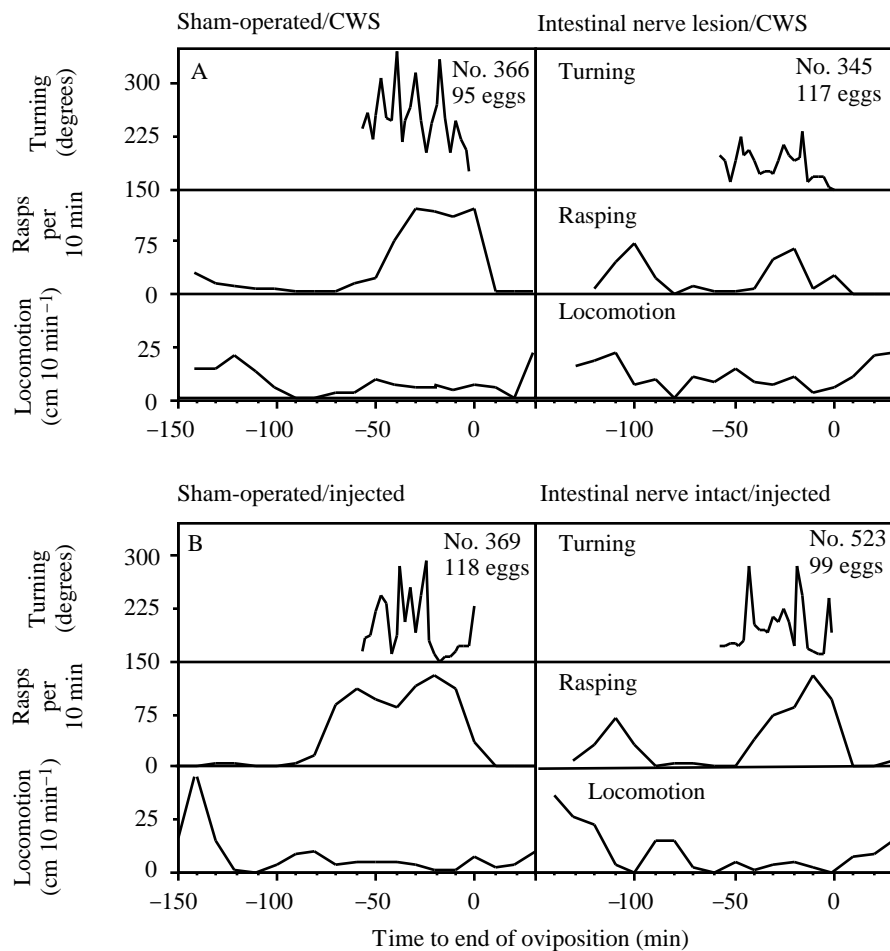
Fig. 3. Time course of egg-laying behaviour in control animals, animals with the intestinal nerve cut and animals in which the intestinal nerve was the only visceral ganglion nerve intact. Cutting the intestinal nerve (A, right) affected turning-phase behaviour by causing a reduction in shell movement and rasping and an increase in locomotion in comparison to the control animal (A, left). When the intestinal nerve was left intact and the other three visceral ganglion nerves were cut (B, right), the normal pattern of turning-phase behaviour occurred and egg laying was similar to that of the control animal (B, left). In A, egg laying was induced by a CWS and, in B, by an injection of CDCH. Time 0 indicates the end of oviposition. Data were sampled every 15s, as in Fig. 1, but they were averaged over every 10min.

In Fig. 3A, the CWS was used to induce egg laying (animals were transferred to clean water at the start of the plots). The control animal showed typical turning-phase behaviour, whereas the animal with the intestinal nerve cut showed lower levels of rasping and shell turning, despite laying a larger egg mass. When the intestinal nerve was the only visceral nerve left intact and egg laying was induced with an injection of CDCH, the three behavioural patterns were similar in both timing and levels to that of the control animal (Fig. 3B).

In summary, it was found that lesioning pathways between the reproductive tract of the animal and the CNS prevents *Lymnaea stagnalis* from entering the turning phase of overt egg-laying behaviour. This shows that the animal uses neuronal feedback to achieve the proper sequence of the egg-laying behaviour pattern. This means that sequential release of peptides by the CDCs or differences in the time courses of the effects of the various peptides need not be invoked to explain the orderly course of egg-laying behaviour. Thus, as with many other complex stereotyped behaviours (e.g. locust flight, Wendler, 1974; walking in cats and cockroaches, Forssberg *et al.* 1975; Wong and Pearson, 1976; lamprey swimming, Grillner *et al.* 1981), input into the CNS is necessary for expression of the full behavioural pattern.

Lesions of the pleuroparietal connectives caused a greater change in turning behavioural patterns than lesions of the intestinal nerve, so the possibility that there are other peripheral pathways by which the sensory input reaches the CNS cannot be ruled out. The intestinal nerve would, however, seem to be the most important pathway. This is because (i) lesions of other visceral and right parietal nerves had no significant effect on turning behaviour and (ii) normal turning behaviour occurred when the intestinal nerve was left intact and the other visceral ganglion nerves were lesioned.

An important consideration, however, is whether the effects seen after lesioning the intestinal nerve could have been due to some cause other than the disruption of a sensory pathway between the reproductive tract and the CNS. Notably, the fact that the intestinal nerve contains the axons of heart motor neurones (Buckett *et al.* 1990) may have confounded our results because cutting it might disrupt cardiovascular regulation and thus alter egg-laying behaviour. We do not believe, however, that this can explain the present results. Nor is it likely that lesions of the intestinal nerve would disrupt the motor pathways producing the behavioural patterns of the turning phase. This is because the motor neurones innervating the muscles used during rasping, shell turning and locomotion are located in the buccal, cerebral and pedal ganglia (Benjamin *et al.* 1985; Jansen and Ter Maat, 1985; Winlow and Haydon, 1986). Furthermore, animals could still rasp (and eat normal amounts of food), turn their shell and move after the lesion.

The resting phase of egg-laying behaviour shows little variation in duration, irrespective of the number of eggs deposited. It is thought to be under peptidergic control (Jansen and Ter Maat, 1985). When the CDCs fire a discharge, an identified interneurone (the ring neurone) becomes active (Jansen and Bos, 1984). This neurone has synaptic connections with pedal ganglion neurones involved in the control of locomotion and shell position, and it might cause an inhibition of locomotion and the adoption of the posture typical of the resting phase (Jansen and Ter Maat, 1985). Although peptidergic control may be sufficient to coordinate resting, it is less likely to be able to coordinate turning fully. The most probable function of turning behaviour is cleaning of the substratum where the eggs will be deposited (Ter Maat *et al.* 1989). Given that the size of individual egg masses can vary from less than 10 to more than 200 eggs, it would seem plausible that, by utilising neuronal input from the reproductive tract to control turning-phase behaviour, animals can prepare an area of the substratum appropriate for the number of eggs to be deposited with more precision than if the turning phase were solely under peptidergic control. An interesting question is whether the input from the reproductive

tract is in itself sufficient to cause turning-phase behaviour or whether its effects are conditional on the presence of the peptides that are released during the CDC discharge, which may have modulatory effects on the neuronal circuits underlying the behaviour pattern.

During the present study, animals laid eggs in a clean glass chamber. It was observed that the egg masses laid by animals that did not show normal turning-phase behaviour were much less securely attached to the substratum than is normally the case (in some cases, even becoming detached from the substratum at the end of the oviposition phase). This would seem to provide additional support for the previous suggestion that the adaptive role of turning-phase behaviour is to clean the substratum and to facilitate good adhesion of the egg mass.

In *Aplysia californica,* the discharge of the neurosecretory bag cells (which are similar to the CDCs of *Lymnaea stagnalis* and release an ovulatory hormone and other peptides; Kupfermann, 1967; Rothman *et al.* 1983) is sufficient to initiate the appetitive phase of egg-laying behaviour, but, as in *Lymnaea stagnalis*, sensory input from the ovulated eggs (*via* pathways yet to be determined) is necessary for the occurrence of consummatory egg-laying behavioural patterns (Cobbs and Pinsker, 1982; Ferguson *et al.* 1986). It may be a general feature of the egg-laying behavioural pattern of all gastropod molluscs that input from the genital tract is necessary for the full expression of the behaviour. Further comparative studies are necessary, however, before this can be stated with confidence.

References

BENJAMIN, P. R., ELLIOTT, C. J. H. AND FERGUSON, G. P. (1985). Neural network analysis in the snail brain. In *Model Neural Networks and Behavior* (ed. A. I. Selverston), pp. 87–108. New York: Plenum Press.

BUCKETT, K. J., PETERS, M., DOCKRAY, G. J., VAN MINNEN, J. AND BENJAMIN, P. R.(1990). Regulation of heartbeat in *Lymnaea* by motoneurons containing FMRFamide-like peptides. *J. Neurophysiol.* **63**, 1426–1435.

COBBS, J. S. AND PINSKER, H. M. (1982). Role of bag cells in egg deposition of *Aplysia brasiliana.* II. Contribution of egg movement to elicited behaviors. *J. comp. Physiol.* A **147**, 537–546.

DE VLIEGER, T. A., KITS, K. S., TER MAAT, A. AND LODDER, J. C. (1980). Morphology and electrophysiology of the ovulation hormone producing neuroendocrine cells of the freshwater snail *Lymnaea stagnalis* (L.). *J. exp. Biol.* **84**, 259–271.

ELO, J. E.(1938). Das Nervensystem von *Limnaea stagnalis* (L.). *Lam.-Ann. Zool. Vanamo* **6**, 1–40.

FERGUSON, G. P., PARSONS, D. W., TER MAAT, A. AND PINSKER, H. M.(1986). Spontaneous and elicited bag cell discharges in gonadectomized *Aplysia. J. exp. Biol.* **123**, 159–173.

FORSSBERG, H., GRILLNER, S. AND ROSSIGNOL, S.(1975). Phase dependent reflex reversal during walking in chronic spinal cats. *Brain Res.* **80**, 103–107.

GERAERTS, W. P. M., TER MAAT, A. AND HOGENES, TH. M.(1984). Studies on the release activities of the neurosecretory Caudo-Dorsal Cells of *Lymnaea stagnalis*. In *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones* (ed. J. Hoffmann and M. Porchet), pp. 44–50. Heidelberg: Springer.

GERAERTS, W. P. M., TER MAAT, A. AND VREUGDENHIL, E. (1988). The peptidergic neuroendocrine control of egg laying behaviour in *Aplysia* and *Lymnaea*. In *Invertebrate Endocrinology*, vol. 2, *Endocrinology of Selected Invertebrate Types* (ed. H. Laufer and R. Downer), pp. 144–231. New York: Alan Liss.

GERAERTS, W. P. M., VREUGDENHIL, E., EBBERINK, R. H. M. AND HOGENES, TH. M. (1985). Synthesis of multiple peptides from a larger precursor in the neuroendocrine caudo-dorsal cells of *Lymnaea stagnalis*. *Neurosci. Lett.* **56**, 241–246.

GRILLNER, S., MCCLELLAN, A. AND PERRET, C. (1981). Entrainment of the spinal pattern generators for swimming by mechano-sensitive elements in the lamprey spinal cord *in vitro. Brain Res.* **217**, 380–386.

JANSEN, R. F. AND BOS, N. P. A. (1984). An identified neuron modulating the activity of the ovulation hormone producing caudo-dorsal cells of the pond snail *Lymnaea stagnalis. J. Neurobiol.* **15**, 161–167.

JANSEN, R. F. AND TER MAAT, A. (1985). Ring neuron control of columellar motor neurons during egg-laying behavior in the pond snail. *J. Neurobiol.* **16**, 1–14.

KITS, K. S. (1980). States of excitability in ovulation hormone producing cells of *Lymnaea stagnalis* (Gastropoda) and their relation to the egg-laying cycle. *J. Neurobiol.* **11**, 397–410.

KUPFERMANN, I. (1967). Stimulation of egg-laying: possible neuroendocrine functions of bag cells of abdominal ganglion of *Aplysia californica. Nature* **216**, 814–815.

MAYERI, E. AND ROTHMAN, B. S. (1985). Neuropeptides and the control of egg-laying behavior in *Aplysia*. In *Model Neural Networks and Behavior* (ed. A. I. Selverston), pp. 285–301. New York: Plenum Press.

PINSKER, H. M. AND DUDEK, F. E. (1977). Bag cell control of egg-laying in freely behaving *Aplysia. Science* **197**, 490–493.

ROTHMAN, B. S., MAYERI, E., BROWN, R. O., YUAN, P.-M. AND SHIVELY, J. E. (1983). Primary structure and neuronal effects of α-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia. Proc. natn. Acad. Sci. U.S.A.* **80**, 5753–5757.

SCHELLER, R. H. AND AXEL, R.(1984). How genes control an innate behavior. *Scient. Am.* **250**, 54–62.

SCHELLER, R. H., JACKSON, J. F., MCALLISTER, L. B., ROTHMAN, B. S., MAYERI, E. AND AXEL, A.(1983). A single gene encodes multiple neuropeptides mediating a stereotyped behavior. *Cell* **32**, 7–22.

SIGVARDT, K. A., ROTHMAN, B. S., BROWN, R. D. AND MAYERI, E. (1986). The bag cells of *Aplysia* as a multitransmitter system: identification of alpha bag cell peptide as a second transmitter. *J. Neurosci.* **6**, 803–813.

TER MAAT, A., DIJCKS, F. A. AND BOS, N. P. A. (1986). *In vivo* recordings of neuroendocrine cells (caudo-dorsal cells) in the pond snail. *J. comp. Physiol.* A **158**, 853–859.

TER MAAT, A., LODDER, J. C. AND WILBRINK, M. (1983). Induction of egg laying in the pond snail *Lymnaea stagnalis* by environmental stimulation of the release of ovulation hormone from the caudo-dorsal cells. *Int. J. Invert. Reprod.* **6**, 239–247.

TER MAAT, A., PIENEMAN, A. W., GOLDSCHMEDING, J. T., SMELIK, F. AND FERGUSON, G. P. (1989). Spontaneous and induced egg-laying behavior of the pond snail *Lymnaea stagnalis. J. comp. Physiol.* A **164**, 673–683.

VREUGDENHIL, E., JACKSON, J. F., BOUWMEESTER, T., SMIT, A. B., VAN MINNEN, J., VAN HEERIKHUIZEN, H., KLOOTWIJK, J. AND JOOSSE, J. (1988). Isolation, characterization and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the freshwater snail *Lymnaea stagnalis. J. Neurosci.* **8**, 4184–4191.

WENDELAAR BONGA, S. E. (1970). Ultrastructure and histochemistry of neurosecretory cells and neurohaemal areas in the pond snail *Lymnaea stagnalis* (L.). *Z. Zellforsch. mikrosk. Anat.* **108**, 190–224.

WENDLER, G. (1974). The influence of proprioceptive feedback on locust flight coordination. *J. comp. Physiol.* **88**A, 173–200.

WINLOW, W. AND HAYDON, P. G. (1986). A behavioural and neuronal analysis of the locomotory system of *Lymnaea stagnalis. Comp. Biochem. Physiol.* **83**A, 13–21.

WONG, R. AND PEARSON, K. G. (1976). Properties of the trochanteral hair plate and its function in the control of walking in the cockroach. *J. exp. Biol.* **64**, 233–249.