*Invertebrate Reproduction and Development*, 36:1–3 (1999) 123–127
Balaban, Philadelphia/Rehovot
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# An *in vivo* electrophysiological study of mating behaviour in the snail *Helix aspersa*

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**Summary**

The mating behaviour of the garden snail *Helix aspersa* consists of introductory behaviour, dart shooting and copulation. Earlier electrophysiological, morphological and immunocytochemical studies indicated that mating behaviour of this simultaneous reciprocal hermaphrodite is under control of the neurons of the mesocerebrum. With the use of implanted fine wires, *in vivo* recordings and stimulation of the right mesocerebrum were performed. The recorded neurons showed increased activity upon tactile stimulation of certain skin areas, during courtship and some also during copulation. Electrical stimulation of the right mesocerebrum evoked a gradual eversion of the genital pore. By combining this electrical stimulation with tactile stimulation of the everted genital pore, penial eversion and sometimes dart shooting could be evoked. The injection of APGWamide, a neuropeptide involved in the control of mating in other molluscs, caused an eversion of the genital pore resembling that seen in nature. FMRFamide has been suggested to be responsible for dart shooting, but its injection had no obvious effect. It is concluded that the mesocerebrum, with APGWamide as an important neuropeptide, has a central role in the execution of mating behaviour, but that other areas of the central nervous system might also be involved.

***Key words***: Mollusc, copulation, electrophysiology, neuropeptides, behaviour

## Introduction

The bizarre dart shooting behaviour, part of the complex and extensive courtship of the hermaphrodite garden snail *Helix aspersa* (Adamo and Chase, 1988), has been the subject of a lot of the attention that this animal has received over the years. The love dart is a sharp calcareous structure that is used to pierce the skin of the partner at the end of courtship before spermatophore transfer. Many scientists have tried to explain this phenomenon [reviewed by Kothbauer (1988)]. We have recently shown that the mucus that is transferred with the dart is of more biological significance than the dart itself (Koene and Chase,

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*Proceedings of the 8th International Congress on Invertebrate Reproduction and Development, Amsterdam, The Netherlands, 10–14 August 1998.*

1998a, 1998b). When this mucus comes into contact with the female organs, it causes contractions of the bursa tract diverticulum and the copulatory canal in such a way that the transport of donated sperm to the spermathecal sacs can be facilitated (Koene and Chase, 1998b).

The left and right mesocerebra of the central nervous system are thought to be responsible for mating behaviour (Chase, 1986). The right mesocerebrum is significantly larger, reflecting the fact that most reproductive structures of the snail are located in the right side of the body cavity. This resembles the situation of the anterior lobes in the cerebral ganglia of *Lymnaea stagnalis* which show the same asymmetry and are also involved in mating behaviour (De Boer et al., 1997). Evidence for the function of the mesocerebrum has come from *in vitro* electrophysiology and morphological results. Based on these findings, it has been suggested that the cells of the mesocerebrum are involved in both dart shooting and penial eversion (Chase, 1986). Li and Chase (1995) suggested, from immunocytochemical results, that there are different groups of cells in the mesocerebrum that are responsible for the different components of mating behaviour: one group containing APGWamide responsible for penial eversion and one containing FMRFamide responsible for dart shooting. This is partly supported by the fact that in *Lymnaea stagnalis* APGWamide evokes eversion of part of the penial complex, the preputium (De Boer et al., 1997).

All the previous work showed the function of the mesocerebrum indirectly. In order to obtain direct evidence for the role of the right mesocerebrum in mating behaviour, we made use of *in vivo* fine wire recordings (Parsons et al., 1983; De Boer et al., 1997). By recording from the mesocerebral cells during natural behaviour and by stimulating the mesocerebrum electrically *in vivo*, we tried to confirm the role of the mesocerebrum in mating. With the injection of known peptides we were able to test the above-mentioned hypothesis proposed by Li and Chase (1995).

## Material and Methods

The individually housed snails were fed lettuce, carrots and chalk. They were kept moist at 20°C with a light–dark cycle of 16:8 h. The animals were in isolation for at least 2 weeks before being used. The sexual activity of the snails was tested by putting them in a group box for 1–2 h. The snails showing sexual activity were selected for experiments.

Fine wire electrodes (25 $\mu$m diam, Teflon-coated stainless steel) were used to stimulate and record from the right mesocerebrum. Snails were anaesthetized at the beginning of the operation with the injection of 2–3 ml $MgCl_2$ (60 mM) into the back of the foot just underneath the shell. A 2-mm incision was made slightly on the left of the dorsal midline at the height of the genital pore. The basic procedure was similar to the one used for *L. stagnalis* (Hermann et al., 1994; Yeoman et al., 1994). Because *H. aspersa* is a land snail, two adaptations needed to be made. Firstly, a second fine wire was inserted into the body cavity, serving as the reference electrode. Secondly, the incision was sutured serving to hold the two fine wires, held together with silicone glue, in position. After injection of 2–3 ml of saline the animal was transferred to an individual box and kept overnight to recover from the operation.

The experiments included either electrical stimulation through the implanted wire or recording from the implanted wire. For the stimulation of the right mesocerebrum the voltage was set at 50% of the voltage that evoked skin contraction. Then, repeated stimulations of 5 ms were done at 2 Hz. Some of the electrical stimulations were combined with a tactile stimulation of the genital pore. These tactile stimulations were done with a fine plastic filament.

For recordings of the mesocerebral activity during tactile stimulation of the skin, the same plastic stimulator was used. In this way a sensitivity map could be made. For the recording of the mesocerebrum activity during natural behaviour, sexually active snails that had formed pairs in the group box were used. One of the individuals had a fine wire implanted while the other was kept intact. The electrical activity as well as the behaviour was recorded on super-VHS videotape.

Injections of 50 $\mu$l of APGWamide or FMRFamide were applied in the back of the foot, like the anaesthetic. Animals were injected with different concentrations ($10^{-3}$ to $10^{-7}$ M). Also a mix of APGWamide and FMRFamide, with equal final concentrations as the single injections, was injected (50 $\mu$l) in several animals. Saline injections were used as a control.

## Results

Electrical stimulations were performed to see whether this would cause eversion. In most cases this stimulation evoked an eversion of the genital pore within a few minutes after the start of stimulation. The eversion usually reached stage 5 and was similar to that

Table 1. Evoked eversion of the genital pore of *Helix aspersa.* Electrical stimulation of the right mesocerebrum caused an eversion of the genital pore similar to that seen during courtship. Electrical stimulation combined with tactile stimulation usually evoked penial eversion, and in two cases dart shooting. Injections of APGWamide caused similar genital eversions as during courtship which lasted several minutes. FMRFamide had no obvious effect, but injection of a combination of APGWamide and FMRFamide evoked fewer eversions than APGWamide alone. Injections of equal volumes of saline were used as a control. Results are reported as numbers of snails observed.

| | Electrical | Elect. + tactile | APGW | FMRF | APGW/FMRF | Saline |
|---|---|---|---|---|---|---|
| Eversion | 26 | 8 | 22 | 0 | 5 | 0 |
| No effect | 18 | 2 | 2 | 9 | 10 | 5 |

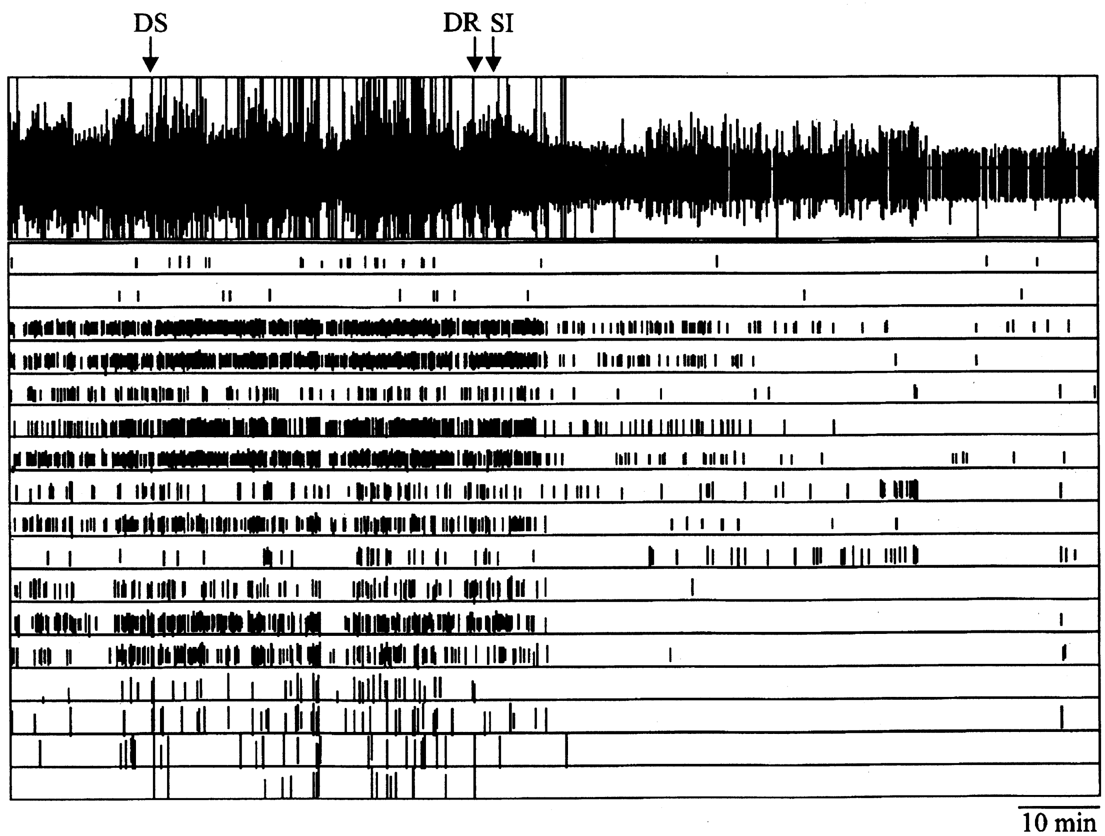


Fig. 1. *In vivo* recording of the right mesocerebrum during mating behaviour. The top trace shows the recording including courtship and part of the copulation. The lower traces show 17 separate units, ranked by size. These units were selected using a spike sorting program (Jansen and Ter Maat, 1992), and each unit represents a cell from the mesocerebrum. The times of dart shooting (DS), dart receipt (DR), and simultaneous intromission (SI) are indicated with the arrows.

seen during normal courtship (Adamo and Chase, 1988). The eversion was maintained as long as the electrical stimulation continued. Once the stimulus was ended, the genital eversion disappeared. In the animals with a stage 5 eversion tactile stimulation of the everted genital pore usually evoked penial eversion (eight of ten animals). In two of these cases dart shooting was observed before penial eversion. Table 1 summarizes these results in the first two columns. Snails that were grouped with a sexually active snail while being electrically stimulated ($N$=2) performed normal copulatory behaviour.

Tactile stimulation of the skin of animals implanted with a fine wire showed that the cells of the right mesocerebrum are sensitive to touching of the skin in the head region. On the left side, the sensitivity is limited to the whole length of the tentacles including the base. Touching of the skin in between the tentacles also evokes spiking activity. On the right side, the sensitive area stretches from the tentacle base to the genital pore. Typically, when these areas are touched, a short train of spikes is observed in the electrical recording.

APGWamide and FMRFamide have been suggested to be involved in the mating behaviour of *H. aspersa*, respectively, in penial eversion and dart shooting (Li and Chase, 1995). To test their involvement in mating behaviour, they were injected into animals that had not been operated upon. As expected, APGWamide (50 $\mu$l of $10^{-5}$ M or greater) evoked an eversion of the genital pore similar to the one seen during normal courtship behaviour, lasting 9 min on average. Contrary to prediction, FMRFamide did not have any obvious effect when it was injected. However, with the mix of APGWamide (25 $\mu$l of $2\times10^{-5}$ M) and FMRFamide (25 $\mu$l of $2\times10^{-5}$ M), considerably fewer animals showed genital eversion. The above results are summarized in the second part of Table 1.

In only one case did an operated snail go through complete courtship and copulation behaviour during recording of the activity of the right mesocerebrum. In one other recording the animals separated at the end of courtship. Fig. 1 shows part of the complete recording (top trace) with the individual units (bottom traces) that were selected using a spike sorting program (Jansen and Ter Maat, 1992). Each unit represents the activity of one cell in the upper trace. The occurrence of dart shooting, dart receipt and simultaneous intromission are indicated. It can be seen that most units increase their activity during the courtship period. Once simultaneous intromission is achieved, activity decreases, changes, or stops completely. Some units start to show occasional bursts of activity during the hours of copulation (not all shown).

## Discussion

The results of the *in vivo* recording confirm that the neurons of the mesocerbrum are involved in the execution of the mating behaviour. Most of the recorded neurons show increased activity during the courtship period. This activity decreases soon after simultaneous intromission is achieved. Some units show occasional trains of spikes during the hours of copulation, while others stopped spiking altogether. The low number of neurons staying active during copulation suggests that there might be other brain areas involved in the transfer of the spermatophore.

From the electrical stimulation it can be concluded that the neurons of the right mesocerebrum can contribute to the eversion of the genital pore. The electrically evoked eversion did not pass stage 5, but penial eversion or dart shooting could be elicited when tactile stimulation was applied at the same time. The necessity of tactile stimulation for penial eversion or dart shooting might imply that there are more areas in the central nervous system involved in the execution of the behaviour. It might also indicate that a sensory feedback to the mesocerebrum is needed for the animal to continue its behaviour. This would suggest that the animal will only continue mating behaviour when tactile stimulation from the partner is present. Indeed, penial eversion or dart shooting never occurs when the mating partners are not in close body contact. This can explain why the cells of the mesocerebrum are sensitive to touching of certain areas of the skin. The tentacles and the skin directly around the genital pore are often touched by the partner during courtship.

Mesocerebral neurons are not the only neurons sensitive to tactile stimulation. The information about tactile stimulation is processed at least partly in a neural network in the parietal ganglia, which is responsible for avoidance behaviour. Balaban and Chase (1990) have shown that the network underlying this behaviour is inhibited by neurons of the mesocerebrum. This explains why the snail withdraws only partly from strong stimuli received from the partner. Some of these stimuli, like biting, would evoke complete withdrawal under normal circumstances, but during courtship these signals are needed for continuation of the behaviour.

As expected from previous work [*Lymnaea*: De Boer et al. (1997); *Aplysia*: Fan et al. (1997)], the injection of APGWamide evoked an eversion of the genital pore. APGWamide seems to have a similar function in many molluscs, suggesting that it is a conserved neuropeptide. FMRFamide, on the other hand, did not evoke any obvious behaviour by itself. This latter peptide was originally discovered as a cardio-excitatory peptide (Price and Greenberg, 1977). Later it was found to have other functions as well, including the induction of contractions in the penis retractor muscle of *H. aspersa* (Lehman and Greenberg, 1987). This latter effect could explain why the injections with a mix of APGWamide and FMRFamide evoked only a few genital eversions. The peptides seem to have opposite effects on the genital machinery, although they may act at different loci. Of course, other neuropeptides of the mesocerebrum, such as the recently identified neuropeptide GFAD (Poteryaev et al., 1998), also play a role.

The fact that hardly any of the operated snails showed sexual activity is probably due to the fact that mating behaviour is under motivational control. Normally, 10 days of isolation increases the sexual activity of *H. aspersa* (Adamo and Chase, 1988). However, disturbances obviously affect the sexual state of the animal. The implantation of a fine wire clearly induces enough disturbance to decrease sexual activity, as observed in fine wire experiments done with *L. stagnalis* (De Boer et al., 1997).

In summary, we investigated the role of the mesocerebrum in mating behaviour in the snail *Helix aspersa* using *in vivo* recordings and stimulations, tactile stimulations and neuropeptide injections. The mesocerebral neurons seem to have both a motor and sensory function, and APGWamide is one of the important neuropeptides. We conclude that the right mesocerebrum plays a key role in the control of mating behaviour, but some other areas of the central nervous system are probably also involved.

## Acknowledgements

We thank A.W. Pieneman for technical assistance and P.A.C.M. de Boer for valuable comments.

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