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A CONSERVED LOCATION FOR THE CENTRAL NERVOUS SYSTEM CONTROL OF MATING BEHAVIOUR IN GASTROPOD MOLLUSCS: EVIDENCE FROM A TERRESTRIAL SNAIL

JORIS M. KOENE[1,*], RENÉ F. JANSEN[2], ANDRIES TER MAAT[2] AND RONALD CHASE[1]

[1]*Department of Biology, McGill University, 1205 Dr Penfield Avenue, Montréal, Québec, H3A 1B1 Canada* and [2]*Faculty of Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands*

*e-mail: jkoene@BIO1.LAN.mcgill.ca

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Summary

We have investigated the role of the right mesocerebrum in the expression of mating behaviour in the garden snail *Helix aspersa*. Using an *in vivo* stimulation and recording technique, we provide evidence for both sensory and motor functions in the mesocerebral neuronal population. Some neurones were specifically sensitive to tactile stimuli delivered to the skin on the superior tentacles and around the genital pore. Electrical stimulation of the right mesocerebrum evoked genital eversion and, in combination with tactile stimulation, dart-shooting and penial eversion. Genital eversions were also elicited by injections of APGWamide. During courtship, one recorded unit increased its activity only in correlation with penial eversion, while six other units increased their activity only during dart-shooting. Three additional units increased their activity during both types of behaviour. In addition, most of the recorded units showed increased neuronal activity during times of contact with a partner. Comparison of our results with available data from other molluscs leads us to conclude that the right anteromedial region of the cerebral ganglion is an evolutionarily conserved region of the gastropod brain specialised for the control of male mating behaviour. It is striking to find such functional conservation in the central nervous system of phylogenetically distant gastropods given the large differences in behaviour during mating.

Key words: *Helix aspersa*, *Lymnaea stagnalis*, *Aplysia californica*, mesocerebrum, sexual behaviour, APGWamide.

Introduction

The large size of neuronal cells in gastropod molluscs has allowed many neurones of the central nervous system (CNS) to be identified as unique individuals. The identifiability of neurones has, in turn, contributed greatly to the assignment of function and, hence, to the understanding of behavioural control. While the functions of the various ganglia are known in broad outline, the regional localisation of function within the ganglia is poorly understood. The present experiments were designed to identify a function for a single lobe in the brain of the common garden snail *Helix aspersa*, which is a representative of the order Stylommatophora in the subclass Pulmonata. By implication, our results also provide insights into the ganglionic organisation of two other taxa, namely the Basommatophora, represented by *Lymnaea stagnalis*, and the Opisthobranchia, represented by *Aplysia californica*. Some of the data presented here have appeared in the *Proceedings of the Eighth International Congress of Invertebrate Reproduction and Development* (Koene et al., 1999).

The cerebral ganglion of *Aplysia californica* is generally thought to be organised into eight cell clusters (Jahan-Parwar and Fredman, 1976). For *Lymnaea stagnalis*, three lobes are recognised (De Boer et al., 1996), and in *Helix aspersa* three to five lobes are recognised (Bullock and Horridge, 1965). Only in a few cases are the functions of the lobes and clusters known. Thus, the procerebrum is an olfactory lobe in *Helix aspersa* (Ratté and Chase, 1997), the caudo-dorsal cells initiate egg laying in *Lymnaea stagnalis* (Ter Maat et al., 1986) and the anterior lobe regulates the expression of male sexual behaviour in *Lymnaea stagnalis* (De Boer et al., 1997). Significantly, homologous representations of the clusters and lobes have not been identified across taxa. It has been argued, however, that neurones variously located in the right mesocerebrum of *Helix aspersa*, the right anterior lobe of *Lymnaea stagnalis* and the H-cluster of *Aplysia californica* are homologous on the basis of a common expression of the neuropeptide APGWamide (Fan et al., 1997). Here, we present additional evidence that these neuronal populations are homologous using the criterion of function.

Helix aspersa is a simultaneous reciprocal hermaphrodite. Mating is preceded by a protracted courtship that involves repeated mutual contacts of the tentacles, lips and genitalia (Adamo and Chase, 1988). The peripheral genital structures are

progressively everted through recognisable stages. After 30–60 min, the calcareous 'love' dart is thrust into the skin of the mating partner (stage 5). Recently, it was shown that the dart is used to introduce a bioactive substance into the blood of the partner to influence the female reproductive organs (Koene and Chase, 1998b). After dart-shooting, the animals attempt to achieve simultaneous intromission (stage 6). If successful, they enter the copulatory phase, which requires 6–8 h for complete transfer of the spermatophore.

Most of the reproductive organs are located on the right side of the animal, which is reflected in the bilateral size asymmetry of the mesocerebral lobes. The mesocerebrum is believed to have a function in mating behaviour on the basis of its afferent and efferent connections, studied *in vitro* (Chase, 1986; Chase and Li, 1994). It has been suggested that some neurones contain the neuropeptide FMRFamide and mediate dart-shooting, while other neurones contain APGWamide and mediate penial eversion (Li and Chase, 1995).

Materials and methods

Specimens of the garden snail *Helix aspersa* Müller were kept moist at 20 °C and on a light:dark cycle of 16 h:8 h. They were fed lettuce, carrots and chalk every other day. The snails were housed in isolation for at least 2 weeks before being used in experiments.

Implantation of the fine wire electrode

A stainless-steel fine wire electrode (California Fine Wire Company), diameter 25 μm, was implanted on the right mesocerebrum for electrical stimulation and extracellular recording. The Teflon coating was removed from the end of the wire, and the naked ending was bent into a small loop (diameter approximately 200 μm). The loop was then bent by 90 ° relative to the rest of the wire (Fig. 1A). The procedure was similar to that used previously in *Lymnaea stagnalis* (Hermann et al., 1994; Yeoman et al., 1994).

For implantation, a snail was anaesthetised with 2–3 ml of 60 mmol l^{-1} $MgCl_2$ (pH 7.8) injected into the back of the foot just beneath the shell. The animal was pinned down with two small pins through the front of the foot, while the shell was held back with a larger pin. At the anterior–posterior position of the genital pore, a 2-mm incision was made in the skin near the dorsal midline. The cut was made approximately 2 mm to the left of the dorsal midline to avoid the reproductive organs.

The cerebral ganglia were gently pulled out through the incision and stabilised on a plastic hook attached to a micro-manipulator. The overlying connective tissue was carefully freed from the right mesocerebral neurones using two pairs of fine forceps. The mesocerebrum was then dried with a weak jet of air, and the fine wire was glued in place (Instant SuperGlue, World Precision Instruments). The glue covered the mesocerebral neurones and some of the surrounding connective tissue. The site of implantation is shown in Fig. 1A. The glue was dried in air, after which some $MgCl_2$ solution was added to it, and it was then air-dried again until the glue turned white. The brain was then lowered back into the body cavity. A second fine wire with a naked ending was inserted into the body cavity to serve as a reference electrode.

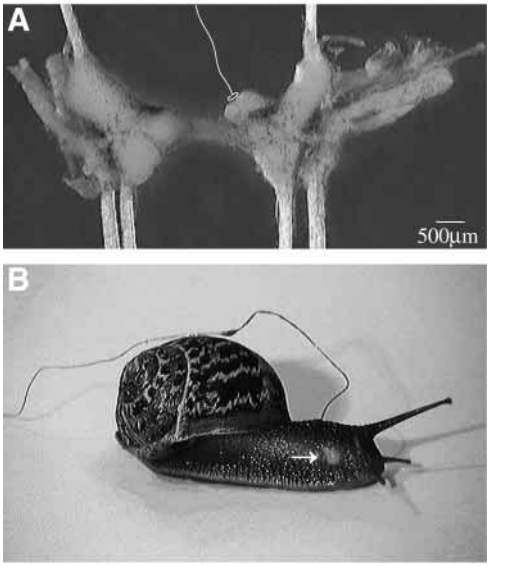

Fig. 1. Implantation of the fine wire. (A) The cerebral ganglia are shown with the connective tissue removed. A fine wire is drawn near the right mesocerebrum to indicate its size, shape and site of implantation. (B) Photograph of a snail implanted with a fine wire. The arrow points to the partially everted genital pore.

The incision was sutured (Braun Mirafil, 0.2 mm) to close the body cavity and, especially, to hold the fine wires in position. A length of wire was left inside the animal to avoid stress on the glued junction. The pair of wires was held together with silicone elastomere glue (KwikCast, World Precision Instruments). The wires were attached to the shell, leaving enough length for the animal to extend fully and to withdraw fully into its shell. Beyond the glued site, the wires were held together with light-bodied polyvinylsiloxane impression material (Kerr Extrude), and they were fitted with Harwin plugs at the ends. The snail was then injected with 2–3 ml of saline (Prescott et al., 1997) and allowed to recover. Fig. 1B shows an animal after implantation. The animals were killed after completion of the experiments to verify the position of the electrodes.

In vivo *recording*

Only sexually active snails were implanted with wires (N=166). To determine sexual activity, groups of 12–24 snails, all previously isolated, were placed in a small chamber. When courting pairs formed, one snail was taken for implantation of a fine wire while the other snail was returned to an isolation chamber. The next day, after recovery of the operated animal, the two snails were put back together again. If courtship behaviour was observed, the electrical activity and behaviour were recorded on super-VHS videotape. The electrical signal from the fine wire was fed through a DAM-80 differential amplifier (100× amplification, bandpass 3 Hz to 10 kHz; World

Precision Instruments) before it was stored on the hi-fi track of the video tape. Every video frame was provided with a time code (VITC, Alpermann and Velte) to synchronise the data. The electrical activity was digitised (Cambridge Electronic Design, model 1401, 12-bit analog-to-digital converter), and a program using a template-matching algorithm for waveform recognition (Jansen and Ter Maat, 1992) was used to reconstruct individual spike trains from the multi-neuronal recording. A group of wave forms associated with one template is referred to as a unit.

Tactile and electrical stimulation

If no courtship behaviour occurred after implantation, the animals were used for tactile and electrical stimulation experiments. Several skin areas were stimulated with a hand-held fine plastic filament to test for mesocerebral responses. For electrical stimulation of the right mesocerebrum through the fine wire, 5 ms pulses were delivered at 2 Hz for 3–6 min. The voltage was set at 50 % of the threshold for a single pulse to evoke a visible local skin contraction. Electrical stimulation was sometimes combined with a tactile stimulation of the genital pore.

Neuropeptide injection

Neuropeptides were injected into non-sexually active snails at the back of the foot just beneath the shell. The volume of the blood of *Helix aspersa* is estimated to be approximately 2.0–2.5 ml (Koene and Chase, 1998b). Each injection contained a 50 µl solution of peptide(s) dissolved in saline. A snail was injected only once with a single dose (10^{-3} to 10^{-7} mol l^{-1}) of APGWamide (Ala-Pro-Gly-Trp-NH_2; American Peptide), FMRFamide (Phe-Met-Arg-Phe-NH_2; Peninsula) or a mixture of APGWamide and FMRFamide at equal concentrations. Saline injections were used as a control. Approximate final concentrations in the blood were 10^{-5} to 10^{-9} mol l^{-1}. APGWamide concentrations of 10^{-6} mol l^{-1} and higher have been shown to inhibit contraction of the penis retractor muscle in *Lymnaea stagnalis* (Croll et al., 1991).

Data analysis

For behavioural analysis, the mating components were defined as follows. The stages of genital eversion, from 1 to 6, were scored according to Adamo and Chase (1988). The duration of the dart-shooting event was the time from when the dart first began to emerge from the shooting animal to when the dart sac was again fully withdrawn. Dart receipt was taken to occur between the time when the dart hit the skin of the recipient and when the shooter withdrew its dart sac. The duration of penial eversion was the time from the initial externalisation of the penis and its ensuing retraction. Simultaneous intromission was defined as intromission that was mutually successful and sustained.

The data are reported as means ± standard deviations (S.D.) wherever applicable. For the *in vivo* recordings, the correlation between spiking activity and the different types of behaviour was investigated by means of permutation tests, as described elsewhere (Jansen et al., 1997). In short, randomisation techniques were used to calculate the probability that an experimentally found relationship was due to chance by sampling a large number of random possibilities (4000 permutation runs). Experimentally obtained bin counts were compared with a 'population' of bin counts obtained from the same recording by data permutation. Because every individual bin of the experimentally obtained histogram was compared with this 'population', we made multiple comparisons, with the number of comparisons being equal to the number of bins in the histogram. Since we wish to accept, for every comparison, an alpha error of P=0.05, the actual significance level used in each test was adjusted by dividing P by the number of bins in the histogram. A detailed description of this analytical procedure is available elsewhere (Jansen et al., 1999).

Results

Tactile and electrical stimulation

Areas of the skin were stimulated with a mechanical probe while mesocerebral activity was recorded. In this way, we determined the area of the skin to which the recorded mesocerebral neurones were responsive (Fig. 2A). On the left side of the animal, sensitivity is confined to the superior tentacle, but includes the entire length of the tentacle as well as its base. Touching the skin between the superior tentacles also evoked spiking activity. On the right side, the sensitive skin area stretches from the base of the tentacle to the genital pore. Responses fell sharply as the probe was moved away from the genital pore. Typically, a short burst of spikes was observed when the skin was stimulated in the sensitive skin area (Fig. 2B).

To test whether neurones of the right mesocerebrum can mediate part of the mating behaviour, electrical stimulation trials were performed. In many cases, stimulation evoked an eversion of the genital pore (Table 1, first column). The

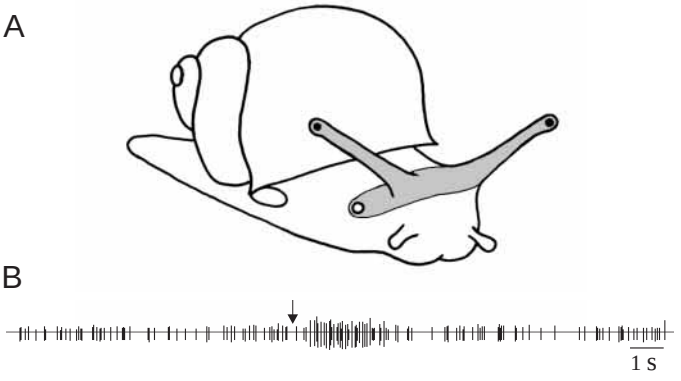

Fig. 2. The *in vivo* sensitivity of mesocerebral neurones to tactile stimulation. (A) The shaded area indicates the sensitive skin area of the mesocerebrum for stimulation with a plastic filament (N=13 snails tested). (B) A typical response to tactile stimulation (arrow) shown here for stimulation of the right tentacle. The trace is digitised and multi-unit.

Table 1. *Evoked eversion of the genital pore by electrical stimulation of the right mesocerebrum with and without tactile stimulation*

	Evoked eversion (%)		
	Electrical only (*N*=44)	Electrical + tactile (*N*=10)	Tactile only (*N*=13)
Genital eversion	52.3	20	0
Genital eversion and penial eversion	6.8	60	0
Genital eversion, dart shooting and penial eversion	0	20	0
No effect	40.9	0	100

Electrical stimulation caused eversion of the genital pore similar to that seen during courtship. In two cases, this was followed by penial eversion. When electrical stimulation was combined with tactile stimulation on the genital pore, penial eversion was reliably evoked and, in two cases, dart-shooting also occurred. Tactile stimulation alone evoked neither genital eversion nor penial eversion.

Percentage values indicate successful trials relative to total trials.

eversion usually reached an advanced state (stage 5) within a few minutes of continuous stimulation at 2 Hz. Except for the fact that they developed faster, the observed eversions looked identical to those seen during normal courtship (Adamo and Chase, 1988). The eversions were maintained as long as the electrical stimulation continued. Once the stimulus was terminated, the genitalia retracted to their normal positions within 1 min. Electrical stimulations of the right metacerebrum (also known as postcerebrum) did not evoke genital eversion (*N*=5).

Tactile stimulation of the everted genital pore, when combined with simultaneous electrical stimulation of the mesocerebrum, evoked penial eversion (Table 1, second column). In two cases, the combination of electrical and tactile stimulation elicited dart-shooting as well as penial eversion. Tactile stimulation alone did not evoke any eversion (Table 1, third column). When an electrically stimulated snail was paired with a sexually active partner, the stimulated snail performed normal courtship and copulation behaviour (*N*=2; not shown in Table 1). This last result is especially striking in the light of the fact that only two of 166 implanted animals courted and mated in the absence of electrical stimulation (see below).

Injection of APGWamide elicits genital eversion

The peptides APGWamide and FMRFamide have been implicated in the mating behaviour of *Helix aspersa*. Specifically, it has been suggested that APGWamide mediates penial eversion whereas FMRFamide mediates dart-shooting (Li and Chase, 1995). To test this hypothesis, the peptides were injected into the blood of unoperated animals. Using this method, when combined with nerve lesions, APGWamide has been shown to act peripherally on the penial complex of *Lymnaea stagnalis* (De Boer et al., 1997).

Injection of APGWamide (50 µl at 10^{-5} mol l^{-1} or greater) evoked an eversion of the genital pore identical to that seen during normal courtship behaviour (similar concentrations evoked eversion in *Lymnaea stagnalis*; De Boer et al., 1997). The eversion developed quickly to stage 5, as with electrical stimulation, and it lasted 8.59±2.97 min (*N*=24). There was no additional effect when tactile stimulation of the genital pore was combined with the APGWamide injection. Contrary to the expectation that FMRFamide mediates dart-shooting, injections of FMRFamide (50 µl at 10^{-5} mol l^{-1} or greater) had no obvious effect on the animal. However, when a mixture of APGWamide and FMRFamide was injected (both dosages 25 µl at 2×10^{-5} mol l^{-1}), significantly fewer animals showed genital eversion than when APGWamide alone was injected ($P<0.0001$; Fisher Exact test; Table 2).

Fine wire recordings during mating behaviour

The electrical signal from the right mesocerebrum was generally silent during locomotion and feeding. Although most of the operated animals remained active and looked healthy, only two of them engaged in courtship and copulation. Unfortunately, the signal-to-noise ratio recorded from one of the animals was inadequate, leaving just one animal for the analysis of electrical events during mating behaviour.

Fig. 3 shows the first 2.3 h of the recording during a natural sequence of courtship and mating. The top trace shows the analog multi-neuronal recording, with markings to indicate the time of occurrence of dart-shooting, dart receipt and simultaneous intromission. The overall spiking frequency dropped sharply from 0.52 spikes s^{-1} during courtship to

Table 2. *Eversion of the genital pore evoked by neuropeptide injections*

	Evoked eversion (%)			
	APGWamide (*N*=24)	FMRFamide (*N*=9)	APGWamide + FMRFamide (*N*=15)	Saline (*N*=5)
Genital eversion	91.7	0	33.3	0
No effect	8.3	100	66.7	100

Injections of APGWamide caused genital eversions with mean durations of 8.59±2.97 min (mean ± S.D.). FMRFamide had no obvious effect, but injections combining APGWamide and FMRFamide evoked fewer eversions than APGWamide alone, with mean durations of 9.12±1.79 min. Injections of similar volumes of saline were used as a control.

Percentage values indicate successful trials relative to total trials.

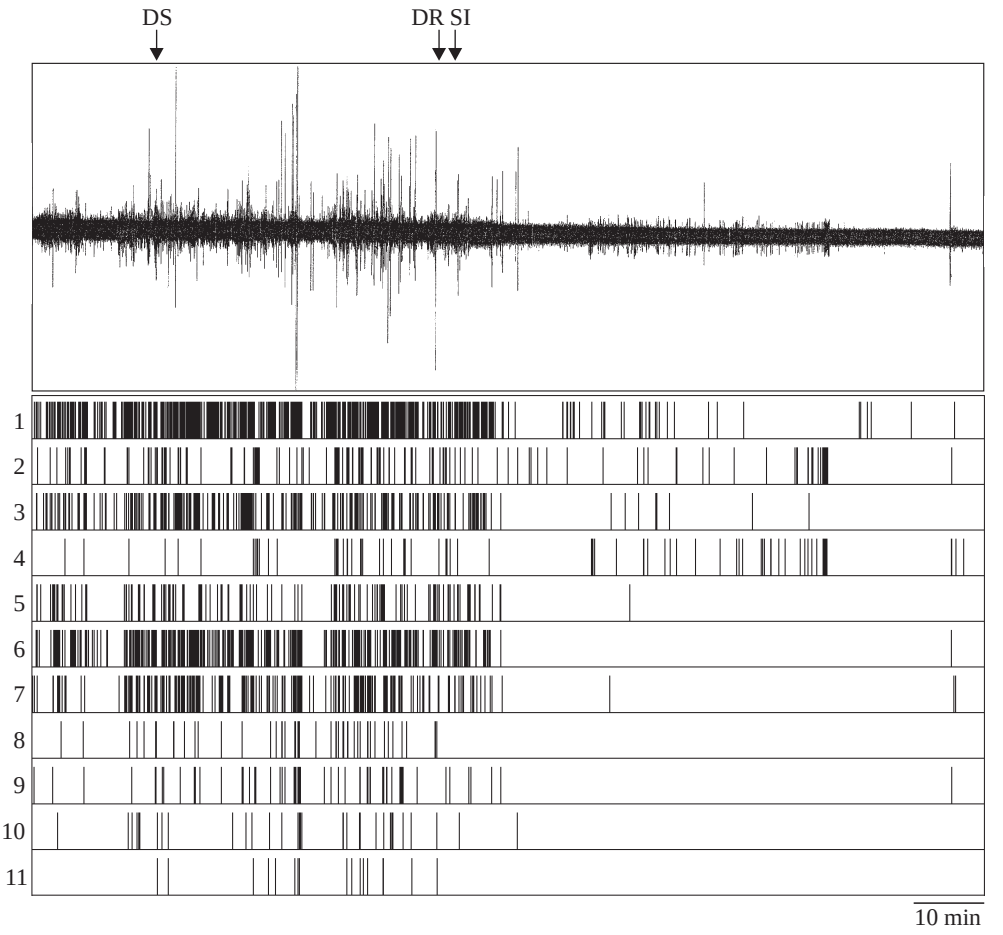

Fig. 3. A recording of neural activity in the right mesocerebrum during mating behaviour. The top trace shows the analog multi-unit activity recorded before and after the transition from courtship to copulation (simultaneous reciprocal intromission). Arrows above the recordings indicate times for dart-shooting (DS), dart receipt (DR) and simultaneous intromission (SI). The lower traces represent activity in 11 units extracted from the multi-unit recording using a spike-sorting program (Jansen and Ter Maat, 1992). The units are ordered top to bottom by increasing action potential amplitude.

0.06 spikes s^{-1} after simultaneous intromission. The numbered traces in Fig. 3 are the reconstructed spike trains of individual units that were extracted from the original recording using a spike-sorting program (Jansen and Ter Maat, 1992). Only those units selected by the spike-sorting program with an amplitude above the noise level were used for analysis, because spikes with smaller amplitudes are not well discriminated (Jansen and Ter Maat, 1992). The reconstructed traces indicate that most units were highly active during courtship, but that their activity decreased, changed or stopped completely soon after simultaneous intromission (SI).

The aggregate neuronal activity in the mesocerebrum during specific times of interest is shown in Fig. 4, where it is correlated with four types of behaviour or events attributable to the implanted animal: contact with the partner, dart-shooting, dart receipt and penial eversion. Dart-shooting and dart receipt each occurred just once, whereas there were numerous contacts with the partner and multiple penial eversions before simultaneous intromission. Neural activity was therefore averaged for the latter two events. Pronounced increases in activity were correlated with all events except dart receipt (Fig. 4C). The dart was received in the right side of the animal 5 mm behind the genital pore outside the sensitive skin area of the mesocerebral neurones (Fig. 2A). Contact (Fig. 4A) and dart-shooting (Fig. 4B) were both correlated with peaks of

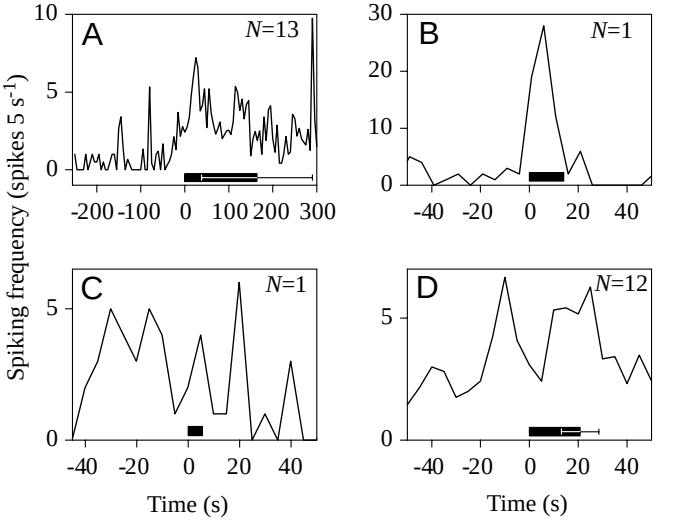

Fig. 4. Aggregate spiking frequencies in relation to four different types of behaviour. Time zero indicates the start of each type of behaviour. The horizontal bars indicate the duration of the behaviour. Dart-shooting (B) and dart receipt (C) were unique events, whereas contact with the partner (A) and penial eversion (D) were repeated events whose mean durations are indicated. Values of duration are means ± S.D.

neural activity at the start of each type of behaviour. For penial eversion (Fig. 4D), an initial peak of activity can be seen just prior to the start of the behaviour followed by a second peak near the end of the behaviour. To investigate the activity patterns of individual units, these were plotted separately relative to each behavioural event. None of the units changed its activity in any significant way at the time the animal received a dart (data not shown). The changes during dart-shooting, penial eversion and contact with the partner were subjected to further analysis, as described below.

To investigate the idea that dart-shooting and penial eversion are separately controlled by different neurones in the mesocerebrum, spike counts during these two types of behaviour are plotted together for each unit in Fig. 5. One unit increased its activity only during penial eversions (unit 3), while other units increased their activity only during dart-shooting (units 1, 5, 7–10). Three other units increased their activity during both types of behaviour (units 2, 4 and 6) and, for each of these, there was a statistically significant correlation between spiking activity and penial eversion. One unit was unaffected by either behaviour (unit 11). The activity of several units (units 2–4 and 6) showed the same double peak during penial eversion as seen in the aggregate neural activity of Fig. 4D. The peaks occur on either side of time zero in the plots of these units (Fig. 5). By examining the patterns of spiking activity in individual units relative to contact with the partner, we found that most units increased their activity during times of contact, but that only the increases in units 5 and 9 were significant (Fig. 6) with the statistical method we used (Jansen et al., 1999). This result is consistent with results from the sensory mapping experiment (Fig. 2) and the analysis of aggregate activity (Fig. 4A).

Discussion

The results presented here suggest that neurones of the right mesocerebrum play a key role in controlling the mating behaviour of *Helix aspersa*. Our data confirm that the mesocerebrum should be regarded as an integrative centre with both sensory and motor functions (Chase and Li, 1994). The demonstration that APGWamide can mediate genital eversion in *Helix aspersa* links these findings to previous studies of mating behaviour in related gastropod molluscs and leads us to conclude that the localisation of function is evolutionarily conserved in this group of animals.

During courtship, the tentacles of the snails repeatedly touch and interlock (Adamo and Chase, 1988). Thus, it was expected that mesocerebral activity would be especially responsive to tactile stimulation of the tentacles, and this was the case. The observed sensory function of the mesocerebrum is consistent with the finding of putative mechanosensory neurones in the anterior lobe of *Lymnaea stagnalis* (Steffensen et al., 1995); this lobe is homologous to the mesocerebrum (see below). However, there is also a great deal of lip contact during courtship (Adamo and Chase, 1988), and the lip nerves provide strong excitatory input to the mesocerebrum (Chase, 1986). Despite this, tactile stimulation on the lips in the present experiments did not evoke neural activity in the

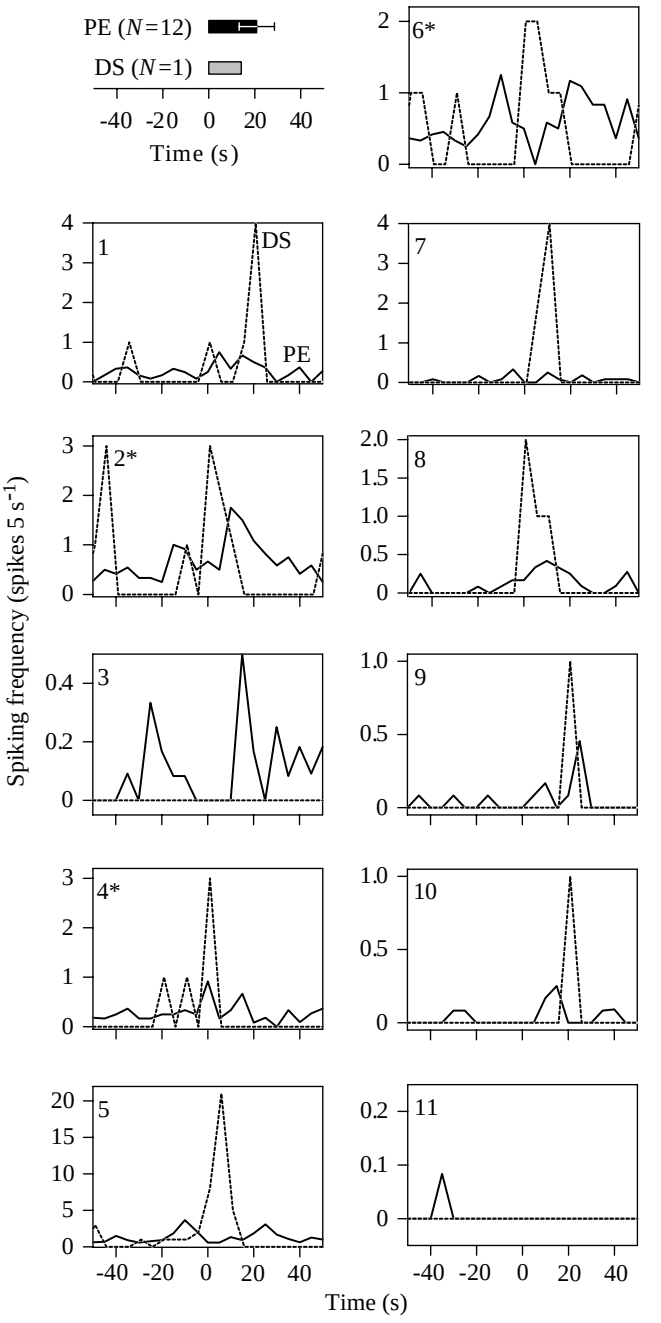

Fig. 5. Comparison of spiking activity in 11 units during penial eversion and dart-shooting. The timing of each type of behaviour relative to the spike counts is shown at the top left, where the same time scale is used as in the unit graphs. Penial eversion (PE) and dart-shooting (DS) begin at time zero, and their durations are indicated by horizontal bars. The bar for penial eversion shows the mean duration with the standard deviation. Each graph illustrates activity in a different unit; the unit number is indicated in the upper left corner. Spiking frequencies are shown during penial eversion (black) and dart-shooting (dashed). The statistical significance of frequency changes during penial eversion is indicated by an asterisk (P<0.05).

mesocerebrum. The neurones of the mesocerebrum have a diameter of between 25 and 80 μm (Li and Chase, 1995), and the area of the mesocerebrum in which activity was recorded was approximately 200 μm^2 in diameter. Thus, the activity of only a small sample of the total population of approximately 140 right mesocerebral neurones (Chase, 1986) was recorded with the fine wire. Possibly, sensory information from lip contacts is selectively processed by mesocerebral neurones located at sites not recorded by the implanted wire. Alternatively, since the animals used for recording sensory responses were not sexually aroused, it is possible that lip stimulation only elicits activity in mesocerebral neurones when the animal is sexually aroused. The fact that the receipt of a dart does not seem to evoke activity in the mesocerebrum is consistent with the idea that the mechanical action of the dart is only incidental to its function (Koene and Chase, 1998a,b).

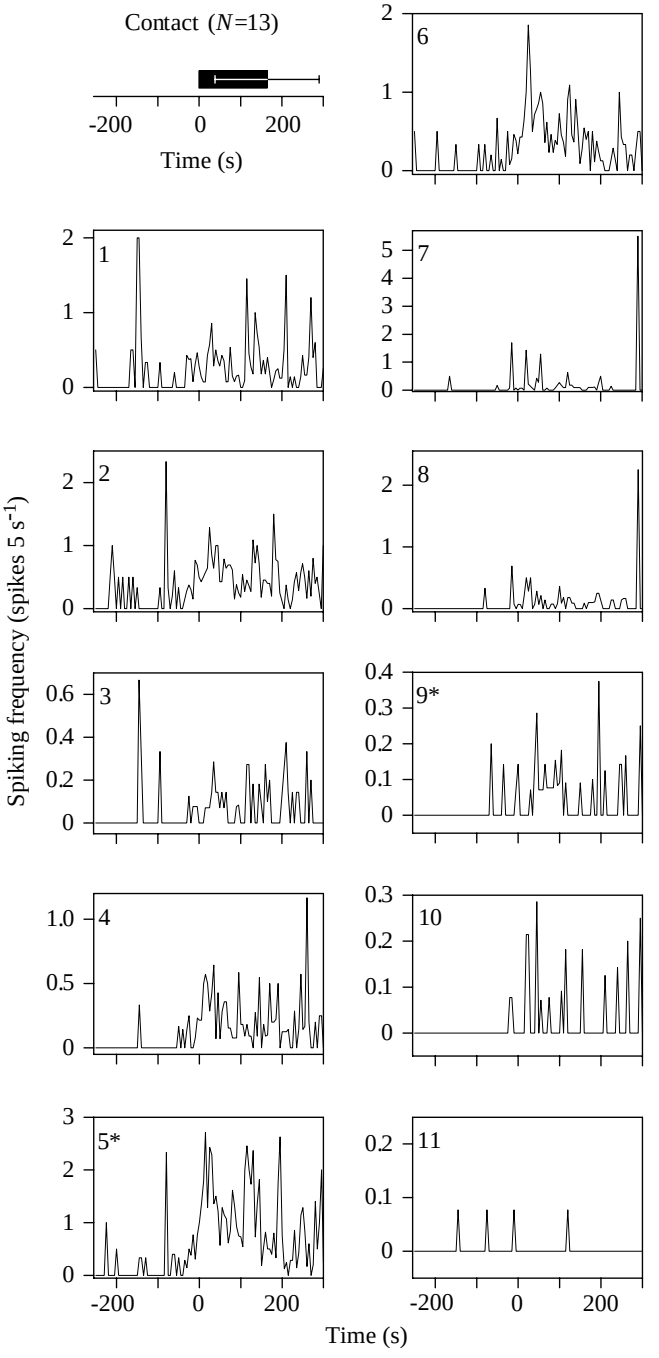

Fig. 6. The spiking activity of 11 units in relation to contacts with the mating partner during courtship. Contact begins at time zero, and its duration is indicated by the horizontal bar, which shows the mean duration with the standard deviation. Each graph illustrates activity in a different unit; the unit number is indicated in the upper left corner. The statistical significance of frequency changes during contact is indicated by an asterisk (P<0.05).

The suggested motor function for the mesocerebrum (Chase, 1986) was confirmed by electrical stimulation *via* the implanted wire, although penial eversion was rarely observed unless a tactile stimulus was added to the electrical stimulus (Table 1). The tactile stimulus had to be applied specifically to the everted genital pore to evoke penial eversion and, in some cases, dart-shooting. Possibly, skin stimulation provides a key input to a second motor control centre, in the pedal ganglion, as discussed below. It is noteworthy that the artificially evoked instances of penial eversion were not always preceded by dart-shooting. This is consistent with behavioural observation that dart-shooting is occasionally omitted even in natural matings (J. M. Koene and R. Chase, in preparation).

The injections of neuropeptides yielded some unexpected results: APGWamide evoked genital eversion but not penial eversion, while FMRFamide, which has been proposed to be responsible for dart-shooting (Li and Chase, 1995), had no overt effect. We tested whether dart-shooting required a pre-established genital eversion by injecting a combination of both peptides. Again, we observed no dart-shooting; moreover, we observed fewer genital eversions than with APGWamide alone. The most likely explanation for the latter result is that APGWamide and FMRFamide have opposite effects on the penial retractor muscle. Whereas APGWamide relaxes the muscle (*Lymnaea stagnalis*, De Boer et al., 1997), FMRFamide contracts it (*Helix aspersa*, Lehman and Greenberg, 1987). It therefore seems likely that APGWamide is responsible for everting the genitalia, while FMRFamide is responsible for retracting them after mating and possibly for holding them inside the snail. The role of FMRFamide in dart-shooting, if any, remains to be determined. Some care must be taken with the interpretation of the FMRFamide results because its presence in the mesocerebrum of *Helix* spp. is controversial (Elekes and Nässel, 1990; Cottrell et al., 1992; Li and Chase, 1995). Also, the injection method might not be appropriate because of the involvement of FMRFamide in other types of behaviour.

The fact that very few of the operated snails showed sexual activity is probably a result of the loss of sexual motivation caused by the operation. Similar effects were observed during *in vivo* experiments with *Lymnaea stagnalis* (De Boer et al., 1997). While our data on the correlation between neural and behavioural activity are derived from just a single animal, this does not affect the validity of the positive results. Caution must be exercised, however, with respect to negative results.

Most of the recorded units increased their activity during dart-shooting and/or penial eversion (Fig. 5). The units active during both types of behaviour were anticipated by earlier *in vitro* tests of motor function (Chase, 1986) and by immunohistochemical and anatomical findings suggesting that some mesocerebral neurones may be multifunctional (Li and Chase, 1995).

Together with other evidence, our data suggest that the neural control of mating has evolved conservatively in the gastropod subclasses Opisthobranchia and Pulmonata (Fig. 7). To summarise the evidence for *Helix aspersa*, the right mesocerebrum is an integrative centre for mating behaviour on the basis of the following evidence: anatomical studies of axon projections (Chase and Li, 1994), recorded responses to peripheral stimulation (Chase, 1986, and present study), direct electrical stimulation (present study) and fine wire recordings during mating behaviour (present study). In addition, the demonstration here that the neuropeptide APGWamide can mediate genital eversion is consistent with the immunohistochemical localisation of APGWamide in the mesocerebrum (Li and Chase, 1995).

In *Lymnaea stagnalis*, a representative of the Basommatophora, backfills of the nervus penis demonstrate a strong projection from the anterior lobe of the right cerebral ganglion (De Boer et al., 1997). Electrical recordings from the lobe, using the implanted fine wire technique, show increased activity coincident with eversion of the preputium, a part of the male copulatory apparatus (De Boer et al., 1997). Eversion can be evoked by direct electrical stimulation of the lobe, and it is mediated by APGWamide, which is expressed in the lobe (Croll and Van Minnen, 1992). It is significant that the mesocerebrum of *Helix aspersa* and the anterior lobe of *Lymnaea stagnalis* are both located at the same anteromedial position in the cerebral ganglion, and both regions are larger on the right side than on the left side (Fig. 7A,B).

In *Aplysia californica*, a representative of the Opisthobranchia, the H-cluster is also located at the anteromedial margin of the right cerebral ganglion; there is no counterpart in the left cerebral ganglion (Fig. 7C). The neurones of this cluster send processes to the penial complex *via* the lower labial nerve (Jahan-Parwar and Fredman, 1976). An early study found that electrical stimulation of the right cerebral ganglion causes contractions in the penial complex (Bottazzi and Enriques, 1900). Recently, it has been reported that the neurones of the H-cluster show strong immunoreactivity for APGWamide (Fan et al., 1997) and that APGWamide can evoke penial eversion in a reduced preparation (Yu and Blankenship, 1997). In summary, the evidence from *Helix aspersa*, *Lymnaea stagnalis* and *Aplysia californica* indicates that there is a homologous group of neurones at the anteromedial margin of the right cerebral ganglion and that these neurones are responsible, at least in part, for the control of mating behaviour.

The immunohistochemical localisation of APGWamide in several additional species of gastropod molluscs suggests that the homology is robust within the class Gastropoda. In a comparative study, De Lange and Van Minnen (1998) found immunoreactivity for APGWamide in clusters of neurones at the anteromedial margin of the cerebral ganglia in the basommatophore *Bulinus truncatus*, the stylommatophores *Arion ater* and *Limax maximus* and even in the prosobranch *Littorina littorea*.

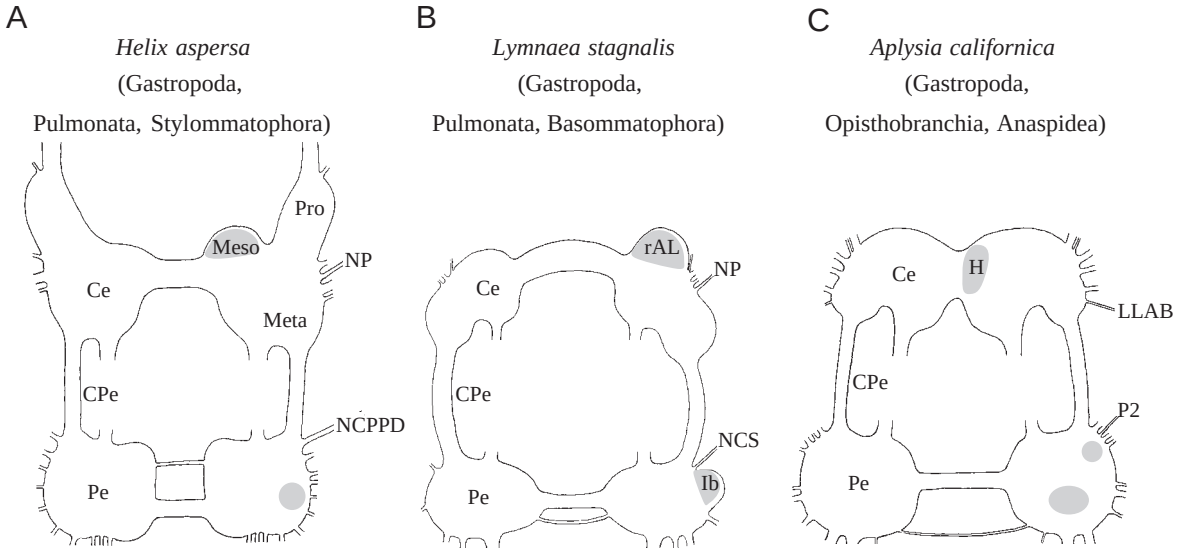

Fig. 7. Regions of the central nervous system controlling mating behaviour in three species of gastropod mollusc. Dorsal views are shown. The shading indicates regions with conserved function, as explained in the Discussion. Nerves are labelled if they are mentioned in the Discussion. Ce, cerebral ganglion; CPe, cerebro-pedal connective; H, H-cluster; Ib, pedal Ib-cluster; LLAB, lower labial nerve; Meso, mesocerebrum; Meta, metacerebrum; NCPPD, nervus cutaneus pedalis primus dexter; NCS, nervus cervicalis superior; NP, nervus penis; P2, anterior tegumentary nerve; Pe, pedal ganglion; Pro, procerebrum; rAL, right anterior lobe.

There is another cluster of neurones, located in the lateroventral region of the right pedal ganglion, that also has a role in the expression of mating behaviour. Most of these cells have projections into the penial nerve and seem to be motoneurones controlling muscles of the penial complex. Their approximate locations are shown in Fig. 7 (*Helix aspersa*, Eberhardt and Wabnitz, 1979; Li and Chase, 1995; *Lymnaea stagnalis*, De Boer et al., 1996: *Aplysia californica*, Rock et al., 1977). Some of the pedal motoneurones project not into the penial nerve but into a pedal nerve, which is itself a pathway to the penial complex. In *Helix aspersa*, this nerve is the NCPPD (Li and Chase, 1995), in *Lymnaea stagnalis*, the NCS (Elo, 1938), and in *Aplysia californica*, a branch of nerve P2 (Kandel, 1979).

Although the particular function, or functions, of the pedal ganglion neurones have yet to be determined, these cells should be regarded as constituting another centre for motor control of the penial complex. Whether they operate downstream from right anteromedial cerebral ganglion neurones, or independently, is unclear, although some axons of right anteromedial neurones do terminate in the pedal ganglion (*Helix aspersa*, Li and Chase, 1995; *Lymnaea stagnalis*, De Boer et al., 1997: *Limax maximus* and *Arion ater*, De Lange and Van Minnen, 1998). If the pedal neurones must be activated for full motor expression and if their excitation depends on inputs additional to those from the mesocerebrum, this might explain why simple electrical stimulation of the right mesocerebrum elicits only partial genital eversion. In *Lymnaea stagnalis*, peripheral sensory neurones in the penial complex send processes to the pedal Ib-cluster (Croll et al., 1999). If a similar pathway exists in *Helix aspersa*, it could provide the additional input necessary for the complete expression of the behaviour.

Our data establish the existence of an evolutionarily conserved region of the gastropod brain that is responsible for the central control of the male genitalia. The pulmonates and the opisthobranchs diverged from their prosobranch ancestors sometime in the Carboniferous period, approximately 350 million years ago. Since then, mating behaviour has evolved and differentiated. For example, *Helix aspersa* mates as a simultaneous reciprocal hermaphrodite, *Lymnaea stagnalis* is a serial reciprocal hermaphrodite and *Aplysia californica* is a simultaneous non-reciprocal hermaphrodite. There are numerous differences in the details of courtship and copulation, including the unique dart-shooting behaviour of *Helix aspersa*. It will now be interesting to investigate how the circuitry and cellular properties of sex-related neurones have evolved to accommodate the different mating strategies.

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