Functional Role of Peptidergic Anterior Lobe Neurons in Male Sexual Behavior of the Snail *Lymnaea stagnalis*

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1Department of Organismal Neurobiology, Faculty of Biology, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands; 2Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada; and 3Department of Biology, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-03, Japan

De Boer, Pamela A.C.M., Andries Ter Maat, Anton W. Pieneman, Roger P. Croll, Makoto Kurokawa, and René F. Jansen. Functional role of peptidergic anterior lobe neurons in male sexual behavior of the snail *Lymnaea stagnalis*. *J. Neurophysiol.* 78: 2823–2833, 1997. A morphologically defined group of peptidergic neurons in the CNS of the hermaphroditic snail, *Lymnaea stagnalis*, is concerned with the control of a very specific element of male sexual behavior. These neurons are located in the anterior lobe of the right cerebral ganglion (rAL). By using chronically implanted electrodes, we show that the rAL neurons are selectively active during eversion of the penis-carrying structure, the preputium. The preputium is normally contained inside the body cavity and is evaginated during copulation in the male role. Electrical stimulation of the rAL neurons through the implanted electrodes, induced eversion of the preputium in vivo. Injection of APGWamide (Ala-Pro-Gly-Try-NH2), a small neuropeptide that is present in all rAL neurons, induced eversion of the preputium. Application of APGWamide to in vitro preparations of the preputium caused relaxation of this organ. In contrast, injection of the neuropeptide conopressin, which is co-localized with APGWamide in 60% of the rAL neurons, did not induce any behavior associated with male sexual activities. These results show that the neurons of the rAL can induce an eversion of the preputium as occurs during male copulation by release of APGWamide during a period of electrical activity.

**INTRODUCTION**

Considerable evidence indicates that a large number of neuropeptides are involved in the control of male sexual behavior in various species (e.g., Argiolas and Melis 1995; De Boer et al. 1996; Dorman and Malsbury 1989). Because sexual behavior is often not a simple cyclic pattern but a complex behavioral sequence comprising several elements, it is difficult to elucidate the specific role of peptidergic neurons and of the neuropeptides they contain. Invertebrates, such as the hermaphroditic snail *Lymnaea stagnalis*, can serve as valuable model systems for the study of peptidergic control of sexual behavior because their CNS consists of a limited number of neurons that are often large and identifiable. Moreover, the well-described, restricted behavioral repertoire of this snail makes it easier to characterize specific neuronal functions. Still, even in these animals copulation is not a fixed action pattern but rather a chain of acts that is varied in duration and is modulated by the motivational state of the animal (De Boer et al. 1997). Here we describe a distinct function for a group of peptidergic neurons and their co-localized neuropeptides in the control of male sexual behavior of *L. stagnalis*.

*L. stagnalis* is a simultaneous hermaphrodite (Holm 1946) in which each adult possesses the complete neural circuitry and endocrine centers necessary for ovulation and egg laying as well as spermatiation and copulation in both the female and male roles. The stereotyped egg laying behavior has been studied in great detail in *Lymnaea*, and large parts of the neural circuitry of egg laying behavior have been described (reviewed in Ter Maat et al. 1992), as well as the neuropeptides that are involved with the different aspects of the behavior (Hermann et al. 1994). Also, the considerably more complex male copulation behavior has now been studied on the behavioral, neural, and molecular levels. The appetitive male copulatory behavior consists of a number of elements that have variable durations and do not always appear in the same sequence (De Boer et al. 1996; Van Duivenboden and Ter Maat 1988). In addition, motivational factors have been shown to affect the behavioral sequence (De Boer et al. 1997; Van Duivenboden and Ter Maat 1985). The complex copulation behavior is thought to be under the control of at least 10 different neurotransmitters and neuropeptides (reviewed in De Boer et al. 1996; De Lange et al. 1997). Although the functions of the different messenger molecules are not yet clearly defined, there is a strong indication that it is difficult to deduce specific functions for the peptides on the basis of morphological and physiological evidence obtained so far. Here we concentrate on the co-localized neuropeptides, APGWamide (Ala-Pro-Gly-Try-NH2) and the vasopressin/oxytocin related conopressin. APGWamide has been shown to inhibit serotonin- and dopamine-induced contractions of retractor muscles in a dose-dependent fashion (Croll et al. 1991; Li et al. 1992). The male copulatory organ, which comprises the preputium, penis, and retractor muscles, is evaginated during copulation putatively by relaxation of the retractor muscles. APGWamide-containing axons and terminals are located in muscles of the male copulatory apparatus, the prostate gland, the vas deferens, and on the body wall surrounding the male gonopore (Croll and Van Minnen 1992; Croll et al. 1991). Conopressin is co-localized with APGWamide in neuronal fibers on the vas deferens (Van Golen et al. 1995). Application of conopressin induces contractions in the vas deferens and this action is inhibited in the presence of APGWamide (Van Kesteren et al. 1995a, b). These data suggest that APGWam-
ide and conopressin are involved in the control of various elements of male copulation behavior. Neurons of the anterior lobe of the right cerebral ganglion (rAL) of the CNS are the major site of expression of the APGWamide and the conopressin genes (Croll and Van Minnen 1992; De Lange et al. 1997; Smit et al. 1992; Van Golen et al. 1995). All rAL neurons express the APGWamide gene, whereas in 60% of the neurons the conopressin gene is co-expressed (De Lange et al. 1997; Van Golen et al. 1995). Retrograde stainings have identified those rAL neurons that send axons in the sole nerve innervating the male copulatory apparatus, the penis nerve (Smit et al. 1992; Van Duivenboden 1984). These results suggest that the rAL neurons have an important role in the control of male copulation in *L. stagnalis*.

In this paper we analyze the function of the rAL neurons and the co-localized neuropeptides APGWamide and conopressin in the control of male copulation of *L. stagnalis* through the use of in vivo and in situ recordings and injection experiments. We demonstrate that the neurons of the rAL can control the eversion of the male sexual organ and that APGWamide induces such an eversion after injection into the body cavity.

**Methods**

**Animals**

Adult, laboratory-bred specimens of *L. stagnalis* (shell heights 28–33 mm) were used. The snails were bred and kept in tanks with continuous water change at 19–21°C (SE) (Van Der Steen et al. 1969). The animals used for the continuous recording with fine wire electrodes were housed individually for 8 days before the implantation. This procedure induces an increase in male sexual drive in these animals (De Boer et al. 1997; Van Duivenboden and Ter Maat 1985), thereby causing more animals to copulate during the recording. A 12:12 h light:dark cycle was maintained and lettuce leaves were provided ad libitum.

**Retrograde and anterograde staining**

The central ganglia were dissected and pinned out in saline (pH 7.8). The saline had the following composition (in mM): 4.0 CaCl₂, 1.7 KCl, 1.5 MgCl₂, 3.0 NaCl, 5.0 NaHCO₃, 10.0 NaCH₃SO₄, and 10.0 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES). The technique for axonal backfilling was modified after the procedure described by Fredman (1987). The cut stump of the penis nerve was drawn tightly into the end of a finely tipped glass pipette of the appropriate diameter. Saline within the pipette was replaced with a solution of nickel-lysine (1.7 g NiCl₂·6H₂O and 3.5 g l-lysine free base in 20 ml H₂O). This preparation was maintained at room temperature for 18–24 h before the pipette was removed and the ganglia were washed in fresh saline. Nickel was precipitated by adding 5–10 drops of a saturated alcohol solution of rubanie acid (dithiooxamide) to the 10 ml saline bath. After 20–30 min, the ganglia were fixed overnight in 4% paraformaldehyde in saline. After several rinses of saline, the ganglia were dehydrated through an ascending alcohol series, cleared in methyl salicylate and mounted in Entellan (Merck).

Morphological details of individual cells were obtained by intracellular staining with Lucifer yellow as described by Stewart (1978).

**Electrophysiology**

Extracellular recordings of the penis nerve were made with a suction electrode. Intracellular recordings of various cells were made with glass microelectrodes (resistance 5–50 MΩ), which were filled with a saturated K₂SO₄ solution. Conventional electrophysiological apparatus was used.

A stainless steel fine wire electrode (25 μm diam, California Fine Wire Company) was used for extracellular recording of electrical activity from the intact rAL in vivo. The procedure of implantation has been described elsewhere (Hermann et al. 1994; Yeoman et al. 1994). After the electrode was implanted in the animal it was connected to an amplifier with a high-impedance head stage (DAM80, WPI). The animal was placed in a glass tank (20 cm high, 30 cm long, and 6 cm wide), which was continuously perfused with water. Camcorders (Blaupunkt) on both sides of the tank continuously recorded the animal’s behavior. These recordings together with the electrical activity of the rAL neurons were stored on super-VHS videotape. A stimulus isolator (Neurolog NI-800, Digitimer) was used for electrical stimulation via the fine wire electrode. The electrical recordings were digitized with a Cambridge Electronic Design A/D converter (model 1401, 12-bit).

**Behavioral experiments**

Injections of 20 μl of saline, APGWamide (American peptide company), or conopressin (Saxon Biochemicals, Germany) solutions were made through the sole of the foot into the body cavity. After injection all snails were placed in individual containers and held with the foot in contact with the wall of the container until they attached themselves. When the snails are in this position the observer has the clearest view of the male aperture. Animals that did not attach were placed on the bottom of the container in such a way that any eversion of the preputium would be visible. The behavior was stored on super-VHS tape. During the experiment the observer was unaware of the treatment each snail had undergone.

**Surgery**

The penis nerve together with the penis artery were lesioned in animals anesthetized with 1.5 ml of 50 mM MgCl₂. The penis nerve originates from the right cerebral ganglion near the base of the median lip nerve (Fig. 1). A network of fine branches of the trunk of the columellar muscle. The penis sheath is attached by retractor muscles (Holm 1946) to the main trunk of the columnellar muscle. The penis sheath is attached at the
tip of the preputium. The penis cannot be seen because it is enveloped by the penis sheath. The preputium was dissected and attached to a length transducer with fine hooks made out of insect pins (0.15 mm diam) in a 0.5-ml chamber filled with saline. The preputium was held under a constant tension of 0.28 g. The preparations were continuously perfused with saline at a flow rate of 0.5 ml/min by using a pump (Minipuls 3, Gilson, France). Data were stored on a DAT-recorder modified to record DC-signals (JVC, Japan). Experimental and control solutions were applied in 1-ml volumes by continuous perfusion. Two minutes after the start of application, when the volume of the chamber was exchanged twice, the perfusion was stopped for 1 min. Then the pump was turned on again to wash out the sample solution with fresh saline.

RESULTS

rAL neurons project into the penis nerve

Both tract tracing experiments and electrophysiology were used to identify the neurons that project into the penis nerve. Retrograde staining with nickel-lysine was used to localize neurons with axons in the penis nerve. Five groups of neurons could be identified, all restricted to the right half of the CNS (Fig. 2). They were located in the anterior and ventral lobes of the right cerebral ganglion, the pedal Ib cluster (PeIb)-cluster of the right pedal ganglion, and in scattered populations within the right pleural and parietal ganglia. In the rAL ~60–70 backfilled somata were identified. Generally about 75% of the neurons at the surface were stained. The stained neurons were found scattered over the entire lobe.

To determine the projections of the rAL neurons electrophysiologically, we combined intracellular recordings with extracellular recordings of the penis nerve. In an isolated central ganglia ring the rAL was easily recognized by its outer layer of relatively large (35–70 μm diam) and uniformly pale orange to yellow cells. Intracellular recordings showed that the neurons were generally silent, with resting membrane potentials between −70 and −80 mV. At intracellular stimulation with depolarizing square current pulses, the neurons generated action potentials and either small or large spikes could be recorded in the nerve. Occasionally a neuron was recorded that showed spontaneous activity (Fig. 3).

From the 125 somata impaled in the anterior lobe (26 prepa-
FIG. 3. Identification of neurons projecting into penis nerve. A: neurons in the rAL that have a projection in penis nerve (black somata) or have not (white somata), as revealed by simultaneous recording of neurons and penis nerve (PN). B: simultaneous intracellular recording of rAL neurons (bottom) and extracellular recordings of penis nerve (top). Examples of a spontaneously active neuron (right) and of electrically silent neurons that show either a small or large component in penis nerve after depolarization of the soma (left and middle, respectively). All spikes recorded from somata were followed 1 for 1 with spikes in nerve at constant latencies. DB, dorsal body; rCG: right cerebral ganglion.

115 somata had spikes which were followed one for one with spikes in the nerve at constant latencies. In one preparation 18 somata were impaled, 14 of which had a corresponding element in the penis nerve. Electrical stimulation of the penis nerve induced action potentials in the rAL neurons. In four preparations a repetitive firing pattern (afterdischarge) occurred as a result of the stimulation. This feature has been reported earlier by Chase and Li (1994).

Several rAL neurons were also injected with Lucifer yellow (n = 12). Figure 4 is an example of a staining of a rAL neuron with axonal projections into the ventral lobe of the right cerebral ganglion and from there into the penis nerve and into the cerebropedal connective toward the Pelb-cluster and into the superior cervical nerve. In one preparation only one axonal branch extended from the cell body, projected toward the right ventral lobe, and from there into the penis nerve. In the other 11 preparations a single axonal branch extended from the cell body and projected to the right ventral lobe where the initial branch split into two or three branches. In eight of the preparations one or two of these axonal branches projected into the cerebropedal connective and the rest projected into the penis nerve. In the other preparations all branches projected into the penis nerve. In four preparations an axonal branch was found in the Pelb-cluster.

These data indicate that most cells at the outer surface of the rAL send axons into the penis nerve. This is in accordance with the results of the nickel-lysine backfills where only some cells at the surface of the lobe were left unstained. The Lucifer yellow stainings show that individual rAL neurons do not solely project into the penis nerve. Furthermore, the branching patterns suggest that rAL neurons communicate with the neurons in the right ventral lobe and Pelb-cluster.

**rAL neurons are electrically active during eversion of the preputium**

The electrical activity of rAL neurons was studied in freely behaving animals over 2-day periods by means of chronically implanted fine wires. During this period all the animals (n = 41) appeared healthy and exhibited a variety of ongoing behaviors (e.g., egg laying, rasping, and lung ventilation). However, only two recordings were made during successful copulation (i.e., where sperm transfer had taken place) and 2 were made during sham copulation (preputium eversion without sperm transfer).

The electrical recordings, including those from the animals that did not copulate, showed few and infrequent spikes not associated with the execution of any (noncopulatory) behavior. Figure 5A, top, shows a digitized recording of the rAL made from an animal while it crawled about in the tank,
CONTROL OF MALE SEXUAL BEHAVIOR IN LYMNAEAE

FIG. 4. Camera lucida drawing of a rAL neuron filled with Lucifer yellow. Ventral view of anterior right side of central ganglia ring. PeGb, pedal Ib-cluster; PN, penis nerve; rCG, right cerebral ganglion; rPeG, right pedal ganglion; rVL, ventral lobe of the right cerebral ganglion; SCN, superior cervical nerve.

Although normally the preputium is only everted during male copulatory activities, one animal was recorded while the preputium was everted in the absence of a female copulation partner. The animal everted its preputium for 5 min during which the activity in the rAL neurons was increased (Fig. 6).

These findings show that the neurons of the anterior lobe are active during preputium eversion. This suggests that the neurons of the rAL are involved in the control of preputium eversion. Furthermore, it was demonstrated that the different units in the recording do not become active simultaneously during the eversion of the preputium.

Stimulation of the anterior lobe neurons induces eversion of the preputium in vivo

To investigate whether or not the rAL neurons can indeed control the eversion of the preputium, the rAL neurons were stimulated via an implanted fine wire in another group of animals (n = 24). Initial recording from the fine wire electrodes revealed the same extracellular activity of the rAL neurons in all animals: generally silent with only occasional spikes. The implanted fine wires were then used to stimulate the rAL neurons. Three of the 24 animals responded to the electrical stimulation by fully evertting the preputium, 20 showed partial eversion (ranging from 25 to 50%), and 1 did not exhibit any overt effect. The eversion started within 30–60 s after the onset of the stimulation and the preputium was retracted again within 10 s after the end of stimulation. The stimulations lasted 5–10 min.

These results show that stimulation of the rAL neurons can cause eversion of the preputium, thus further indicating that these neurons are involved in the control of the penial complex.

Injection of APGWamide induces eversion of the preputium

Almost all rAL neurons contain APGWamide, whereas in 60% of the rAL neurons APGWamide is coexpressed with the conopressin gene (De Lange et al. 1997). Because little is known about the release sites, injections of these neuropeptides provide a first approximation of their function. Injection experiments were performed to investigate the role of APGWamide and conopressin in the control of preputium eversion.

APGWAMIDE INJECTIONS. Three different concentrations of APGWamide were tested: 10^{-5} M (n = 7), 5 \times 10^{-6} M (n = 6), and 10^{-6} M (n = 4). Control injections (n = 4) consisted of saline only. Saline injected animals attached to the container wall and remained stationary for 5–10 s. Thereupon, the animals started moving around the container in an apparently normal manner. Within the first minute after all APGWamide injections, animals were distinguishable from saline-injected animals by their decreased locomotory activity. All of the snails injected with either 10^{-5} M or 5 \times 10^{-6} M and two of the four animals injected with 10^{-6} M APGWamide showed eversion of the preputium. In five snails injected with 10^{-5} M APGWamide the eversion was full; in the other animals the eversion was partial. Two of the snails injected with 10^{-6} M APGWamide did not show any eversion. A typical full eversion is shown in Fig. 7.
Within 2 min the partially everted preputium became visible at the right side of the animal (3). About 1 min later, the preputium was fully everted (6). This eversion lasted 10–15 s, after which the preputium was partially retracted into the body cavity. After 9 min, the preputium is fully retracted and locomotion started again (9). Generally the eversion started within 2–7 min after the injection. Within 10 min, the preputium was retracted back into the body cavity in all animals and the animals started moving again. Within another 5 min, all animals had resumed normal behaviors.
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**FIG. 7.** Effect of APGWamide injection in vivo. Images of a snail taken after injection with 20 μl of 5 × 10⁻³ M APGWamide. Animal is placed against transparent side of experimental container. Within 2 min after injection, preputium is everted and becomes visible as a white structure at right side of animal (→, starting point). Preputium become fully everted (6) and are retracted again. After ~9 min, animal is crawling around again (9). Time interval between images is 1 min for 1st 3 images, 20 s for next 5 images, and 5 min and 20 s for last image.

There was no effect of the different concentrations on the temporal pattern of the eversion and retraction of the preputium. From these data it can be concluded that APGWamide injection induces eversion of the preputium.

**CONOPRESSIN INJECTIONS.** Three different concentrations of conopressin were tested: 10⁻⁶ M (n = 4), 10⁻⁷ M (n = 6), and 10⁻⁸ M (n = 2). Conopressin induced a strong contraction of the foot. During this contraction, the foot was folded with the tip of the posterior end contacting the anterior side. Possibly as a result of this the animals did not attach to the wall of the container after injection. The contraction of the foot lasted 2–10 min for the animals injected with either 10⁻⁷ M or 10⁻⁸ M and 8–18 min for the animals injected with 10⁻⁶ M. Within 1–2 min after the relaxation of the foot, the animals attached to the surface and started to crawl around again, in an apparently normal manner. Two of the six animals injected with 10⁻⁷ M conopressin, showed minimal eversion of the preputium. Less than ~20% of the preputium everted. However, none of the other animals showed any eversion.

**COMBINED INJECTIONS.** APGWamide and conopressin are co-localized in neurons of the rAL. In previous studies the neuropeptides were found to have opposite effects on muscle tension. To investigate whether conopressin could alter the effect of APGWamide described above, combined injections of 10⁻³ M APGWamide and 10⁻⁷ M conopressin were performed. The effect on the behavior was a combination of the behaviors seen after injections of the separate peptides. All animals (n = 7) showed eversion of the preputium and simultaneously contraction of the foot, which again prevented attachment. This suggests that conopressin does not alter the gross effect of APGWamide when simultaneously injected.

*Lesion of the penis nerve prevents retraction of the preputium, not eversion*

Eversion of the preputium might be caused either by a peripheral effect of APGWamide on the male copulatory organs or by a central effect of APGWamide on the neurons in the CNS and thereby mediating an eversion. To investigate whether or not eversion of the preputium can be induced by peripheral actions of APGWamide, we injected 20 μl of 10⁻³ M APGWamide into animals that had their penis nerve cut 3 or 4 days earlier (n = 7). Because the penis artery is attached to the penis nerve, a lesion of the penis nerve implies also a lesion of the penis artery which supplies the preputium with blood. To investigate possible effects of a disturbed blood supply on the ability to evert and retract the preputium, we also injected APGWamide in animals that had only the penial artery cut (n = 3). Finally, we also injected APGWamide into sham-operated animals (n = 4).

The sham-operated animals behaved similarly to the nonoperated animals. Two of the animals showed a partial eversion, whereas the other two showed a total eversion of the preputium. All lesioned animals, except for one with only the artery cut, showed a total eversion of the preputium. Generally, the eversion started within 2–4 min after the injection. Preputium length in the animals with both penis nerve and artery cut appeared to be increased as compared with the artery-lesioned, nonoperated, and sham-operated animals (not quantified). Animals with both the artery and penis nerve lesioned did not retract their preputium into the body cavity. By contrast, the artery-lesioned animals did retract the preputium within 10 min after the injection.

These results show that the ability to evert the preputium after injection with APGWamide persists after a lesion of the penis nerve and artery. It is concluded that eversion of the male copulatory apparatus can be induced by the peripheral action of APGWamide. Retraction of the preputium is abolished by lesions of the penis nerve but not the penis artery. From these data we conclude that retraction of the penial complex is an active process requiring the activity of a central circuit.
Effect of APGWamide and conopressin on muscles of the preputium

The effect of APGWamide and conopressin on the relaxation or contraction of the preputium was tested by application of these peptides to the preputium in vitro while the length of this organ was measured continuously with a length transducer. Seven different concentrations of APGWamide were tested, ranging from $10^{-7}$ M to $10^{-3}$ M ($n = 5$ for $10^{-7}$ to $10^{-4}$ M, $n = 4$ for the other concentrations). Bath application of APGWamide induced a relaxation of the preputium at a concentration of $10^{-8}$ M. The relaxation was maximal at a concentration of $10^{-4}$ M (Fig. 8B). An example of a recording made during application of $10^{-5}$ M is given in Fig. 8A, top. Within 30 s after the start of the application the preputium starts to relax. Sixteen minutes after the wash out of sample solution, the preputium has resumed its initial length. The relaxation of the preputium was dose-dependent (Fig. 8B). The half-maximal effective concentration ($EC_{50}$) value was $3.63 \times 10^{-6}$ M.

Conopressin was tested in three different concentrations ranging from $10^{-6}$ to $10^{-4}$ M ($n = 3$ for each concentration). Bath application of conopressin had no effect on the relaxation or contraction of the preputium (Fig. 8A, bottom). We also tested whether or not conopressin could alter the effect of APGWamide by simultaneous application of APGWamide ($10^{-3}$ M) and conopressin ($10^{-4}$ M) ($n = 4$). No effect of conopressin was found (Fig. 8B, Δ). From these data it can be concluded that the effect of APGWamide on the preputium is not modulated in the presence of conopressin.

Role of the rAL neurons in the eversion of the preputium

The electrophysiological experiments showed that spikes in rAL somata were followed one for one with spikes in the penis nerve. Direct projections of rAL neurons in the penis nerve were not checked by blocking the synaptic activity. However, both the constant latencies of the nerve spikes with the somata spikes and the morphological demonstration of projections of rAL neurons in the penis nerve are consistent with direct projections. The projections of the rAL neurons were studied earlier. With the use of intracellular Lucifer yellow staining, Khennak and McCrohan (1988) demonstrated that at least 45% of the neurons send projections into the penis nerve. Here we show with retrograde tract tracing, Lucifer yellow stainings, and electrophysiological recordings that this number must be higher. The results from intracellular recording indicate that this number is ~80%.

The electrical activity of the rAL neurons is correlated with the eversion of the preputium. Both intra- and extracellular recordings showed that the neurons are generally electrically silent. During the eversion of the preputium the electrical activity of ~10 units was increased. This amount is very likely the maximum that can be recorded from in this configuration, because the recording electrode covered about one-fifth of the total amount of somata at the surface of the rAL. Furthermore, the number of units that was recognized was similar in all recordings, although the precise position of the electrode varied between animals. Therefore, we conclude that the majority of the rAL neurons are probably active during the eversion of the preputium. Neurons controlling male copulation have been recorded from in some other gastropods. In Aplysia californica pedal cluster neurons (the “primary cluster”) were found to produce one for one excitatory potentials in the penis retractor muscle (Rock et al. 1977). In the terrestrial snail Helix aspersa, neurons in the right mesocerebrum have projections in the penial nerve (Li and Chase 1995). Stimulation of these neurons causes contractions of either the dart sac, the penis, or both (Chase 1986). In this animal, the mesocerebrum is asymmetrical, the right hand side being appreciably larger than the left side. Furthermore, neurons in this cluster were found to display APGWamide-like immunoreactivity (Griffond et al. 1992). In this respect, the mesocerebrum resembles the anterior lobe of Lymnaea.
Although the in vitro studies indicate a possible role for central neurons, this is the first report of in vivo activities of neurons controlling male copulation behavior in gastropods. Of the 41 animals with an implanted electrode only 4 showed male copulation activities. The animals that did not engage in such reproduction were not apparently ill. In other studies in which in vivo electrical recordings were made there is no report of such a low percentage of animals showing a specific behavior (Begnoche et al. 1996; Hermann et al. 1994; Yeoman et al. 1994). Male copulation behavior of 
Lymnaea
 was demonstrated to be under the control of motivational factors (De Boer et al. 1997). In previous studies in which individually housed animals (without any implanted electrodes) were used, 40% of the animals showed intromission (De Boer et al. 1997; Van Duivenboden and Ter Maat 1985), a percentage much higher than reported here. The performance of the more complex male copulation behavior is easily disrupted by relative small disturbances. Chronically implanted electrodes obviously induce enough disturbance to cease male copulation behavior.

Functional role of APGWamide

In 
Lymnaea
 several different neuropeptides have been demonstrated in the neurons that project into the penis nerve (reviewed in De Boer et al. 1996). APGWamide is the neuropeptide that has been studied most extensively. Practically all neurons in the rAL contain APGWamide. The current experiments show that injection of APGWamide induces an eversion of the preputium not only in intact animals but also in animals with the penis artery lesioned and in animals with both the penis nerve and artery lesioned. These data suggest that the injected APGWamide exerts its action via receptors in the periphery.

The presence of the peptide has been demonstrated in the periphery. Immunoreactive fibers are located in the penis retractor muscle, throughout the preputium, along the inner surface of the penis sheath, and in the body wall surrounding the male gonopore. (Croll and Van Minnen 1992; De Lange et al. 1997). The overall action of the APGWamide in 
Lymnaea
 seems to be relaxation of muscles. The neuropeptide inhibits contractions of the retractor muscle that are induced by serotonin and dopamine (Croll et al. 1991; Li et al. 1992), it inhibits the spontaneous contractions of the vas deferens (Van Golen et al. 1995) and relaxes the preputium. Recently, the presence of cDNA-encoding APGWamide has been demonstrated in 
Aplysia
 (Fan et al. 1997; Nagle et al. 1996). The cDNA was found almost exclusively in the right cerebral ganglion and in male reproductive organs. This suggests a functional role of APGWamide in male copulation of 
Aplysia
. There are studies on the functional role of APGWamide in mollusks other than 
Lymnaea
, although these studies did not relate to male sexual activities. In the prosobranch snail 
Fusinus ferrugineus
, APGWamide potentiates twitch contractions in the radula retractor muscle and shows an inhibitory action on the radula protractor muscle (Kuroki et al. 1990). In 
Mytilus edulis
, APGWamide inhibits the electrically induced contractions of the byssus muscles (Muneoka and Kobayashi 1992). These reports suggest that APGWamide has a general function in the modulation of muscles in mollusks.

In addition to peripheral effects of APGWamide, there is also evidence that it has central actions. Just as in the periphery, immunoreactivity and effects of APGWamide are found in the CNS. In addition to the rAL neurons, the peptide has also been demonstrated to occur in some cells of the anterior lobe of the left cerebral ganglion, the ventral lobe of the right cerebral ganglion, and in the ring neuron (Croll and Van Minnen 1992; Croll et al. 1991). This suggests that APGWamide is not exclusively involved in preputium eversion. This idea is supported by the tract tracing, immunocytochemical, and electrophysiological data. Croll and Van Minnen (1992) and De Lange et al. (1997) describe that APGWamide positive neurites of the rAL neurons project in a fascicle toward the right ventral lobe and from there into both the penis nerve and the right cerebral-pedal connective. Furthermore, they describe APGWamide-immunoreactive terminals surrounding somata of the PeIb-cluster of the right pedal ganglion. These terminals originate from neurons of the rAL. Our data show that these neurones do indeed project to the PeIb-cluster. Together, these results suggest that the rAL neurons have APGWamidergic inputs on the PeIb-cluster of the right pedal ganglion. Central effects of APGWamide have been found in other gastropods. In the stylomatophoran snails 
Achatina fulica
 and 
H. aspera
, it was demonstrated that the peptide has a hyperpolarizing effect on neurons (Chen and Walker 1992; Liu et al. 1991).

In 
Lymnaea
, APGWamide induces an inhibition of the light green cells (H. Van Tol-Steye and K. S. Kits, personal communication) and also an inhibition of the caudodorsal cells (Croll et al. 1991) which control ovulation and oviposition (Ter Maat et al. 1992).

Now that the functional role of the rAL neurons and their major neuropeptide APGWamide becomes more and more clear, it is interesting to know what function the co-localized peptides have in male sexual behavior. APGWamide is co-localized with conopressin in 60% of the rAL neurons (De Lange et al. 1997; Van Golen et al. 1995). Because conopressin has excitatory effects on muscles of the vas deferens (Van Golen et al. 1995; Van Kesteren et al. 1995a) Van Kesteren and colleagues (1995a) suggested that conopressin may be involved in the control of ejaculation of semen during intromission in 
Lymnaea
. However, this idea cannot yet be confirmed by the injection experiments described in this paper. Although the animals were observed carefully, no ejaculation was observed after injection of conopressin alone or in combination of APGWamide. Possibly conopressin has a functional role in the CNS. The receptor for conopressin, LSCPR, is present on rAL neurons (Van Kesteren et al. 1995b). Recent data show that conopressin excites the rAL neurons (Van Soest and Kits 1997). More experiments have to be done to elucidate the function of conopressin in male copulation behavior.

Control of preputium eversion

On the basis of the present data we postulate the following model of preputium eversion as induced by APGWamide. A discharge of the rAL neurons causes APGWamide to be released in parts of the penial complex. This release induces the simultaneous relaxation of the retractor muscles and preputium muscles. The preputium is everted inside out starting...
at the base of the preputium (unpublished data). Therefore, and because APGWamide immunopositive fibers were found near the male gonopore, it is likely that the gonopore is relaxed by APGWamide.

It is possible that additional factors play a role in the eversion of the preputium. In *Lymnaea*, the preputium is supplied with blood by the penis artery. Preliminary data indicate that the blood pressure of the penis artery is increased during a normal eversion of the preputium (M. Kurokawa, personal observations). This aspect has to be examined further. Another aspect that cannot be excluded is the possible role of the other neurons that send projections in the penis nerve in the eversion of the preputium (e.g., the neurons of the right ventral lobe, Pdb-cluster, and those scattered throughout the right pleural and right parietal ganglia). However, the effect of stimulation of the rAL neurons and the APGWamide injections were very explicit; no behavioral acts other than eversion were observed. This suggests that the rAL neurons are the primary group of neurons responsible for eversion. Male copulation behavior is comprised of more elements other than eversion (i.e., probing and retraction of the preputium, intromission). Therefore, it is likely that the other groups of neurons with a projection to the penial complex have a function in the execution of these other elements.

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