Role of Neuropeptides Encoded on CDCH-1 Gene in the Organization of Egg-Laying Behavior in the Pond Snail, *Lymnaea stagnalis*

PETRA M. HERMANN, ROBERT P. J. DE LANGE, ANTON W. PIENEMAN, ANDRIES TER MAAT, AND RENE F. JANSEN

Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Faculty of Biology, 1081 HV Amsterdam, The Netherlands

Hermann, Petra M., Robert P. J. de Lange, Anton W. Pieneman, Andries ter Maat, and Rene F. Jansen. Role of neuropeptides encoded on CDCH-1 gene in the organization of egg-laying behavior in the pond snail, *Lymnaea stagnalis*. *J. Neurophysiol.* 78: 2859–2869, 1997. Egg laying in the pond snail *Lymnaea stagnalis* is triggered by a discharge of the neuroendocrine caudodorsal cells (CDCs). The CDCs expresses three different caudodorsal cell hormone (CDCH) genes. This gene family expresses, in total, 11 different peptides among which is the ovulation hormone. Besides the CDCs, the gene family is expressed in other central and peripheral neurons. In this study, we investigated the roles of the different CDCH peptides play in the organization of egg-laying behavior. Egg-laying behavior is a sequence of stereotyped movements in which three phases can be distinguished: resting, turning, and oviposition. We have used the excitation of right pedal N (RPeN) motor neurons as a simple analogue of shell-turning behavior, one of the elements of egg-laying behavior. RPeN motor neurons were inhibited during the resting phase of egg laying but were subsequently excited at the onset of and during the turning phase. The excitatory effect could be evoked by application of beta3-CDCP on RPeN motor neurons in the CNS as well as in isolation but not by the ovulation hormone, alpha-CDCP or Calfluxin, the other CDCH-1 peptides tested. The ovulation hormone itself caused inhibition of RPeN motor neurons. Anti-CDCH-1 positive fiber tracts were found close to the cell bodies and axons of the RPeN motor neurons. Electrical stimulation of a nerve that contains these fibers resulted in excitation of the RPeN motor neurons. The effects of injection of CDCH-1 peptides into intact animals correlated well with the effects of these peptides on RPeN motor neurons. Injection of beta3-CDCP or alpha-CDCP into intact animals resulted in immediate turning behavior in the absence of egg laying itself. The ovulation hormone and Calfluxin had no immediate effect on the behavior. Furthermore, our data indicate that the individual CDCH-1 peptides act on different targets.

INTRODUCTION

A recurrent theme in neurobiology is the regulation of complex behavior by families of neuropeptides. Convenient model systems to study neuropeptidergic regulation of behavior are the egg-laying behaviors in the gastropods mollusks *Lymnaea stagnalis* and *Aplysia*. The egg laying behaviors of *Lymnaea* and *Aplysia* long have been thought of as behaviors triggered in a preprogrammed way by the central release of multiple peptide messengers that are encoded on a small family of genes (Scheller et al. 1984). The release of these neuropeptides from neuroendocrine centers in the brain triggers egg laying and egg-laying behavior (Kupfermann 1967; Scheller et al. 1983; ter Maat et al. 1986). Egg laying in *Lymnaea* is triggered by a discharge of spiking activity in the caudodorsal cells (CDCs), a group of neuroendocrine cells in the cerebral ganglia of the CNS. The CDCs express three different caudodorsal cell hormone (CDCH) genes, CDCH-1 and -2 (Li et al. 1992; Vreugdenhil et al. 1988) and the recently discovered CDCH-3 gene (A. B. Smit, personal communication). The CDCH-1 gene gives rise to nine different peptides (Li et al. 1994), among which is caudodorsal cell hormone-1, the ovulation hormone. The CDCH-2 gene encodes for the same peptides except for caudodorsal cell hormone-2, which is different from the ovulation hormone (Li et al. 1992). Immunocytochemical and in situ hybridization experiments have shown that both the CDCH-1 and -2 genes are expressed in neurons in the CNS other than the CDCs. These genes also are expressed in peripheral neurons located along the male and female reproductive tract. Additionally, a large number of fiber tracts in the CNS and in the periphery stain positive for CDCH gene products (van Minnen et al. 1989a,b). Several studies described physiological effects of individual CDCH-1 peptides. It has been shown that the ovulation hormone is released into the blood where it acts as a hormone (Geraerts and Bohilken 1976; Geraerts et al. 1983). Injection of this hormone into the animal evokes ovulation and egg mass formation and triggers the last two phases of egg-laying behavior (Geraerts and Bohilken 1976; ter Maat et al. 1989). The ovulation hormone and alpha-CDCP appear to have an auto-excitatory function (Brussaard et al. 1990). Calfluxin, one of the other CDCH-1 peptides, causes an influx of Ca$^{2+}$ into the mitochondria of the female accessory albumen gland (Dictus and Ebberink 1988; Dictus et al. 1987). However, despite these studies little is known of the role the CDCH gene family plays in the regulation of the specific behavioral patterns during egg laying in *Lymnaea*.

The behavioral events of egg laying include the resting, turning, and oviposition phases, each characterized by a specific pattern of locomotion, buccal rasping, and movements of the shell (Hermann et al. 1994; ter Maat et al. 1989). In short: the shell is moved forward and kept over the head/foot complex during the resting phase, and there is a decrease in rasping movements and a decrease in the speed of locomotion. During the turning phase, the animals make long-lasting turns of their shells through >60° relative to the head/foot. These turns only occur in this phase of egg laying. Furthermore, during this phase, the number of rasping movements is increased (see also ter Maat et al. 1986, 1989). The motor...
neurons of the right pedal N cluster have been shown previously to be necessary for shell turning behavior. These neurons project into the right inferior cervical nerve and innervate muscles of the head/foot that are connected to the shell (Ferguson and Benjamin 1991; Hermann et al. 1994). Firing patterns that are appropriate for shell motor neurons can be recorded from the right inferior cervical nerve in freely behaving animals, and a lesion of this nerve totally abolishes spontaneous and egg-laying–related shell turns (Hermann et al. 1994). In this study, we investigated the effects of the application of the ovulation hormone and the CDCH-I peptides alpha-CDCH, beta3-CDCH, and Calfluxin on the activity of the right pedal N motor neurons. Furthermore, we show that the effects of these CDCH-I peptides on the motor neurons correspond well with the effects of these peptides on the behavior of intact animals.

METHODS

Animals

Adult specimens of *Lymnaea stagnalis* (L.), age 4–6 mo, shell length 25–35 mm, bred under standard laboratory conditions (Van der Steen et al. 1969) were used in all experiments described below. The animals were housed in perforated jars placed in a large tank with running fresh water (20°C) and were kept under a 12 h-12 h light-dark cycle and fed daily ration of lettuce.

Analysis of behavior

The three phases of egg laying are characterized by the speed of locomotion, the frequency of buccal rasping, and turning movements of the shell. The animal’s behavior was recorded onto S-VHS videotape. Turning movements of the shell as well as the speed of locomotion of the animal were quantified by digitizing one video frame every 10 s using a Data Translation DT255 video digitizer. An image-processing program (NIH-Image) with a 1-bit look-up table was used to determine the outline of the animal in the jar. The speed of locomotion (the distance traveled per 10 s) was calculated from 15 min before until 15 min after the moment of injection. Every 10 s, a “turn index” was determined as a measure of the position of the shell with respect to the body of the animal. To this end, the ellipse best fitting the outline of an animal was determined. The index that measures the shell turn then was calculated by dividing the length of the short axis of the ellipse by the length of the long axis of the ellipse. The position of the shell in the normal position (backward) corresponds with a turn index of 0.5. Shell turning will change the shape of the animal: as the long axis becomes shorter the index will increase from 0.5 toward 1.0 (Fig. 8C). The length/width ratio of an animal is such that by retraction without shell turning (e.g., the typical position during resting phase) the shell index varies between 0.5 and 0.6 (see also Fig. 8). The correlation between this turn index and shell angles measured manually (Hermann et al. 1994) was 0.82 in a test series where both variables determined. Thus this automated method gives us an accurate estimate of the shell angle during different phases of egg laying.

Injection experiments

Thirty minutes (t = –30) before the actual start of the experiment the animals were placed in the experimental tank. After an acclimatization period of 30 min (t = 0), the animal’s behavior was recorded on video tape. A time code generator (TC 30, Alpermann and Velte) was used to provide every video frame with a time code to be able to synchronize the behavior stored on the two video tapes. Fifteen minutes after the actual start of the experiment (t = 15) the animal was taken out of the tank and 50 μl of a particular CDC-peptide was injected into the foot of the animal. After the injection, the animal was replaced gently in the experimental tank. Locomotion of the animals returned within 30–60 s, indicating that the injection did not induce long-term withdrawal (i.e., stress) responses. The animal’s behavior was recorded until 15 min after the injection (t = 30).

We have tested the ovulation hormone CDCH (Applied Biosystems) (Ebberink et al. 1985) and the CDC-peptides Calfluxin (Dicos and Ebberink 1988), alpha-CDCP (Applied Biosystems) (Li et al. 1994) and beta3-CDCP (Applied Biosystems) (Li et al. 1994). All injected peptides were dissolved in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-buffered saline (see below for composition; pH = 7.8) except the ovulation hormone. For this hormone, stock solutions (10−3 M) were prepared at pH = 11, then diluted (1:100 and 1:1,000) in HEPES-buffered saline (pH = 7.8). Animals that laid eggs during the experiment or during the 2 h after an injection were not used for the analysis except in the case of injections with the ovulation hormone. Effective final concentrations of the injected peptides were estimated to be 10−7 and 10−6 M for the ovulation hormone, and 10−6 or 10−5 M for the other peptides (injection volume of 50 μl and a total blood volume of 2.0 ml). Saline injections were used as controls. For every animal, the mean of the three behavioral variables (speed of locomotion, rasping frequency, and the shell movements) during the 15 min period before and after an injection was determined. The change in the value of each variable with regard to the preinjection period was then calculated. The averages (±SE) of these changes of each variable were calculated.

Recording of electrical activity

Isolated CNS were pinned down in a recording chamber lined with a silicon elastomer (Xantopren-Blue, Bayer). The outer layer of connective tissue of the CNS was removed carefully to expose the CDCs and the right pedal N (RPeN) motor neurons. No proteases were used in the preparation of the CNS. The preparations were maintained in HEPES-buffered saline (HBS) of the following composition (in mM) 30.0 NaCl, 1.7 KCl, 1.5 MgCl2, 4.0 CaCl2, 5.0 NaHCO3, 10.0 NaCH3SO4, and 10.0 HEPES; adjusted to pH 7.8 with NaOH. Except for NaCH3SO4 (Merck), the chemicals used in the saline were obtained from J. T. Baker (J. T. Baker Chemical; Deventer, The Netherlands). The neurons were impaled with glass microelectrodes (GC150F-10, Clark Electromedical; impedance 50–60 MΩ) filled with 0.5 M KCl. RPeN neurons with an axon in the right inferior cervical nerve were motor neurons of the dorsal longitudinal muscle (Ferguson and Benjamin 1991; Hermann et al. 1994). Therefore, to identify RPeN motor neurons, we recorded the electrical activity in the right inferior cervical nerve with a suction electrode. During the experiments, the recording chamber (2 ml) was supplied continuously with fresh saline using a peristaltic pump (speed 0.6 ml/min; Gilson). The state of electrical excitability of the CDCs (excitable/inhibited) was determined in each experiment, with a brief suprathereshold electrical stimulation (20–40 1-nA pulses of 100 ms at 3 Hz) that induce depolarizing afterpotentials (DAPs). DAPs only occur when the CDCs are in an excitable state (Brussaard et al. 1988; Kits 1980). Unless otherwise described, electrical activity was recorded starting 15 min after the neurons were impaled. After this period (t = 0), the electrical activity of the neurons and nerves was recorded for ±120 min and stored on FM tape (TEAC XR-310; bandwidth dc –1.250 Hz). Only one RPeN neuron per animal was studied.

Local application of peptides to the somata of RPeN motor neurons was done with a microelectrode using a picospritzer (General Valve). In all experiments, the peptides were delivered from
a micropipette located over the cell body of the impaled neuron by a series of short (50 ms) pressure pulses at a rate of 2 Hz. The pipette concentration of the peptides was 2 × 10^{-6} M for the ovulation hormone and 2 × 10^{-7} M for alpha-CDCP, beta3-CDCP, and Calfluixin. Local application of saline was used as a control. To visualize the application of peptides on the somata of neurons, all the solutions were colored with a red dye (Amaranth; Merck).

Peptides were applied 30 min after the start of the experiment (τ = 30) for a period of 30–60 s.

To determine the electrical activity of the RPeN neurons during the turning phase of egg laying, animals were given a clean water stimulus (ter Maat et al. 1983). After 45–50 min, the animals were killed. At that point, some animals were still in the resting phase, whereas in other animals, egg mass formation already had started and these animals had entered the turning phase. The presence of eggs in the oothecal gland indicated that these animals were dissected when they were in the turning phase of egg laying (n = 6). The animals with no eggs in the oothecal gland were used as controls (n = 7). The procedure of CNS isolation and impaling of a neuron lasted 25 min.

**Isolation and recording of individual neurons**

RPeN motor neurons were isolated under aseptic conditions according to a procedure modified from Ridgway et al. (1991). In short: the CNS were incubated for 20–21 min in defined medium (DM) with 0.67 mg/ml trypsin (Sigma, type III) and 0.67 mg/ml collagenase / dispase (Boehringer Mannheim) at 20–22°C. The DM used was serum free 50% Leibowitz L-15 medium (GIBCO, special order) with added inorganic salts [concentration (in mM): 40.0 NaCl, 1.7 KCl, 4.1 CaCl₂, and 10.0 HEPES; pH 7.9] and 20 µg/ml of gentamicin. After enzyme treatment, the brains were rinsed for 15 min in a DM containing 0.67 mg/ml solution of soybean trypsin inhibitor (Sigma, type I-S). Isolated cells were plated on 35 mm poly-styrene tissue culture dishes (Falcon 3001) filled with 2 ml DM. Intracellular recordings of the isolated RPeN neurons were made 24–30 h after plating. The cells were impaled with glass microelectrodes (TW150 F-6, WPI; resistance 50–60 MΩ) filled with 0.5 M K-acetate and 0.01 M KCl. The recording chamber was perfused continuously with HBS (exchange rate 0.5–1 times/min). Beta 3-CDCP (10^{-6} M pipette concentration) was applied locally by means of pressure ejection.

**Electrical nerve stimulation**

For electrical whole nerve stimulation, the cut end of the nerve was sucked into a glass micropipette that was just large enough for the nerve to fit. Voltage current pulses (period 2 ms, 0.2-ms rise time, 0.6-ms total duration) were fed into a stimulus isolation unit (Neurolog systems, NL 800). Amplitude of the current stimulus was set at 5 µA.

**Immunocytochemistry**

Double labeling with two fluorescent dyes was used to investigate the branching pattern of anti-CDCH positive fibers close to the somata and axons of RPeN motor neurons. The motor neurons were stained with a 5% aqueous solution of Lucifer yellow. The dye was injected into the cells using a pressure injector.

Polyclonal antibody against CDCH-1 raised in rabbits against the synthetic peptide LERQKENL-RKFEL-NH₂ (amino acids 21–36 of CDCH-1) was used. The specificity of the antibody has been verified (Van Minnen et al. 1989a,b). The pedal ganglia with the Lucifer yellow-stained motor neurons were separated from the other ganglia and fixed overnight at 4°C in Bouin’s solution. The ganglia were washed seven times for 1 h in tris(hydroxy-methyl)aminomethane (Tris)-buffered saline (0.1 M Tris; 0.15 M NaCl, pH 7.4) and 2% triton X-100, followed by incubation for 16–18 h at 4°C in primary antiserum in TBS-triton-gelatin (TBS-tg: TBS and 0.5% triton x-100 and 0.25% gelatin). Seven, 60-min washes in TBS-tg preceded a 18-h incubation at 4°C in Texas red-conjugated swine anti-rabbit peroxidase (1:50 in TBS-tg; Dako, Denmark). Finally the preparations were washed five times for 1 h in TBS, dehydrated in graded alcohols, and cleared in methylsilicate. The pedal ganglia were mounted between two coverslips and photographed using a confocal laser-scan microscope (Zeiss LSM, Axiovert 135 M, Argon-ion laser: 488 and 514 nm).

**Statistical techniques**

Throughout this paper group means always are presented with the standard error of mean. Before all tests, data were tested for normality (Kolmogorov-Smirnov) and homogeneity of variances (Fmax). If necessary, data were logarithmic transformed. Transformed data distributions were tested and did not differ significantly from normal. A factorial analysis of variance (ANOVA) followed by comparisons of means was used to evaluate the changes in behavior after injection of a peptide against the controls. Because we were testing eight groups (4 peptides, 2 concentrations) against the control group, the minimum criterion of statistical significance was P < 0.005 (i.e., joint confidence probability). Repeated measures ANOVA was used to evaluate effects of the different peptides on the spiking frequency of the RPeN motor neurons. The minimum criterion of statistical significance was P < 0.05 (see also Sokal and Rohlf 1981; Winer et al. 1991).

**RESULTS**

**Electrical activity of RPeN shell motor neurons during egg laying**

We recorded the firing patterns of RPeN neurons during turning behavior. Because the somata of the RPeN neurons are small (20–30 µm) and located on the ventral side of the pedal ganglion, the cell bodies are not accessible for fine wire electrodes used to record from freely behaving animals. We therefore investigated the electrical activity of the RPeN neurons in vitro. Animals were given a clean water stimulus (CWS) to induce egg laying (ter Maat et al. 1983). Animals that responded to the CWS with resting phase behavior were selected and killed after 45–50 min. At that point, some animals were still in the resting phase, whereas in other animals, egg mass formation already had started: these animals had entered the turning phase. The increased frequency and duration of shell movements during the turning phase do not occur in most experiments during the first 5–10 min of this phase (ter Maat et al. 1989) (see also Fig. 9). We therefore used the presence of eggs in the oothecal gland when these animals were dissected as an indication that they were in the beginning of the turning phase of egg laying. After dissection, which took 20–25 min (see METHODS), the spontaneous electrical activity of neurons in the RPeN cluster was recorded for a period of 15 min. Thus the spiking activity was recorded starting approximately halfway during the turning phase.

Of the 13 animals used, 7 were still in the resting phase, whereas the remaining 6 animals were in the turning phase. The differences in spontaneous firing were dramatic (Fig. 1). Figure 1A (bottom) shows an example of the electrical activity of a RPeN neuron during the turning phase of egg laying. The firing rate of the neurons of animals that were
motor neurons during the resting phase is significantly lower than in nonegg-laying animals. Furthermore after egg mass formation has started, the RPeN motor neurons show electrical activity at a rate ~10-fold higher than before egg mass formation had started. Thus it appears that the RPeN motor neurons are inhibited during the resting phase and subsequently are excited during the turning phase of egg-laying behavior.

The next question that needed to be addressed was: what causes these changes in activity of the RPeN motor neurons? Because the rate of spontaneous spiking activity of the motor neurons in the RPeN cluster was found to be correlated with the state of electrical excitability of the CDCs (Hermann et al. 1994; Kits 1980) (see also Figs. 2 and 5), the state of the CDCs (excitable or inhibited) was determined before each experiment below (see METHODS).

**Ovulation hormone, CDCH inhibits RPeN motor neurons**

Local application of the ovulation hormone, CDCH, on the somata of the RPeN neurons in a pipette concentration of $2 \times 10^{-6}$ M induced a decrease in the firing frequency regardless of the state of excitability of the CDCs. Figure 2A shows an example of the response of a RPeN motor neuron after ovulation hormone application in a preparation in which the CDCs were in an excitatory state. Almost immediately after application there was a decrease in the firing frequency of this neuron (Fig. 2A and B; $P < 0.05, n = 6$). This effect could not be reversed by continuous washing with saline. Application of this hormone to a RPeN neuron from a CNS in which the CDCs were inhibitory electrically yielded similar responses (Fig. 2B, $P < 0.05, n = 6$). Con-
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FIG. 3. Effect of application of beta3-CDCP on the electrical activity of the RPeN neurons (2 different preparations). A: example of a transient excitation of a RPeN neuron after local application of beta3-CDCP. There is an increase in the firing frequency of this neuron ~14 s after the application. Top: intracellular recording of an RPeN neuron. Middle: bar graph of the firing rate of this neuron. Bottom: moment of application. In all experiments, the peptides were delivered from a micropipette, located over the cell body of the neuron recorded from, by a series of short (50 ms) pressure pulses a rate of 2 Hz. B: example of a delayed long-lasting excitation of a pedal N motor neuron after local application of beta3-CDCP. There is an increase in the firing frequency of this neuron, starting several minutes after the application. This excitation is continued for ~20 min. The pressure pulses are indicated in the middle trace. Width of the bins is the same as in A. C: faster runout of the spiking data shown in B immediately before application of beta3-CDCP (left, *) and at peak response (right, **).

control experiments where RPeN neurons were recorded for a similar length of time before and after application of saline did not show a change in firing rate. We conclude that the spiking activity of the RPeN neurons is inhibited by the ovulation hormone.

Beta3-CDCP excites RPeN shell motor neurons

Local application of beta3-CDCP in a pipette concentration of \(2 \times 10^{-5}\) M to the RPeN cell bodies induced an excitatory response in all the neurons tested (\(n = 9\); Fig. 3). However, the latencies and the duration of the excitatory responses varied. In some preparations (\(n = 5\)), the application of beta3-CDCP was followed within seconds by an excitation that lasted 1–5 min (Fig. 3A). In the other four preparations, there was a delayed (3–5 min) but long-lasting (>20 min) increase in the firing frequency (Fig. 3, B and C). The two types of excitatory responses in the different preparations did not correlate with the state of excitability of the CDCs.

The long delay in the second type of response suggested an indirect action. Furthermore, because it is impossible to ascertain whether neuromodulatory agents act directly on a neuron within the complex environment of the CNS, we tested beta3-CDCP on acutely isolated RPeN neurons (see METHODS). Isolated RPeN neurons were silent (7 of 9) or were spiking at an irregular and low rate (2 of 9). Local application of Beta3-CDCP evoked spiking activity in two of the seven silent neurons (Fig. 4A) and increased the spiking activity of both spontaneously active cells (Fig. 4B). Five of the silent neurons did not respond (Fig. 4C). The changes in activity started 10–30 s after application and lasted several minutes, outlasting the presence of the peptide. Application of saline never resulted in changes in spiking activity.
activity (not shown). Thus it appears that beta 3-CDCP has a direct excitatory effect on a subset of RPeN motor neurons.

**Calcitonin gene-related peptide (CGRP)**

Local application of Calfluxin on the somata of RPeN neurons (2 × 10⁻⁵ M pipette concentration) had neither transient nor long-lasting effects on the electrical activity of the RPeN neurons, regardless of the state of electrical excitability of the CDCs (Fig. 5; left: CDC excitable, n = 6; right: CDCs inhibited, n = 6).

Local application of alpha-CDCP (2 × 10⁻⁵ M pipette concentration) on the somata of RPeN neurons also had no effect on the electrical activity of these cells. Figure 5 shows the spiking frequency of the RPeN neurons of preparations in which the CDCs were in the excitable state (left) and in the inhibited state (right). There was no significant effect of the application of alpha-CDCP on the electrical activity of these neurons compared with the controls (CDCs inhibited, n = 6; CDCs excitable, n = 6).

**Projections of CDCH-1 positive fibers**

Because beta 3-CDCP appears to have a direct effect on RPeN motor neurons, we addressed the question of how this peptide would reach the somata and/or axons of these neurons. Therefore, a double labeling experiment was done. In single preparations, several (2–3 per animal) RPeN neurons were stained with Lucifer yellow by means of pressure ejection from a microelectrode. Subsequently, the pedal ganglia were labeled with polyclonal anti-CDCH-1 (n = 3 animals). In all the preparations, staining of CDCH positive fibers (Fig. 6B) could be observed within tens of micrometers of the axons and/or the somata of the RPeN neurons (Fig. 6A). This demonstrates that axonal projections of neurons that express the CDCH-1 gene are found close to the cell bodies and axonal projections of RPeN neurons.

**Electrical stimulation of the intestinal nerve causes excitation of RPeN motor neurons**

The intestinal nerve, which innervates the female reproductive tract, previously has been found to be necessary for the turning and oviposition phase during egg laying (Ferguson et al. 1993). Furthermore, neurons in the female reproductive tract do express the CDCH-1 gene (Van Minnen et al. 1989a,b). Our results showed that the firing rate of the RPeN motor neurons is ~10-fold higher during the turning phase than it is during the resting phase. Therefore, we investigated whether axonal projections in the intestinal nerve originating from the female tract can serve as part of a pathway that causes excitatory input onto the RPeN motor neurons.

Stimulation of the intestinal nerve induced an excitation of the RPeN motor neurons in all preparations (n = 5). The effects of stimulation on the electrical activity of the motor neurons lasted longer than the stimulation itself. Figure 7 shows an example of the effect of a single, short train nerve stimulation on the activity of an RPeN neuron. After the stimulation, the neuron was excited for ~40 s. Short stimulation of other visceral nerves (the anal nerve or the cutaneous pallial nerve; n = 4) resulted in a short increase in spiking activity during the stimulation itself, followed by a long inhibition. Thus this provides circumstantial evidence that the intestinal nerve can serve as a part of a pathway that excites the RPeN motor neurons.

**Beta3-CDCP and alpha-CDCP induce shell turning and buccal rasping in intact animals**

The results described above demonstrated that some, but not all, of the CDCH-1 peptides modulate part of the neuronal elements involved in egg-laying behavior. Thus the next question we addressed was: do these CDCH-1 peptides have the appropriate effect on the behavior of intact animals?

The known release sites of the CDCs in *Lymnaea* are characterized by the absence synaptic specialization, and
release from the CDCs to the brain is thought to occur in a nonsynaptic fashion. This is probably similar to the situation in Aplysia (Mayeri et al. 1985). In such cases, injection of the substance involved is a good way of exploring the biological activity of neuropeptides and neurotransmitters in intact animals. To optimize the putative action of the CDC peptides, each peptide was injected in two concentrations. These concentrations can be only an approximation of the normal concentrations found in the animal because the actual concentrations in the blood or local concentrations in the CNS during egg laying are unknown.

Figure 8 shows the results of normal egg-laying behavior induced by a CWS (see also ter Maat et al. 1983). The animal’s behavior was analyzed until 30 min after the end of oviposition. The start and end times of the three phases of egg-laying behavior, resting, turning, and oviposition, are indicated by the dashed lines and were determined as described earlier (Fig. 8A) (ter Maat et al. 1989). Figure 8B shows the average changes in shell turning, rasping frequency, and speed of locomotion during the three phases of egg laying (n = 4). Note that the turn index is averaged over the entire turning period (~60 min in this example), and the average increase in this variable (0.035, Fig. 8B) is therefore small compared with the absolute size of the turning variable (between 0.5 and 1, see Fig. 8C).

Figure 9 shows the changes in the turn index, the rasping frequency, and the speed of locomotion with regard to the preinjection period for each of the individual CDC-peptides. Injection of alpha-CDCP (10^{-5} M, n = 8 and 10^{-6} M, n = 5) or beta3-CDCP (10^{-6} M, n = 6 and 10^{-5} M, n = 5) into animals induced a significant increase in the shell movements and in the rasping frequency and also a significant decrease in the speed of locomotion compared with the control animals (P < 0.005). Within 1–2 min after an injection of alpha-CDCP or beta3-CDCP, the animals started turning their shells. One or two turns were made and each turn lasted 6–15 min. These shell turns were of longer duration than typically was seen during normal, spontaneous turning-phase behavior (compare Fig. 8A with Fig. 9). Consequently, the average increase in the shell turning variable was larger after injection than during spontaneous egg laying (0.08 for Beta3-CDCP in Fig. 9 and 0.035 in Fig. 8B, respectively).

The animals also showed an increase in the frequency of rasping movements within 1–2 min after the injection. The changes in shell movements and rasping frequency lasted 10–15 min, after which rasping and turning slowly returned to preinjection levels, and the animals behaved as the control animals (not shown). None of the injected animals laid eggs within 120 min after the injection. Injection of beta3-CDCP into animals that have the intestinal nerve lesioned also increased in a similar way shell turning and the frequency of rasping movements (changes in turn index: 0.087 ± 0.056; rasping frequency/10 min: 65.88 ± 14.47; n = 5).

**Ovulation hormone and Calfluin have no immediate effects on behavior**

Injection of the ovulation hormone, CDCH (10^{-6} M, n = 6 and 10^{-7} M, n = 5), had no significant effect on the turn index, the rasping frequency, or the speed of locomotion within 15 min after the injection (Fig. 9). The sizes of the changes that occurred were comparable with those of the control animals, which were injected with saline (n = 15). Note that the effects of injections are measured as differences with respect to the preinjection period (see METHODS). Also, injection of Calfluin (n = 8, 10^{-5} M; and n = 8, 10^{-6} M)
FIG. 8. Quantification of spontaneous egg-laying behavior. A: shell movements (top), the rasping frequency (middle), and the speed of locomotion (bottom) were analyzed from 30 min before the onset of egg-laying behavior until 30 min after end of oviposition. Shell movement just before the start of the turning phase is caused by the animal breathing at the water surface, not by a shell turn proper. B: changes in the 3 variables that characterize egg-laying behavior, measured during the resting phase, the turning phase, and the oviposition phase. For each variable, the change in the average value with respect to the 30 min period before egg laying was calculated (n = 4). C: analysis of shell turning. Shell position was determined every 10 s by calculating a “turn index.” Ellipse best fitting the outline of the animal was determined (see METHODS). Turn index was calculated by dividing the short axis of this ellipse by the long axis. An index of ~0.5 indicates that the shell is pointing backward, i.e., the long axis is twice the length of the short axis (left). Turning of the shell decreases the long axis, therefore, increases the turn index (right).

had no effect on these three variables compared with the saline injected control animals (Fig. 9). The two concentrations of the peptides used also did not significantly differ in their effects on the behavior. It should be noted that the assay used in this study was designed to measure only effects that occur immediately (that is: within minutes) after the injection. Therefore, the effects of injection of the ovulation hormone on the behavior that are seen after ~50 min (ter Maat et al. 1989) specifically are excluded from the current results. A lack of effect of the ovulation hormone was not due to failed injections because all the ovulation hormone-injected animals showed oviposition within 120 min after injection.

**DISCUSSION**

In this paper, we have investigated the role that peptides, encoded on the CDCH-1 gene, play in the organization of egg-laying behavior in the snail, *L. stagnalis*. The key observations in this study were the activity of RPeN motor neurons is inhibited during the resting phase and excited during the turning phase; application of the ovulation hormone inhibits the activity of RPeN motor neurons whereas application of beta3-CDCP induces an excitation of RPeN motor neurons; and injection of beta3-CDCP and alpha-CDCP induces shell turning and rasping movements in the intact animal.

RPeN motor neurons have been shown earlier to be necessary for the execution of shell movements during egg-laying behavior (Hermann et al. 1994). The data shown in the present paper are consistent with the observation that RPeN motor neurons are essential to some aspects of egg-laying behavior. The spontaneous firing rate in RPeN motor neurons is decreased when animals are in the resting phase. Subsequently, the firing rate increased ~10-fold in animals...
that are in the turning phase of egg-laying behavior. The firing rates measured in animals that were in the turning phase are also much higher than spontaneous firing rates of RPeN motor neurons in nonlaying animals (Hermann et al. 1994). During the resting phase, the shell is moved forward but there are no shell turnings. In contrast, during the turning phase, the animals make long-lasting turns of their shells through 60° relative to the longitudinal axis of the head/foot. These turns only occur during this phase of egg laying. Thus the resting phase would correspond with an inhibition, whereas the turning phase corresponds with an excitation of the RPeN motor neurons. Taken together, these findings allowed us to use the activity of the RPeN neurons as a simple in vitro model of the regulation of shell turning that occurs during egg-laying behavior. This model enabled us to investigate the role the CDCH-1 peptides play in the organization of egg laying.

Previous experiments have shown that CDC discharges causes long-lasting inhibition of RPeN motor neurons (Hermann et al. 1994). In this paper, we showed that the ovulation hormone caused an inhibition of the RPeN motor neurons. The effects of CDC discharges on RPeN motor neurons are very similar to the inhibition of RPeN motor neurons caused by the ovulation hormone. This suggests that release of the ovulation hormone during a CDC discharge causes the inhibitory effects on RPeN motor neurons. These inhibitory effects are associated with resting phase behavior during egg laying.

The effects of injection of the individual CDCH-1 peptides into intact animals in general correspond well with the effects of direct application onto the somata of RPeN motor neurons. Application of beta3-CDCP excites RPeN motor neurons and also caused shell turning on injection. The other peptide from the CDC-1 gene that resulted in shell turning was alpha-CDCP. However, alpha-CDCP did not result in changes in firing of RPeN neurons. Possibly, the behavioral effect of alpha-CDCP is due to an indirect action on the RPeN motor neurons or direct action on other, unidentified motor neurons involved in shell turning.

In addition, beta3-CDCP and alpha CDCP injection caused buccal rasping behavior. This behavior is, together with shell turning, normally associated with turning/oviposition phase behavior. This shows that these two peptides, in addition to a direct action on RPeN neurons, also excites either directly or indirectly the buccal motor neurons. This suggests that there may be a larger network of beta3-CDCP sensitive neurons that are involved in triggering all turning-phase behaviors. Thus of the tested CDC-1 peptides, beta3-CDCP (and alpha-CDCP) appear to induce turning-phase behavior and are able to evoke the appropriate response in RPeN motor neurons.

Our previous data (Hermann et al. 1994) indicated that during and after a CDC discharge no effects are induced in the RPeN motor neurons that can be attributed to either beta3-CDCP or alpha-CDCP release. It is conceivable that differential release of these peptides from the CDCs toward the end of a discharge contributes to the onset of turning and oviposition behavior in intact animals. We do not think this is the case because then it would be expected that the RPeN motor neurons will show an increased spiking activity at the end or after a discharge of the CDCs. However, such an increase was never observed (Hermann et al. 1994). Furthermore, recent studies indicated that the release of all CDC peptides in vivo takes place during the discharge itself, i.e., alpha-CDCP, beta3-CDCP, and the ovulation hormone are released simultaneously during the CDC discharge (Jiménez 1997). One possible scenario is that beta3-CDCP and alpha-CDCP are released at the start of the turning phase by neurons other than the CDCs.

Injection of the ovulation hormone alone into the intact animal has been shown to cause turning and oviposition behavior (ter Maat et al. 1989) although the resulting turning behavior does not start (on average) until 50 min after the injection. It is demonstrated that the total duration of turning and oviposition behaviors is highly correlated with the number of eggs in the egg mass (ter Maat et al. 1986). Our hypothesis is that turning and oviposition behaviors are an indirect effect of injection of the ovulation hormone and that the primary cause of turning and oviposition behaviors during egg laying are internal sensory signals originating from the eggs moving in the female tract. Several sensory structures in the female tract (De Jong-Brink and Goldschmeding 1983; Van Minnen et al. 1989a) are associated with neurons and varicosities that express the CDCH-1 gene but not the CDCH-2 gene (Van Minnen et al. 1989a,b). This finding raised the possibility that CDCH-1
gene-expressing neurons in the female tract are involved in the processing of sensory information coming from the female tract. Van Minnen et al. (1989a) showed that the CDCH-1 positive fibers in the right pedal ganglion are part of a network that extends beyond the CNS. The RPeN motor neurons also found to be associated closely with fibers that are positive only for CDCH-1 gene products (this paper; van Minnen et al. 1988). It is, therefore, not unlikely that these fibers in the pedal ganglion are the source of beta3-CDCP that excites these motor neurons during turning behavior in intact animals. The origin of these fibers is, however, still unclear. However, two lines of evidence suggest that these fibers do not originate from the CDCs themselves. First, the CDCs express both the CDCH-1 and -2 genes, whereas the fiber tracts in the pedal ganglia only stain with antibodies against CDCH-1 gene products. Second, repeated attempts (by means of Lucifer yellow staining of the CDCs) to show that the CDCs project to the pedal ganglia failed (ter Maat, unpublished data). These CDCH-1 positive fibers, however, can be traced to the intestinal nerve tract in whole-mount preparations (Van Minnen et al. 1988; ter Maat and Jansen, unpublished results). The intestinal nerve innervates the female tract and ovotestis and was found to be necessary for the turning and oviposition phase (Ferguson et al. 1993). In that respect, it is suggestive to notice that electrical stimulation of the intestinal nerve resulted in excitation of the RPeN motor neurons. Thus this suggests that the turning/oviposition phase depends on internal sensory input from the female tract and ovotestis via the intestinal nerve. A role for internal sensory feedback in the organization of egg laying is also described for the marine mollusc Aplysia (Ferguson et al. 1986, 1989; ter Maat and Ferguson 1996).

Taken together, the data presented here together with previous data indicate that individual CDCH-1 peptides act on different targets and several CDCH peptides play a role in the regulation of the behavioral patterns during egg laying in Lymnaea. Furthermore, some CDCH peptides have a differential effect depending on the timing and source of release.

We hypothesize that the sequence of egg-laying events in Lymnaea would be as follows: 1) A CDC discharge triggers the start of egg laying, i.e., the resting phase. During the discharge, the CDCs release all peptides encoded on the CDCH-genes. Release of the ovulation hormone causes an inhibition of RPeN motor neurons. Of the other CDCH peptides, alpha-CDCP and the ovulation hormone act as autoexcitatory transmitters and Calfluxin induces an influx of Ca$^{2+}$ into the mitochondria of the female accessory albumen gland. In addition, the ovulation hormone causes ovulation and packaging of ripe eggs in the female tract. 2) Packaging of ripe eggs is sensed by ciliated cells in the tract, and a nervous signal is sent to the CNS via the intestinal nerve. This signal triggers the release of beta3-CDCP and alpha-CDCP and causes/contribute to turning-phase behavior (i.e., shell turning and rasping movements). Beta3-CDCP induces an excitation of the activity of RPeN motor neurons. In addition, alpha-CDCP and beta3-CDCP cause excitation of other motor neurons involved in shell movements and buccal rasping. These CDCH-1 peptides are released by cells other than the CDCs. This process continues as long as eggs are transported, thereby causing the turning/oviposition behavior to be correlated with the size of the egg mass.

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Present address of P. M. Hermann: Dept. of Medical Physiology, Faculty of Medicine, University of Calgary, Health Sciences Centre, 3330 Hospital Dr. NW, Calgary, Alberta T2N 4N1, Canada.

Address for reprint requests: R. F. Jansen, Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

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