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AN ENDOGENOUS PEPTIDE MODULATES THE ACTIVITY OF A SENSORY NEURONE IN THE LEECH *HIRUDO MEDICINALIS*

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Summary

Sensory and neurosecretory innervation of each leech excretory complex, a nephridium and its bladder, is accomplished by a single neurone, the nephridial nerve cell (NNC). The NNC monitors the extracellular Cl^- concentration, which ranges between 20 and 100 mmol l^{-1} depending on the physiological state. The NNC contains FMRFamide in its soma and sensory terminals in the nephridium. Bath or focal application of FMRFamide leads to hyperpolarization and decreases the rate of firing of the NNC, suggesting autoregulation of peptide release. Experiments under single-electrode current-clamp and voltage-clamp show that FMRFamide turns off the receptor-specific Cl^- current of the NNC, indicating that FMRFamide also modulates the receptor gain.

Key words: *Hirudo medicinalis*, leech, FMRFamide, receptor gain control, autoregulation, peptide modulation.

Introduction

The presence of peptides and small-molecule transmitters in the sensory terminals of receptor neurones opens the possibility of modulation of their sensitivity through autoregulation. For example, the sensory cells in the lobster oval organ, a stretch receptor organ of the second maxilla, are sensitive to their endogenous peptide proctolin, which affects the receptor potential and the spike generation (Pasztor and Bush, 1989).

Sensory and neurosecretory innervation of the leech excretory system (34 nephridia with their associated bladders) is accomplished by 34 peripheral neurones, the nephridial nerve cells (NNCs), which monitor the extracellular Cl^- concentration (Wenning, 1989) and contain the tetrapeptide FMRFamide (Wenning *et al.* 1993*b*). The NNC soma lies on the urinary bladder and sends a thick axon into the segmental ganglion. The dendritic projections in the nephridium are both sensory and neurosecretory and extend between the basal infoldings of the urine-secreting cells (Wenning and Cahill, 1986).

FMRFamide-related peptides, first identified by Price and Greenberg (1977) as cardioactive peptides in molluscs, are widespread throughout the animal kingdom and modulate the activity of neurones and muscles. In the leech, FMRFamide-like immunoreactivity has been found in a number of motor neurones, where it serves as a cotransmitter (Kuhlmann *et al.* 1985*a*,*b*; Norris and Calabrese, 1987; Evans and Calabrese, 1989). Five neuropeptides have been isolated and identified in the segmental ganglia (Evans *et al.* 1991). FMRFamide modulates currents in leech neurones and muscles. In the heart interneurones, for example, FMRFamide alters the steady-state activation and inactivation characteristics of an outward K^+ current (Simon *et al.* 1992) and modulates fast synaptic transmission between the same neurones (Simon *et al.* 1994). FMRFamide is the cotransmitter of the heart motor and accessory neurones (Kuhlmann *et al.* 1985*a*) with inotropic effects on the hearts (Thompson and Calabrese, 1992). It elicits barrages of action potentials in the neurosecretory Retzius cells (Sahley *et al.* 1993). Recently, a new FMRFamide-related peptide was isolated from ganglia of the reproductive segments in a pharyngobdellid leech, where it affects water balance (Salzet *et al.* 1994).

The NNC is the first sensory neurone in the leech that has been shown to contain FMRFamide (Wenning *et al.* 1993*b*). As shown here, FMRFamide modulates the excitability of the NNC, implying autoregulation of the receptor gain and autoregulation of peptide release.

Part of the results reported here have already appeared in abstract form (Wenning and Calabrese, 1993, 1994).

Materials and methods

Leeches (*Hirudo medicinalis* L.) were obtained from commercial suppliers and kept in artificial pond water at 15 °C. Experiments were carried out at room temperature (20–22 °C).

The preparation is described in detail in Wenning and Calabrese (1991). Briefly, a single nephridium and the dorsal part of its urinary bladder were dissected out and transferred to a Sylgard-lined dish. For extracellular recordings, the bladder

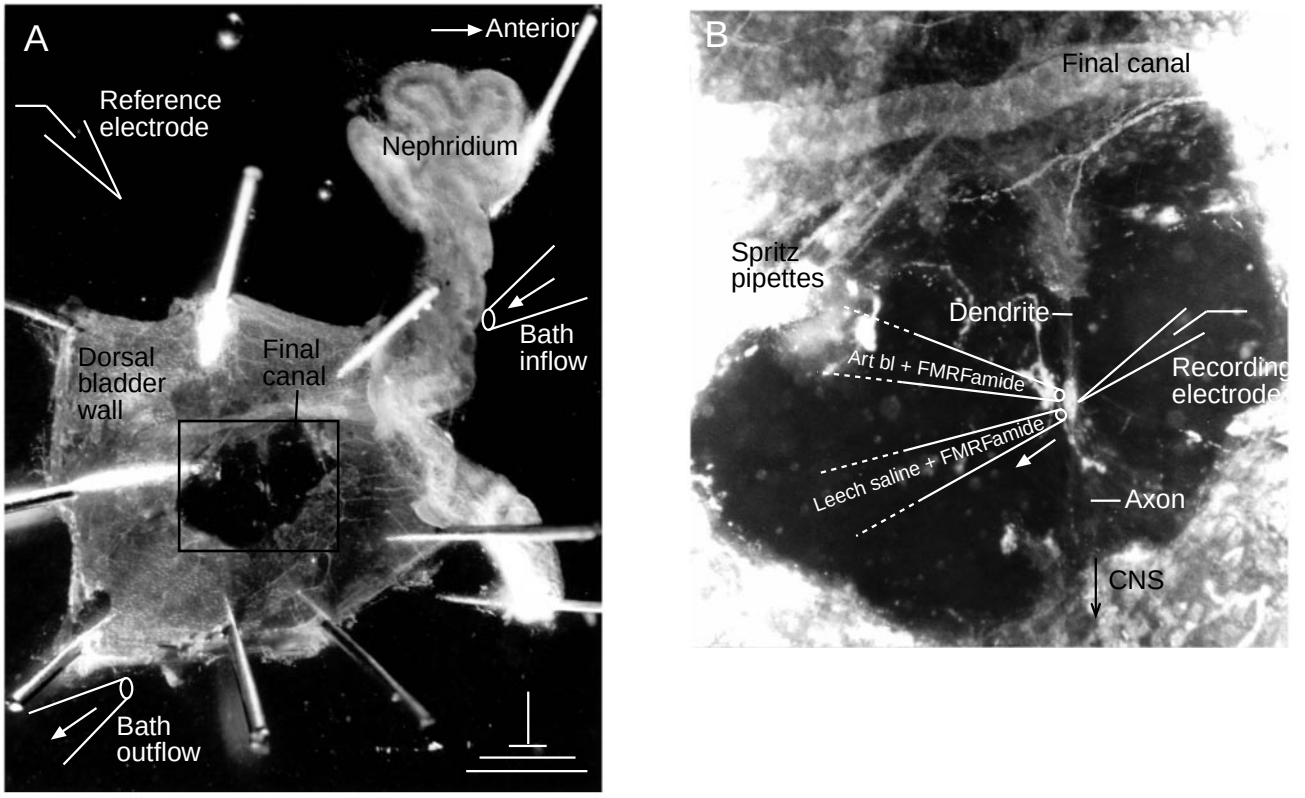


Fig. 1. Tissue preparation from *Hirudo medicinalis.* A nephridium and the dorsal part of the bladder were dissected out. For intracellular recordings, the bladder wall was pinned inside out and the soma of the nephridial nerve cell exposed by a small incision near the final canal. Recordings were made differentially with a second microelectrode as reference electrode and a separate system ground. The preparation was constantly superfused (A). FMRFamide was focally applied. The effect of the peptide at different Cl^- concentrations (for example, artificial blood, art bl, and leech saline) was tested in the same preparation using two pipettes placed close to the nerve cell soma (B). For extracellular recordings, the same preparation was used with the bladder pinned outside up. A suction electrode was then placed on the nerve cell soma.

wall was pinned flat with the NNC soma on the upper surface. For intracellular recordings, the bladder wall was pinned inside out and the NNC was exposed by a small cut through the bladder wall (Fig. 1). Unless otherwise noted, dissections were carried out in leech saline (for the composition of solutions see Table 1). Electrophysiological techniques used were conventional. A detailed description of the extracellular recordings is given by Wenning (1989), and that for intracellular recordings under discontinuous current-clamp and single-electrode voltage-clamp by Wenning and Calabrese (1991).

Extracellular recordings from the NNC and bath application of FMRFamide

A glass suction electrode was placed onto the soma of the NNC. After establishing a control rate of activity from the

Table 1. *Salines used for extra- and intracellular recordings*

	Salt concentrations (mmol l^{-1})			
	NaCl	KCl	$CaCl_2$	Other substances
Artificial blood[a] (40 mmol l^{-1} $[Cl^-]_o$)	20	4	1.8	40 mmol l^{-1} disodium malate, 10 mmol l^{-1} disodium succinate; pH adjusted with NaOH or malic acid
Na^+-free artificial blood (40 mmol l^{-1} $[Cl^-]_o$)	–	4	1.8	120 mmol l^{-1} *N*-methyl-D-glucamine, 20 mmol l^{-1} HCl, pH adjusted with HCl
Leech saline[b] (130 mmol l^{-1} $[Cl^-]_o$)	115	4	1.8	10 mmol l^{-1} glucose

All solutions were buffered with 10 mmol l^{-1} Tris–HCl and adjusted to pH 7.4.
[a]Hoeger *et al.* (1989); [b]Muller *et al.* (1981).

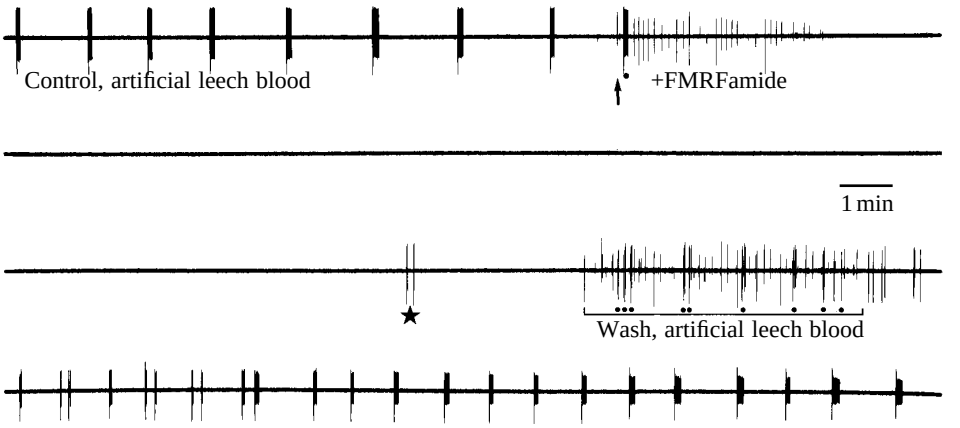
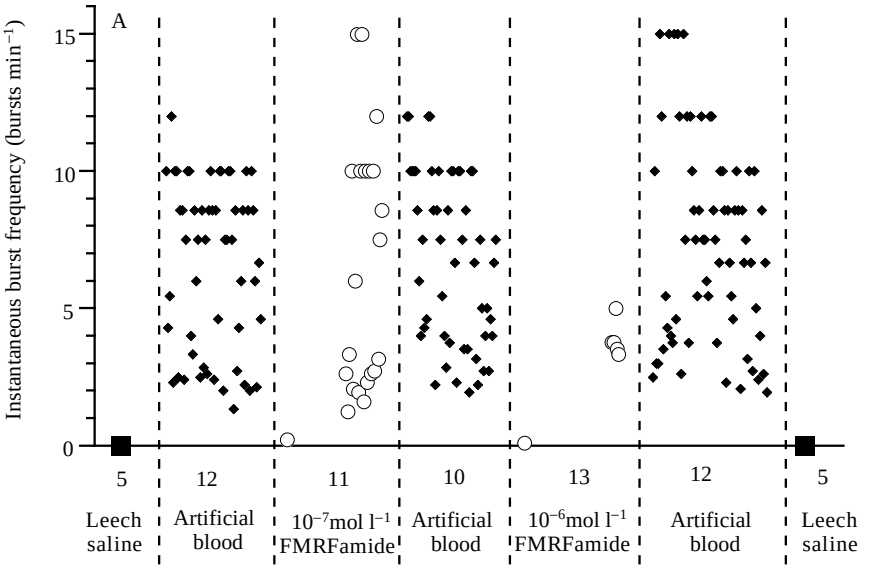


Fig. 2. Continuous extracellular recording from the nephridial nerve cell (NNC) bathed in artificial blood (40 mmol l^{-1} $[Cl^-]_o$). The NNC stopped spiking upon bath application of FMRFamide (arrow; 10^{-6} mol l^{-1}) (a dot marks the last burst). It fired two bursts after about 17 min (★) and resumed firing upon washout. When played back at high speed (not shown), spikes (dots) can be distinguished from wash artefacts by their shape.



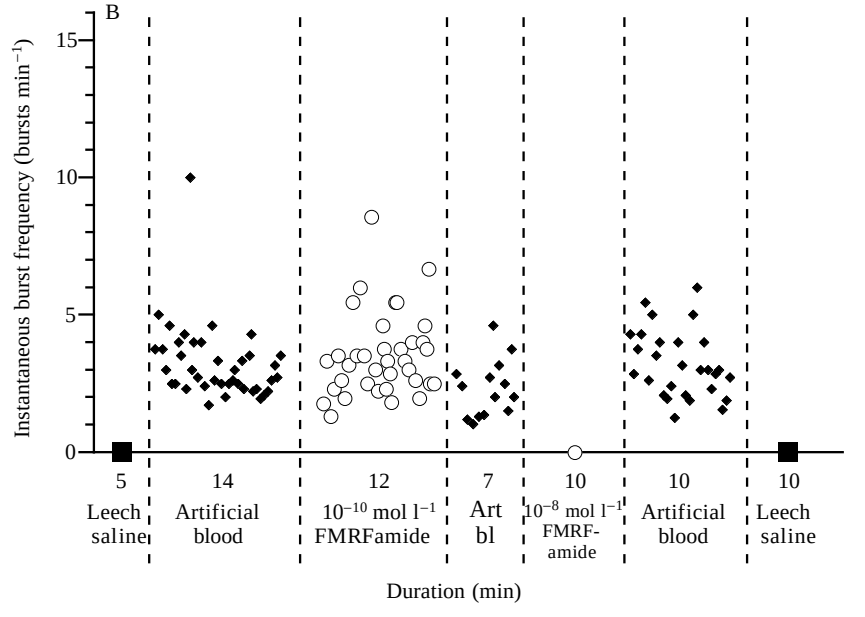


Fig. 3. The sensitivity of the NNC to FMRFamide varied, as seen in the two complete experiments shown here. The instantaneous burst frequency is given for each burst. Recording began in leech saline (130 mmol l^{-1} $[Cl^-]_o$) (no bursts for 5 min) which was then replaced with artificial blood (control). In the experiment shown in A, the NNC was insensitive to 10^{-7} mol l^{-1} FMRFamide, but stopped spiking for 11 min in 10^{-6} mol l^{-1} FMRFamide. It then resumed firing. In the example shown in B, 10^{-10} mol l^{-1} FMRFamide had no effect on the activity of the NNC, whereas spiking was blocked for 10 min in the presence of 10^{-8} mol l^{-1} FMRFamide. When artificial blood was replaced with leech saline at the end of each experiment, the NNC stopped spiking.

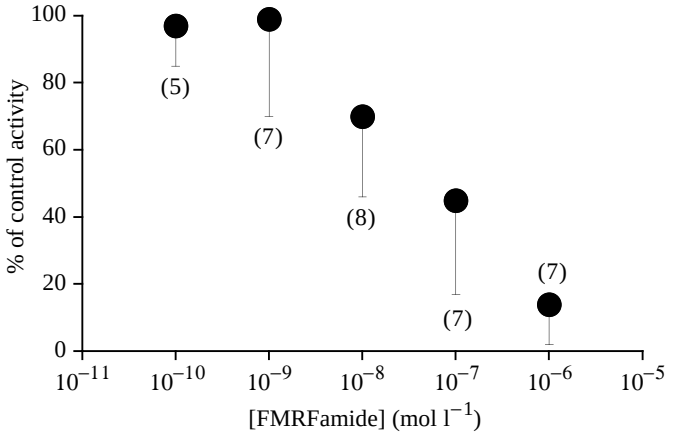


Fig. 4. The inhibitory effect of FMRFamide on the NNC was dose-dependent, as seen from extracellular recordings. Bath concentrations of FMRFamide above 10^{-8} mol l^{-1} caused inhibition of spiking when compared with the burst frequency of the NNC during the previous control period of 10 min (taken as 100 %). Data are expressed as mean burst frequency + S.E.M. The number of experiments is indicated in parentheses for each data point.

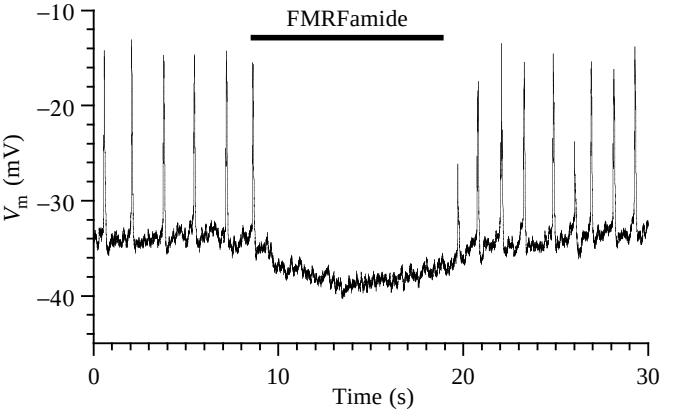


Fig. 5. Intracellular recording of membrane potential V_m from one NNC in leech saline. During focal application of FMRFamide (10^{-5} mol l^{-1}), the NNC hyperpolarized and stopped firing. We chose a cell that was firing as a result of electrode damage to illustrate the inhibitory effect that was observed even in high-Cl^- leech saline.

NNC for 10–20 min in artificial blood (Table 1), FMRFamide (Bachem, Heidelberg, Germany) at various concentrations was added to the bathing medium. After 10–15 min, the peptide was removed by exchange of the bathing medium, and a new control rate was measured before a second FMRFamide concentration was tested. Depending on the sensitivity of the preparation, one or more concentrations of FMRFamide were tested, starting with the lowest concentration. At the end of each experiment, the response of the NNC to changes of extracellular Cl^- concentrations was tested by exchanging artificial blood for leech saline.

Intracellular recordings and focal application of FMRFamide

Intracellular recordings began in leech saline. The preparation was constantly superfused (Fig. 1), which allowed complete changes of the bathing medium within 1–2 min. Glass microelectrodes were filled with a mixture of 4 mol l^{-1} potassium acetate and 20 mmol l^{-1} KCl and had resistances of 20–30 MΩ. To lower their capacitance, they were dipped into dimethyl-polysiloxane (Sigma). To compensate for junction potential changes due to changes of the Cl^- concentration in the bathing medium, the two functions of the indifferent electrode (current return and stable reference potential) were assigned to separate electrodes (Fig. 1A; Wenning and Calabrese, 1991).

For measurements under voltage-clamp, the discontinuous single-electrode voltage-clamp mode of the Axoclamp-2A (Axon Instruments Inc., Burlingame, USA) was used with a gain setting of 0.8–2.5 nA mV^{-1}. FMRFamide (10^{-5} mol l^{-1}) was focally delivered onto the NNC soma *via* glass pipettes, using a pneumatic picopump (WPI, USA). To check for successful peptide application, Fast Green (Sigma) was added to the solutions in the pipette. The Cl^- concentration in the pipettes matched the Cl^- concentration in the bathing medium. When testing for a response to FMRFamide at different Cl^- concentrations in the same preparation, two pipettes with appropriate Cl^- concentrations were used (Fig. 1B). To minimize any effects due to leakage of peptide, the pipette was withdrawn when not in use.

Control experiments showed that Fast Green had no effects on the NNCs.

Data acquisiton, storage and analysis

Data from extracellular recordings were stored on an FM tape recorder (Racal Recorders Ltd, England) and transferred to a chart recorder for the preparation of figures.

Data from intracellular recordings were stored on a VHS video cassette recorder modified for FM recordings (Vetter, model 240). For voltage protocols and data acquisition, pClamp software (Clampex and Axotape respectively; Axon Instruments Inc.) was used in combination with a personal computer. Data were then transferred to an Apple Macintosh computer. Kaleidagraph (Abelbeck Software) was used for analysis and display purposes.

Data are expressed as mean ± S.E.M. Student's *t*-test was used to assess significant differences.

Results

Response of the NNC to FMRFamide: extracellular recordings

The NNC was spontaneously active when recorded extracellularly in artificial blood (Cl^- concentration, $[Cl^-]_o$, 40 mmol l^{-1}; Table 1) (Wenning, 1989). Bath application of FMRFamide decreased spontaneous activity of the NNC within 1–2 min (Fig. 2). The effect was fully reversible, long-lasting (Fig. 3) and dose-dependent (Fig. 4). The sensitivity to FMRFamide varied. In some preparations, bursting of the

NNC was initially inhibited completely at lower peptide concentrations, but started again after a few minutes. In the example shown (Fig. 3A), the NNC stopped spiking for 5 min upon application of 10^{-7} mol l^{-1} FMRFamide and for 11 min after application of 10^{-6} mol l^{-1} FMRFamide. Other preparations were more sensitive and the inhibitory effect persisted until washout (Fig. 3B, 10^{-8} mol l^{-1}). To establish the dose–response relationship, the activity of the NNC in the first 5 min after application of FMRFamide was compared with the activity during the previous 10 min control period (Fig. 4). As the NNC is the only neurone present in the preparation, the effects of FMRFamide are mediated by autoreceptors.

Response of the NNC to FMRFamide: characterization of the FMRFamide-specific current

The NNC hyperpolarizes upon increase of the extracellular Cl^- concentration (Wenning and Calabrese, 1991). At any given Cl^- concentration, FMRFamide also hyperpolarizes the NNC. Focal application of FMRFamide (10^{-5} mol l^{-1}) onto the NNC soma or the dendrites caused hyperpolarization for as long as the peptide was applied (Fig. 5).

To determine whether the FMRFamide-induced hyperpolarization was due to the turning off of an inward current or to the activation of an outward current, we performed voltage-clamp experiments. In the example shown (Fig. 6), the NNC was held at −40 mV in 85 mmol l^{-1} $[Cl^-]_o$.

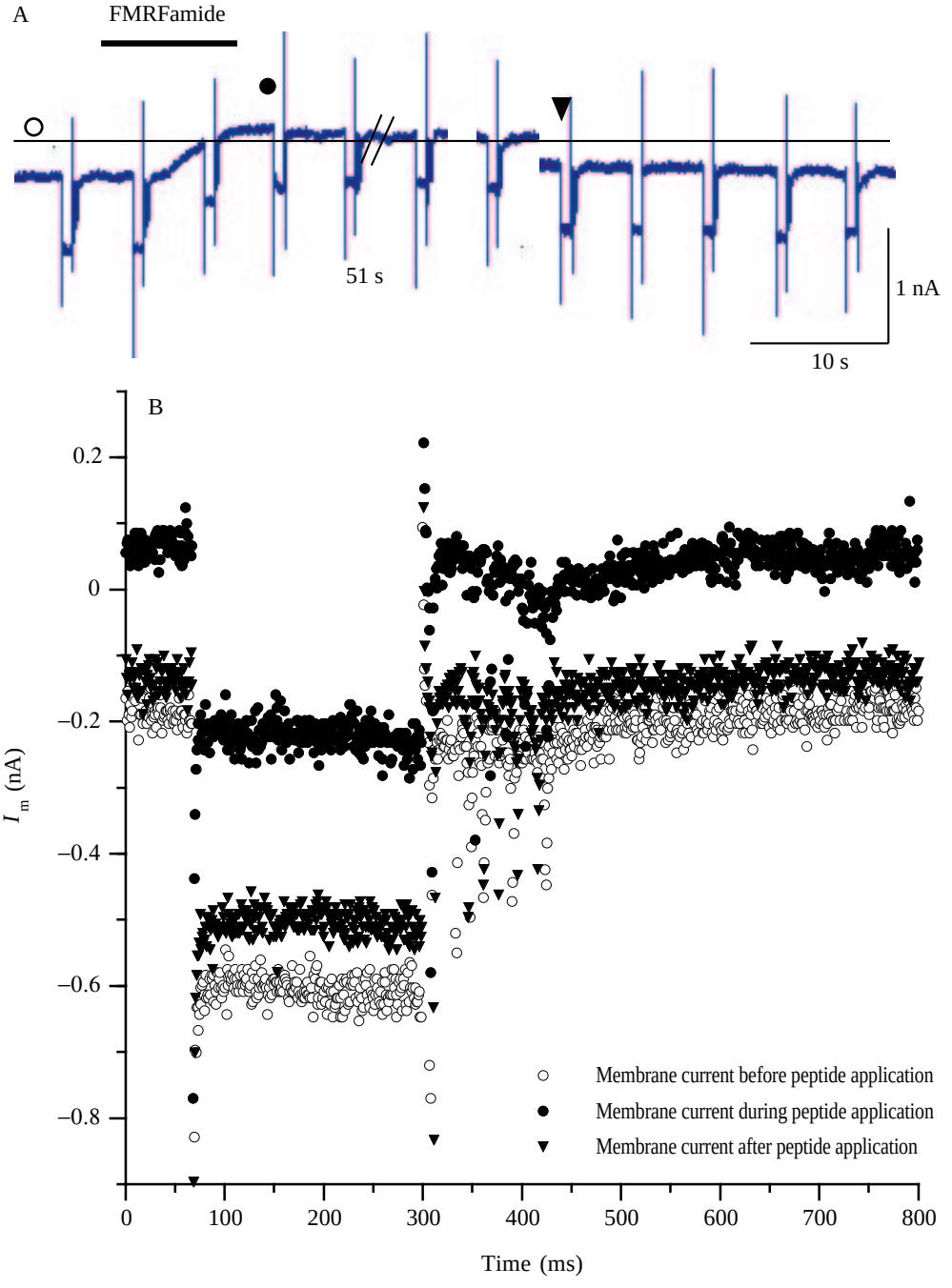


Fig. 6. Voltage-clamp experiment to reveal conductance changes upon focal application of FMRFamide (10^{-5} mol l^{-1}). (A) The NNC was held at −40 mV in 85 mmol l^{-1} $[Cl^-]_o$. Voltage steps (−10 mV) were applied at 0.25 Hz. The horizontal line represents 0 nA holding current. Note that the holding and step currents did not fully return to the control value within 60 s. (B) Individual voltage steps as indicated by symbols in A [before (○), during (●) and after (▼) FMRFamide application] expanded, synchronized (t=50 ms) and superimposed to show the peptide-induced changes of the holding current and the membrane conductance. I_m, membrane current.

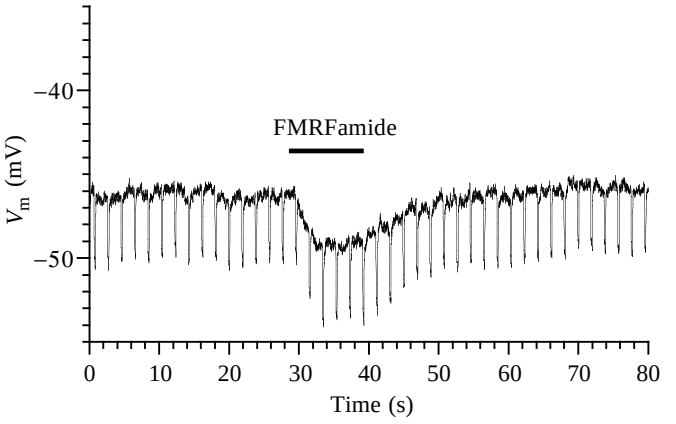


Fig. 7. Intracellular recording from the NNC in Na^+-free artificial blood in discontinuous current-clamp (current pulses −0.1 nA, 200 ms). During focal application of FMRFamide (10^{-5} mol l^{-1}), the NNC hyperpolarized and its input resistance increased.

Voltage steps (−10 mV, 250 ms, 0.25 Hz) were applied to monitor the resting conductance (Fig. 6A). The conductance decrease and change in the membrane current became obvious when voltage steps before, during and after peptide application were compared (Fig. 6B). Note that the holding current and the step current did not return fully to their original values in the record shown.

These voltage-clamp experiments show that FMRFamide turns off a persistent inward current and thereby hyperpolarizes the cell. High extracellular Cl^- concentrations also turn off a persistent inward current in the NNC, which is carried by Cl^- moving out of the cell (Wenning and Calabrese, 1991). Hence, the inward current turned off by FMRFamide could be either the receptor-specific Cl^- current or a current carried by cations, which flow inwards. The response to FMRFamide was therefore tested in Na^+-free artificial blood (Table 1). In addition, the reversal potential of the peptide-induced current was determined in different Cl^- concentrations.

If FMRFamide turns off a Na^+ current, the response should disappear in Na^+-free artificial blood. If the current turned off by FMRFamide is a nonspecific cation current, with K^+ and Na^+ as the charge carriers, the NNC should depolarize in Na^+-free solutions upon application of FMRFamide. Turning off a pure K^+ current would depolarize the cell and thereby shift the membrane potential (V_m) closer to the equilibrium potential for Cl^- (E_{Cl}; see Fig. 11). In the example shown, the NNC was impaled in leech saline which was then exchanged for Na^+-free artificial blood. As shown here under current-clamp, the NNC hyperpolarized and its resistance increased upon focal application of FMRFamide (Fig. 7). The result shows that FMRFamide does not turn off a Na^+ current or a nonspecific cation current.

If FMRFamide turns off the receptor-specific Cl^- current in the NNC, the reversal potential of the peptide-induced current should shift to more negative values with increasing external Cl^- concentration. We determined the reversal potential in four solutions with different Cl^- concentrations: artificial blood (40 mmol l^{-1} $[Cl^-]_o$), leech saline (130 mmol l^{-1} $[Cl^-]_o$) and two mixtures of artificial blood and leech saline in concentrations similar to those determined in leech blood during postprandial diuresis (85 and 100 mmol l^{-1} $[Cl^-]_o$) (Zerbst-Boroffka *et al.* 1982). Since the step current reaches steady state quickly and does not show any transients (Fig. 6B), we used voltage ramps rather than series of voltage steps to obtain the current–voltage relationship. That voltage ramps are faster and less damaging to the cells is especially important, because we attempted to determine the reversal potential at two different Cl^- concentrations in a single preparation.

In the example shown (Fig. 8), the NNC was held at −40 mV in 100 mmol l^{-1} $[Cl^-]_o$. Ramps were applied 15 s before and during focal application of FMRFamide (Fig. 8A). The response to FMRFamide was not voltage-dependent in the range −30 to −90 mV (Fig. 8B). The long downward slope of the ramp was used for calculation of the current–voltage relationship of the peptide-induced current. Because the membrane current after washout did not always return to the value seen before peptide application (Fig. 6), the current before peptide application was used as a control and was subtracted from the current during peptide application. The reversal potential was then determined by linear regression (Fig. 9). The reversal potential of the current turned off by FMRFamide indeed shifted significantly and more than predicted by the Nernst equation. This is evident when comparing the values obtained at 40 and 100 mmol l^{-1} $[Cl^-]_o$ ($P<0.001$) and 85 and 100 mmol l^{-1} $[Cl^-]_o$ ($P<0.001$) (Fig. 10). There was no shift in the reversal potential between 40 and 85 mmol l^{-1} $[Cl^-]_o$, suggesting that intracellular $[Cl^-]$ might have been changing (see Discussion). If FMRFamide and high extracellular Cl^- concentration act similarly on the same current, then most of the Cl^--gated Cl^- channels would already be turned off in high-Cl^- leech saline and the effect of FMRFamide would therefore be small. This possibility is reflected in the high variability of the peptide response observed in leech saline (Fig. 10).

Discussion

Mechanism of FMRFamide action on the NNC

The high resting conductance for Cl^- and its non-passive distribution are pre-requisites for the NNC's sensitivity to changes in the extracellular Cl^- concentration (Wenning and Calabrese, 1991). Several lines of evidence suggest that both FMRFamide (the NNC's endogenous peptide) and an increase of the extracellular Cl^- concentration turn off the same Cl^- current (Fig. 11). First, FMRFamide and high extracellular Cl^- concentrations turn off an inward current and thereby hyperpolarize the NNC (Fig. 6; Wenning and Calabrese, 1991). Second, the inhibitory response of the NNC to changes of extracellular Cl^- concentrations and FMRFamide persists in Na^+-free artificial blood (Fig. 7; Wenning and Calabrese, 1991). Third, the reversal potential of the current turned off by FMRFamide and by changes of Cl^- concentration shifts to more negative values with increasing extracellular Cl^-

concentrations (Fig. 10; Wenning and Calabrese, 1991). The shift, however, deviates from the slope predicted by the Nernst equation (dotted line in Fig. 10). In particular, the reversal potential does not change between $[Cl^-]_o$ values of 40 and 85 mmol l^{-1}, suggesting that the intracellular Cl^- concentration of the NNC increases upon the change to 85 mmol l^{-1} $[Cl^-]_o$. In the NNC, Cl^- is not distributed passively, and active transport mechanisms are assumed to regulate internal Cl^- concentration (Wenning, 1989). If these transport mechanisms work near saturation, internal Cl^- concentration would increase with increased Cl^- conductance, which might explain the precipitous shift in reversal potential of the FMRFamide response observed between 85 and 100 mmol l^{-1} $[Cl^-]_o$. Changes of intracellular Cl^- concentration associated with changes of extracellular Cl^- concentration and/or Cl^- conductance have recently been demonstrated in Retzius cells (Munsch *et al.* 1995).

Hyperpolarization of the NNC due to focally applied FMRFamide (10^{-5} mol l^{-1}) occurred over the physiological range of Cl^- concentrations (40–100 mmol l^{-1}) and even in

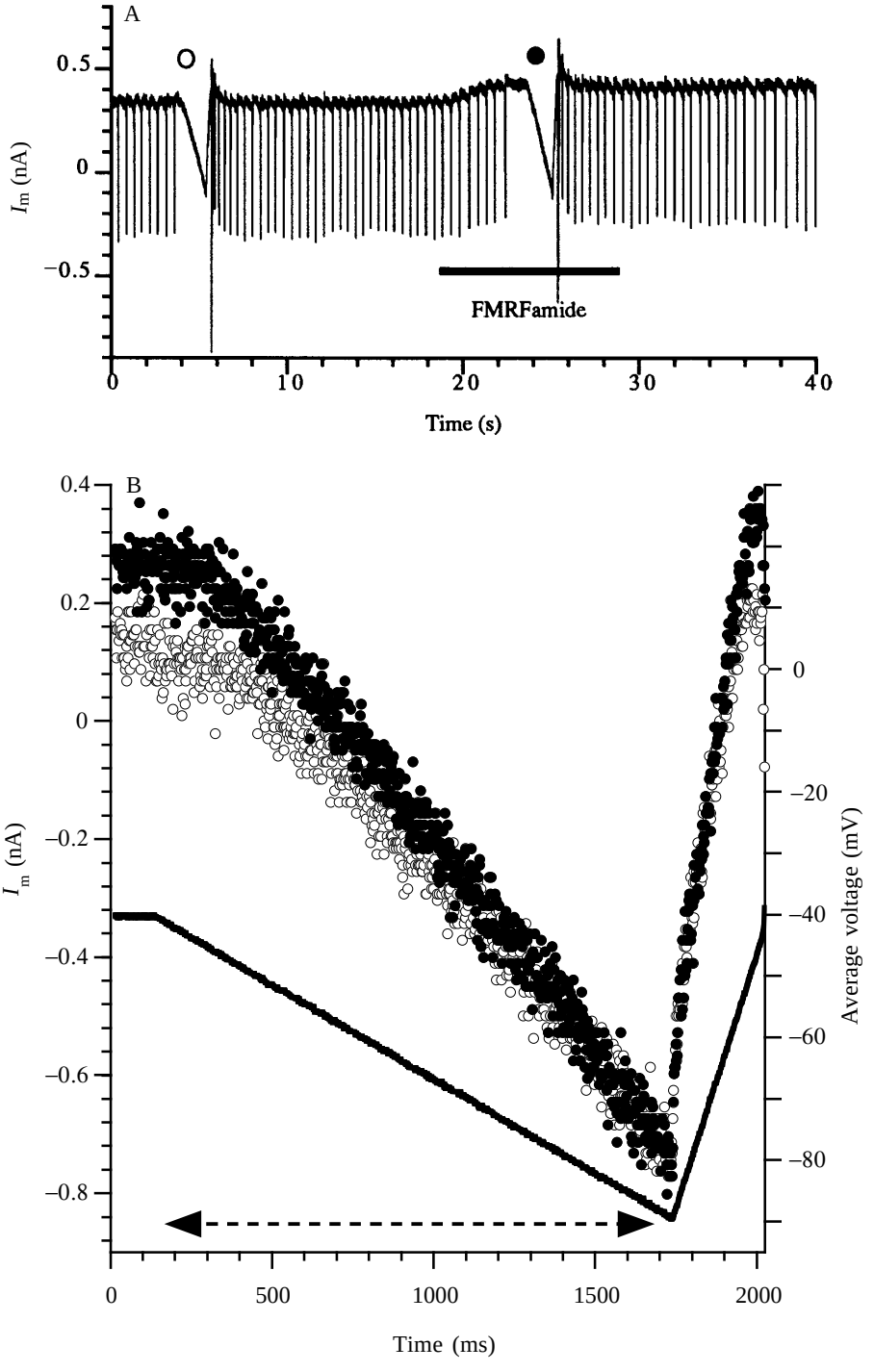


Fig. 8. Voltage-clamp experiment to determine the reversal potential of the current turned off by FMRFamide. (A) The NNC was held at −40 mV in 100 mmol l^{-1} $[Cl^-]_o$. The regular rapid downward deflections are unclamped action potentials. Voltage ramps were performed before (○) and during (●) focal application of FMRFamide (10^{-5} mol l^{-1}) (command voltage shown in B). (B) The downward slope of the voltage ramp (dashed line between arrowheads) was used for calculation of the reversal potential of the peptide-specific current. The current before peptide application (○) was used as the control current and subtracted from the current during peptide application (●) to calculate the peptide-specific current.

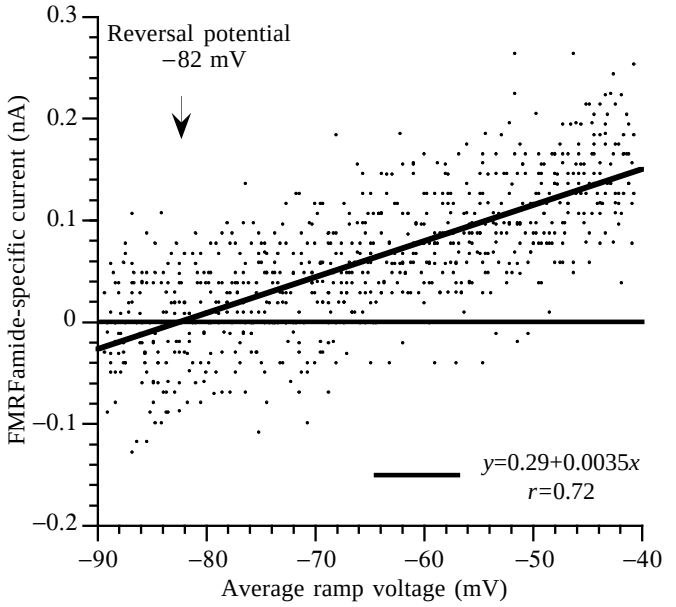


Fig. 9. Calculation of the reversal potential from the experiment shown in Fig. 8. The current–voltage relationship of the peptide-specific current is shown as the difference between the current before and that during peptide application. The reversal potential was obtained by linear regression (see equation).

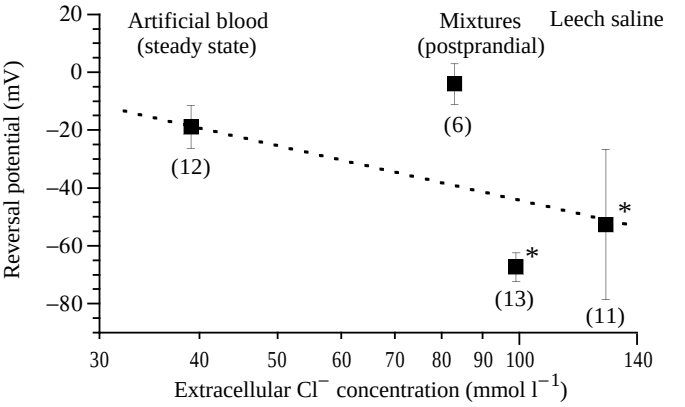


Fig. 10. Changes in the reversal potential of the current turned off by FMRFamide. Cl^- concentrations in leech blood are normally 40 mmol l^{-1} (steady state) and increase to 95–100 mmol l^{-1} after a meal (postprandial). Values around 130 mmol l^{-1} are observed when leeches invade brackish water (leech saline). Data are expressed as mean ± S.E.M. and the number of experiments is indicated in parentheses. Statistical significance ($P \leqslant 0.001$, asterisks) compared with steady state was assessed using an unpaired *t*-test. The dotted line represents the slope predicted by the Nernst equation.

leech saline (130 mmol l^{-1}) (Figs 2, 5, 6, 8). Extracellular recordings from the NNC show that FMRFamide causes long-lasting and complete inhibition of spiking at lower peptide concentrations (e.g. Fig. 3B, 10^{-8} mol l^{-1} FMRFamide). In contrast to focally applied peptide, bath-applied FMRFamide reaches not only the soma but also the dendrites in the nephridium and therefore might exert stronger effects. Further, the NNC, albeit 30–40 μm in diameter, is fairly thin and easily damaged during impalement, which may counteract the effect of FMRFamide.

The response of the NNC to its endogenous peptide is probably mediated by autoreceptors. The isolated preparation we used consists of the nephridium with the sensory and neurosecretory arborizations of the NNC, and the urinary bladder with the NNC soma and part of its axon. The NNC has been shown to be the only neurone projecting into the nephridium (Wenning *et al.* 1993*a*,*b*).

Autoregulation of excitability by endogenous transmitters has been shown for another set of neurosecretory neurones in the leech, the Retzius cells. Serotonin (5-HT), the endogenous transmitter, increases their Cl^- conductance. Since Cl^- is not distributed passively in the Retzius cells and E_{Cl} is kept more negative than V_m, opening of Cl^- channels causes hyperpolarization and reduction of excitability (Munsch and Schlue, 1993). Interestingly, a Cl^- current in the Retzius cells of the reproductive segments is turned on by acetylcholine (Szcupak *et al.* 1993). Apparently, the Cl^- conductance of the Retzius cells of the reproductive segments and that of the other body segments are modulated by different transmitters.

Source of FMRFamide in the nephridium

The NNC is the only FMRFamide-containing neurone projecting into the nephridium, and the neurosecretory endings of the NNC at the urine-forming cells contain FMRFamide (Wenning *et al.* 1993*a*,*b*). In addition, systemically released FMRFamide could reach the nephridial projections of the NNC through the dense capillary network and also act on the NNC and its peripheral arborizations. However, leeches lack neurohaemal release sites and their inner organs are directly innervated. The only transmitter that has been conclusively shown to enter the bloodstream is 5-HT. It is released by the giant Retzius cells in central ganglia and its level fluctuates with the status of feeding and motor activity (Lent *et al.* 1989; O'Gara *et al.* 1991). FMRFamide is the cotransmitter of the heart motor and accessory neurones (Kuhlmann *et al.* 1985*a*,*b*). These innervate the lateral hearts and their afferent vessels, which are in close proximity to the nephridium and the bladder. However, FMRFamide is released at the neuromuscular junction of the vessel envelope and is unlikely to enter the bloodstream. Furthermore, the observed initial phasic response of the NNC to changes of extracellular Cl^- concentration (see below) suggests that endogenous FMRFamide modulates the receptor gain.

Functional significance of receptor gain control through modulation by endogenous peptides and small-molecule transmitters

Modulation of proprioceptive feedback is one way of biasing motor behaviour and might be mediated by endogenous peptides, as shown for the stretch-sensitive neurones of the lobster oval organ (Pasztor and Bush, 1989; Pasztor and Golas, 1993). Presynaptic modulation of the release of endogenous transmitters at central synapses of sensory neurones also

contributes to receptor output control (e.g. Trimmer and Weeks, 1989; Le Corronc and Hue, 1993). Endogenous acetylcholine blocks transmitter release at the central synapse between sensory hairs of the cerci and giant interneurone 2 in the cockroach (Le Corronc and Hue, 1993) and at the central synapse between the sensory neurones of the planta hairs of larval *Manduca sexta* and the central motor neurones (Trimmer and Weeks, 1989). In both cases, autoregulation of transmitter release is assumed, since membrane potential, input resistance and the sensitivity of postsynaptic acetylcholine receptors did not change in the respective postsynaptic neurone.

In the NNC, the response to its endogenous peptide is clearly mediated by autoreceptors (see above). Interestingly, FMRFamide and the receptor's adequate stimulus, the extracellular Cl^- concentration, act on the same current. An important difference, however, is that E_{Cl} shifts with the increase of extracellular Cl^- but not in the presence of FMRFamide (Fig. 11). Consequently, when FMRFamide is no longer present, the NNC returns to its former membrane potential and thereby to the original receptor gain. Autoregulation of the sensitivity of the NNC to Cl^- might explain the initial phasic response of the NNC to the change from high to low extracellular Cl^- levels: the frequency of bursts is higher in the first minutes after the change than after 30 min (Wenning, 1989). Presumably, more FMRFamide is released initially, closing some of the Cl^- channels which had just opened in response to low $[Cl^-]_o$ (Fig. 11). The decrease in Cl^- conductance reduces the sensitivity to Cl^-. Conversely, when extracellular Cl^- concentration increases and spontanous activity decreases, FMRFamide is no longer released, thereby turning up the receptor gain.

The Cl^- concentration in the extracellular fluid of jawed leeches in the steady state is 40 mmol l^{-1} (Zerbst-Boroffka, 1970). In metabolic compromise, it ranges from 20 mmol l^{-1} in hypoxia to 100 mmol l^{-1} after a blood meal (Zerbst-Boroffka *et al.* 1982; Hildebrandt and Zerbst-Boroffka, 1992; Schmidt and Zerbst-Boroffka, 1993). Changes of blood Cl^- concentration persist for several hours in postprandial diuresis and during recovery from hypoxia. A tonic Cl^- receptor neurone, such as the NNC, is therefore advantageous for an animal with low blood Cl^- concentration and a Cl^--rich diet.

FMRFamide and nephridial performance

Previous anatomical data suggest that the FMRFamide released in the nephridium may affect urine formation. The neurosecretory terminals of the NNC extend between the basal infoldings of the urine-secreting cells (Wenning and Cahill, 1986). Preliminary results indicate that the NNC is involved in the control of salt output rather than volume output. The NNC densely innervates that part of the nephridium in which the final urine is formed by ion reabsorption (A. Wenning and I. Zerbst-Boroffka, unpublished observations). In freshwater species such as leeches, final urine concentration is kept low to prevent salt loss in the steady state, and ion reabsorption is high. In postprandial diuresis, in contrast, salt reabsorption decreases to facilitate salt excretion. The NNC fires bursts of action potentials in the steady state, and the released FMRFamide may speed up ion recycling. In postprandial diuresis, extracellular Cl^- concentration is high and both the inter- and intraburst frequencies of the NNC *in vivo* decrease substantially (Wenning, 1989). This decrease is likely to slow down peptide release. Indeed, NaCl reabsorption decreases from 85 % in the steady state to less than 10 % in postprandial diuresis (Zerbst-Boroffka *et al.* 1982). Further, when leeches

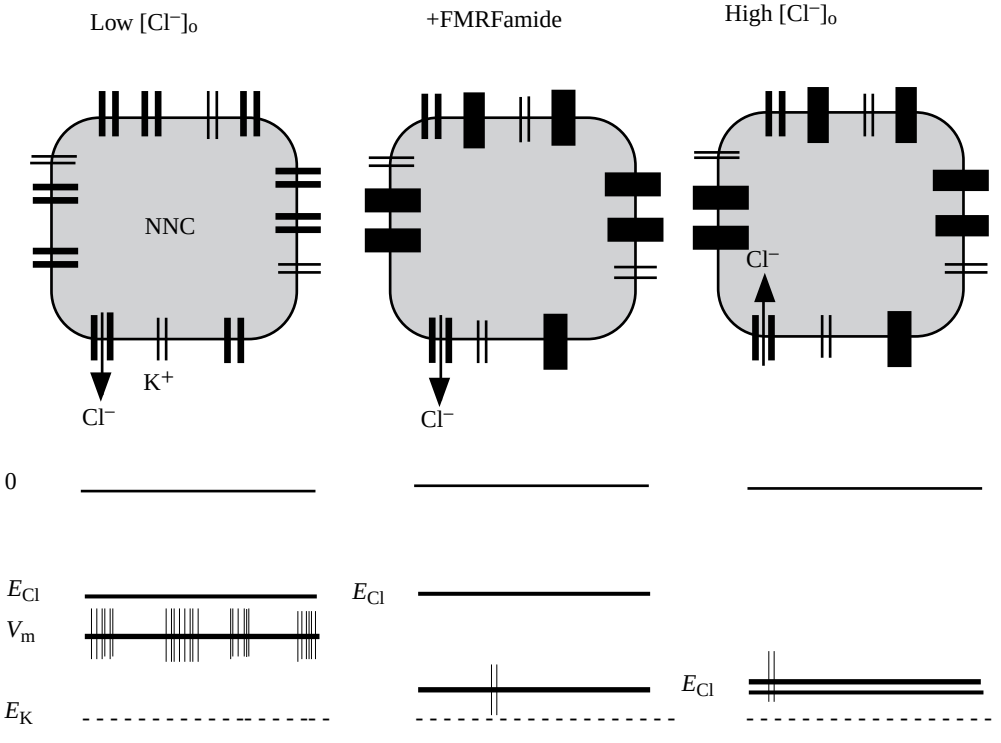


Fig. 11. A schematic model of the Cl^- sensitivity of the NNC and the effect of FMRFamide. At normal, low extracellular Cl^- concentration (low $[Cl^-]_o$), the NNC is spontaneously active and the membrane potential V_m is governed by the Cl^- conductance g_{Cl} (indicated here by many open Cl^- channels), which leads to a constant outflow of Cl^- (V_m moves towards the Cl^- equilibrium potential E_{Cl}). The endogenous peptide FMRFamide (+FMRFamide) turns off g_{Cl} (many closed Cl^- channels). The NNC hyperpolarizes because V_m is now governed by g_K (relatively more open K^+ channels, V_m moves toward E_K). $[Cl^-]_o$ triples after a blood meal. High $[Cl^-]_o$ reduces g_{Cl} of the NNC (many closed Cl^- channels) and, at the same time, E_{Cl} shifts to more negative values. Again, g_K governs V_m and the NNC hyperpolarizes.

invade brackish water, they lose water and gain salt. Under these conditions, the Cl^- concentration increases to 138 mmol l^{-1} while the frequency of bursts decreases in the NNC (Wenning, 1986).

In the sea slug *Aplysia californica*, the cholinergic motor neurone B15 additionally synthesizes small cardioactive peptides, which are more likely to be released during bursts of spikes than in response to single action potentials. Hence, B15 would function as a pure cholinergic motor neurone when firing slowly and as a cholinergic/peptidergic neurone when firing rapidly (Whim and Lloyd, 1989). In the NNC, in addition to the dense-cored vesicles associated with FMRFamide, small clear vesicles are present in its terminals in the nephridium (Wenning and Cahill, 1986; Wenning *et al.* 1993*b*). This suggests the presence of a second transmitter. In postprandial diuresis, the NNC is still active, although the inter- and intraburst frequency, as well as the number of action potentials, decreases (see Fig. 5 of Wenning, 1989). Hence, a second, small-molecule transmitter still might be released under these conditions.

FMRFamide, the endogenous peptide of the NNC, not only regulates the receptor gain by modulating the excitability of the NNC but at the same time regulates its own release. Increasing the receptor gain is important in postprandial diuresis to monitor the slow return to the normal, low extracellular Cl^- concentration. Autoregulation of endogenous peptide release may be important when blood Cl^- concentration decreases – during hypoxia, for example – because it limits the putative effects of the NNC on ion transport in the nephridium.

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References

EVANS, B. D. AND CALABRESE, R. L. (1989). Small cardioactive peptide-like immunoreactivity and its colocalization with FMRFamide-like immunoreactivity in the central nervous system of the leech *Hirudo medicinalis*. *Cell Tissue Res.* **257**, 187–199.

EVANS, B. D., POHL, J., KARTSONIS, N. A. AND CALABRESE, R. L. (1991). Identification of RFamide neuropeptides in the medicinal leech. *Peptides* **12**, 897–908.

HILDEBRANDT, J.-P. AND ZERBST-BOROFFKA, I. (1992). Osmotic and ionic regulation during hypoxia in the medicinal leech, *Hirudo medicinalis*. *J. exp. Zool.* **263**, 374–381.

HOEGER, U., WENNING, A. AND GREISINGER, U. (1989). Ion homeostasis in the leech: contribution of organic anions. *J. exp. Biol.* **147**, 43–51.

KUHLMANN, J. R., LI, C. AND CALABRESE, R. L. (1985*a*). FMRF-amide-like substances in the leech. I. Immuncytochemical localization. *J. Neurosci.* **5**, 2301–2309.

KUHLMANN, J. R., LI, C. AND CALABRESE, R. L. (1985*b*). FMRF-amide-like substances in the leech. II. Bioactivity on the heartbeat system. *J. Neurosci.* **5**, 2310–2317.

LE CORRONC, H. AND HUE, B. (1993). Electrophysiological evidence for the modulation of acetylcholine release by endogenous acetylcholine in the cockroach central nervous system. *J. exp. Biol.* **175**, 305–310.

LENT, C. M., DICKINSON, M. H. AND MARSHALL, C. G. (1989). Serotonin and leech feeding behavior: obligatory neuromodulation. *Am. Zool.* **29**, 1241–1254.

MULLER, K. J., NICHOLLS, J. G. AND STENT, G. S. (1981). (ed.) *Neurobiology of the Leech*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

MUNSCH, T., REUSCH, M. AND DEITMER, J. W. (1995). Intracellular chloride activity of leech neurons and glial cells in physiological, low chloride saline. *J. comp. Physiol.* A **176**, 273–280.

MUNSCH, T. AND SCHLUE, W. R. (1993). Intracellular chloride activity and the effect of 5-hydroxytryptamine on the chloride conductance of leech Retzius neurones. *Eur. J. Neurosci.* **5**, 1551–1557.

NORRIS, B. J. AND CALABRESE, R. L. (1987). Identification of motor neurones that contain a FMRFamide-like peptide and the effects of FMRFamide on longitudinal muscle in the medicinal leech *Hirudo medicinalis*. *J. comp. Neurol.* **266**, 95–111.

O'GARA, B. A., CHAE, H., LATHAM, L. B. AND FRIESEN, W. O. (1991). Modification of leech behavior patterns by reserpine-induced amine depletion. *J. Neurosci.* **11**, 96–110.

PASZTOR, V. M. AND BUSH, B. M. (1989). Primary afferent responses of a crustacean mechanoreceptor are modulated by proctolin, octopamine and serotonin. *J. Neurobiol.* **20**, 234–254.

PASZTOR, V. M. AND GOLAS, L. B. (1993). The modulatory effects of serotonin, neuropeptide F1 and proctolin on the receptor muscles of the lobster abdominal stretch receptor and their exoskeletal muscle homologues. *J. exp. Biol.* **174**, 363–374.

PRICE, D. A. AND GREENBERG, M. J. (1977). Structure of a molluscan cardioexcitatory peptide. *Science* **197**, 670–671.

SAHLEY, C. A., STRONG, J. A. AND KLEINHAUS, A. L. (1993). FMRFamide modulates the electrical activity of the leech Retzius cell. *Neurosci. Lett.* **164**, 37–40.

SALZET, M., BULET, P., WATTEZ, C. AND MALECHA, J. (1994). FMRFamide-related peptides in the sex segmental ganglia of the pharyngobdellid leech *Erpobdella octoculata*. Identification and involvement in the control of hydric balance. *Eur. J. Biochem.* **221**, 269–275.

SCHMIDT, H. AND ZERBST-BOROFFKA, I. (1993). Recovery after anaerobic metabolism in the leech (*Hirudo medicinalis* L.). *J. comp. Physiol.* **163**, 574–580.

SIMON, T. W., OPDYKE, C. A. AND CALABRESE, R. L. (1992). Modulatory effects of FMRF-NH_2 on outward currents and oscillatory activity in heart interneurones of the medicinal leech. *J. Neurosci.* **12**, 525–537.

SIMON, T. W., SCHMIDT, J. AND CALABRESE, R. L. (1994). Modulation of high-threshold transmission between heart interneurones of the medicinal leech by FMRF-NH_2. *J. Neurophysiol.* **71**, 454–466

SZCUPAK, L., JORDAN, S. AND KRISTAN, W. B., JR (1993). Segment-specific modulation of the electrophysiological activity of leech Retzius neurones by acetylcholine. *J. exp. Biol.* **183**, 115–135.

THOMPSON, K. J. AND CALABRESE, R. L. (1992). FMRFamide effects on membrane properties of heart cells isolated from the leech, *Hirudo medicinalis*. *J. Neurophysiol.* **67**, 280–291.

TRIMMER, B. A. AND WEEKS, J. C. (1989). Effects of nicotinic and muscarinic agents on an identified motoneurone and its direct afferent inputs in larval *Manduca sexta*. *J. exp. Biol.* **144**, 303–337.

WENNING, A. (1986). Nervous control of salt and water balance in the leech, *Hirudo medicinalis* L. *Zool. Beitr. N.F.* **30**, 379–392.

WENNING, A. (1989). Properties of a set of internal receptors in the medicinal leech: the nephridial nerve cells monitor extracellular chloride concentration. *J. exp. Biol*. **143**, 115–132.

WENNING, A. AND CAHILL, M. A. (1986). Nephridial innervation in the leech, *Hirudo medicinalis* L. *Cell Tissue Res.* **245**, 397–404.

WENNING, A., CAHILL, M. A., GREISINGER, U. AND KALTENHÄUSER, U. (1993*a*). Organogenesis in the leech: Development of nephridia, bladders and their innervation. *Roux's Arch. devl Biol.* **202**, 329–340.

WENNING, A., CAHILL, M. A., HOEGER, U. AND CALABRESE, R. L. (1993*b*). Sensory and neurosecretory innervation of leech nephridia is accomplished by a single neurone containing FMRFamide. *J. exp. Biol.* **182**, 81–96.

WENNING, A. AND CALABRESE, R. L. (1991). Mechanism of Cl^- sensitivity in internal ion receptors of the leech: an inward current gated off by Cl^- in the nephridial nerve cells. *J. comp. Physiol.* A **168**, 53–61.

WENNING, A. AND CALABRESE, R. L. (1993). A sensory neuron is sensitive to its endogenous peptide. *Soc. Neurosci. Abstr*. **19**, 929.

WENNING, A. AND CALABRESE, R. L. (1994). Regulation of peptide release: FMRFamide and Cl^- inhibit receptor-specific Cl^- currents in the nephridial nerve cells of the leech, *Hirudo medicinalis* L. *Verh. dt. zool. Ges.* **87/1**, 151.

WHIM, M. D. AND LLOYD, P. E. (1989). Frequency-dependent release of peptide cotransmitter from identified cholinergic motor neurons in *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9034–9038.

ZERBST-BOROFFKA, I. (1970). Organische Säurereste als wichtigste Anionen im Blut von *Hirudo medicinalis* L. *Z. vergl. Physiol.* **70**, 313–321.

ZERBST-BOROFFKA, I., WENNING, A. AND BAZIN, B. (1982). Primary urine formation during diuresis in the leech, *Hirudo medicinalis* L. *J. comp. Physiol.* **146**, 75–79.