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Neuromodulation of the locust frontal ganglion during the moult: a novel role for insect ecdysis peptides

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Summary

In insects, continuous growth requires the periodic replacement of the exoskeleton during the moult. A moulting insect displays a stereotypical set of behaviours that culminate in the shedding of the old cuticle at ecdysis. Moulting is an intricate process requiring tightly regulated physiological changes and behaviours to allow integration of environmental cues and to ensure the proper timing and sequence of its components. This is under complex hormonal regulation, and is an important point of interaction between endocrine and neural control.

Here, we focus on the locust frontal ganglion (FG), an important player in moulting behaviour, as a previously unexplored target for ecdysis peptides. We show that application of 10^{-7} mol l^{-1} ecdysis-triggering hormone (ETH) or 10^{-7} mol l^{-1} and 10^{-6} mol l^{-1} Pre-ecdysis-triggering hormone (PETH) to an isolated FG preparation caused an increase in bursting frequency in the FG, whereas application of 10^{-6} mol l^{-1} eclosion hormone (EH) caused an instantaneous, though temporary, total inhibition of all FG rhythmic activity. Crustacean cardioactive peptide (CCAP), an important peptide believed to turn on ecdysis behaviour, caused a dose-dependent increase of FG burst frequency. Our results imply a novel role for this peptide in generating air-swallowing behaviour during the early stages of ecdysis. Furthermore, we show that the modulatory effects of CCAP on the FG motor circuits are dependent on behavioural state and physiological context. Thus, we report that pre-treatment with ETH caused CCAP-induced effects similar to those induced by CCAP alone during pre-ecdysis. Thus, the action of CCAP seems to depend on pre-exposure to ETH, which is thought to be released before CCAP *in vivo*.

Key words: ecdysis, neuropeptides, neuromodulation, crustacean cardioactive peptide, frontal ganglion, *Schistocerca gregaria*.

Introduction

Insect growth occurs through multiple stages. At the end of each stage the animal must moult to produce a new exoskeleton (cuticle) for the next stage, then shed the remains of the exoskeleton from the previous stage using a behaviour called ecdysis. The periodic shedding of the insect's cuticle is a complex yet stereotyped process that demands careful control and precise coordination. A successful moult is critical to an insect's survival; it requires that the physiological changes and the behaviours occurring at that time are tightly regulated so that they occur in the right sequence. Ecdysial behaviour can be divided into distinct phases, which have been extensively characterised in crickets (Carlson, 1977), locusts (Hughes, 1980a), moths (Reynolds, 1980; Truman and Endo, 1974; Weeks and Truman, 1984; Miles and Weeks, 1991) and flies (Park et al., 2002; Park et al., 2003; Clark et al., 2004): (i) the pre-ecdysis phase, which includes motor patterns that are believed to loosen the old and new cuticles, and (ii) ecdysis, during which the insect extricates itself from its old cuticle by means of anteriorly directed peristaltic contractions.

At least four different peptide hormones are involved in the control of these two phases (Hewes and Truman, 1994; Gammie and Truman, 1997; Truman et al., 1997; Zitnan et al., 1999): Pre-ecdysis triggering hormone (PETH) and ecdysis triggering hormone (ETH), both of which are co-released from the Inka cells in the peripheral epitracheal glands; eclosion hormone (EH), which, depending on the species, is synthesised by one or two pairs of ventral medial (VM) neurones located in the protocerebral region of the brain; and crustacean cardioactive peptide (CCAP), which is expressed in 1–2 pairs of neurones in each ganglion. Though still somewhat controversial (Ewer et al., 1997; Kingan et al., 1997; Gammie and Truman, 1999; Zitnan and Adams, 2000), the generally accepted model of the hormonal interactions leading to ecdysis behaviours is as follows: the decline of the moulting hormone, 20-hydroxyecdysone triggers the onset of a reciprocal interactions between EH and ETH, with each peptide causing

the release of the other. PETH and ETH act on the central nervous system (CNS) to initiate pre-ecdysis behaviours. Centrally released EH is believed to then trigger ecdysis behaviour, either directly (Hesterlee and Morton, 1996) or by a subsequent release of CCAP within the central nervous system, in a cGMP-dependent manner. CCAP then triggers the motor activities necessary to complete ecdysial behaviour (Truman et al., 1997; Zitnan et al., 1999; Gammie and Truman, 1999; Park et al., 2002; Park et al., 2003) (for a review, see Ewer and Reynolds, 2002). This model is consistent with most of the available data. However, recent reports suggest that the endocrine control of ecdysis might be even more intricate (McNabb et al., 1997; Park et al., 2003; Clark et al., 2004). Thus, ecdysis offers an important point of interaction between endocrine and neural control, allowing integration of environmental cues, in order to ensure the proper timing and sequence of its behavioural components.

We have previously described a novel central pattern generator (CPG) network situated in the locust frontal ganglion (FG), and the motor patterns it generates (Ayali et al., 2002; Zilberstein and Ayali, 2002). In the desert locust, *Schistocerca gregaria*, FG neurones innervate foregut dilator muscles and play a critical role in the control of foregut motor patterns in different physiological and behavioural states (Ayali, 2004). We have recently presented the FG as an important target for chemical modulation (Zilberstein et al., 2004). During the moult, the foregut and FG are involved in air-swallowing behaviour. By filling the gut with air, the larval locust can generate enough internal pressure on the body wall to eventually split open the old cuticle, and to then stretch and shape the new adult cuticle and wings after the old cuticle has been shed at ecdysis (Bernays, 1972). Frontal ganglionectomy abolishes air-swallowing and results in difficulty or failure in eclosion and wing expansion (Bell, 1983; Zilberstein and Ayali, unpublished). A role for the FG during moulting has been reported for a number of insect species (Bounhiol, 1938; Clarke and Langley, 1963; Penzlin, 1971; Hughes, 1980a; Carlson and O'Gara, 1983; Bell, 1983; Bestman et al., 1997; Miles and Booker, 1998).

Here we focus on the FG CPG as a previously unexplored target for ecdysis peptides. We identified CCAP as a potent modulator of the locust FG motor patterns, and our results imply a novel role for this peptide in generating air swallowing behaviour during the early stages of ecdysis. We show that the modulatory effects of CCAP on the FG motor circuits are dependent on the behavioural state and physiological context.

Material and methods

Animals

Schistocerca gregaria (Forskal) were reared under crowded conditions, in 60 l metal cages (Hunter-Jones, 1961). The cages were kept at 30°C under a 12 h:12 h L:D regime. Additional heat, including direct radiant heat, was supplied during the daytime by incandescent electric bulbs to bring the day temperature up to 36–38°C. Locusts were fed daily with fresh Kikuyu grass and flaked oats. Adult and fifth instar locusts of both sexes were used. Animals were staged relative to ecdysis as described by Hughes (Hughes, 1980a).

Electrophysiology

Locusts were briefly anaesthetised with CO_2, and their wings and legs removed. The FG and the nerves leaving it are easily accessible by cutting out a window in the head cuticle (frons). For the *in vitro* preparation, the FG and nerves leaving it were dissected out, pinned in a Petri dish lined with Sylgard (Dow Corning, Midland, MI, USA), and continuously superfused with locust saline at 26–27°C. Extracellular recordings were made with suction electrodes. Data were recorded in real time using a four-channel differential AC amplifier (model 1700, A-M Systems Inc., Carlsborg, WA, USA) and stored using an A-D board (Digidata 1320A, Axon instruments, Molecular Devices, Sunnyvale, CA, USA) and Axoscope software (Axon instruments).

Cross-correlation analysis

Cross correlation analyses were carried out as described previously (Zilberstein et al., 2004; Segev et al., 2004). Briefly, peak detection was performed on each burst of action potentials. A time window was selected that included the entire burst duration. A smoothened representation of the burst activity profile (the activity of all the neurones firing throughout the burst) is then convoluted with a normalised Gaussian filter. A matrix representing all recorded convoluted bursts is composed and the cross-correlation between all pairs of bursts is computed using a standard algorithm under a MatLab environment (The MathWorks Inc., Natick, MA, USA). A colour code is then assigned to the values of the cross-correlation. The correlation values of sets of bursts (distinct physiological or experimental conditions) can be averaged and the significance of the results can be determined.

Tissue processing for CCAP immunoblotting

Brain, and frontal and abdominal ganglia were collected over dry ice and stored at –70°C until use. Tissues were then homogenised on ice in 1.5 ml Eppendorf tubes containing homogenisation buffer (40 mmol l^{-1} Tris (pH 7.4), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA 1 μg ml^{-1} PMSF, 0.01% Triton X-100). Following microcentrifugation at 3360 ***g*** for 5 min at 4°C, the supernatant was removed and analysed for CCAP by immuno dot blot, as described below.

Immuno dot blotting

Nitrocellulose membranes were spotted with 3 μl of ganglion extracts. After air drying, the membrane was blocked for 1 h with Tris-buffered saline (0.05 mol l^{-1} Tris 0.9% NaCl, pH 7.4) containing 0.1% Tween 20 (TBST) and 2.5% BSA. The membrane was then washed three times, for 10 min each, in TBST and incubated overnight at 4°C in primary antiserum against CCAP, diluted 1:5000 [code antiCCAP 1TB, kindly provided by Prof. Heinrich Dircksen, Stockholm University, Sweden; Dircksen and Keller (Dircksen and Keller, 1988)].

Following incubation, the membranes were washed three times with TBST. Immunoreactivity was assessed using a secondary goat anti-rabbit antibody, coupled to horseradish peroxidase, and visualised with a chemiluminescence reaction (EZ-ECL chemiluminescence detection kit for HRP; Biological Industries, Beit Haemek, Israel) on Kodak X-OMAT low sensitive film. Films were scanned and relative CCAP content was estimated by densitometry of the immunoreactive dots using the Scion Image analysis program (Scion Corporation, Maryland, USA). The specificity of the primary antiserum has been established previously in several immunocytochemical and radioimmunological studies on crustacean and insect tissues (Dircksen and Keller, 1988; Stangier et al., 1989; Dircksen et al., 1991; Ewer and Truman, 1996). An additional control was performed by omitting the incubation in primary antiserum. No signal was detected after this procedure.

Immunohistochemistry

CCAP and cGMP immunohistochemistry was carried out as described by Ewer and Truman (Ewer and Truman, 1996). Animals were anaesthetised with CO_2, ganglia dissected and fixed in 4% buffered paraformaldehyde overnight at room temperature. They were then rinsed three times for 10 min in phosphate-buffered saline (PBS) + 0.3% Triton X-100 (PBSTX) at room temperature and incubated in 0.5–1.0 mg ml^{-1} collagenase (Type IV; Sigma Chemical Co., St Louis, MO, USA) in PBSTX for 30–60 min at room temperature to aid in antibody penetration. They were then rinsed three times for 10 min in PBSTX and incubated simultaneously in rabbit anti-CCAP and sheep anti-cGMP. Anti-CCAP was kindly provided by H.-J. Agricola (Universität Jena, Jena, Germany) and used at a dilution of 1:5000 (cf. Park et al., 2003). Anti-cGMP was a generous gift from J. de Vente (Limburg University, Maastrich, The Netherlands) and was used at a dilution of 1:500 (cf. Clark et al., 2004). Primary antibodies were diluted in 2% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBSTX. Tissues were incubated in primary antibody for 24–36 h at 4°C, rinsed four times for 10 min in PBSTX and incubated overnight in Texas-Red donkey anti-rabbit + FITC donkey anti-sheep (Jackson ImmunoResearch, Inc.) in 2% NDS. Tissues were then rinsed three times for 20 min in PBSTX, twice for 10 min in PBS, mounted onto polylysine-coated slides, and dehydrated, cleared and mounted under DPX (Fluka). The preparations were then examined using a ZEISS LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). Data from all ganglia were collected during the same session using the same gain and settings. Horizontal optical sections were taken at 3 μm steps through each ganglion, and the resultant *z*-series was then projected as a flat image. The CCAP-immunoreactive arborization boundaries in the ganglion neuropil and the innervated axons were than analysed using LSM 5 Image Browser (Carl Zeiss, Jena, Germany).

Saline and chemicals

Locust saline consisted of 147 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} NaOH (Frutarom, Haifa, Israel) 10 mmol l^{-1} Hepes (Biological industries, Beit Haemek, Israel) pH 7.2 (Abrams and Pearson, 1982; Penzlin, 1985).

Synthetic *Manduca sexta* PETH, ETH and EH were a generous gift from Dr Dušan Žitnan (Slovak Academy of Sciences, Slovakia). CCAP was purchased from Bachem (Bubendorf, Switzerland). Although the sequence of locust EH, ETH and PETH is currently unknown, the synthetic CCAP used is identical to locust CCAP (Stangier et al., 1989). All chemicals were prepared at different final concentrations, as noted, in normal saline just before bath application. The FG pattern was examined before, during, and after 5–10 min of application, as well as after washing for at least 30 min.

The significance of results was tested using one-way analysis of variance (ANOVA) test, followed by Tukey post test. Kruskal–Wallis test was used for nonparametric tests (Instat, GraphPad software Inc, San Diego, CA, USA).

Results

Effects of the PETH, ETH, EH and CCAP peptides on the FG rhythm

FG, dissected from feeding animals and isolated from all descending and sensory inputs, showed a robust and consistent rhythmic motor output that was recorded as multi-unit bursts of action potentials from its efferent nerve, the frontal connective (Fig. 1A; control). Bath application of 10^{-7} mol l^{-1} synthetic *Manduca* ETH [peptides doses were chosen following Zitnan et al. (Zitnan et al., 1996) and Gammie and Truman (Gammie and Truman, 1999)] caused an acceleration of the rhythm (Fig. 1A). Synthetic *Manduca* PETH also had an excitatory, albeit more modest, effect on the FG rhythm. Fig. 1B summarises the excitatory effects of ETH and PETH on the *in vitro* FG burst frequency. Data are presented as the average change in percentages from control. 10^{-7} mol l^{-1} and 10^{-6} mol l^{-1} PETH significantly increased the burst frequency by 28.5±12.5% and 31.8±16.4%, respectively (N=6). The effect of the two doses did not differ significantly from each other. Application of 10^{-7} mol l^{-1} ETH significantly increased the frequency by 54.1±16.6% (N=9; P<0.05). Thus, the responsiveness of the FG to ETH is much higher than that to PETH. In contrast to the previous effects of PETH and ETH, application of 10^{-6} mol l^{-1} EH generated an instantaneous, though transient, total inhibition of all FG rhythmic activity (Fig. 1C; N=5).

Fig. 2A demonstrates that synthetic 10^{-5} mol l^{-1} crustacean cardioactive peptide (CCAP) exerted a strong excitatory effect on the FG burst frequency (0.41±0.27 Hz, an increase of ~80% compared to control; see also Fig. 3). Furthermore, application of 10^{-5} mol l^{-1} CCAP directly on the FG of an intact adult animal through a small opening in the head capsule resulted in clear swallowing movements and strong activation of gut motor patterns [as seen during the pre-ecdysis behaviour (Zilberstein and Ayali, 2002)]. When the experiment was performed on animals in which the foregut was exposed we

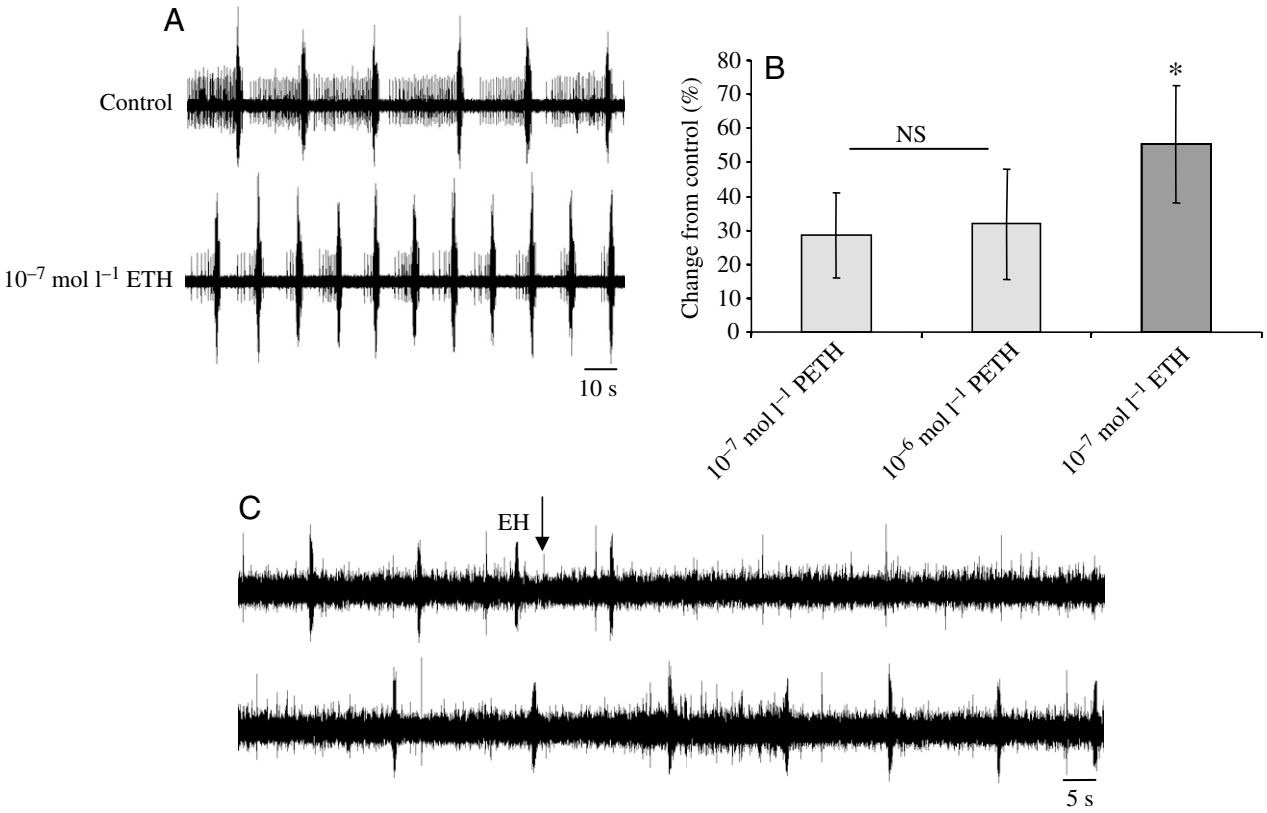

Fig. 1. (A) Extracellular recording from the frontal connective in an isolated frontal ganglion (FG) preparation. Bath application of 10^{-7} mol l^{-1} *Manduca* ecdysis-triggering hormone (ETH) increased the burst frequency. (B) *Manduca* pre-ecdysis-triggering hormone (PETH) and ETH effects on the FG rhythm. Results are shown as the change in burst frequency relative to control (means ± s.d., N=6, 6 and 9, respectively; *P<0.05; NS, not significant). (C) *Manduca* EH transiently inhibited the FG rhythm. Continuous extracellular recording (as in A; arrow indicates time of EH application).

also observed subsequent appearance of air bubbles in the crop (Fig. 2B). These results indicate that CCAP is a potent modulator of the FG rhythm and since air-swallowing behaviour starts before the onset of ecdysis behaviour (Hughes, 1980c), they may indicate an early role for CCAP in generating air-swallowing behaviour during the moult.

State-dependency of the excitatory dose-dependent effect of CCAP

We further examined the dose-dependent effects of CCAP on the rhythmic activity of FG dissected out from feeding *versus* (*vs*) moulting (i.e. in the stage where the gut was already filled with air and the old cuticle started to split) animals. We found that CCAP caused a significantly greater acceleration of the FG rhythm in moulting *vs* feeding animals (Fig. 3A). In Fig. 3B we used a recently introduced advanced cross-correlation analysis technique (Zilberstein et al., 2004) to determine the effects of CCAP on the temporal characteristics of the FG rhythm (spike timing within the burst; data marked by dashed line in Fig. 3A were used). Fig. 3B shows a cross-correlation matrix of a sequence of bursts recorded under three experimental conditions: 10^{-6} mol l^{-1} CCAP and 10^{-5} mol l^{-1} CCAP in feeding animals, and 10^{-6} mol l^{-1} CCAP in moulting animals (separated by dashed line). Three separate correlated clusters can be seen: as expected, each group of bursts (experimental conditions) was most strongly correlated to itself (shades of red; $\bar{R}$>0.95). Interestingly, the next strongest correlation was between feeding animals treated with 10^{-5} mol l^{-1} CCAP and moulting animals exposed to 10^{-6} mol l^{-1} (cross-correlation value of 0.89±0.04). The lowest correlation was obtained in animals from the two different stages treated with 10^{-6} mol l^{-1} CCAP ($\bar{R}$=0.79±0.05). Thus, there was a developmental stage as well as dose dependency in the effect of CCAP. This dependency was manifest not only at the level of cycle period (Fig. 3A), but also in other burst characteristics (Fig. 3B).

In order to explore the bases of this state dependency, we pre-exposed ganglia dissected from feeding locusts to the ETH peptide prior to CCAP application. ETH is situated upstream of CCAP in the ecdysis hormonal cascade (see Introduction). In addition, ETH receptors have recently been located in FG neurones (M. Adams, UC Riverside, USA, personal communication), suggesting that the FG may be a target of this peptide. The FG was first treated with ETH, and 10 min later (once the rhythm had stabilised) with CCAP. We found that in feeding animals pre-exposure of the FG to ETH increased the CCAP response of the FG to levels similar to those induced by CCAP alone on the FGs of moulting animals (53.2±13.3

and 59.6±8.7, respectively, compared with 31.8±9.8 for 10^{-6} mol l^{-1} CCAP treated FGs from feeding animals, Fig. 4). Even though the increased responsiveness induced by CCAP on the ETH-pretreated group was similar to that induced on the FGs of the moulting groups, cross-correlation analysis revealed a low correlation between these two responses ($\bar{R}$=0.77±0.05). This low correlation suggests that ETH may not be sufficient to fully 'prepare' the frontal ganglion for its ecdysis-related task.

Pre-exposure of the ganglion to PETH did not consistently change the effects of subsequent addition of CCAP (not shown).

CCAP levels in the FG and other ganglia during the moult

We used dot-blot analysis to measure CCAP levels in the FG, abdominal ganglia and brain from the following five stages: I, mid fifth larval instar; II, fifth larval instar, pre-eclosion (less than 12 h); III, early stages of eclosion (from air-swallowing to cuticle rupture); IV, advanced stages of eclosion; V, adult. We found that CCAP levels in abdominal ganglia showed a tendency to increase as the animal approached the time of eclosion, but this increase was not statistically significant. CCAP levels then showed a statistically significant drop after eclosion (1.3±0.2; N=5 *vs* 0.6±0.1; N=9, in stages IV and V, respectively; P<0.01; Fig. 5A). This is consistent with findings from *Manduca,* which show that CCAP activates the ecdysis motor program and its immunoreactivity drops post-ecdysis in the abdominal ganglia neuropil (Gammie and Truman, 1997). Fig. 5B demonstrates that the significant decrease in CCAP levels occurred earlier (prior to ecdysis) in FG than in the abdominal ganglia (1.2±0.07, N=8 *vs* 0.9±0.09, N=5 and 0.77±0.07, N=9; stages III–V, respectively; stage III *vs* stage IV, P<0.05; stage III *vs* stage V, P<0.001; Fig. 5B). In the brain the time course of the changes in CCAP levels was similar to that seen in abdominal ganglia (data not shown).

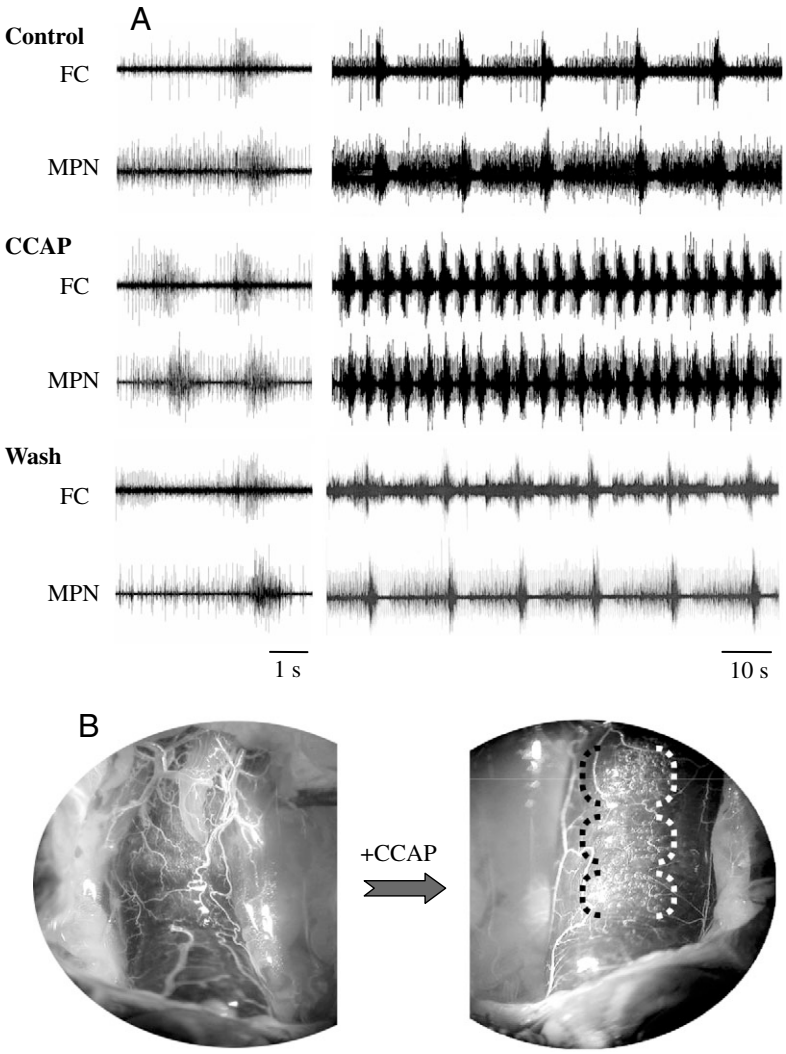

Fig. 2. (A) Simultaneous extracellular recordings from two of the frontal ganglion (FG) efferent nerves: the frontal connective (FC) and medial pharyngeal nerve (MPN), in an isolated FG preparation. Addition of crustacean cardioactive peptide (CCAP; 10^{-5} mol l^{-1}) reversibly increased the burst frequency. The left-hand traces show records played at a higher sweep speed to reveal phase relations between different components of the FG central pattern generator. (B) Application of 10^{-5} mol l^{-1} CCAP on an *in situ* FG resulted in appearance of air bubbles in the crop (broken outline).

In order to obtain an independent measure of the levels of CCAP in the FG, we used antibodies to measure the levels of CCAP immunoreactivity in the FG of animals at different stages of the moult (Fig. 6). As reported earlier by Dircksen and Homberg (Dircksen and Homberg, 1995), we found one to three CCAP-immunoreactive neurones in the tritocerebrum that project through the frontal connective nerve and give rise to extensive arborization within the neuropil of the FG. Fig. 6A demonstrates the variation in the number of CCAP immunoreactive axons in the FG neuropil at the different stages examined. In mid-larvae only one to two (mostly one) fibres were stained, whereas at the air-swallowing stage at least three afferent fibres were visible. As ecdysis progressed the number of stained axons then declined. A quantitative analysis of size of the CCAP-immunoreactive neuropil was consistent with the results obtained using dot blot. As exemplified in Fig. 6B and quantified in Fig. 6C, we found that the area of CCAP-immunoreactive neuropil increased during air-swallowing and decreased at late ecdysis.

One to three CCAP-immunoreactive cell bodies were observed in the locust FG. The axons of these cells did not branch within the ganglion. No difference was found in the staining of these cell bodies at the different stages of the moult (not shown).

cGMP immunoreactivity was not observed in the tritocerebrum and FG CCAP cells at different stages. However,

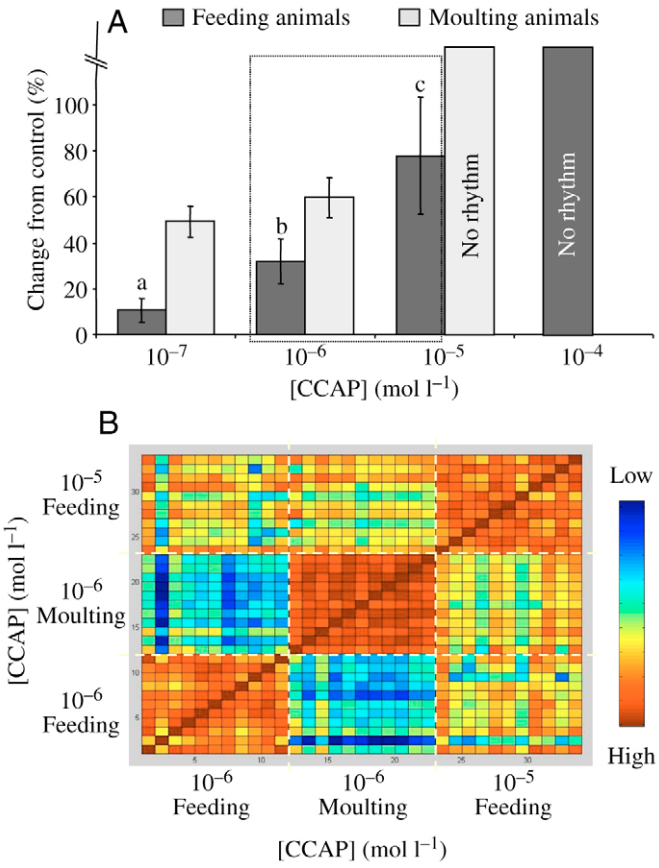

Fig. 3. (A) State dependency of the effect of crustacean cardioactive peptide (CCAP) on the frontal ganglion (FG) burst frequency. Data show the average change in the FG burst frequency as percentage of control. In the column labelled 'No rhythm', the treatment produced enhanced and tonic spiking activity, preventing a determination of burst frequency. Data outlined by a dotted box were used for the cross-correlation analyses shown in B. Pairs of bars marked by different letters differ significantly (values are mean ± s.d.; P<0.05; N=5–10). (B) Cross-correlation analysis matrices based on the FG bursts' temporal characteristics. The y and x axes represent the burst index number (colour code scale is shown on the right). The matrix was constructed from a total sequence of 33 bursts. The first 11 bursts were recorded after application of 10^{-6} mol l^{-1} CCAP to an FG dissected from feeding animals. The next 11 bursts correspond to recordings made after application of 10^{-6} mol l^{-1} CCAP to an FG dissected from moulting animals, and the last 11 bursts represent the activity after application of 10^{-5} mol l^{-1} CCAP to FG from feeding animals. See text for details.

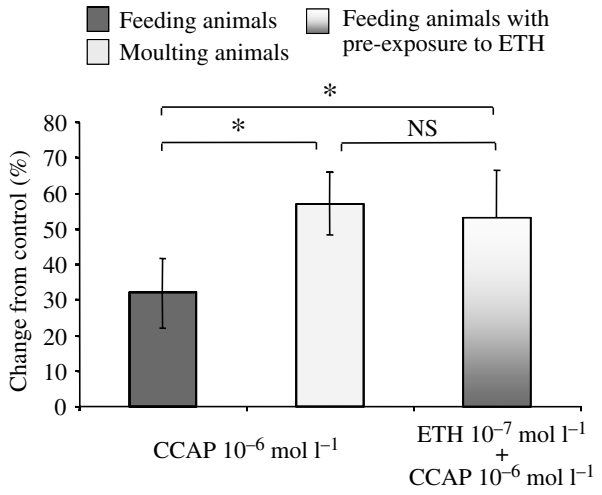

Fig. 4. Effect of pre-exposure to ecdysis-triggering hormone (ETH) on response of frontal ganglion (FG) to crustacean cardioactive peptide (CCAP). Results are presented as the average change in burst frequency relative to control. In the ETH 10^{-7} mol l^{-1} + CCAP 10^{-6} mol l^{-1} column the average increase shows the CCAP effect only (after the ETH effect was stabilised) (means ± s.d.; *P<0.05; NS, not significant; N=6).

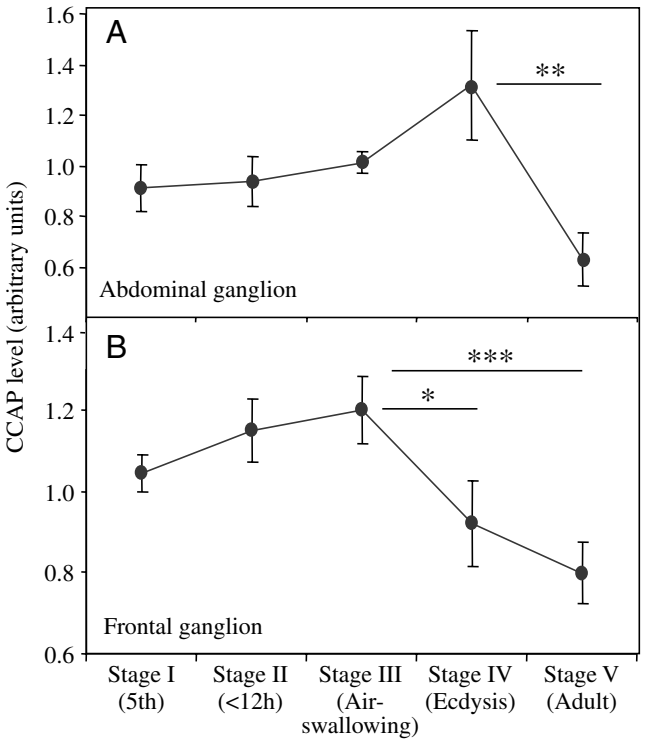

Fig. 5. Average crustacean cardioactive peptide (CCAP) levels at different stages in extracts from single abdominal ganglia (A) and the frontal ganglion (B) (values are means ± s.e.m., for N values, see text. *P<0.05; **P<0.01; ***P<0.001).

one of the two pairs of CCAP-immunoreactive cells in the abdominal ganglia showed elevation of cGMP during ecdysis (data not shown), as observed during the ecdysis to the first instar (Truman et al., 1996).

Discussion

Chemical modulation is very well established as an important factor in the generation and control of motor patterns and behaviours (Harris-Warrick and Marder, 1991; Harris-Warrick, 1994; Grillner et al., 1994; Marder et al., 1994; Marder and Thirumalai, 2002). Insect ecdysis offers a model for the intricate interactions between endocrine and neural

control. It is composed of a series of motor patterns whose timing must be precisely coordinated to ensure the proper execution of its vital outcome.

The imaginal ecdysis of the desert locust has been described in details (Hughes, 1980a). As the moult approaches, the last larval instar locust ceases to feed (approximately 24 h prior to shedding the old cuticle). The abdomen acts as a ventilatory pump, performing characteristic movements that may help to loosen the old cuticle, which are described as the pre-emergence behaviour. During this time the FG acts in full synchrony with the ventilation rhythm (Zilberstein and Ayali, 2002). This synchronisation is momentarily switched off at the specific time of air swallowing activity, during which the endogenous FG activity emerges (Zilberstein and Ayali, 2002). Gut inflation proceeds intensively until the old cuticle splits. As the gut fully inflates, the FG pattern again becomes synchronous with the ventilation rhythm. After eclosion, the gut remains inflated throughout the 'expansional motor program' (Hughes, 1980b) that serves to expand the new cuticle into its final form and to expand the wings of the emerged locust.

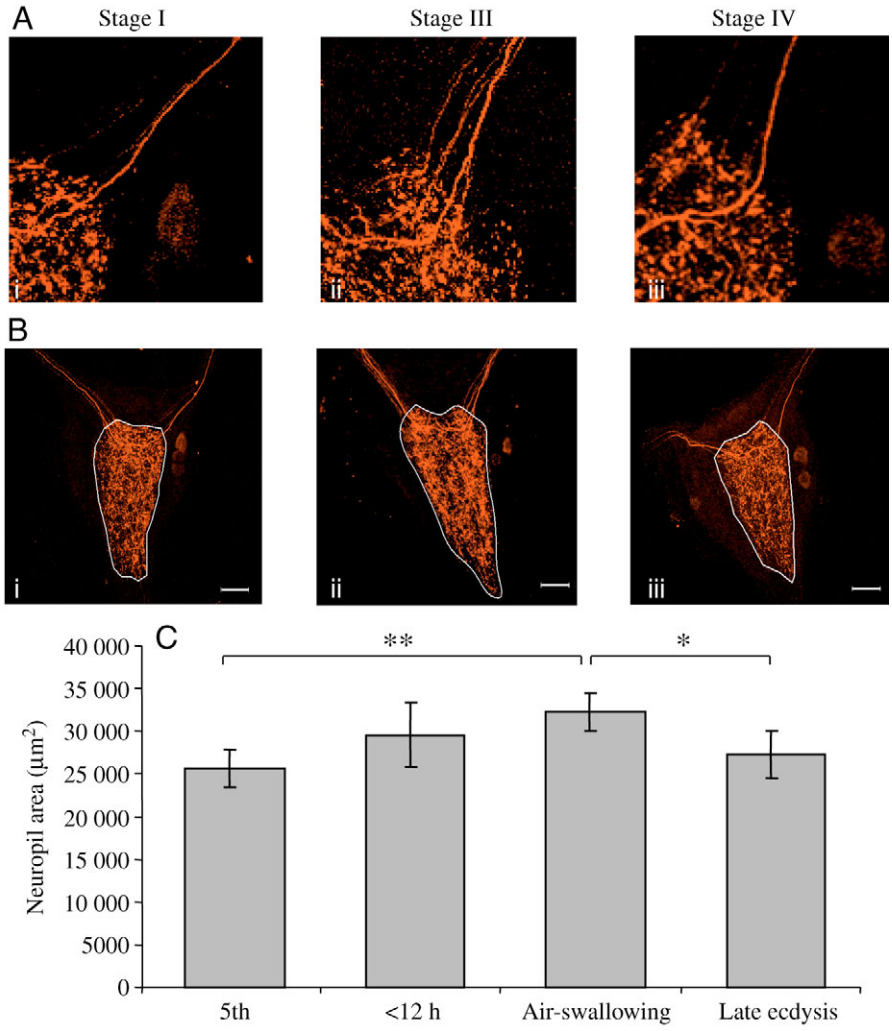

Fig. 6. Pattern of crustacean cardioactive peptide (CCAP) immunoreactivity in the frontal ganglion of locusts dissected at three out of four different stages tested: I, mid last larval instar; III, air-swallowing; IV, late ecdysis. (A) Immunoreactive axons that innervate the frontal ganglion (FG) neuropil. (B) CCAP-immunoreactive area in the ganglion neuropil. (C) Graphic representation of the CCAP-immunoreactive neuropil area (mean ± s.d. N=6; *P<0.05; **P<0.01).

As mentioned previously, the FG plays a critical role in the moult (Bounhiol, 1938; Clarke and Langley, 1963; Penzlin, 1971; Hughes, 1980a; Carlson and O'Gara, 1983; Bell, 1983; Bestman et al., 1997; Miles and Booker, 1998; Zilberstein and Ayali, 2002). In this study we focused on the FG as a previously unexplored target for ecdysis peptides. We first screened the known ecdysis peptides to look for a potent FG modulator and a candidate for activating the air swallowing behaviour. Using an *in vitro* preparation, we found that both PETH and ETH accelerated the FG rhythm, whereas EH transiently inhibited it. We have previously reported that haemolymph collected from non-feeding pre-moult larvae inhibited the FG rhythmic activity (Ayali et al., 2002). In *Manduca* there is also evidence for foregut and FG modulation during the moult, and haemolymph collected from moulting larvae and applied to a larval FG-foregut preparation alters the ongoing feeding motor pattern to resemble that observed in moulting larvae (Bestman et al., 1997). Interestingly, a rhythmic motor pattern that resembles air swallowing could be generated in isolated *Manduca* heads 24–30 h before eclosion by application of eclosion hormone (EH) (Miles and Booker, 1998). Carlson and O'Gara (Carlson and O'Gara, 1983) reported that the cricket FG could generate activity *in vitro* only if isolated from near-moult, ecdysing, or expanding insects. This state washed out within 1 h, during which the ganglion generated an air-swallowing pattern.

These diverse, sometimes contradictory, observations could be explained in light of our current results by considering the different and complex effects of the various insect ecdysis-related peptides (Truman et al., 1997; Zitnan et al., 1996; Kingan et al., 1997), and by suggesting that the timing of peptide application can cause significantly different responses.

We found that CCAP was the most potent modulator of the FG rhythm. CCAP was originally isolated and sequenced from the pericardial organs of the shorecrab *Carcinus maenas*. It has been shown to increase the amplitude and frequency of the

crayfish heart constrictions (Stangier et al., 1987). Further studies revealed an extensive network of CCAP-IR neurones in the central nervous system of *Carcinus maenas*, suggesting that the peptide may also function as a neurotransmitter (Dircksen and Keller, 1998) or neurohormone (Dircksen et al., 1991; Donini et al., 2001). CCAP has since been discovered in insects, including the tobacco hawkmoth, *Manduca sexta* (Cheung et al., 1992), and the migratory locust, *Locusta migratoria* (Stangier et al., 1989; Dircksen and Homberg, 1995). The pattern of CCAP-IR cells and fibres was described in the brain and the nervous system of *L. migratoria* (Dircksen et al., 1991; Dircksen and Homberg, 1995). Most importantly, CCAP has been implicated in the onset of insect ecdysis behaviour (Gammie and Truman, 1999). Furthermore, the sequence of the CCAP peptide is identical in all arthropods sequenced to date (including the locust) (Stangier et al., 1989; Veelaert et al., 1997) Thus, the synthetic CCAP we applied *in vitro* corresponds to locust peptide.

In order to investigate the exact role of CCAP in the execution and regulation of ecdysis, Park et al. (Park et al., 2003) used transgenic *Drosophila* bearing targeted ablations of CCAP neurones. These insects expressed specific defects at ecdysis, and at adult eclosion showed a failure in abdominal inflation. These results are consistent with our current data on the role of CCAP early in ecdysis, during air-swallowing (e.g. gut inflation) behaviour. This is a novel effect of CCAP, which takes place earlier than the traditional role attributed to this neuropeptide in the final stages of the moult.

We were not able to fully mimic, in our *in vitro* preparation, the motor patterns observed during the ecdysis *in vivo* (Zilberstein and Ayali, 2002). In addition to being due to complex effects of the various insect ecdysis-related peptides, this could also be due to another important finding of the current study, i.e. that the modulatory effect of CCAP is strongly dependent on physiological context. We found the effects of CCAP on FG burst frequency to be greater in moulting than in feeding animals. The doses we used for moulting animals were consistent with those reported in the literature (Zitnan and Adams, 2000), whereas higher doses were needed to affect the FG activity of feeding animals. Interestingly, pre-treatment of FGs from feeding animals with ETH increased the effect of CCAP to levels similar to those obtained in moulting animals. Thus, the response to CCAP seems to depend on pre-exposure to ETH, which is thought to be released before CCAP *in vivo* (for a review, see Ewer and Reynolds, 2002; Mesce and Fahrbach, 2002). Practically nothing is known about the ETH in locusts; however, evidence in the literature suggests that, although Inka cells of different insects are very variable in number and morphology, they have a conserved function in activation of the pre-ecdysis and ecdysis motor programs within the CNS (e.g. Zitnan et al., 2003).

An animal's physiological and behavioural state is known to be an important factor affecting the specific actions of neuromodulators in the case of modulation of sensory pathways (Kay and Laurent, 1999; Christensen et al., 2000; Cardin and Schmidt, 2004). Whether the same applies to modulation of motor circuits has been unclear, as fewer examples are known of context-dependent modulation of motor output (Nusbaum and Marder, 1989). Thus, our results strengthen the importance of insect ecdysis as a model of neuromodulation for behaviour.

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