DYNAMICS OF CYCLIC GMP LEVELS IN IDENTIFIED NEURONES DURING ECDYSIS BEHAVIOUR IN THE LOCUST *LOCUSTA MIGRATORIA*

JAMES W. TRUMAN[2,1,*], JOHN EWER[1] AND ELDON E. BALL[2]

[1]*Department of Zoology, University of Washington, Seattle, WA 98185-1800, USA* and [2]*Research School of Biological Sciences, Australian National University, PO Box 475, Canberra, ACT 2601, Australia*

Summary

A grasshopper hatches from its egg, which is laid in soil, as a vermiform larva. This larva continues the stereotyped hatching behaviour as it digs through the egg pod, which provides a passageway to the soil surface. Once at the surface, shedding, or ecdysis, of the vermiform cuticle is initiated. When this process is complete, the first-instar cuticle is expanded to assume the form of the first-instar hopper. We have demonstrated, using immunocytochemical techniques, that these behaviour patterns are associated with dramatic increases in intracellular levels of cyclic GMP in sets of identified neurones in the ventral central nervous system.

The most prominent cyclic-GMP-expressing cells are 34 neurones that appear to contain crustacean cardioactive peptide (CCAP). These CCAP cells show no detectable cyclic GMP at hatching or while the vermiform larva digs through the soil. Upon reaching the surface and freeing itself, the larva initiates ecdysis and associated air-swallowing and tracheal filling within about 1 min. These changes are immediately preceded by the appearance of cyclic GMP in the CCAP cells. Cyclic GMP levels in these neurones peak by 5 min and then decline back to basal levels by 20–30 min. Conditions that cause ecdysing animals to resume digging prolong the elevation of cyclic GMP levels. Once animals have assumed their 'hopper' form, however, external stimuli can no longer affect the time course of the cyclic GMP response. The neurones containing elevated cyclic GMP levels probably influence the air-swallowing, tracheal filling and circulatory changes that are associated with ecdysis behaviour.

Pairs of descending midline neurones in abdominal segments 2–4 also become cyclic-GMP-immunoreactive, but they show peak expression after cyclic GMP levels in the CCAP cells have declined. Also, neurones in the caudolateral region of the abdominal ganglia often become cyclic-GMP-immunoreactive when ecdysing animals are forced to resume digging for an extended period.

Key words: cyclic GMP, ecdysis, hatching, *Locusta migratoria*, identified neurones.

Introduction

Ecdysis in insects involves a stereotyped sequence of behaviours that is initiated with the release of the neuropeptide eclosion hormone (EH; Truman, 1992). Studies on various Lepidoptera have shown that this peptide acts directly on the central nervous system (CNS) to evoke the behavioural cascade that brings about the shedding of the old cuticle (Truman, 1978; Hewes and Truman, 1991). Besides its behavioural effects, EH also causes the release of other neuropeptides that orchestrate the physiological changes that accompany ecdysis. Among these effectors are the cardioacceleratory peptides (CAPs) that increase heart rate at the time of ecdysis (Tublitz and Truman, 1985; Tublitz and Evans, 1986). One of the CAPs (CAP2a) in *Manduca sexta* was subsequently sequenced and found to be identical to crustacean cardioactive peptide (CCAP; Cheung *et al.* 1992),

a peptide first discovered in crustaceans (Dircksen and Keller, 1988) but later found to be distributed widely in insects (Dircksen, 1994). Neurones that contain CCAP in *M. sexta* show a striking increase in intracellular 3′5′-cyclic guanosine monophosphate (cyclic GMP) levels shortly before the onset of ecdysis (Ewer *et al.* 1994). This rise in intracellular cyclic GMP levels causes an increase in excitability of these neurones (Gammie *et al.* 1994; S. C. Gammie and J. W. Truman, unpublished observations), presumably facilitating the release of CCAP. Diverse insect species, with the exception of members of the higher Diptera, show a similar increase in intracellular cyclic GMP levels in CCAP-containing neurones at ecdysis (Ewer and Truman, 1996).

One of the best timed and characterized ecdyses in insects is that exhibited by locusts shortly after hatching. The hatching

---

*Permanent address: Department of Zoology, University of Washington, Seattle, WA 98185-1800, USA.

locust is covered by the second embryonic cuticle and is termed the vermiform larva (Bernays, 1971). The vermiform larva utilizes a specialized behaviour to escape from the chorion and dig upwards through the egg pod to the surface of the soil (Bernays, 1971, 1972*a*). As the anterior end of the larva comes free, the animal ceases hatching/digging movements and rapidly shifts to ecdysis behaviour. The second embryonic (vermiform) cuticle is shed within minutes (Fig. 1), and the larva assumes the shape and proportions of the first-stage hopper. This dependence of ecdysis on the freeing of the larva makes it possible to control precisely the time of onset of ecdysis behaviour and makes hatching locusts excellent animals in which to explore further the relationship between ecdysis and the changes in cyclic GMP levels that occur in the CCAP neurones.

Materials and methods

Experimental animals

A laboratory colony of *Locusta migratoria* was maintained on a diet of wheat germ and sprouted wheat seedlings. Breeding females were provided access to cups of moist sand

Fig. 1. As the hatchling locust emerges from its egg pod, it first sheds the eggshell (top) and then ecdyses its embryonic cuticle (middle and bottom).

into which they deposited their egg pods. The oviposition cups were removed weekly and maintained at 30 °C. Intact individual egg pods were removed from the sand and maintained between layers of moist cheesecloth in Petri dishes. Individual eggs were dissected at intervals to determine the developmental stage of each clutch. Staging of embryos was based on the system of Bentley *et al.* (1979), with additional criteria added for late embryos (E. E. Ball and J. W. Truman, in preparation).

Fixation and immunocytochemistry

In order to obtain an accurate picture of the cyclic GMP response at specific times, we dissected animals as rapidly as possible in 4 % formaldehyde (in phosphate-buffered saline, PBS). Embryos or hatching larvae were stripped out of the chorion, immersed in fixative at room temperature and rapidly opened mid-dorsally. Following removal of the gut, the body wall was pinned out flat. Fixation was generally for about 2 h at room temperature or overnight at 4 °C. To improve reagent access after fixation, the CNS was dissected free from most of the body wall and processed as an isolated nerve cord. Adhering muscle tissue in the meso- (T2) and metathoracic (T3) regions of some nervous systems reduced the quality of immunostaining in these areas. Consequently, our analyses focus primarily on the prothoracic (T1) ganglion and the unfused abdominal ganglia (A4–A8).

Cyclic GMP levels were determined *in situ* using the immunocytochemical methods developed by De Vente *et al.* (1987). After repeated washes, fixed tissues were pre-blocked in 5 % normal goat serum (NGS) in PBS with 0.3 % Triton X100 (PBS-TX), and then incubated with a 1:4000 dilution of a rabbit anti-cyclic-GMP antiserum (generously provided by Dr Jan De Vente) in PBS-TX with 1 % NGS. After 12–36 h at 4 °C, the tissues were repeatedly washed and then incubated overnight with a peroxidase-conjugated goat anti-rabbit IgG (Kierkegaard and Perry Labs) in PBS-TX with 1 % NGS. After additional rinses, the location of the antibody complexes was revealed by reaction with diaminobenzadine (DAB, Sigma) and glucose oxidase (Watson and Burrows, 1981) or H_2O_2. The developing solution contained 0.03 % nickel chloride to yield a black reaction product. Tissues were then dehydrated, cleared in methyl salicylate, and mounted in Permount (Fisher Labs). We scored the intensity of cyclic GMP staining in the cell body of some of the neurones as follows: 0, no staining; 1, cell body can be distinguished as pale grey; 2, cell body is a moderate grey; 3, cell body is dark grey to black.

Behavioural manipulations

Individual egg pods were partially opened and monitored to assess the progress of development. When hatching began, larvae were timed from the beginning of emergence from the chorion or from the time that they initiated ecdysis movements. Ecdysing larvae were placed in individual containers until the time of their dissection.

Hatching animals showed sustained 'digging' behaviour when placed under two layers of moist cheesecloth in a plastic

Petri dish. The metathoracic legs of *digging vermiform larvae* retained the position that they had occupied while in the egg, with the femur extended posteriorly but the tibia pointed anteriorly. We obtained *digging ecdysing larvae* by placing animals that had just initiated ecdysis under cheesecloth. Their posture differed from that of digging vermiform larvae in that the metathoracic tibiae projected posteriorly and trailed the digging animal with the exuvium collected around the distal end of the legs.

Image processing

Video images of the histological preparations were captured using a Sony DXC-960MD video camera mounted on a Nikon Optiphot microscope. Two-dimensional projections of neurones within a ganglion were constructed from stacks of optical sections that were imported into Adobe Photoshop 3.0. The stack was assembled using the layering palettes with out-of-focus elements being deleted. The flattened images were then printed on a Tektronix dye sublimation printer.

Results

Neurones that show cyclic GMP accumulation around the time of ecdysis

CCAP cells

Thirty-four cells distributed from the suboesophageal ganglion (SOG) to A7 (Ewer and Truman, 1996) show elevated levels of intracellular cyclic GMP in ecdysing locusts (Fig. 2). Four pairs of neurones are present in the SOG, two pairs each in ganglia T1–T3 and one pair in abdominal ganglia A1–A7.

The cell body positions and axon trajectories of all 34 cells identify them as members of the group of cells that contain CCAP (Dircksen *et al.* 1991; Dircksen and Homberg, 1995). The posterior three pairs of SOG neurones and the segmental neurones from T1 to A7 are type 1 and type 2 CCAP neurones. In the maxillary SOG neuromere (S2), one pair of neurones sends axons posteriorly through contralateral descending lateral tracts (Fig. 2A) and, thus, these are type 2 neurones. The S2 and S3 type 1 cells appear to be CN2 and CN3, respectively (Dircksen and Homberg, 1995); these neurones project *via* the brain to pharyngeal dilator muscles (Bräunig, 1990). In each thoracic ganglion, one of the two pairs of cyclic-GMP-immunoreactive neurones is also of type 2 since these cells have the characteristic contralateral axonal bifurcation, with one axon exiting to the periphery *via* nerve 5 and the other axon joining the SOG axon in the descending lateral tract (Fig. 2B). The axons of these thoracic type 2 cells project the length of the CNS and arborize in the terminal ganglion. The abdominal ganglia also have type 1 and type 2 CCAP cells (Dircksen *et al.* 1991). In contrast to their thoracic homologues, the abdominal type 2 cells failed to show detectable levels of cyclic GMP at ecdysis, whereas the abdominal type 1 cells showed a robust increase (Fig. 2D). The type 1 cells in both the thoracic and abdominal segments are characterized by an initial process that extends ventrally to the midline and then projects dorsally and forms a contralateral axon. In most instances, the axon then splits, with one branch projecting posteriorly to the next ganglion where it exits to the periphery *via* the contralateral intersegmental nerve. The other axon projects anteriorly to the intersegmental nerve in the ganglion of origin (Fig. 3A). The posterior axon projects to the alary muscles of the heart (Dircksen *et al.* 1991).

This splitting of the axon of the type 1 cell has not been observed except in the S3 neuromere (Dircksen *et al.* 1991; Dircksen and Homberg, 1995). Indeed, in a few of our animals,
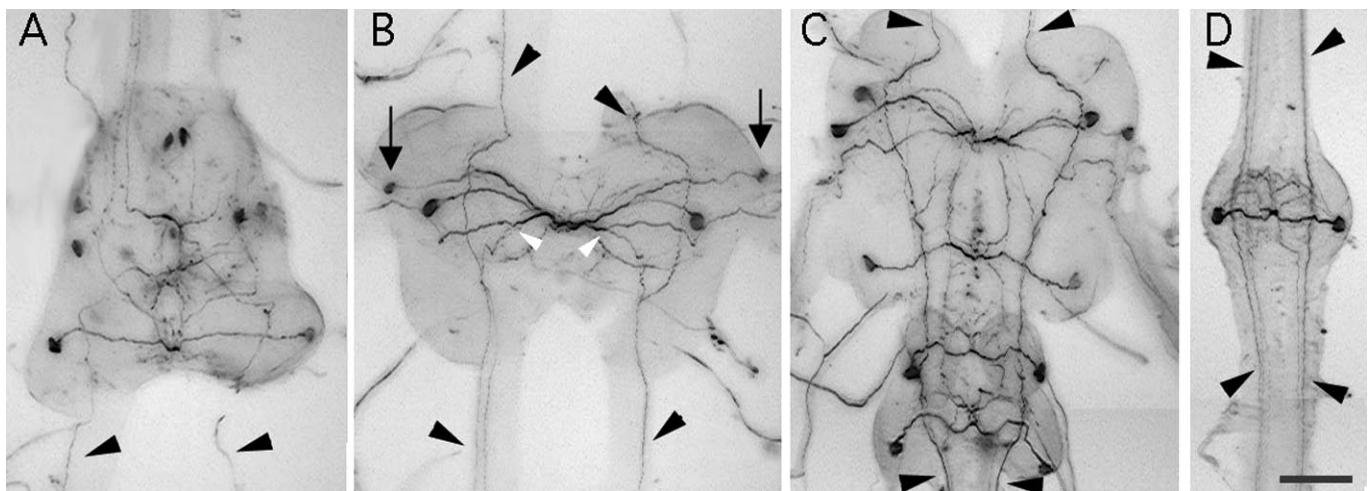

Fig. 2. Video micrographs of selected ganglia in an ecdysing grasshopper showing neurones with intense cyclic GMP immunoreactivity. Each image is a video montage of numerous optical sections combined to show neurone morphology optimally. (A) Suboesophageal ganglion showing one, two and one pairs of neurones in the mandibular, maxillary and labial neuromeres, respectively. (B) Prothoracic (T1) ganglion showing the pair of type 1 and type 2 (arrows) CCAP neurones; white arrowheads show the axonal bifurcation characteristic of type 2 neurones. (C) The fused T3–A3 ganglia; type 1 and type 2 neurones are present in T3 but only type 1 neurones in A1–A3. (D) Ganglion A5 showing the single pair of type 1 cells. Black arrowheads mark the immunostained axons of the type 2 neurones in the descending lateral tract. Scale bar, 100 μm.
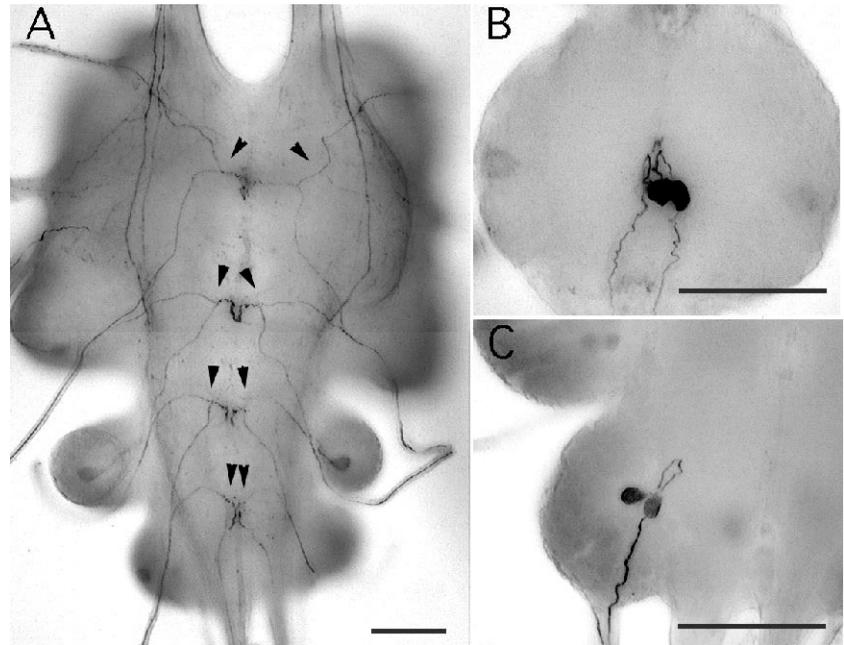
Fig. 3. Video montages of ganglia in grasshopper nervous systems showing cells that are cyclic-GMP-immunoreactive. (A) Dorsal region of the fused T3–A3 ganglia showing the axons of the paired type 1 cells from each segment. Each axon crosses the midline, extends dorsally and then splits (arrowheads) into axons that exit in the segment of origin and the next posterior segment. (B) The paired midline cells in A4. (C) The caudolateral cells in ganglion A3 of a partially ecdysed digging grasshopper. Scale bar, 100 μm.

the anterior or posterior axon in a particular segment was missing. We do not know whether this is a stage-dependent (Dircksen *et al.* 1991 used adults) or a strain-dependent difference.

The descending axons of the thoracic type 2 neurones appear to contact the initial processes of the type 1 neurones. A similar feature is evident in *M. sexta* and a number of other insects (Ewer *et al.* 1994; Ewer and Truman, 1996). This anatomy suggests that the cells may interact directly with one another.

Midline cells

Paired cells near the ventral midline in ganglia A2–A4 also show increases in intracellular cyclic GMP levels (Fig. 3B). These cells do not contain CCAP. Their axons descend the ipsilateral connectives to unknown termination sites.

Caudolateral cells

The last set of cells that shows cyclic GMP immunoreactivity consists of a group of two or three paired cells situated in the caudolateral regions of ganglia A1–A7. They have ipsilateral axons that loop around the lateral tract and leave the ganglion *via* the ipsilateral segmental nerve (Fig. 3C). These cells are in the position of the lateral neurosecretory cells that project to the perivisceral neurohaemal organs (Taghert and Truman, 1982; Dircksen *et al.* 1991).

Time course of cyclic GMP accumulation at the time of ecdysis

Normally, a hatching locust digs through the soil as a vermiform larva and initiates ecdysis only after it reaches the surface. If the anterior end of the egg is exposed, however, the hatching larva can dispense with digging altogether. While still partially in the chorion, it switches to ecdysis behaviour, which

then frees it from both the chorion and the old cuticle (Bernays, 1971, 1972*a,b*).

We collected larvae that were in various stages of hatching from partially dissected egg clutches and examined their cyclic GMP responses. The progression of cyclic GMP staining in the CCAP cells as a function of time after the start of ecdysis is shown in Table 1. We focused on the type 1 and 2 cells in T1, the type 2 cell axons (from the SOG and T1–T3) in the abdominal lateral tract, and the type 1 cells in A4–A7. The neurones of the SOG are omitted from quantitative consideration because this ganglion was often damaged during dissection. We did not quantify the intensity of cyclic GMP expression for individual cells in this group.

Of 20 animals examined within the first minute after starting to emerge from the chorion, seven had begun ecdysis and were dissected immediately following their first ecdysis movement. All had air in their gut or tracheal system and all showed cyclic GMP staining in their CCAP neurones. The remaining 13 hatching larvae were fixed prior to the onset of ecdysis movements. There was no air in either the gut or tracheae of these animals. Eight of the 13 were devoid of any trace of cyclic GMP immunoreactivity (cGMP-IR) in the CCAP network (e.g. Figs 4, 5; −1 min). The remaining five animals all showed distinct cyclic GMP staining in the CCAP neurones. In the thoracic cells, cGMP-IR was evident only in the medial neurites, with little or no staining in the cell bodies. The type 2 cell axons in the abdominal portion of the lateral tract were stained along their entire length. The type 1 cells in A4–A7 showed a weak response in their cell bodies and processes without any evidence of an anterior-to-posterior gradient. In four of five animals, cGMP-IR was also evident in some of the SOG cells, including axons that projected to the brain.

Animals at the start of ecdysis, fixed only seconds after the preceding set of animals, showed the same pattern of staining

Table 1. *Time course relative to the time of ecdysis of the appearance of cyclic GMP immunoreactivity in identified neurones of* Locusta migratoria

Time (min)	N	Percentage of cells that are cyclic-GMP-immunopositive					
		T1 CCAP cells[a]		Abd LT[b]	Abd CCAP cells[c]	A4 midline	
		Arborizations	Somata			%	Score[d]
−1 to 0[e]	8	0	0	0	0	62	0.6
−1 to 0[e]	5	100	40	100	100	80	1.2
0	7	100	25	100	100	50	0.5
2	5	100	65	100	100	100	0.8
5	5	100	80	100	100	80	0.9
10	5	100	50	100	100	100	1.2
20	3	100	17	100	100	100	1.5
30	3	0	0	0	35	100	3.0
45	8	0	0	0	6	100	2.0

[a]Includes both type 1 and type 2 CCAP cells.

[b]Axons in the abdominal portion of the descending lateral tract.

[c]Type 1 CCAP cells in ganglia A4–A7.

[d]Average score based on intensity of cyclic GMP staining: scores of 0, 1, 2 and 3 refer to no, weak, moderate and strong staining, respectively.

[e]Unconfined locusts in the period between hatching and the start of ecdysis. They had no air in either their guts or tracheae. Groups are separated into those that showed no cyclic GMP staining and those showing cyclic GMP staining throughout the CCAP network.

(Table 1; Figs 4, 5, 0 min). Over the next 2–5 min, the cyclic GMP levels continued to rise within the network (Figs 4, 5; 2–5 min). In the thoracic cells, cGMP-IR invaded the cell bodies by 2 min and by 5 min the cyclic GMP levels appeared maximal. The abdominal cells also reached their highest levels of intracellular cyclic GMP by about 5 min after the start of ecdysis. By 10 min, the overall intensity of the cyclic GMP

response had waned. In the thorax, the cyclic GMP staining was faint in both the cell bodies and the medial neurites (Fig. 4; 10 min). The axons of the type 2 cells that projected through the abdominal ganglia still showed moderate cyclic GMP staining, but their terminations in the terminal ganglion were no longer stained. The abdominal type 1 cells showed moderate cyclic GMP staining (Fig. 5, 10 min). By 20 min,
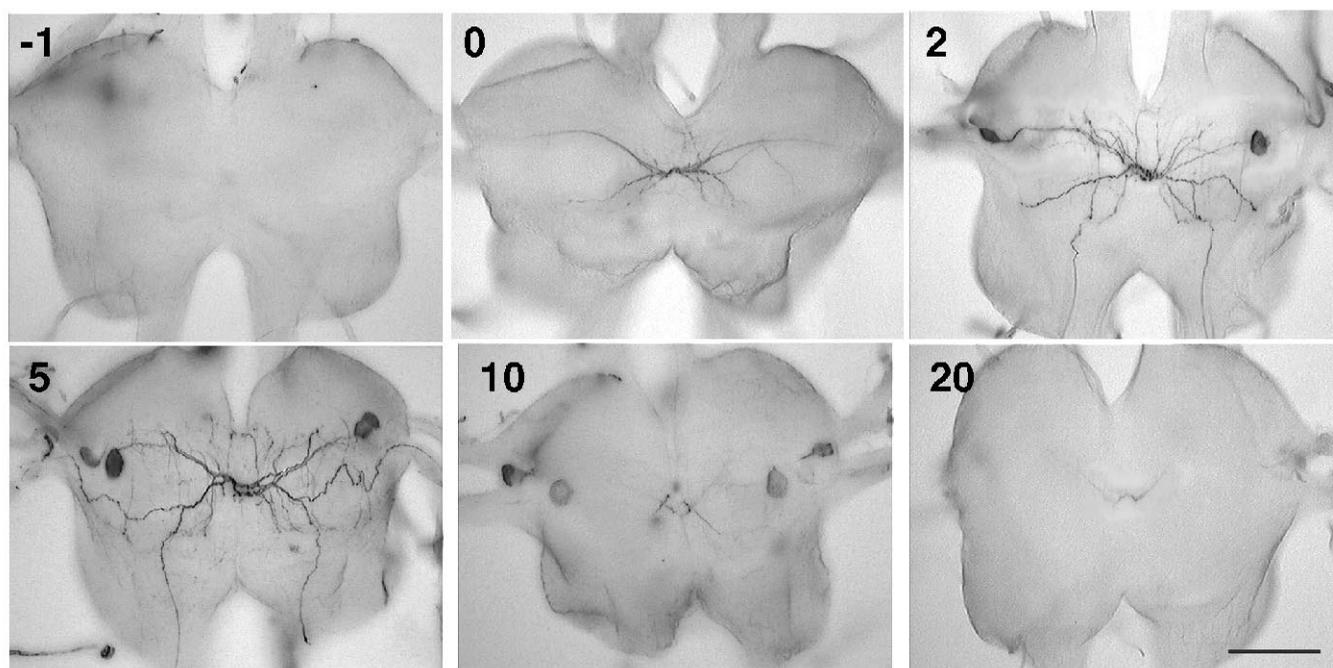

Fig. 4. Video montages of ganglion T1 showing the time course of the appearance and disappearance of cyclic GMP immunostaining relative to the time of onset of ecdysis movements. Numbers refer to time in minutes relative to the start of ecdysis movements. Cyclic GMP is not evident at 1 min (−1 min) before ecdysis, reaches peak levels by about 5 min and is barely visible by 20 min after the start of ecdysis. Scale bar, 100 μm.

Fig. 5. Video montages of ganglion A5 showing the time course of the appearance and disappearance of cyclic GMP immunostaining relative to the time of onset of ecdysis movements. Numbers refer to time in minutes relative to the start of ecdysis movements. These micrographs are from the same nervous systems shown in Fig. 4. Cyclic GMP levels persist slightly longer in the thorax than in the abdomen. Scale bar, 100 μm.

cyclic GMP levels in the thoracic ganglia were reduced to traces of staining in the medial neurites (Fig. 4, 20 min). The descending axons of these cells showed a gradient of weak staining, with cGMP-IR falling to undetectable levels before reaching A7. The abdominal type 1 neurones stained weakly (Fig. 5, 20 min), with those in the posterior ganglia being the most weakly stained. By 30 min, the cyclic GMP levels in the descending axons had fallen below the level of detectability. A few abdominal neurones still showed very low levels of cyclic GMP staining confined to the cell body.

Behavioural modification of the cyclic GMP response in the CCAP neurones

When eggs were placed under moist cheesecloth, the newly hatched vermiform larvae did not ecdyse but rather showed the abdominal movements and cyclical extension of their cervical ampullae characteristic of digging behaviour (Bernays, 1971). We examined five vermiform larvae after 10–60 min of continuous digging. None had air in either their tracheae or gut at the time of dissection. Four were completely lacking cGMP-IR in their CCAP cells (Fig. 6A; Table 2) and one animal at 60 min after hatching showed a trace of immunostaining in the type 1 cells in A4 and A5. Thus, stimuli that delay ecdysis and cause the larva to remain as a vermiform larva also delay the induction of cyclic GMP.

When a digging vermiform larva encountered an open space, it switched from digging to ecdysis behaviour. If it was then covered again, it resumed digging, but this time as a digging ecdysing larva. As described in Materials and methods, these larvae differed from vermiform larvae in their leg position during digging. Also, when dissected, they always

had air in both their gut and their tracheal systems. Such partially ecdysed diggers maintained detectable cyclic GMP levels in their CCAP network for at least twice as long as
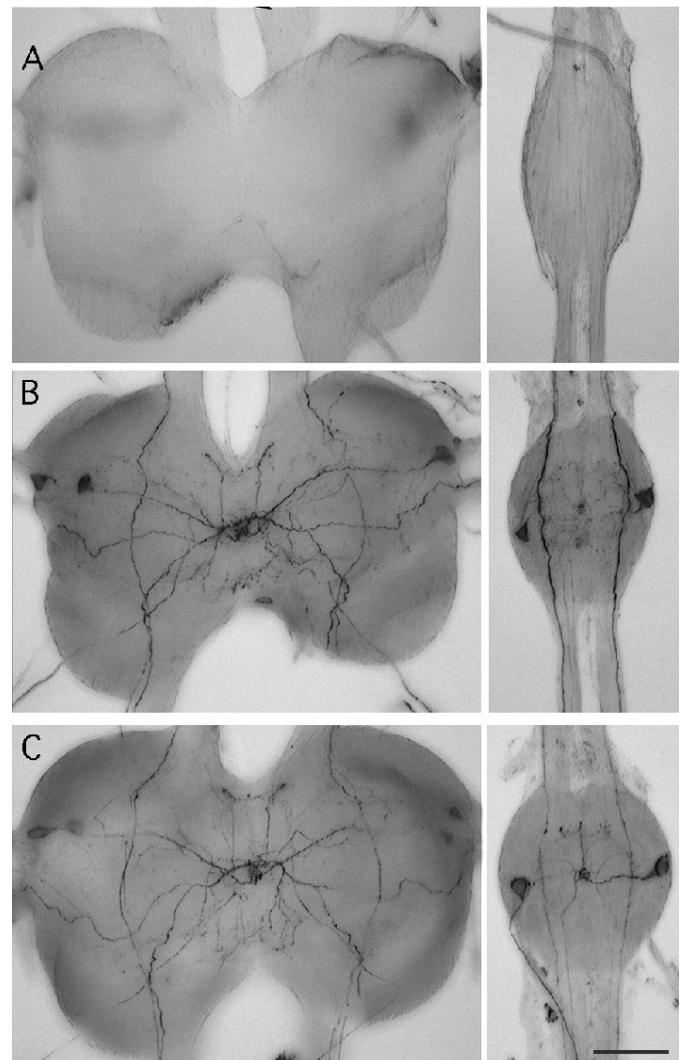

Fig. 6. Video montages of ganglion T1 (left) and A5 (right) showing the cyclic GMP immunoreactivity in newly hatched grasshoppers that were 'digging' under layers of cheesecloth. (A) Ganglia from a digging vermiform larva that had been digging for 35 min. (B,C) Ganglia from partially ecdysed digging grasshoppers that had been digging for 20–30 min and 45–50 min, respectively. Scale bar, 100 μm.

Table 2. *Effects of confining newly hatching locusts under moist cheesecloth on the levels of cyclic GMP in identified neurones in the ventral central nervous system*

			Percentage of cells that are cyclic-GMP-immunopositive					
			T1 CCAP cells[a]		Abd	Abd CCAP	A4 midline	
Stage[b]	Duration (min)	*N*	Arborizations	Somata	LT[c]	cells[d]	%	Score[e]
Vermiform	10–60	5	0	0	0	10	60	0.5
Partially ecdysed	20–30	7	100	92	100	95	86	1.2
Partially ecdysed	40–50	7	86	42	86	86	86	1.3
Partially ecdysed	110	6	0	0	0	0	100	1.6
Hopper	30–45	5	0	0	0	0	80	1.0

[a]Includes both type 1 and type 2 CCAP cells.

[b]Both vermiform larvae and partially ecdysed larvae showed digging behaviour while confined; the hoppers only showed kicking and struggling behaviour.

[c]Axons in abdominal portion of the descending lateral tract.

[d]Type 1 CCAP cells in ganglia A4–A7.

[e]Scoring system for midline cells as in Table 1.

animals freed immediately after ecdysis (Fig. 6; Table 2). In spite of this prolongation of high levels of cyclic GMP expression, the pattern of disappearance of cyclic GMP was similar to that seen in normal animals; namely, a fading of cGMP-IR from the thoracic cell bodies and from the descending axons, starting from their posterior ends. By 120 min, cyclic GMP levels in these digging animals had dropped below our level of detection.

Digging animals have a difficult time walking or even standing when they first reach the surface of the soil. Within a minute or two of being freed, however, these larvae expand and straighten their new cuticle to its proper hopper proportions. These 'hoppers' can now walk and hop, but they can no longer dig. When placed under cheesecloth, they show kicking and struggling movements but no coordinated digging behaviour. As seen in Table 2, the types of confinement that prolonged cyclic GMP expression in the partially ecdysed digging larvae did not do so in these larvae that had become hoppers. Thus, once ecdysed animals make the transition to being hoppers, cyclic GMP levels in the CCAP cells are no longer affected by external stimuli.

Changes in cyclic GMP levels in other neurones during ecdysis

The two other types of neurones that showed cyclic GMP expression around the time of hatching were the midline cells and the caudolateral cells. Cyclic GMP staining in the midline cells was observed only rarely prior to hatching. In embryos taken from egg pods at 3 h (*N*=8), 11 h (*N*=9) or 28 h (*N*=10) prior to hatching, we observed weak cyclic GMP staining in midline cells in only four individuals (all from the −28 h collection). At hatching, over 50 % of the animals showed some level of cyclic GMP in the A4 midline cells. The cyclic GMP response in these cells, however, did not depend on whether the CCAP cells had started to respond. By 2 min after hatching, midline cells were stained in essentially all the animals. The levels of cyclic GMP in these cells continued to

increase while those in the CCAP network rose and then fell. Cyclic GMP levels began to wane in the midline cells by about 45 min post-hatching.

Expression of cyclic GMP in the caudolateral cells was seen only in partially ecdysed larvae that were showing digging behaviour. Immunoreactive cells were observed in one of five animals that had been digging for 20–30 min, two of five that had dug for 40–50 min, and three of five that had done so for 110 min.

Changes in cyclic GMP levels during premature ecdysis behaviour

In Lepidoptera, the mechanical stimulation of peeling the pupal cuticle from developing adult moths can trigger the precocious onset of ecdysis behaviour (Blest, 1960; Truman, 1976; Kammer and Kinnamon, 1974). Likewise, late locust embryos that were removed from their egg shells and placed

Table 3. *Locust embryos showing precocious activation of the CCAP network after being manually removed from the chorion*

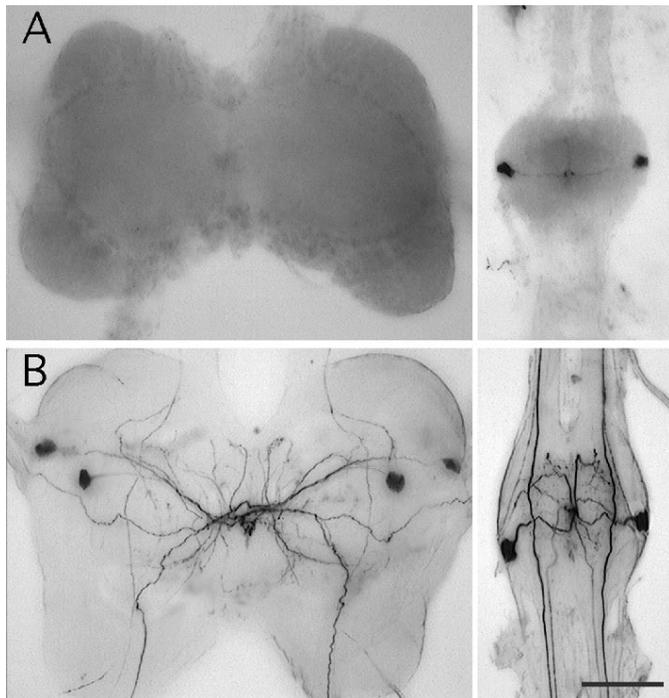|		Number with cyclic-GMP-immunopositive CCAP cells	
Developmental stage[a] (%)	*N*	Abdominal only	Abdominal and thoracic
55–59	9	0	0
60–64	27	0	0
65–69	28	0	0
70–74	12	0	0
75–79	11	2	0
80–84	15	1	0
85–89	15	2	1
90–94	13	1	0
95–99	14	0	7

[a]Percentage of embryonic development (see text).

Fig. 7. Video montages of ganglia in embryos that began spontaneous ecdysis movements within a few minutes after they had been manually removed from the chorion. (A) An embryo at 80–85 % embryogenesis; all the ganglia, including T1 (left), are devoid of cyclic-GMP-immunoreactive cells except for ganglion A7 (right), in which the type 1 CCAP cells show moderate levels of cyclic GMP. (B) Central nervous system from a precociously ecdysing embryo that was taken 5 h before its egg clutch showed spontaneous hatching. Full cyclic GMP expression is evident in ganglia T1 (left) and A5 (right). Scale bar, 100 μm.

in PBS often showed weak ecdysis movements after a delay of a few minutes. These ecdysis movements were also associated with the appearance of cyclic GMP in cells of the CCAP network. As seen in Table 3, the appearance of cyclic GMP in these cells could be induced only during approximately the last 25 % of embryogenesis. Initially, the response was typically confined to the abdominal cells. The number of these cells that responded ranged from only one or two in the younger embryos (Fig. 7A) to the entire abdominal set. Within the last 4–6 h before the hatching of the clutch, the embryos became very sensitive to disturbance. Removing them from the chorion resulted in a very rapid onset of ecdysis movements and activation of the entire network from the SOG to the seventh abdominal ganglion (Fig. 7B). We did not examine how the strength of the precocious ecdysis behaviour was correlated with the extent of the cyclic GMP response in the CCAP cells.

Discussion

Relationship of changes in cyclic GMP levels to the events of hatching

A hatchling locust passes through three behavioural phases during the transition from a hatching vermiform larva to a normally proportioned first-instar hopper. First, the vermiform larva performs a stereotyped hatching behaviour and continues with this behaviour as it digs up through the confining space of the egg pod (Bernays, 1971). Second, when the anterior end of the animal becomes free, the vermiform larva initiates the ecdysis of the embryonic cuticle (Bernays, 1972a). However, given the appropriate conditions, this ecdysing animal can then revert back to digging, albeit with a different leg posture. Third, upon escaping confinement, the animal expands the first-instar cuticle and assumes the proper form of the hopper. Walking and hopping are now possible but digging behaviour can no longer be elicited. Each of these three phases is variable in length and the transition from one to the next is triggered by particular stimuli (or lack thereof). Once made, a transition appears to be irreversible.

The dramatic increase in intracellular cyclic GMP levels seen in the CCAP cells was invariably linked to entry into the ecdysis phase. If the anterior end of the hatching vermiform larva did not contact an obstruction, the insect switched to starting ecdysis within about a minute. Cyclic GMP began to appear in the CCAP cells, and these were invariably cyclic-GMP-immunoreactive by the time of ecdysis (Table 1). This increase in cyclic GMP levels could be delayed for at least an hour by forcing the vermiform larva to dig (Table 2).

The appearance of intracellular cyclic GMP in the CCAP network prior to the onset of ecdysis behaviour has also been observed for larval ecdysis in *M. sexta* (Ewer *et al.* 1994). This relationship in two different species raises the intriguing possibility that the activation of the CCAP network might be the immediate trigger for the start of ecdysis behaviour.

Once initiated, the duration of the cyclic GMP response was subject to modulation by external factors. In animals that were not confined, ecdysis was completed rapidly, and within 2–3 min the hopper had expanded its cuticle to the proper proportions and had started pigmentation. Under these conditions, the cyclic GMP response in the CCAP cells waned within 20–30 min. By contrast, when ecdysis and digging were prolonged, the CCAP cells maintained elevated intracellular cyclic GMP levels for at least twice the normal duration.

In *M. sexta*, the anatomy and peptide content of the cells that show the cyclic GMP increase suggest that these cells mediate physiological changes that occur at ecdysis, such as air-swallowing and increases in heart rate (Ewer *et al.* 1994). In *Locusta migratoria*, both physiological (Bräunig, 1990) and anatomical (Dircksen and Homberg, 1995) studies show that the SOG type 1 cells project to dilator muscles of the pharynx. Importantly, cyclic GMP begins to appear in these cells immediately before the onset of air-swallowing, supporting the involvement of these cells in this behaviour. With respect to circulatory changes, the locust abdominal type 1 CCAP cells project to the heart alary muscles and CCAP has stimulatory effects on the heart (Dircksen *et al.* 1991). Moreover, ecdysing locusts show a dramatic increase in blood pressure, which then gradually falls over the next 30 min (Bernays, 1972a). This change in blood pressure mirrors the changes in cyclic GMP

levels in the abdominal CCAP cells. Besides air-swallowing and circulatory changes, the CCAP cells may also play a role in the filling of the tracheal system. Resorption of fluid and the entry of air into the new tracheae is an important aspect of every ecdysis (Reynolds, 1980). It is especially dramatic, however, at the ecdysis of the embryonic cuticle since the tracheal system of the vermiform larva is filled with fluid, which is rapidly replaced by air when ecdysis begins. The timing of the filling of the tracheal system and the fact that CCAP neurones also project to the spiracles (Dircksen *et al.* 1991) suggest a possible role for CCAP in these tracheal events.

The functions of the medial cells and the caudolateral cells are not known. The time course of cyclic GMP accumulation in the medial cells is intriguing because it starts at about the time of hatching but does not peak until about 30–40 min later, after the CCAP cells have finished their response. These cells are probably involved in some events that occur during the early part of the hopper phase. The caudolateral cells are also interesting because we saw them responding in only one context – as partially ecdysed larvae were digging through the layers of moist cheesecloth. In *L. migratoria*, one pair of these cells also contains CCAP (Dircksen *et al.* 1991). In *M. sexta*, the corresponding cells contain CAPs (Tublitz and Sylwester, 1990) and diuretic hormone (Chen *et al.* 1994) in the larvae and bursicon in the adult (Taghert and Truman, 1982). These cells also occasionally show cGMP-IR during ecdysis in *M. sexta* (J. Ewer and J. W. Truman, unpublished observations). The possibility of affecting diuretic hormone neurones is especially intriguing in the context of an animal digging through a moist environment while showing vigorous swallowing behaviour.

Spread of cyclic GMP through the CCAP network

The ability to induce ecdysis within a minute on command made it possible to examine the time course of changes in cyclic GMP levels within individual cells. The suboesphageal, thoracic and abdominal cells turn on as a unit. Our incomplete staining of the neurones in the SOG precludes a detailed description of the appearance of cyclic GMP in these cells. For the thoracic cells, the first appearance of cyclic GMP was in their medial neurites and their descending axons. Only later did cyclic GMP appear in the cell body. This pattern suggests that the increase in cyclic GMP levels may be initiated by inputs onto the medial neurites of these cells. In *M. sexta*, transection experiments show that the activation of the cyclic GMP response in the abdominal CCAP cells depends on a descending input from the anterior end of the larva (Ewer *et al.* 1994). The axons of the type 2 neurones are perfectly situated to supply such a signal, as they project in the lateral tract through the entire length of the CNS and appear to contact the type 1 cells in each abdominal ganglion. In *L. migratoria*, the appearance of cyclic GMP in these type 2 axons was always accompanied by the induction of cyclic GMP in the type 1 CCAP cells in A4–A7. In this respect, the embryos that showed precocious ecdysis behaviour were interesting because we saw

two types of responses in this network. In some cases, we saw a pattern similar to normal ecdysis, with the axons in the dorsal lateral tract and the abdominal type 1 cells showing the appearance of cGMP-IR. In other cases, we saw only scattered type 1 cells expressing cyclic GMP, but this was in the absence of a response in the descending axons. We think it likely that the rapid, coordinated activation of the abdominal cells is through input from the thoracic type 2 cells *via* their axons in the lateral tract. Consistent with this hypothesis, we find that cyclic GMP in the abdominal cells appears simultaneously in the cell bodies and the medial neurites. These two regions are similar distances away from the proposed contact point with the type 2 axons in the lateral tract.

Relationship of the CCAP network to eclosion hormone

Direct demonstration of the role of EH in triggering the behaviour patterns associated with ecdysis has been made only for Lepidoptera (Truman, 1992). In *M. sexta*, for example, release of EH is followed by the performance of the pre-ecdysis behaviour, with the subsequent activation of the CCAP network and ecdysis occurring 30–40 min later. EH bioactivity has been found in grasshoppers (Truman *et al.* 1981), and recent work using the polymerase chain reaction has provided a partial nucleotide sequence for the EH gene of *L. migratoria* (F. M. Horodyski, personal communication). Nevertheless, we do not know the timing of EH release in *L. migratoria* relative to the events of hatching and ecdysis. EH is probably released prior to hatching. According to this scenario, the hatching and digging behaviour of the vermiform larva would then be comparable to the pre-ecdysis behaviour of *M. sexta*. Although the pre-ecdysis behaviour of larval *M. sexta* is generally considered to be relatively fixed in duration, ecdysis is delayed when pre-ecdysing larvae are placed on smooth substrata that they cannot grip with their prolegs (J. W. Truman, unpublished observations). Thus, even in *M. sexta*, the duration of this initial phase is somewhat flexible and sensitive to external conditions. In both *M. sexta* and *L. migratoria* the onset of ecdysis behaviour is tightly linked to the activation of the cells in the CCAP network. Whether this relationship results from both systems responding to a common input or whether CCAP release is actually the proximal trigger for ecdysis remains to be established.

References

BENTLEY, D., KESHISHIAN, H., SHANKLAND, M. AND TOROIAN-RAYMOND, A. (1979). Quantitative staging of embryonic development of the grasshopper, *Schistocerca nitens. J. Embryol. exp. Morph.* **54**, 47–74.

BERNAYS, E. A. (1971). The vermiform larva of *Schistocerca gregaria* (Forskål): form and activity (Insect: Orthoptera). *Z. Morph. Tiere* **70**, 183–200.

Bernays, E. A. (1972a). The intermediate moult (first ecdysis) of *Schistocerca gregaria* (Forskål) (Insecta, Orthoptera). *Z. Morph. Tiere* **71**, 160–179.

Bernays, E. A. (1972b). The muscles of newly hatched *Schistocerca gregaria* larvae and their possible function in hatching, digging and ecdysial movements (Insecta, Acrididae). *J. Zool., Lond.* **166**, 141–158.

Blest, A. D. (1960). The evolution, ontogeny and quantitative control of settling movements of some New World saturniid moths, with some comments on distance communication by honey bees. *Behaviour* **16**, 188–253.

Braünig, P. (1990). The morphology of suboesophageal ganglion cells innervating the nervus corporis cardiaci III of the locust. *Cell Tissue Res.* **260**, 95–108.

Chen, Y., Veenstra, J. A., Hagedorn, H. and Davis, N. T. (1994). Leucokinin and diuretic hormone immunoreactivity of neurons in the tobacco hornworm, *Manduca sexta* and co-localization of this immunoreactivity in lateral neurosecretory cells of the abdominal ganglia. *Cell Tissue Res.* **278**, 493–507.

Cheung, C. C., Loi, P. K., Sylwester, A. W., Lee, T. D. and Tublitz, N. J. (1992). Primary structure of a cardioactive neuropeptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett.* **313**, 165–168.

De Vente, J., Steinbusch, H. W. M. and Schipper, J. (1987). A new approach to immunocytochemistry of 3′,5′-cyclic guanosine monophosphate: preparation, specificity and initial application of a new antiserum against formaldehyde-fixed 3′,5′-cyclic guanosine monophosphate. *Neurosci.* **22**, 361–373.

Dircksen, H. (1994). Distribution and physiology of crustacean cardioactive peptide in arthropods. *Perspectives in Comparative Endocrinology* (ed. K. G. Davey, R. E. Peter and S. S. Tobe), pp. 139–148. Ottawa: National Research Council of Canada.

Dircksen, H. and Homberg, U. (1995). Crustacean cardioactive peptide-immunoreactive neurons innervating brain neuropils, retrocerebral complex and stomatogastric nervous system of the locust, *Locusta migratoria*. *Cell Tissue Res.* **279**, 495–515.

Dircksen, H. and Keller, R. (1988). Immunocytochemical localization of CCAP, a novel cardioactive peptide, in the nervous system of the shore crab, *Carcinus maenas* L. *Cell Tissue Res.* **254**, 347–360.

Dircksen, H., Muller, A. and Keller, R. (1991). Crustacean cardioactive peptide in the nervous system of the locust, *Locusta migratoria*: an immunocytochemical study on the ventral nerve cord and peripheral innervation. *Cell Tissue Res.* **263**, 439–457.

Ewer, J., De Vente, J. and Truman, J. W. (1994). Neuropeptide induction of cyclic GMP increases in the insect CNS: resolution at the level of single identifiable neurons. *J. Neurosci.* **14**, 7704–7712.

Ewer, J. and Truman, J. W. (1996). Increases in cyclic GMP occur at ecdysis in an evolutionarily conserved insect neuronal network. *J. comp. Neurol.* (in press).

Gammie, S. C., Ewer, J. and Truman, J. W. (1994). An endogenous increase in cGMP is associated with increased excitability in identified neurosecretory cells in *Manduca sexta*. *Soc. Neurosci. Abstr.* **20**, 1604.

Hewes, R. S. and Truman, J. W. (1991). The roles of central and peripheral eclosion hormone release in the control of ecdysis behavior in *Manduca sexta*. *J. comp. Physiol.* A **168**, 697–707.

Kammer, A. E. and Kinnamon, S. C. (1974). Patterned muscle activity during eclosion in the hawkmoth *Manduca sexta*. *J. comp. Physiol.* A **114**, 313–326.

Reynolds, S. E. (1980). Integration of behavior and physiology in ecdysis. *Adv. Insect Physiol.* **15**, 475–595.

Taghert, P. H. and Truman, J. W. (1982). Identification of the bursicon-containing neurones in abdominal ganglia of the tobacco hornworm, *Manduca sexta*. *J. exp. Biol.* **98**, 385–401.

Truman, J. W. (1976). Development and hormonal release of adult behavior patterns in silkmoths. *J. comp. Physiol.* **107**, 39–48.

Truman, J. W. (1978). Hormonal release of stereotyped motor programmes from the isolated nervous system of the *Cecropia* silkworm. *J. exp. Biol.* **74**, 151–173.

Truman, J. W. (1992). The eclosion hormone system of insects. *Prog. Brain Res.* **92**, 361–374.

Truman, J. W., Taghert, P. H., Copenhaver, P. F., Tublitz, N. J. and Schwartz, L. M. (1981). Eclosion hormone may control all ecdyses in insects. *Nature* **291**, 70–71.

Tublitz, N. J. and Evans, P. D. (1986). Insect cardioactive peptides: cardioacceleratory peptide (CAP) activity is blocked *in vivo* and *in vitro* with a monoclonal antibody. *J. Neurosci.* **6**, 2451–2456.

Tublitz, N. J. and Sylwester, A. W. (1990). Postembryonic alteration of transmitter phenotype in individually identified peptidergic neurons. *J. Neurosci.* **10**, 161–168.

Tublitz, N. J. and Truman, J. W. (1985). Insect cardioactive peptides. II. Neurohormonal control of heart activity by two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. *J. exp. Biol.* **114**, 381–395.

Watson, A. H. D. and Burrows, M. (1981). Input and output synapses on identified motor neurones of a locust revealed by intracellular injection of horseradish peroxidase. *Cell Tissue Res.* **215**, 325–332.