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The locust frontal ganglion: a central pattern generator network controlling foregut rhythmic motor patterns

Amir Ayali*, Yael Zilberstein and Netta Cohen
Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
*Author for correspondence (e-mail: ayali@post.tau.ac.il)

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Summary

The frontal ganglion (FG) is part of the insect stomatogastric nervous system and is found in most insect orders. Previous work has shown that in the desert locust, *Schistocerca gregaria*, the FG constitutes a major source of innervation to the foregut. In an *in vitro* preparation, isolated from all descending and sensory inputs, the FG spontaneously generated rhythmic multi-unit bursts of action potentials that could be recorded from all its efferent nerves. The consistent endogenous FG rhythmic pattern indicates the presence of a central pattern generator network. We found the appearance of *in vitro* rhythmic activity to be strongly correlated with the physiological state of the donor locust. A robust pattern emerged only after a period of saline superfusion, if the locust had a very full foregut and crop, or if the animal was close to ecdysis. Accordingly, haemolymph collected at these stages inhibited an ongoing rhythmic pattern when applied onto the ganglion. We present this novel central pattern generating system as a basis for future work on the neural network characterisation and its role in generating and controlling behaviour.

Key words: desert locust, *Schistocerca gregaria*, central pattern generator, frontal ganglion, neuromodulation.

Introduction

To study the generation and control of a specific behaviour, an important first step is to identify the neural networks that coordinate the behaviour. Rhythmic motor patterns, such as locomotion, mastication and ventilation, are types of behaviours that are generated by neural circuits called central pattern generators (CPGs) (Delcomyn, 1980; Getting, 1989; Pearson, 1993; Marder and Calabrese, 1996; Marder, 2000; Marder and Bucher, 2001). CPGs are further defined as ensembles of neural elements whose properties and connectivity can give rise to characteristic patterns of rhythmic activity in the absence of external inputs. The desert locust has served as a leading system for studies of pattern generation and the control of rhythmic motor patterns (e.g. Wilson, 1961; Wolf and Pearson, 1987; Wolf and Pearson, 1989).

In the locust, the frontal ganglion (FG) is part of the stomatogastric nervous system (STNS). The FG lies in the forehead, on the dorsal side of the pharynx, in front of the brain (Fig. 1A). It is a small ganglion connected to the tritocerebrum of the brain by the paired frontal connectives (FC; Fig. 1B). Posteriorly, a recurrent nerve (RN) passes from the FG along the pharynx to the hypocerebral ganglion (HG; Fig. 1B), which is closely associated with the corpora cardiaca. These and other three pairs of efferent nerves (the anterior, median and posterior nerves; APN, MPN and PPN respectively) branch onto the dilator muscles of the gut in a rostrum to caudal order, making the FG the major source of foregut muscles innervation (Fig. 1B; Allum, 1973; Aubele and Klemm, 1977). To date, no studies have examined the neural control of foregut peristalsis and the role of the FG in generating motor patterns associated with locust foregut movements. Descriptions of the fine structure and neuronal organisation of the FG in locusts are also very scarce (e.g. Aubele and Klemm, 1977).

We report here on a series of experiments that demonstrate the presence of a CPG network in the FG of the locust *Schistocerca gregaria*. Our findings, described in this and the accompanying paper, provide insights into the neural basis, control and neuro-endocrine modulation of two fundamental behaviours in the life of locusts: feeding and moulting.

Materials and methods

Animals

Schistocerca gregaria (Forskal) were reared under crowded conditions, in 60-litre metal cages (Hunter-Jones, 1961). The cages were kept at a controlled temperature of 30°C, under a 12 h:12 h L:D lighting regime. Additional heat, including direct radiant heat, was supplied during daytime by incandescent electric bulbs to bring the day temperature to 36–38°C. Locusts were fed daily with fresh Kikuyu grass and flaked oats. Last larval instar and adult locusts of both sexes were used for experiments.

Saline and chemicals

Locust saline contained 147 mmol l^{-1} NaCl, 10 mmol l^{-1}

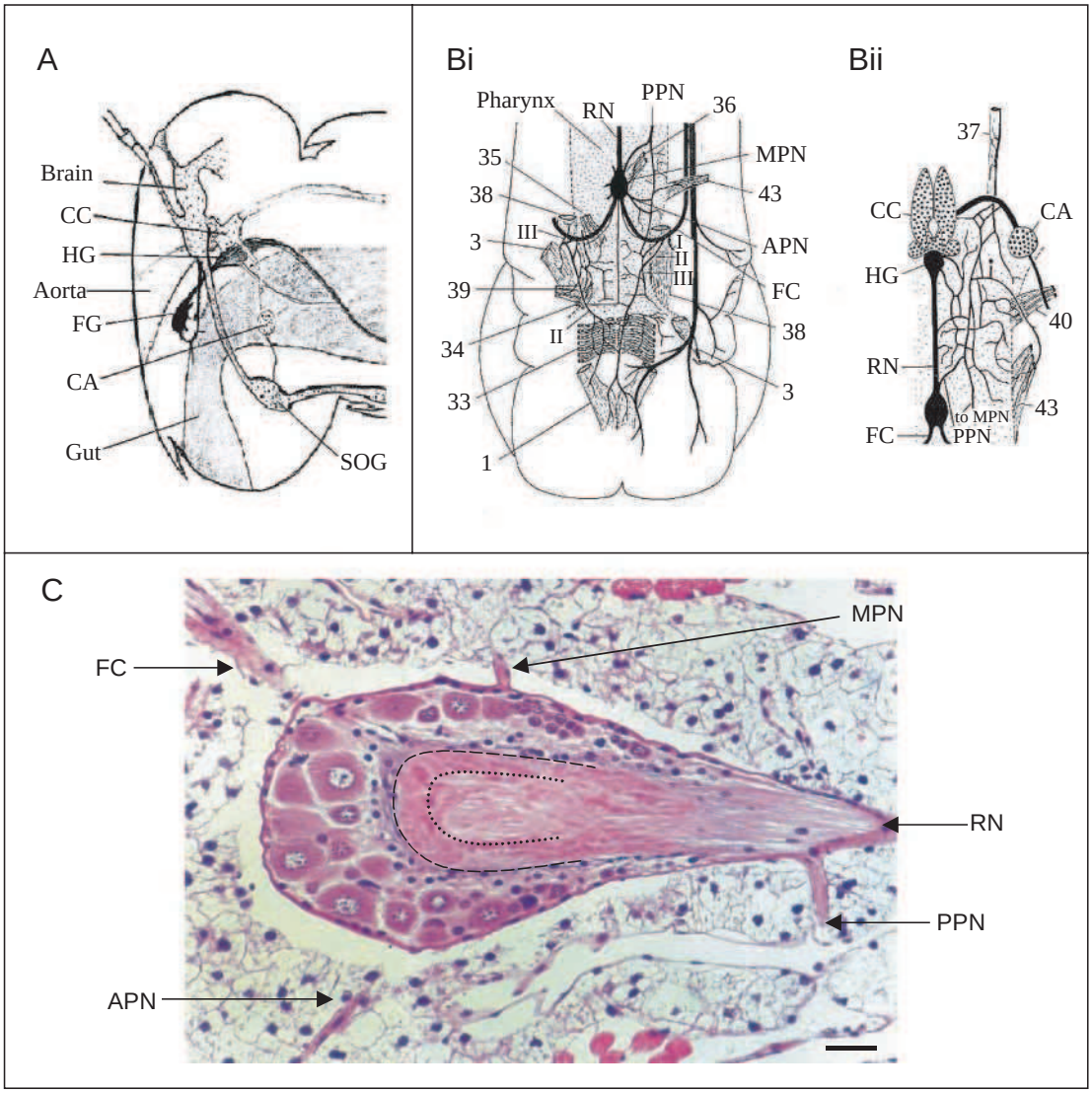

Fig. 1. (A) A sectional diagram of the locust head showing the relative position of the frontal ganglion (FG). CC, corpora cardiaca; HG, hypocerebral ganglion; CA, corpora allata; SOG, suboesophageal ganglion. (Bi, Bii) Schematic drawing of the FG, the nerves leaving it and the muscles they innervate (Bii shows an area more posterior to Bi, after Allum, 1973). RN, recurrent nerve; PPN, posterior pharyngeal nerve; MPN, median pharyngeal nerve; APN, anterior pharyngeal nerve; FC, frontal connective; I, II and III, first, second and third branch of the FC; 1 and 3, muscles of the labrum; 33 and 34, first and second anterior dilators of cibarium; 35, 36 and 37, first, second and third dorsal dilators of pharynx; 39 and 40, first and second lateral dilators of pharynx; 43 second ventral dilator of pharynx. (C) A frontal section through the center of a whole-mount Hematoxylin and Eosin-stained FG. The neuronal cell bodies are in the peripheral zone, surrounding the neuropil (marked by a dashed line). The coarse neuropil in the central core of the ganglion (marked by a dotted line) can be distinguished from the surrounding finer neuropil. The RN and one of each of the paired nerves are visible and marked by arrows. Scale bar, 50 mm.

KCl, 4 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} NaOH, 10 mmol l^{-1} Hepes, pH 7.2–7.4 (Abrams and Pearson, 1982; Penzlin, 1985). All salts were obtained from Frutarom Ltd (Haifa, Israel).

Histology and anatomy

In order to examine the structure and cellular composition of the locust frontal ganglion, whole heads from newly emerged adults were fixed in aqueous Bouin's fixative, embedded in paraffin, sectioned at 8 μm, and stained with Hematoxylin and Eosin.

Physiology

Locusts were anaesthetised in CO_2. The anterior parts of the locust STNS were easily accessed by opening a window in the head cuticle, cutting out most of the frons, and clearing fat tissue and air sacs. Foregut movements were observed or monitored by a force transducer attached to the gut wall. The activity of a specific gut dilator muscle was recorded, using fine (125–175 μm) silver wire electrodes insulated to their tip. The force transducer output or muscle activity recordings were accompanied by extracellular recordings from various FG

nerves using silver wire hook electrodes that were electrically insulated with white petroleum jel (Vaseline).

For the *in vitro* preparation, the FG and nerves leaving it were accessed as above, dissected out, pinned in a Petri dish lined with Sylgard (Dow Corning, Midland, MI, USA), and constantly superfused with locust saline at 26–27°C. Extracellular recordings were made with bipolar stainless-steel pin electrodes. The recording site was insulated from the bath with white petroleum gel.

Haemolymph was drawn with a pipette from a puncture made in the membrane at the base of the metathoracic leg of the chosen donor (see Results for characteristics of the donors), and applied directly on the FG to test modulatory effects. The FG pattern was tested before, during and after 10 min of application, as well as after at least 30-min washout.

Data were recorded using a 4-channel differential amplifier (Model 1700 A-M Systems), played back in real time and stored on the computer using an A-D board (Digidata 1200, Axon instruments) and Axoscope software (Axon instruments).

Data analysis

Burst profiles and phase diagrams were obtained as follows. Peak detection was performed on each signal, yielding a set of peak times and corresponding peak amplitudes (t_i, a_i). From these, cumulative peak amplitudes were calculated

$$A_i = \sum_{j=1}^{i} a_j \, .$$

Plots of the cumulative peak amplitudes as a function of time $A(t)$, where $A_i \equiv A(t_i)$, were used as a visual tool for detecting changes in the intensity of activity. In particular, this measure, combining event density and peak amplitudes, is especially well suited for detecting burst onset times. A sharp rise in slope corresponds to a sudden increase in activity. For convenience, cumulative amplitudes were normalised $A(t) \rightarrow A(t)/A(T)$, where T is the estimated time at which pre-burst activity was regained. Thus, only relative changes in burst intensity were examined. The burst onset times were determined from plots of cumulative amplitudes by linear extrapolation. To quantitatively characterise burst progress in the different nerves, FC burst onset times were used as temporal offsets for superimposing the cumulative amplitude plots for a series of consecutive bursts. For each burst, FC, MPN and PPN cumulative amplitudes at the offset time were used as the corresponding vertical offsets. The characteristic burst profiles, $\bar{A}(t)$, were then obtained by averaging cumulative amplitude plots over different bursts. For each nerve, the following burst parameters could be extracted from the characteristic profiles: burst onset time, end time, pre- and post-burst inhibition and activity recovery time. In addition, the derivative of the cumulative amplitude plots $d/dt\ \bar{A}(t)$ corresponds to the burst energy density function and provides a characteristic burst envelope. All analysis methods were developed by one of us (N.C.) under a Fortran and MatLab (Mathworks) environments.

Results

Anatomy

The locust FG is a small ganglion shaped like a somewhat flattened pear, 200–250 μm in its long axis (Fig. 1). It is encased by a fine perineural sheath, and characterised by a central neuropil surrounded dorsally and laterally by one or two layers of neuronal cell bodies (Fig. 1C). The neuropil can be subdivided into a central region of coarse neuropil and a peripheral finer region (Fig. 1C). The cell bodies range in size from 10 to 50 μm in diameter.

According to a previous electron microscopy study (Allum, 1973) the recurrent nerve and the frontal connectives contains *circa* 2200 and 1200 axon profiles, respectively, as counted in a section made close to their ganglion origin. The smaller nerves have far fewer fibers (32 axon profiles counted in a PPN section). Staining ganglion neurons by back-filling the various nerves indicated that only a small fraction of all these nerve fibers originate in neurons located within the ganglion (data not shown).

The frontal ganglion controls foregut dilators

In order to reconfirm previous work regarding the role of the frontal ganglion in generating foregut movements (see Discussion), we monitored foregut movements while recording the motor output of the FG. In agreement with early results (Allum, 1973, and references within), foregut peristaltic activity was never observed when we either dissected the FG out, or cut all the FG efferent nerves. Fig. 2A demonstrates fixed synchronisation between bursts of action potentials recorded extracellularly from the recurrent nerve (RN), and foregut dilation monitored by a force transducer attached to the

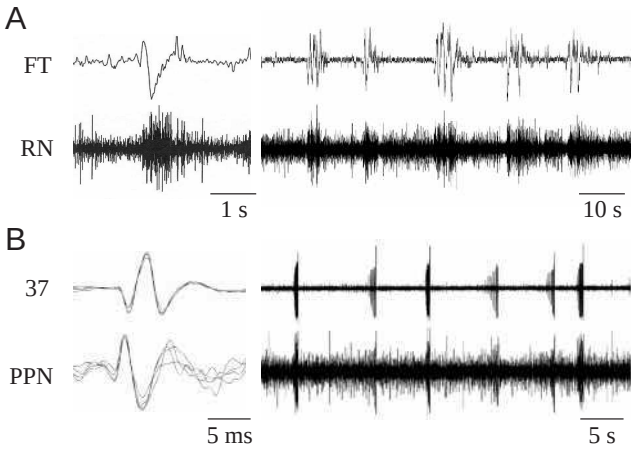

Fig. 2. FG motor output *in vivo* can be correlated to foregut movement and to specific gut dilators. (A) Extracellular recording from the recurrent nerve (RN) and the simultaneous output of a force transducer (FT) connected to the esophagus wall in a fully intact locust. (B) Simultaneous extracellular recordings from the posterior pharyngeal nerve (PPN), and from the third dorsal dilator of the pharynx (37) *in vivo*. Inset on left shows 5 overlaid sweeps triggered by the large unit in the PPN trace, demonstrating 1:1 relationship between this unit and the muscle activity.

oesophagus. This result was confirmed by recordings from the FC or PPN. Activity in the third dorsal dilator of the pharynx (muscle 37) could be correlated to bursts of action potentials recorded extracellularly from the PPN (Fig. 2B). We only had limited access to the gut while recording from the nerves, so could not correlate movements of different areas of the foregut to activity recorded on the different nerves.

The frontal ganglion central pattern generator

In an *in vitro* preparation, isolated from all descending and sensory inputs, the FG generated spontaneous rhythmic activity. Over 90% of all *in vitro* preparations displayed a consistent and robust FG rhythmic pattern that lasted for several hours (cycle period 15.8±5.9 s, mean ± S.D. of 48 preparations, 10 cycles per preparation). In many cases rhythmic activity was not obtained immediately but emerged slowly and gradually (Fig. 3). We found the time of first appearance of bursting activity to be strongly related to the physiological state of the donor locust. In preparations from locusts with a very full foregut and crop at the time of dissection, or from non-feeding larvae close to ecdysis, a robust pattern emerged after up to 2 h of saline superfusion (1.5±0.6 h, *N*=10; Fig. 3). The rhythm was the fastest to appear (10 min or less) when the ganglion was dissected out from a locust just a few minutes after it had begun feeding. Accordingly the application of haemolymph collected from animals in which the alimentary canal was replete throughout its length, strongly inhibited the FG motor output (five different preparations, Fig. 4A). The effects of haemolymph application varied from total inhibition, as in the example shown in Fig. 4A, to inhibition of the bursting activity only. Haemolymph collected from non-feeding pre-moult larvae also inhibited FG rhythmic activity, always giving rise to arrhythmic activity as demonstrated in Fig. 4B (five out of five different FG preparations and fifth instar larva haemolymph donors). Application of haemolymph collected from fifth instar larva just after the initiation of feeding had no inhibitory effect whatsoever (five different preparations, Fig. 4C).

The frontal ganglion rhythm

Spontaneous multi-unit bursts of action potentials could be recorded from all the FG efferent nerves (Fig. 5). Recordings from the bilateral pairs of nerves (FC nerves, MPN and PPN) revealed that they all exhibited fully synchronised activity (including both bursts and inter-burst activity; data not shown). The activity recorded on the APN (three preparations, not shown) was consistent with our observations as described here for the other nerves. APN recordings were rare since this nerve is exceptionally thin and delicate. In the recurrent nerve, bursting activity was often masked by arrhythmic background spiking activity (in four out of ten recordings; not shown).

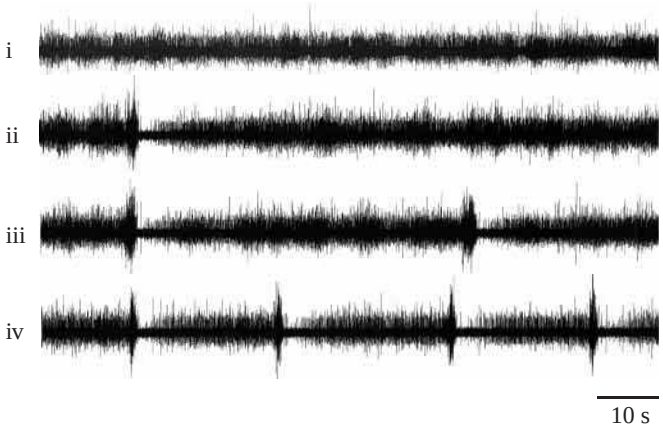

Fig. 3. Emergence of the FG rhythm *in vitro*. A continuous frontal connective recording in a ganglion totally isolated from all descending and sensory inputs, dissected out from a locust with very full crop and gut. (i-iv) 15, 45, 60 and 75 min, respectively, after dissection.

Simultaneous extracellular recordings from the different FG efferent nerves revealed fixed-phase relations in burst onset times, consistent with a rostrum-to-caudal innervation sequence of foregut muscles (i.e. the order FC, MPN, PPN, in efferent nerve bursts, Fig. 5B). A similar pattern *in vivo* would have resulted in a peristaltic anterior-to-posterior wave of muscle contraction along the locust foregut.

As shown in the cumulative amplitude plots of Figs 5 and 6, each nerve displayed a characteristic burst profile. The temporal delineation of burst progression, as recorded

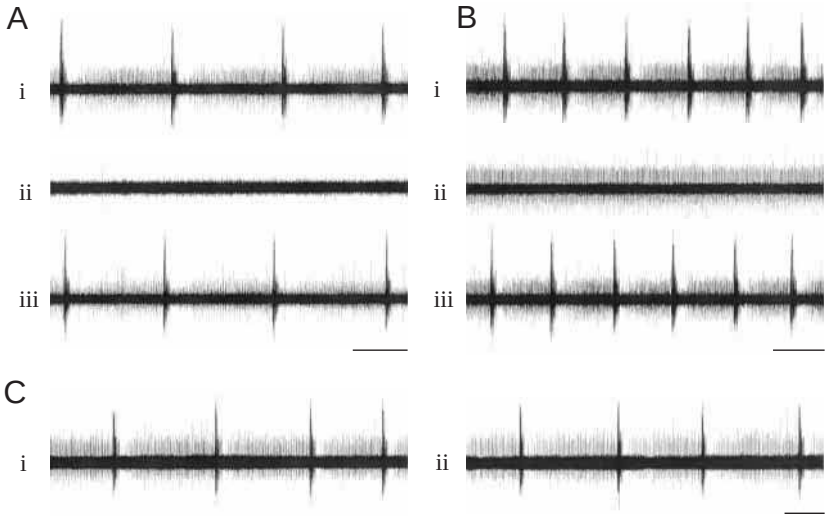

Fig. 4. (A) The effect of haemolymph collected from a fifth instar larva with a very full crop, on an ongoing FG rhythm *in vitro*, as seen in a frontal connective extracellular recording. (B) As in A; haemolymph collected from a non-feeding pre-molt fifth instar larvae. (C) As in A and B; haemolymph collected from a fifth instar larvae just after the initiation of feeding. In all panels, (i) is control; (ii) haemolymph application; (iii) wash. Scale bars, 10 s.

extracellularly, is summarised in Fig. 7. In all our preparations we have recorded at least one of the FC nerves accompanied by simultaneous recordings of minor nerves, MPN or PPN or both. Thus the FC burst was used for alignment and normalization (see figure legend for details). The burst onset appears in the form of intensified activity in the FC nerve (either sudden or gradual), characterised by increased spike density as well as spike amplitudes. This early FC nerve activity typically coincides with strong inhibition on the PPN. About one third of our PPN recordings showed an additional early unit, coinciding with the early units in the FC nerve; all other PPN units were still strongly suppressed at this phase (Fig. 6). Bursting activity followed in the MPN and finally in the PPN. In all nerves, bursting typically ended with a gradual decay of activity, with MPN activity ending first or concomitantly with FC bursting, and PPN activity ending with a slight delay thereafter (Fig. 7). All nerves exhibited strong post-burst inhibition and a slow recovery of inter-burst levels of activity (with the PPN recovering first 3–5 s after the onset of the burst, followed by the MPN, and finally by FC nerves, which typically displayed the slowest recovery of activity, taking as long as 15 s; Fig. 7).

Burst profiles from different experiments were found to be robust and independent of the level of inter-burst activity and of cycle periods (Fig. 7C). In addition the temporal relations between different bursting units (whether recorded on the same or different nerves) were generally conserved. Thus, while burst durations were relatively robust (1.8±0.5 s in FC nerves, and 1.3±0.3 s for MPN and PPN), the fraction of burst to cycle time varied drastically (e.g. 2–33% in FC nerve bursts, N=15). In other words, converting Fig. 7C to a conventional (linear) phase diagram (by normalising the time axis by burst cycle periods) would yield a smeared, highly variable burst profile, in sharp contrast to the robust temporal description. This result demonstrates that during a burst cycle, the phase increases nonlinearly with time. In effect, this description translates to a high phase velocity during the burst itself, and much slower phase increase in the ensuing inter-burst phase. Figs 5–7 were prepared from the recordings most suitable for the type of analysis made. It is important to note that the results of all of the experiments performed were examined for consistency with the data presented.

In addition to the above extracellular nerve recordings, which capture the motoneuron activity in the various efferent nerves, intracellular recordings are needed to elucidate the structure of the underlying neural network, to locate and to characterise the key pattern-generating neurons. In fact, probing many FG neurons with an intracellular pipette in a number of preparations revealed that the large majority of neurons do not show oscillations of membrane potential or any rhythmic pattern of activity, and are either silent or fire tonically. Experimental manipulations with the membrane potential of these cells (strong stimulation and hyperpolarization; data not shown) suggested that only a small number of the FG neurons are part of the CPG circuit. A small number of the recorded neurons did exhibit rhythmic activity. Fig. 8 shows examples of rhythmic inhibition or bursting potentials, as demonstrated by intracellular recordings from FG neurons, while a robust rhythm was recorded extracellularly. The neuron shown in Fig. 8A corresponds to the large units recorded extracellularly on the FC nerve.

Discussion

The current paper presents our results on the hitherto little-explored FG of locusts. The FG is a principal component of the insect stomatogastric nervous system (STNS) in most insect orders (Penzlin, 1985; Chapman, 1985). Ample previous results have indicated that in locusts, as in other insects, the FG is important for the control of food passage through the gut and for crop emptying (Highnam et al., 1966; Hill et al., 1966; Bignell, 1973). We have established the presence of a CPG circuit in the locust FG; a spontaneous rhythmic motor pattern was generated by FG neurons in the total absence of descending or sensory inputs. Previous work in *P. americana* described spontaneous burst activity in the STNS and specifically in the FG (Hertel, 1978; Hertel et al., 1978; Hertel and Penzlin, 1982; Pandey and Habibulla, 1982), yet the function of this activity remained unclear (Penzlin, 1985). We explored the motor pattern by recording extracellularly from all the locust FG efferent nerves. When analysing the temporal delineation of bursts of action potentials recorded on the motor nerves, and considering the muscles innervated by these nerves, the FG rhythmic pattern could be defined as fictive feeding-related or 'food passage' behaviour. The pattern was characterised by fixed phase relations in burst onset times, consistent with a rostrum-to-caudal innervation sequence in foregut muscles. While the FG rhythmic bursting activity was robust and well characterised, the inter-burst tonic activity varied in duration and intensity, both in time and from one preparation to another, but had no apparent effect on the burst profile itself. The two different patterns of burst activity that were observed in the PPN signals may possibly indicate different behaviours, which require closer investigation.

We correlated the FG motor output with foregut movements *in vivo*, thus confirming earlier reports on the FG control of the foregut. In the accompanying paper (Zilberstein and Ayali, 2002) we investigate the FG motor patterns *in vivo* and their role in the control of multiple foregut behaviours.

The locust FG contains around one hundred neurons. Are all these neurons members of the pattern generating network(s)? Many of the FG neurons are neurosecretory cells containing a variety of neuropeptides (Miyoshi and Endo, 1998; Maestro et al., 1998; Duve et al., 1999, 2000). Aubele and Klemm (1977) described 19 neurons located in the FG that send their axons to innervate foregut muscles *via* the FC and FC 1. In most CPG systems investigated the pattern-generating circuit consists of interneurons, though in some preparations the motor neurons themselves participate in generating the rhythm (e.g. the STNS of Crustacea) (for references, see Marder and Bucher, 2001). The rather limited number of rhythmic units in our FG nerve recordings, and our preliminary intracellular survey of FG

neurons in which the majority of neurons proved to be either silent or tonically active, are both consistent with the idea that a relatively small number of FG neurons take part in the FG rhythmic motor pattern. However, this point awaits further characterisation of the CPG.

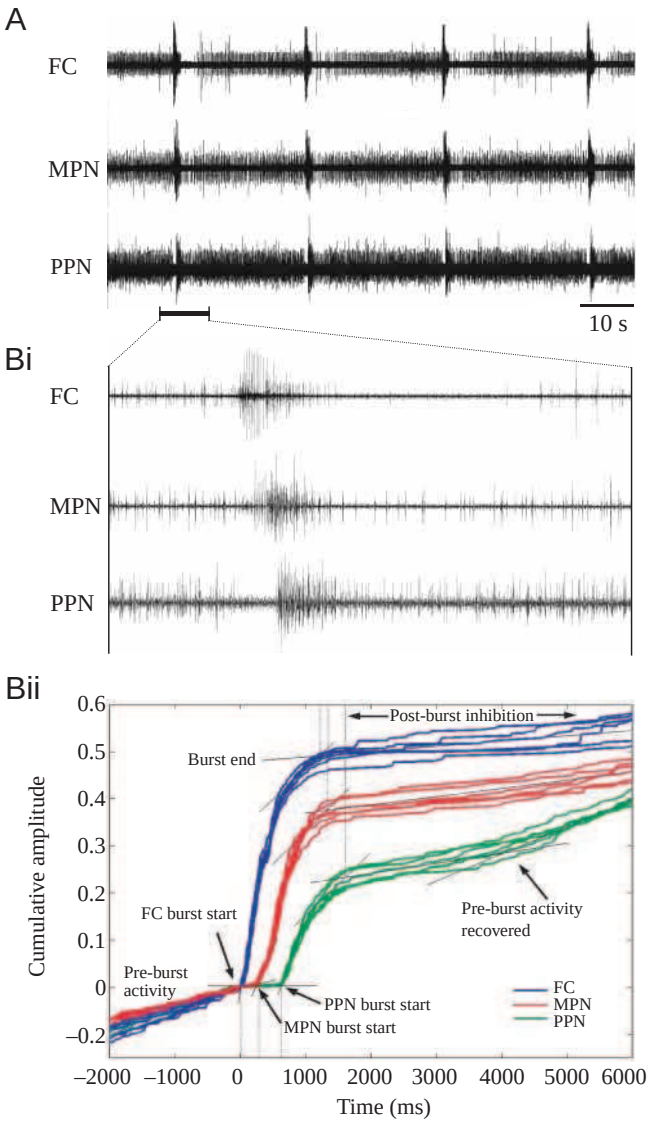

Fig. 5. (A) Simultaneous extracellular recording from three of the FG efferent nerves (see Fig. 1) *in vitro*. Data demonstrate spontaneous rhythmic bursting activity in all the recorded nerves. (B) The area marked by the bar in A is amplified to reveal a three-phase rhythmic pattern of the different members of the FG pattern generator (Bi). The graph in Bii shows burst profiles of the activity recorded on the three nerves in the same time window as in Bi. The cumulative peak amplitudes are plotted as a function of time (see Materials and methods). Six consecutive sweeps as in Bi were normalized and overlaid. Different bursts display similar profiles. Different nerves have distinct profiles and exhibit clear time lags in their onset times, burst end times, and recovery profile. Quantitative parameterization of the burst is performed by linear fits to different phases of the bursts (corresponding to normalized rates) and extrapolation of the crossover times between these linear regimes (marked by dotted lines). These analyses are performed on multi-burst average profiles, and are only schematically shown in the figure.

The concept of neuromodulation is central to our current understanding of central pattern generation. Our results suggest that the FG CPG is modulated *in vivo*; a humoral factor affecting foregut activity by inhibiting the FG rhythmic output is present in the haemolymph of feeding animals, when the entire gut is stuffed with food and the foregut should stop pushing its content backwards, or in pre-moult larvae that do not feed at all. Hence, in our haemolymph application experiments, rhythmic activity was very slow to appear in the isolated FG when the physiological state of the donor animal was one of the above. It is nowadays clear that the nervous system can alter the properties of CPGs, *via* both descending and sensory inputs, to elicit many different motor patterns (e.g. Harris-Warrick and Marder, 1991; Grillner et al., 1994; Harris-Warrick, 1994; Marder et al., 1994; Ayali and Harris-Warrick, 1999).

Recent years have witnessed rekindled interest in the insect STNS. It has been shown to be an important source of

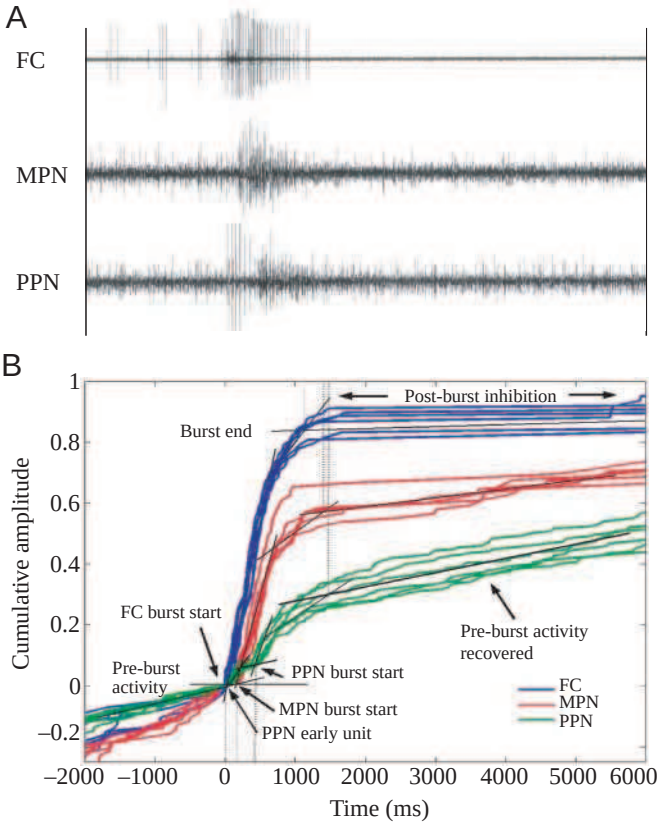

Fig. 6. (A) An example of a simultaneous extracellular recording from three of the FG efferent nerves, in which an additional early unit participated in the PPN burst (large spikes). The time window shown corresponds to that in Fig. 5B. (B) Here, FC nerve and PPN bursts commence together (compare to Fig. 5Bii). The early PPN activity is dominated by a single unit generating 2–4 high-amplitude spikes. Other PPN units appear to be unaffected by this early unit (see, for instance, the return to low spiking rate between the early PPN activity and the main burst).

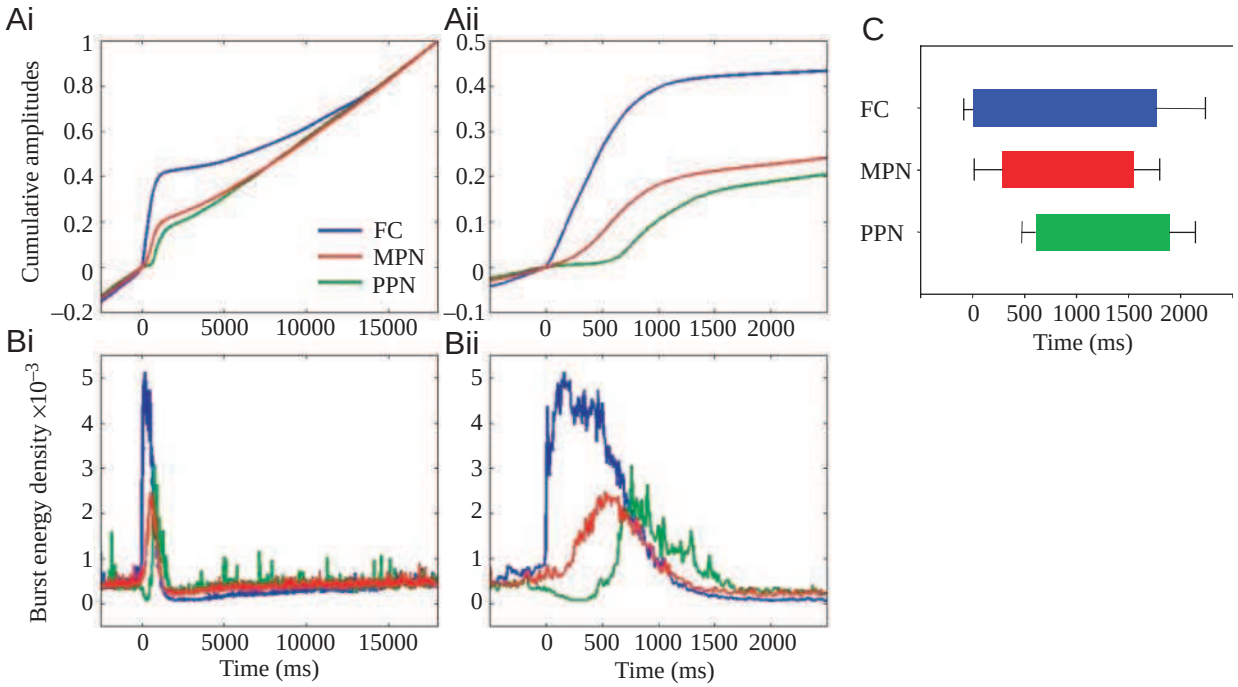

Fig. 7. Burst profile representations, averaged over 15 experiments (chosen from those in which the PPN did not include activity of an early unit). The mean cumulative amplitude burst profiles were calculated for each recording. (Ai) Mean cumulative amplitude burst profiles averaged over all preparations. (Aii) Magnification of the burst time window. (Bi, Bii) The first derivative of the above, normalized to yield burst energy densities. Again, the left and right columns differ only by the horizontal (time) domain. Note the strong pre-burst PPN inhibition and the post-burst inhibition in all three nerves. (C) Time-lag diagram calculated by linear extrapolation of burst onset and termination times in cumulative amplitude traces from each of the recordings. The diagram summarizes burst temporal progression in the three nerves (means ± S.D.). *N*=15, 14 and 5 for FC, MPN and PPN, respectively.

neuropeptides that take part in controlling gut movements in Orthoptern and Lepidopteran insects (Duve et al., 1995, 1999, 2000; Miyoshi and Endo, 1998; Maestro et al., 1998). The insect STNS is also emerging as a model system for nervous system development (e.g. Copenhaver and Taghert, 1989a,b, 1991; Ganfornina et al., 1996; Hartenstein, 1997; Forjanic et al., 1997; Boleli et al., 1998; Gonzalez-Gaitan and Jackle, 2000). Surprisingly, with the notable exception of Miles and Booker (1994, 1998), little research has been conducted on the STNS neural control of gut motor patterns. The current study will help to fill this gap, and provide the necessary physiological basis for this promising model system.

From an evolutionary point of view, it is intriguing to compare the relatively unexplored insect system to the STNS of Crustacea, specifically to the stomatogastric ganglion (STG) of lobster and crabs (Harris-Warrick et al., 1992). The latter has been established as a leading model system for the study of pattern-generating circuits. It has fewer neurons (approx. 30) and, unlike our system, the STG requires chemical modulation in order to exhibit its rhythmic motor output. Further research is needed to elucidate other differences as well as similarities between the two systems.

In summary, our work presents a novel CPG network in the locust FG, and the motor patterns it generates. Together with the adjacent paper, this is part of an investigation into the regulation of motor patterns associated with locust feeding and the control of moult-related gut motor patterns, and thus to fill a gap in our knowledge and understanding of this economically important pest insect.

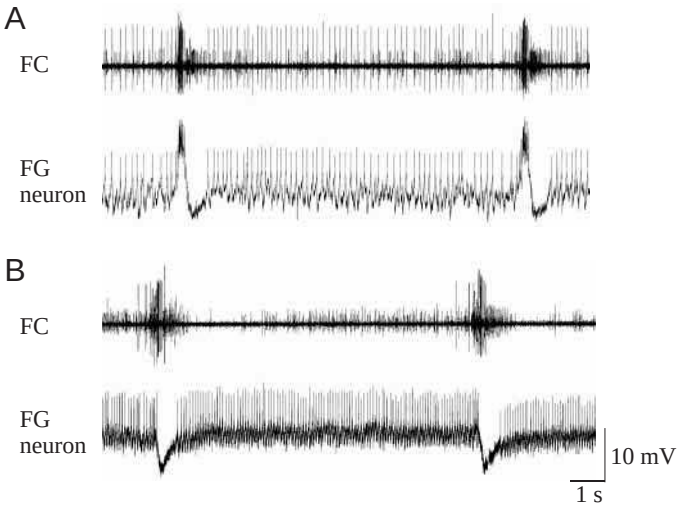

Fig. 8. Simultaneous extracellular recording from the FC nerve, and intracellular recording from a FG neuron. (A) and (B) are from two different preparations. The neuron recorded in A demonstrates rhythmic bursting, which coincide with the large action potentials on the FC recording. The neuron in B shows strong rhythmic inhibition during the FC burst.

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