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CHARACTERIZATION OF OSCILLATORY OLFACTORY INTERNEURONES IN THE PROTOCEREBRUM OF THE CRAYFISH

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Summary

1. We obtained intracellular electrophysiological recordings from local interneurones within the hemi-ellipsoid neuropile of the brain in the freshwater crayfish *Cherax destructor* and *Procambarus clarkii*. The recordings were made from perfused, isolated head preparations that provided several indications of a healthy physiological condition.

2. The hemi-ellipsoid interneurones are spontaneously active, generating bursts of action potentials at regular intervals. The inter-burst period differs among neurones, varying from about 1.0 s at the shortest periods to around 30 s for the longest periods.

3. Evidence from both electrophysiological recordings and from injection of Lucifer Yellow and Neurobiotin dyes into hemi-ellipsoid interneurones suggests that some of the cells in the populations are electrically coupled to one another.

4. Hemi-ellipsoid interneurones are driven postsynaptically by axons within the lateral protocerebral tract. Experiments with focal electrical stimulation strongly suggest that the pathways responsible include axons of the olfactory–globular tract. These findings support our previous electron microscopical data showing that olfactory–globular tract axons are presynaptic to the hemi-ellipsoid interneurones.

5. These findings support the conclusion that hemi-ellipsoid interneurones are an integral link in the central olfactory pathway of the crayfish.

Introduction

Ancestral arthropods were using well-developed chemosensory organs to guide their movements through the aquatic environment before even the most primitive vertebrates had evolved, and modern arthropods have maintained a high degree of

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sensitivity to chemical stimuli in their surroundings. Considerable interest has been focused on the physiology of arthropod chemical senses, especially during the past 20 years. Studies on insect olfaction have been particularly productive, and much has now been learned about the peripheral and central nervous mechanisms involved in the detection of odorants by flies (Stocker *et al.* 1983), moths (Matsumoto and Hildebrand, 1981), bees (Arnold *et al.* 1985) and cockroaches (Ernst and Boeckh, 1983; Boeckh *et al.* 1989). The neural elements concerned with the detection of female pheromones (attractants) have received particular attention. In male *Antherea polyphemus* and *Manduca sexta* moths, most of the olfactory receptors found on the antennae are specifically sensitive to the female pheromone (e.g. Matsumoto and Hildebrand, 1981). The axons of these receptor cells terminate within a unique neuropile in the antennal lobe (the macroglomerular complex) that has come to be regarded as a model for other antennal lobe glomeruli.

Details of chemical senses in crustaceans have been slower to emerge than in insects, perhaps because the olfactory behaviour of these large, primarily aquatic and long-lived arthropods has not received as much attention from neuroethologists. Nevertheless, lobsters, crabs and crayfish use their olfactory receptor organs to detect food as well as sexual partners and, possibly, potential predators. 30–40 % of crayfish brain volume is devoted to the processing of olfactory information.

The major crustacean chemosensory organs – the antennules – are comparatively large, and their olfactory sensory neurones have proved to be amenable to electrophysiological investigations (Ache, 1972; Ache and Derby, 1985; Schmiedel-Jakob *et al.* 1989; McClintock and Ache, 1989). Individual olfactory receptor neurones, at least those in the spiny lobster *Panulirus argus*, are either broadly tuned to respond to complex mixtures of different molecules or narrowly tuned to respond to individual molecular species. Narrowly tuned receptor neurones, moreover, are often inhibited by the presence of other compounds in the stimulus solution, possibly as a result of competition for receptor sites on the cell membrane (Ache *et al.* 1988).

Comparisons between the central olfactory pathways in insects and decapod crustaceans can be instructive. In both groups, primary olfactory sensory neurones on the first antennae project to the deutocerebrum. The target region in insects is the antennal lobe, where axons of the olfactory sensory neurones terminate in a species-specific number of regions of structured neuropile called glomeruli. Here, they synapse with local interneurones that interconnect different glomeruli and activate projection neurones, the axons of which run within the olfactory–globular tract to terminate in the mushroom bodies of the protocerebrum (Ernst and Boeckh, 1983; Boeckh *et al.* 1989; Distler, 1990; Malun, 1991). The projection neurones constitute the only output pathway of the glomeruli. In each antennal lobe of the cockroach *Periplaneta americana* there are about 175 projection neurones – one for each glomerulus – and each output axon terminates bilaterally within the mushroom bodies, in many cluster-like endings. The functional identity

of each antennal lobe glomerulus is thus represented in the protocerebrum at multiple, distributed terminations formed by its own unique projection neurone.

The central olfactory pathways in decapod crustaceans and insects share some common features and differ significantly in others. Crayfish olfactory sensory neurones project to a region of the deutocerebrum called the olfactory neuropile, where they terminate in columns that are also called glomeruli. Olfactory neuropile projection neurones (the globuli cells) connect the columns of the olfactory neuropile with the protocerebrum, but it is not known whether these projection neurones are driven through local neurones as in insects. In contrast to the insects, there are about 100 000 projection neurones on each side of the brain in crayfish. Their axons all project *via* the olfactory–globular tracts in the eyestalks to a circumscribed region of glomerular neuropile (the hemi-ellipsoid neuropile) in the lateral protocerebrum, where they terminate in multiple, distributed knot-like endings. A comparison between the hemi-ellipsoid neuropile of the crustaceans and the mushroom bodies of the insects is tempting because both structures are the protocerebral targets of deutocerebral olfactory projection pathways (Hanstroem, 1925). The large difference in the number of projection neurones found in the two groups is nevertheless striking, and we do not know if this represents real differences in the olfactory capabilities of the two groups.

In some decapod crustaceans there is a second large pair of deutocerebral glomerular neuropiles, the accessory neuropiles. Many projection neurones in the olfactory globular tracts have branches in both the olfactory and the accessory neuropiles (Mellon and Alones, 1992) but a direct functional connection between the olfactory and accessory neuropiles has never been demonstrated. The accessory neuropiles do not appear to receive any primary sensory input or contain any motor elements, and their function remains obscure. There does not appear to be a homologue of the accessory neuropile in the brain of insects.

Protocerebral cells that are the targets of deutocerebral projection neurones have not been investigated in either insects or crustaceans. In the present paper we report findings from electrophysiological and anatomical studies of a population of local interneurones in the hemi-ellipsoid neuropile of freshwater crayfish that are postsynaptic to the olfactory neuropile projection neurones. The response properties of these neurones are highly unusual in that they characteristically exhibit periodic, high-frequency bursts of action potentials, separated by periods of electrical quiescence. Oscillatory field potentials are found in the central olfactory areas of other invertebrate and vertebrate animals, suggesting that common cellular mechanisms based upon oscillatory electrical changes may be a unique requirement for olfactory processing in these disparate groups (Gelperin, 1989; Freeman, 1975).

Materials and methods

Most observations were made on the Australian freshwater crayfish *Cherax destructor* (Clark). Recordings from the hemi-ellipsoid neuropile of *Procambarus*

clarkii (Girard) from Louisiana, USA, revealed no quantitative differences in the responses of the two animals. Likewise, anatomical data obtained from Neurobiotin-filled neurones in *Procambarus* revealed no substantial differences in morphology from those of *Cherax*. All of the physiological and anatomical data shown in this paper are from *Cherax destructor*. In both cases animals were purchased from commercial suppliers and maintained in holding facilities in the laboratory until used. They were fed daily on commercial tropical fish food.

The preparation

Electrophysiological recordings from neurones in the hemi-ellipsoid neuropile were made from 'isolated brain' preparations. Variations of the perfused brain preparation first developed for the crab (Sandeman, 1967) have been used for studies on the chemoreceptive system of *Euastacus armatus* (Ache and Sandeman, 1980) and the motor system of the second antennae of *Euastacus* and *Cherax* (Sandeman and Wilkens, 1983; Sandeman, 1989). In the present study we needed access to the protocerebral neuropiles within the eyecups, so the standard preparation was modified by first fixing the posterior rim of the eyecup to its orbit in the cephalothorax. The eye was twisted slightly about its long axis so that its dorsal surface faced caudally, before glueing it to the posterior wall of the orbit with cyanoacrylate adhesive. The head of the animal was isolated by cutting around the cephalothorax with sharp scissors, starting at a point anterior to the cervical groove and continuing the cut dorsally across to the other side of the animal. The head, still attached to the body ventrally, was then bent forwards to reveal the dorsal longitudinal muscles that attach the gastric mill to the anterior body wall. These muscles were severed, making sure that the underlying gastric mill and the lobes of the digestive gland were not damaged. The stomatogastric system contracts caudally when these muscles are cut. The animal was held tail down while the stomatogastric mill was emptied of its contents by aspiration with a large-capacity syringe. This exposed the brain and the oesophageal connectives. The connectives and the ventral body wall were cut, and the green glands were removed from the now isolated head, which was then clamped rostral end down over a vacuum-aspirated sump. A small-bore, fire-polished glass pipette was then inserted into the severed median dorsal artery, to which it delivered a steady flow of chilled oxygenated crayfish saline (Van Harreveld, 1936) at nominal pressure. A successful perfusion caused immediate swelling of the cor frontale and an upwelling of pooled blood from sinuses in the eyes, antennules and antennae. Saline that had passed through the vascular network gathered in the head capsule and overflowed into the sump or, if the head capsule was submerged, into the surrounding saline bath.

The hemi-ellipsoid neuropile lies within the eyecup, and further dissection was required to expose it. Portions of the anterior lateral carapace and the dorsal wall of the eyecup itself were removed with micro-rongeurs. The hemi-ellipsoid neuropile is a prominent subspherical structure, located slightly posterior and medial to the sinus gland, which is a highly visible, bluish-white opalescent

landmark. The sinus gland tract that originates in the X-organ courses anteriorly and laterally around the base of the hemi-ellipsoid neuropile.

Interruption of the blood supply to the visual and olfactory sensory receptor systems of crustaceans results in the cessation of neural activity after a short period. This problem was overcome in experiments on olfaction in *Euastacus* by the simultaneous perfusion of the anterior median and lateral arteries (Ache and Sandeman, 1980). *Cherax* is smaller than *Euastacus* and cannulation of the anterior median and lateral arteries is time-consuming and difficult in a preparation where ultimate success seems to depend on speedily flushing the blood out of the vascular system. In studies that concentrated on the central reflexes of the motor system of antenna 2 (Sandeman, 1989) only the anterior median artery was cannulated. This was found to be effective in maintaining neural activity. In the present study, however, we relied on good perfusion of both the antennule and the neuropiles in the eyestalks. We therefore thought it prudent to check the flow of saline through these regions by introducing dye into the anterior median artery. Traces of the dye appeared in the sinuses leading from both the eye and the antennule. Cutting off the tips of the antennae and antennules resulted in dye flowing out of the cut ends. In addition, the preparations routinely exhibited reflex eyecup, antennal and antennular withdrawal movements for up to 6 h. Spontaneous flicking of the lateral antennular filament also continued during this period, as did light-evoked activity in 'sustaining' fibres in the lateral protocerebral tract.

Electrophysiology

A small opening was cut in the neural lamella immediately overlying the hemi-ellipsoid neuropile to provide microelectrode access to its centre or base. Further dissection was avoided to minimise damage to the blood supply. Mechanical resistance to penetration by the microelectrode was usually encountered at the periphery of the neuropile, because of a glial connective tissue sheath which surrounds this region of the central nervous system. In most cases it was possible to penetrate this sheath without breaking the electrode tip. We used thick-walled glass micropipettes filled with $1\,\text{mol}\,l^{-1}$ KCl. Electrode resistances, measured in the preparation bath, were 60–90 MΩ. Focal extracellular electrical stimulation of the globuli cell clusters or the lateral protocerebral tracts was accomplished with a suction electrode made from fire-polished capillary tubing, which was pressed down onto the tissue. Stimulating electrodes were coupled to a stimulus isolation unit to restrict current spread. Stimulus pulses were 1 ms in duration, the interior of the stimulating capillary electrode being negative with respect to the outer electrode (a small-diameter silver wire coiled around the capillary tip).

Neuroanatomy

Protocerebral neurones in isolated head preparations were stained with Biocytin (Sigma no. B4261, 2 % in $1\,\text{mol}\,l^{-1}$ KCl), Neurobiotin (Vector Laboratories, Inc. no. SP-1120, 2 % in $1\,\text{mol}\,l^{-1}$ KCl) or Lucifer Yellow (10 % in $0.1\,\text{mol}\,l^{-1}$

LiCl) using ionophoretic injection (Mellon and Alones, 1992). 500 ms rectangular pulses of 2–5 nA were delivered through the recording electrode at a frequency of 1 Hz. In general, we stained each neurone for 1 h, after which the preparation was fixed in either 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (for Lucifer Yellow preparations) or a mixture of 2 % paraformaldehyde and 2 % glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (for Biocytin and Neurobiotin preparations).

Fixation was carried out at 4°C overnight. Lucifer-stained preparations were prepared as whole mounts by dehydration in an ethanol series and clearing in methyl salicylate. Preparations were placed between coverslips in a custom-fabricated aluminium depression slide and were photographed on an Olympus Vanox fluorescence microscope.

Preparations stained in Biocytin or Neurobiotin and fixed were embedded in noble agar and cut at 100 μm on a vibratome. The sections were washed in three separate rinses of phosphate-buffered saline (PBS) and then incubated overnight in a 1:500 solution of Vector Labs A-B-C reagents. After additional rinsing in PBS, the sections were stained with a standard reaction mixture of diaminobenzidine and hydrogen peroxide. The stained sections were mounted on glass microscope slides and dehydrated in ethanol. Coverslips were fixed in place with permount. Individual sections were photographed on the Olympus Vanox microscope using transmitted light from a tungsten filament. In some cases the stained neurones were reconstructed by drawing them using a *camera lucida*.

Results

Spontaneous activity

The intracellular responses of the cells from which we recorded in the hemiellipsoid neuropile were all very much alike and, although described here as two types, probably represent a single class of neurones. We recorded from 45 interneurones in *Cherax* and 5 interneurones in *Procambarus*. When penetrated, the cells behaved in one of two ways. (i) A resting potential of 20–30 mV was accompanied by a steady train of action potentials of about 25 mV amplitude, firing at a frequency of 1–10 Hz. Action potentials of different amplitudes were often recorded, not necessarily firing at the same frequencies. The resting potential usually increased (hyperpolarized) some minutes after penetration and the impulses of different amplitudes merged to be recorded as a single action potential with an amplitude greater than those of either of the individuals and generally active at a frequency that was lower than either of the initial frequencies. Further recovery frequently resulted in a transition of the cells' behaviour to that found in the second type. Records in Fig. 1D are representative of the first stage of this type of response pattern. (ii) The second type of response consisted of a resting potential of 50–70 mV in amplitude accompanied by brief, high-frequency bursts of impulses. In different cells, individual bursts were separated by different interburst intervals: usually these intervals were relatively long, ranging between

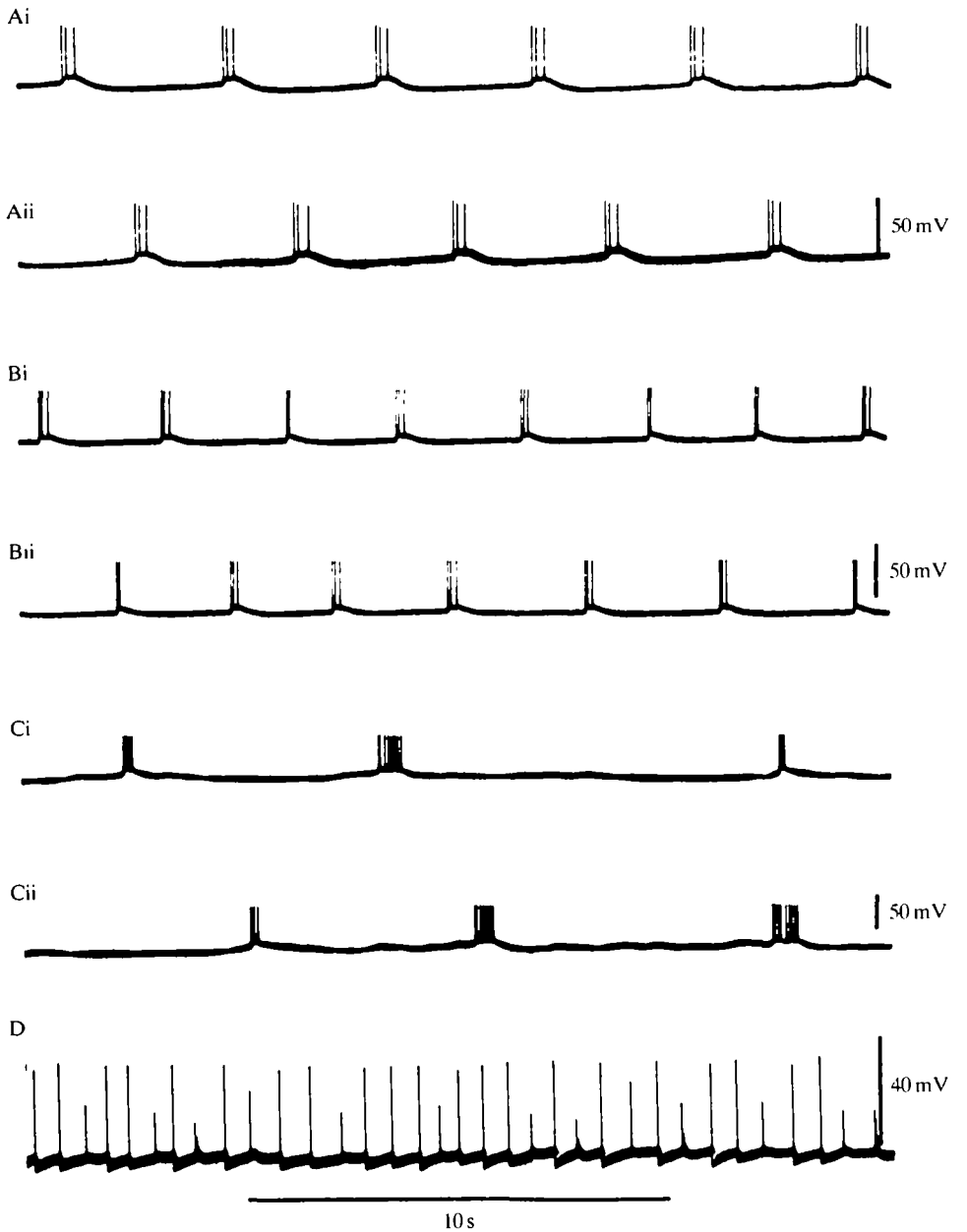

Fig. 1. Characteristic electrical responses recorded intracellulary from hemi-ellipsoid neurones of *Cherax destructor*. (A–C) Three typical examples of autogenically bursting cells. (D) Recording from a different preparation illustrating spontaneous firing and multiple action potential amplitudes. Traces in A–C each continue over two lines.

15 and 30 s. Fig. 1A–C shows examples of cells with interburst intervals which range between 2.5 s and 7 s. The records shown in Fig. 1C are typical of those neurones with longer interburst intervals. Some variability in the interburst interval, as well as in the number and frequency of impulses within the burst, occurred in these cells.

Fig. 2 illustrates excerpts, not necessarily contiguous, from a continuous record of a hemi-ellipsoid neurone with a well-established bursting rhythm and clear slow depolarizing potentials. Variations in interburst interval, number of impulses per burst and impulse frequency within a burst are correlated with differences in, respectively, the onset, time course and peak amplitude of the underlying slow membrane potential changes. In accordance with the terminology used by Selverston *et al.* (1983), we will refer to these events as bursting pacemaker potentials (BPPs). There appear to be two classes of impulse in the records of Fig. 2, based upon amplitude. Records exhibiting two different amplitude classes of impulse bursts were recorded from single recording sites on several occasions; often the two classes of burst were initially asynchronous but then became superimposed (as in Fig. 2). An additional example is shown in the records of Fig. 3. The large-amplitude and small-amplitude bursts sometimes occurred on their own, but more frequently fired in near synchrony, suggesting the existence of a strong functional coupling (Fig. 3C,D). Early in this study it seemed likely that the separate sites of impulse generation resided in different branches of a single hemi-ellipsoid neurone; however, later dye-injection experiments have raised the strong possibility that the different classes of action potential represent impulse activity in neighbouring, electrically coupled nerve cells.

Responses to injected current

Hyperpolarizing a bursting hemi-ellipsoid neurone by passing current through the recording electrode decreased the interburst frequency, whereas depolarizing the membrane increased the frequency (Fig. 4). In no case did injected current affect the frequency of impulses *within* a burst (Fig. 4F–H).

Maintained strong depolarization of some bursting neurones caused them to stop bursting and instead to fire steady trains of action potentials. This finding is interesting because it suggests that the two types of activity seen on penetration of some cells can arise in the same neurone not only as the result of injury but also in response to depolarization of a physiologically stable, bursting neurone. By the same token, natural stimuli that lead to membrane depolarization in hemi-ellipsoid neurones might be expected to initiate continuous firing in a normally bursting cell. Indeed, we occasionally encountered cells that spontaneously alternated their mode of activity between periods of regular firing and periods of bursting. An example is illustrated in Fig. 5. The activity pattern of this cell was to generate four or five short bursts, then to fire single action potentials for an approximately equal period before returning to a bursting mode. No obvious shifts in resting membrane potential occurred during these transitions.

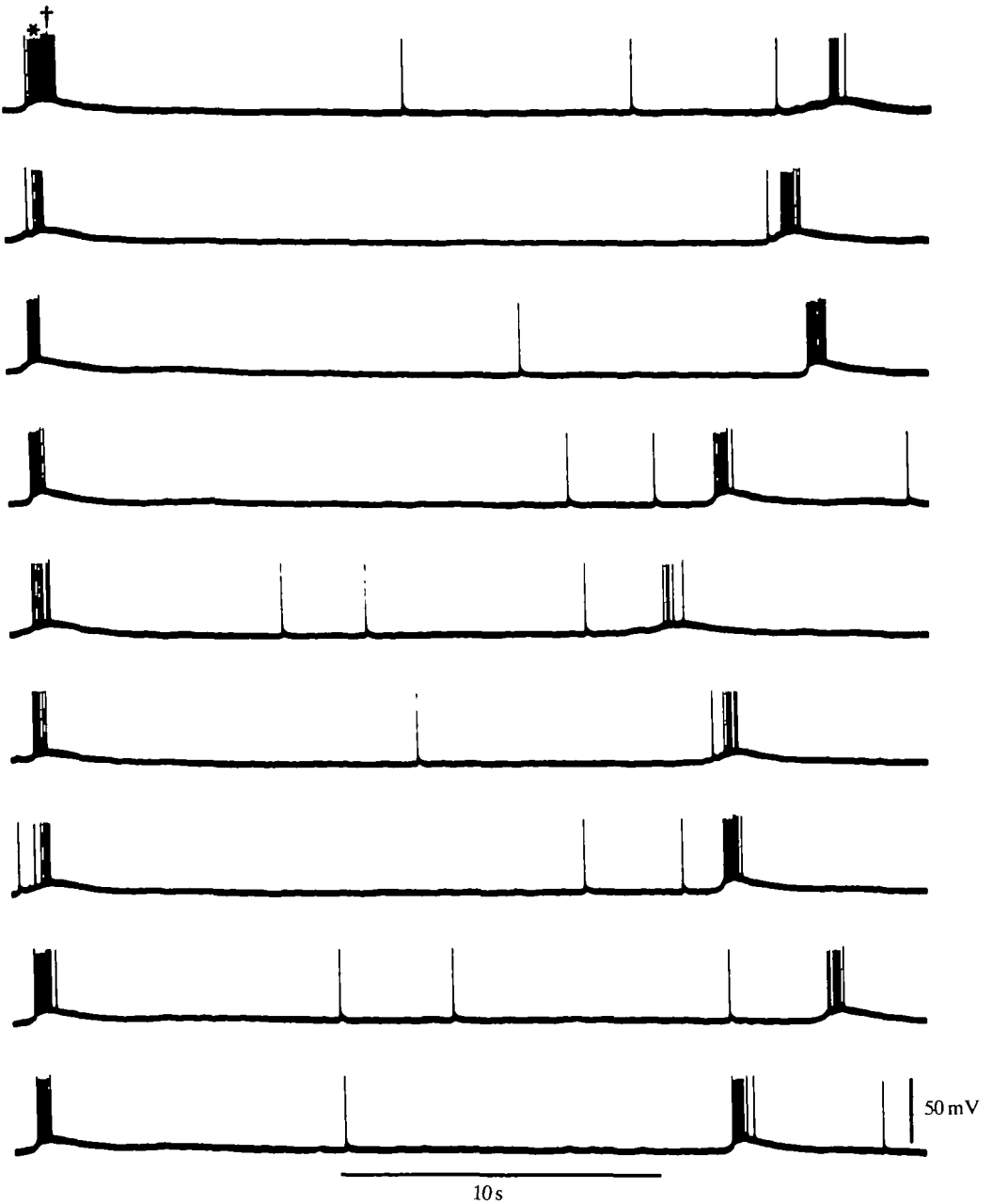

Fig. 2. Records from a hemi-ellipsoid neurone with a long interburst interval. Nine separate excerpts are shown to illustrate interburst timing variability and differences in amplitude and duration of bursting pacemaker potentials. Two different impulse amplitudes (*,†) suggest tight electrical coupling and occasional burst synchronization between neighbouring cells.

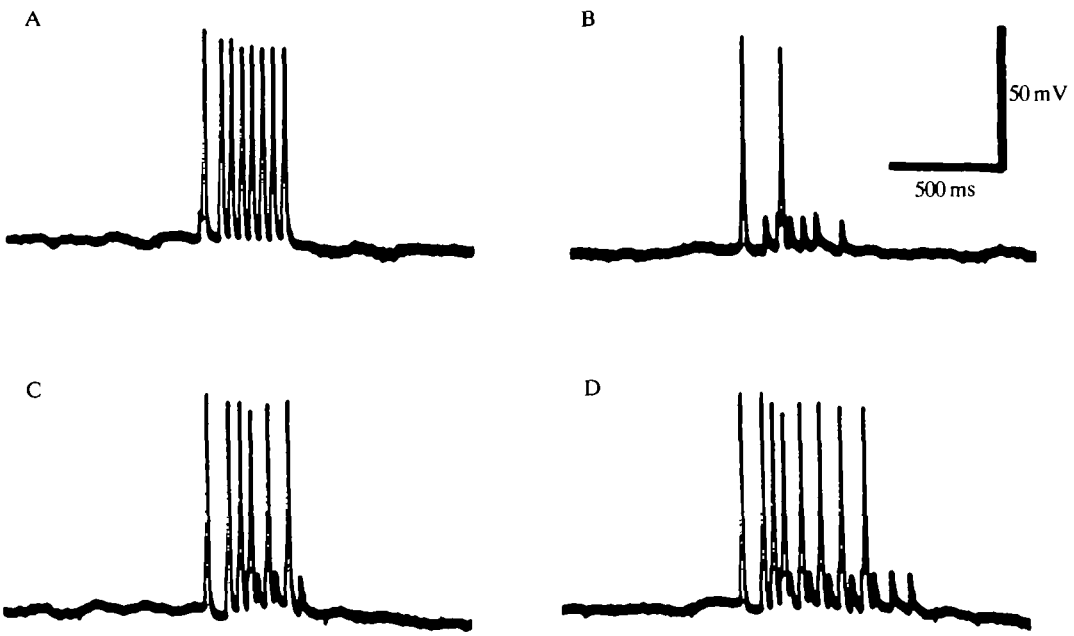

Fig. 3. Records of individual bursts in a hemi-ellipsoid neurone illustrating apparent coupling to a neighbouring cell (B–D). (A) An impulse burst presumably originating in the impaled neurone. (B) Two impulses in the impaled cell are superimposed upon a burst of highly attenuated action potentials, presumably originating in a neighbouring cell. (C,D) Succeeding coincident bursts in the two neurones.

Electrical stimulation of the central olfactory pathway

Oscillatory hemi-ellipsoid neurones are driven synaptically by electrical stimulation of the lateral globuli cell clusters and the projection axons in both lateral protocerebral tracts. The lateral globuli cells and the olfactory–globular tract axons can be clearly visualized, allowing precise placement of the stimulus probe. Lateral displacement of the stimulating electrode from these sites resulted in the failure of the responses evoked in the hemi-ellipsoid interneurones. From this result, we conclude that axons lying adjacent to the olfactory–globular tract do not project to the hemi-ellipsoid interneurones. Fig. 6 shows the responses of a hemi-ellipsoid neurone to single brief shocks of increasing intensity delivered to the lateral protocerebral tract on the contralateral side of the brain. On each side of the brain approximately 100 000 deutocerebral projection neurones send axons to the hemi-ellipsoid neuropiles along each lateral protocerebral tract, *via* the olfactory–globular tract (OGT), and we assume that this is the route of synaptic drive. These axons have a mean diameter of 0.2 μm and a conduction velocity in *Procambarus* of 10 cm s^{-1} at 20 °C. The dramatically broad dynamic range of the postsynaptic responses to incremental changes in stimulus intensity undoubtedly reflects the very large numbers of small OGT axons contributing to the postsynaptic response profile. Further evidence that nerve impulses in this tract

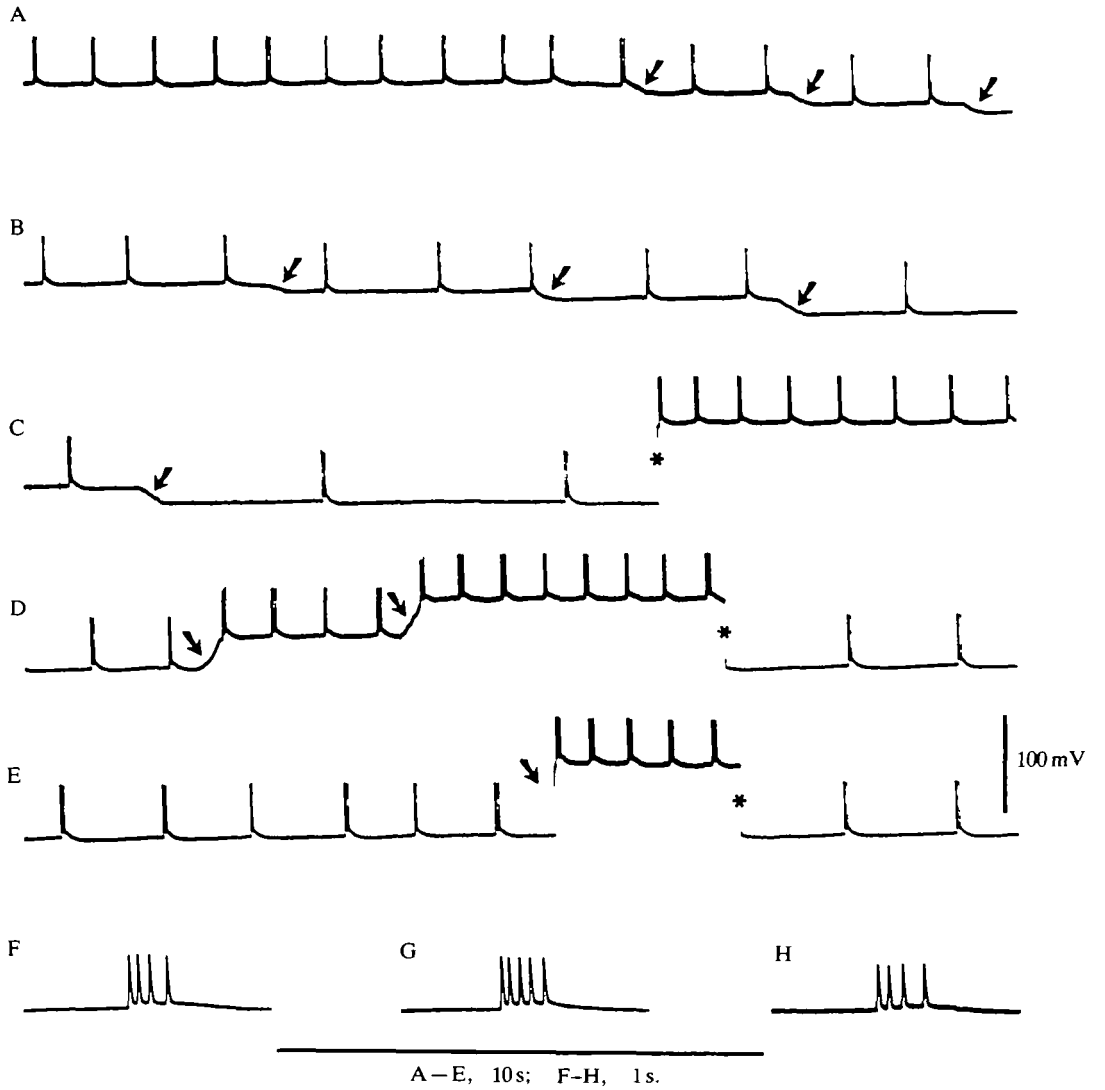

Fig. 4. Responses of a bursting hemi-ellipsoid neurone to injected current. In A–C, the frequency of the bursts is progressively reduced by small, incremental steps of applied hyperpolarizing current (arrows). Cessation of the current (asterisk) in C results in a resumption of the unstimulated bursting frequency. The records in D and E were obtained several minutes later, when the spontaneous burst frequency had declined somewhat, and show the frequency-enhancing effects of injecting depolarising current (arrows) across the membrane. Asterisks indicate return to normal resting potential (current cessation). The traces in F–H are records of bursts filmed at higher speed: (F) at resting potential; (G) during a period of reduced burst frequency induced by injection of hyperpolarising current; and (H) during a period of increased burst frequency induced by injection of depolarising current. It is evident that the burst structure and impulse frequency were not greatly affected by the injected current.

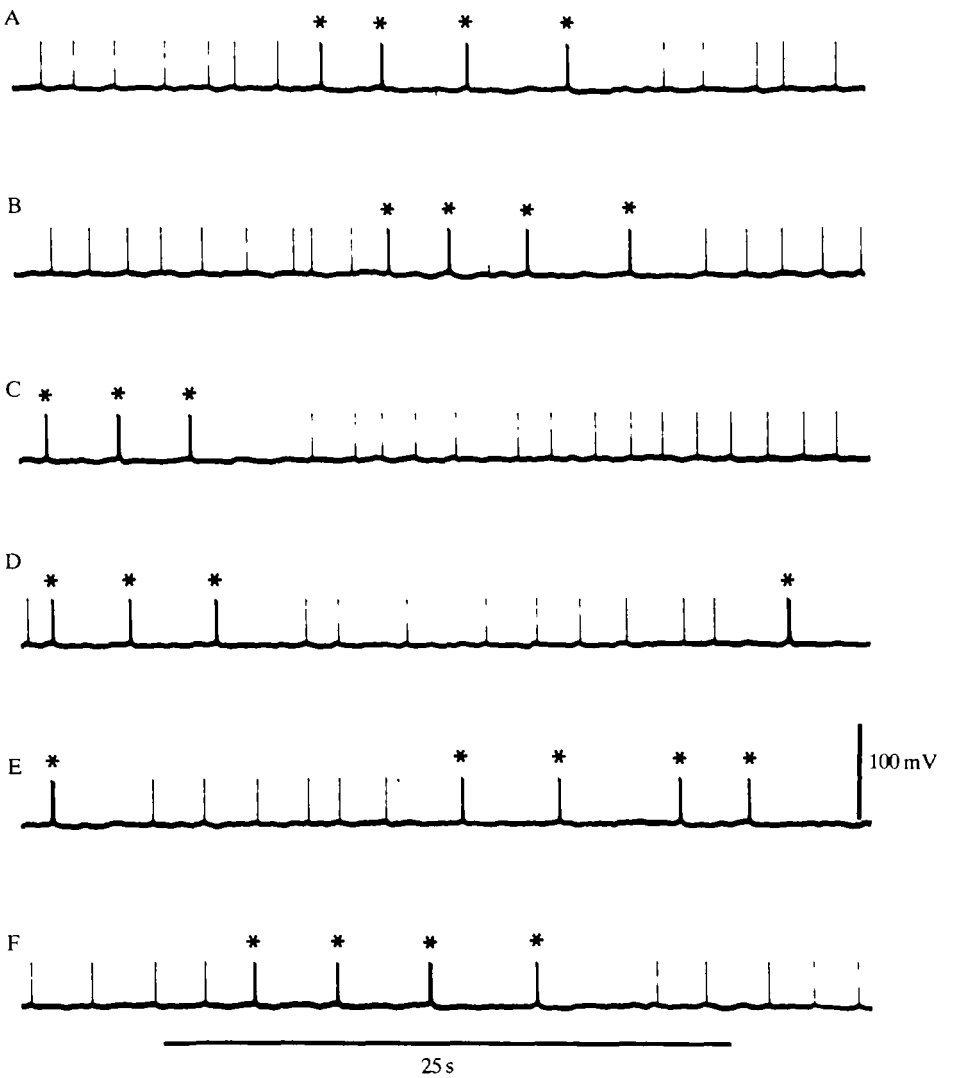

Fig. 5. Intracellular records from a hemi-ellipsoid neurone that exhibited spontaneous switching between bursting (asterisks) and regular firing. A, B and F are instances of periods of bursting inserted in periods of regular firing. C–E are contiguous records showing several cycles of spontaneous switching between the two response modes. Note that no net changes in membrane potential occur in conjunction with the shifts.

are the source of synaptic drive to hemi-ellipsoid cells is suggested by the very long latency of the postsynaptic response, approaching 50 ms, and the continuing synaptic activation even after most of the axons in the lateral protocerebral tract, except those in the OGT, have been severed.

A brief train of high-frequency suprathreshold shocks to the lateral protocerebral tract can delay the activity rhythm in hemi-ellipsoid neurones (Fig. 7).

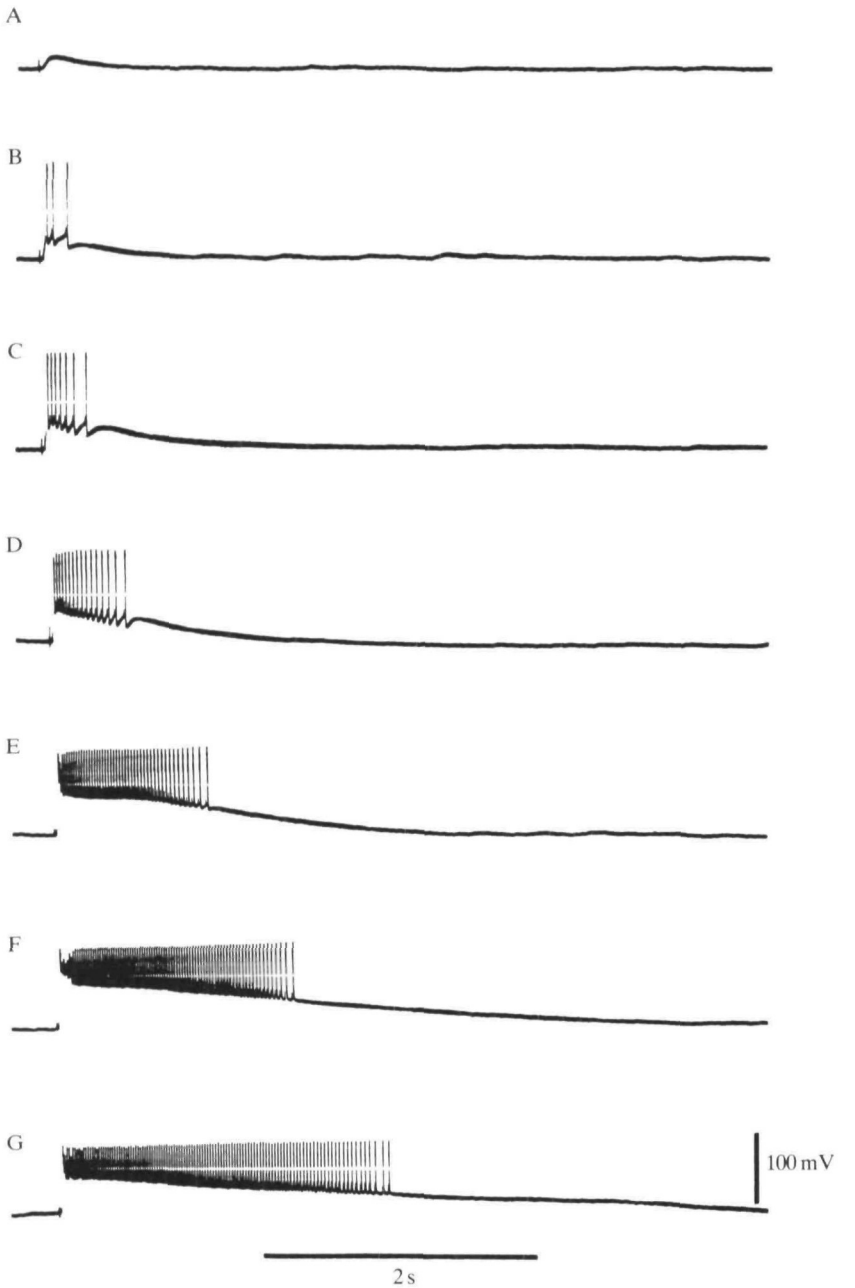

Fig. 6. Responses of a hemi-ellipsoid neurone to approximately equal intensity increments of stimulus voltage (in single, 2 ms electrical pulses) delivered to the contralateral lateral protocerebral tract (small artefacts preceding membrane depolarisation). A large excitatory postsynaptic potential shunts the initial impulses in response to the strongest stimuli (E–G). The membrane depolarisation in response to the stimulus in G persisted for 5 s.

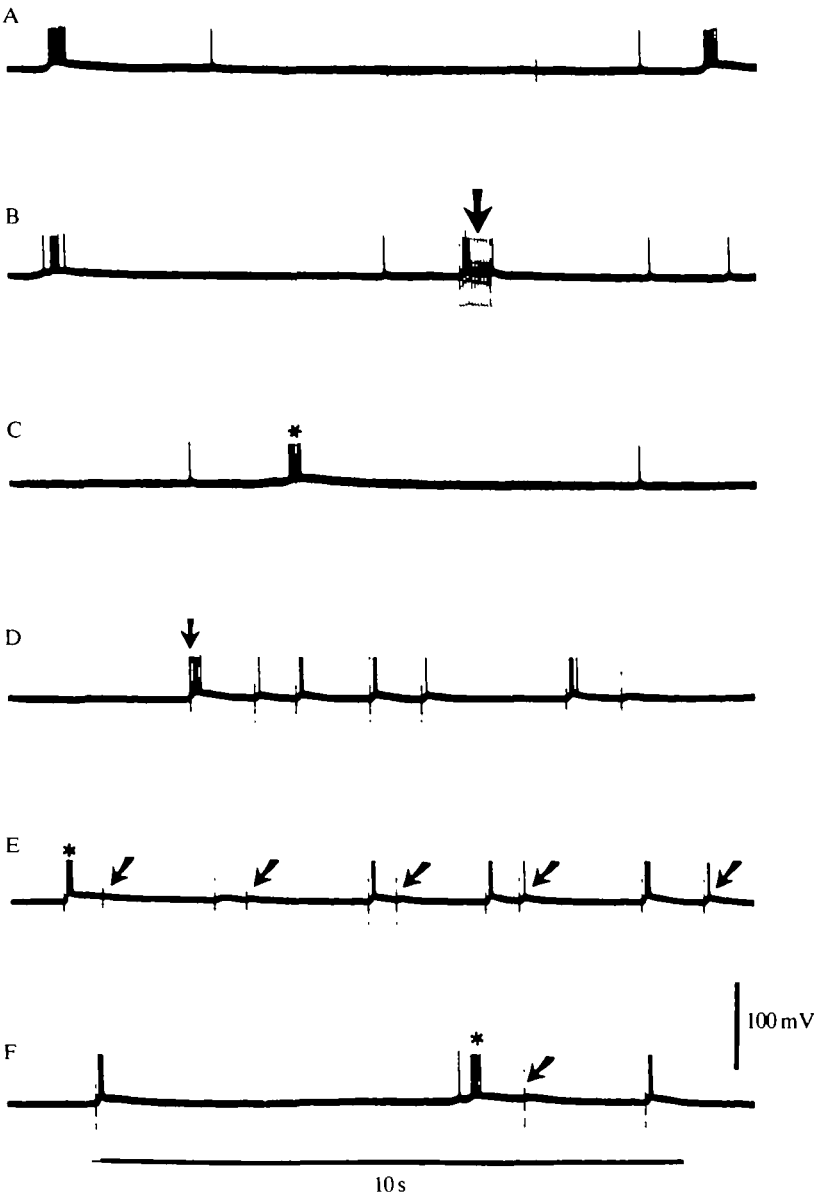

Fig. 7

Fig. 7A is an excerpt from a long continuous record of bursting in a neurone. The interburst interval in this cell varied between 9 and 12 s. A 500 ms train of shocks at a frequency of 20 Hz was delivered to the lateral protocerebral tract approximately 7 s after an impulse burst (arrow, Fig. 7B), which delayed the occurrence of the next spontaneous burst by approximately 5–8 s (Fig. 7C, asterisk). The next spontaneous burst then occurred after an interval of 11 s. Prolonged trains of

Fig. 7. Interactions between endogenous bursting activity and responses to stimulation of the contralateral olfactory globular tract axons. (A) Spontaneous bursts with an interval of around 11 s. (B,C) Continuous records from the same neurone showing effects of a brief train of suprathreshold (but submaximal) electrical stimuli at 20 Hz (arrow). The spontaneous burst in B is aligned with the burst above, in trace A, so that the relative timing of the stimulus train may be compared to the expected time of occurrence of the next spontaneous burst. The next spontaneous burst (asterisk) is delayed by as much as 8 s compared to the second burst in A. (D) The strength of the response to single, maximal electrical stimulus delivered to the lateral protocerebral tract depends upon the timing of the stimulus with respect to the cycle of endogenous bursting. A stimulus (arrow) close to the time of an expected burst generated a strong response. Succeeding stimuli just following the burst generated much smaller responses (fewer action potentials) whereas, later in the burst cycle, stimuli of the same, or even weaker intensity (last pair of stimuli), once again produced strong responses. (E) The second stimulus of a pair (arrows) generates a weaker response than the first, especially when the first stimulus is approximately coincident with a spontaneous burst (asterisk). (F) A strong electrical stimulus (arrow) following a spontaneous burst (asterisk) is incapable of eliciting a response (possibly because of neuronal refractoriness), although the same stimulus earlier or later in the cycle produces a brief burst of action potentials.

stimuli (longer than the interburst interval) applied to the presynaptic pathway suppressed endogenous bursting, which rebounds following termination of the stimulus train (not shown).

We noted that the response amplitude following a single brief electrical shock to the lateral protocerebral tract depended upon the phase of the stimulus relative to the neurone's intrinsic activity rhythm, as well as upon the immediate past history of activity of the neurone. The record of Fig. 7D shows stimuli that were delivered at about the time of the predicted occurrence of the spontaneous burst, as well as at various times during the interburst interval. The records suggest that the response to OGT stimulation is minimal immediately following a burst and becomes increasingly robust around the time predicted for the next burst. Furthermore, as shown by the paired stimuli in Fig. 7E, the response to the second of a pair of electrical stimuli is diminished by a refractory period of as long as 500 ms following the initial shock. We believe that the source of this refractoriness is in the recorded neurone, because we have found that the relative refractory period of OGT axons in *Procambarus* is about 50–75 ms at room temperature. Thus, the absolute refractory period of individual OGT axons must be less than 50 ms. Finally, the records of Fig. 7F confirm that even a strong single shock following a spontaneous burst by just 1 s is ineffective in eliciting a response, suggesting that the refractoriness arises from previous activity in some membrane region of the burst-generating neurone, although not necessarily in that part of the membrane supporting action potentials. These and comparable data from other hemi-ellipsoid oscillatory neurones show that synaptic drive and endogenous oscillatory activity in hemi-ellipsoid neurones interact directly upon the burst-generating mechanism and that the instantaneous state of a neurone can strongly

modulate the effect of the OGT input in producing impulses. By the same token, strong synaptic drive *via* the OGT and its attendant postsynaptic electrical activity appear to be capable of delaying the neurone's endogenous membrane potential oscillator.

Anatomy of hemi-ellipsoid interneurones

Injection of Biocytin, Neurobiotin or Lucifer Yellow into oscillating hemi-ellipsoid neurones revealed a uniformity in their architecture that matched their electrophysiological responses. An idealized cell is illustrated in the drawing of Fig. 8. Its relative postion within the lateral protocerebrum is shown. The inset shows the position of the lateral protocerebrum and the three distal optic ganglia within the crayfish eyecup. The soma of each neurone, 15–30 μm in diameter, is located in a cluster of cell bodies on the side of the terminal medulla opposite the hemi-ellipsoid neuropile. A long neurite from the soma divides into two branches within the terminal medulla. One branch (1) forms small distributed groups of synaptic contacts within the terminal medulla proper; the other (2) enters the hemi-ellipsoid neuropile, where it divides into two or three relatively large-diameter branches, which themselves then subdivide extensively to arborize within all peripheral regions of the hemi-ellipsoid neuropile. Cells with dendritic trees that only partially filled the hemi-ellipsoid structure were occasionally

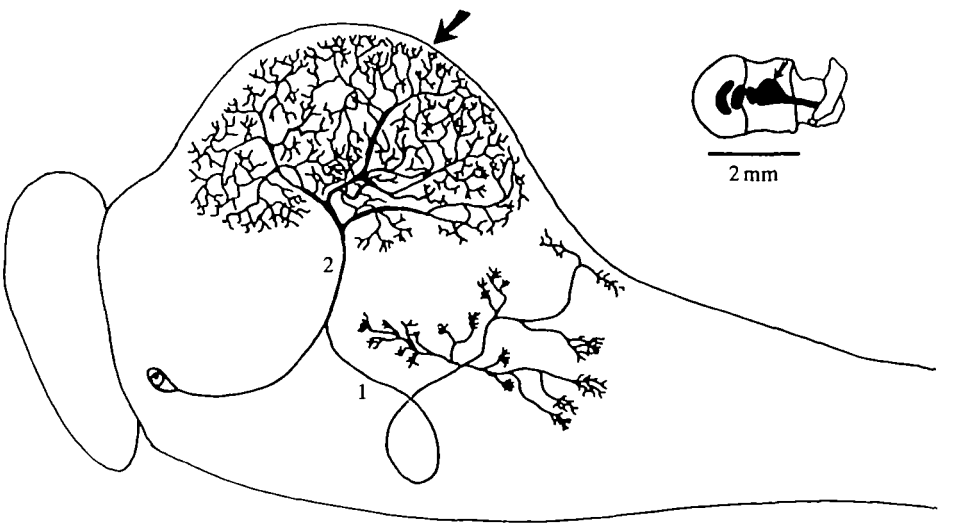

Fig. 8. Idealized drawing of a standard hemi-ellipsoid neurone based upon reconstruction of stained, sectioned preparations. (1) Neurite with distributed endings in the terminal medulla; (2) neurite with distributed arborizations in the hemi-ellipsoid neuropile (large arrow). (Inset) Localization of the hemi-ellipsoid neuropile (small arrow) in relation to the entire neuraxis of a left-hand eyecup of *Procambarus*. See text for further details.

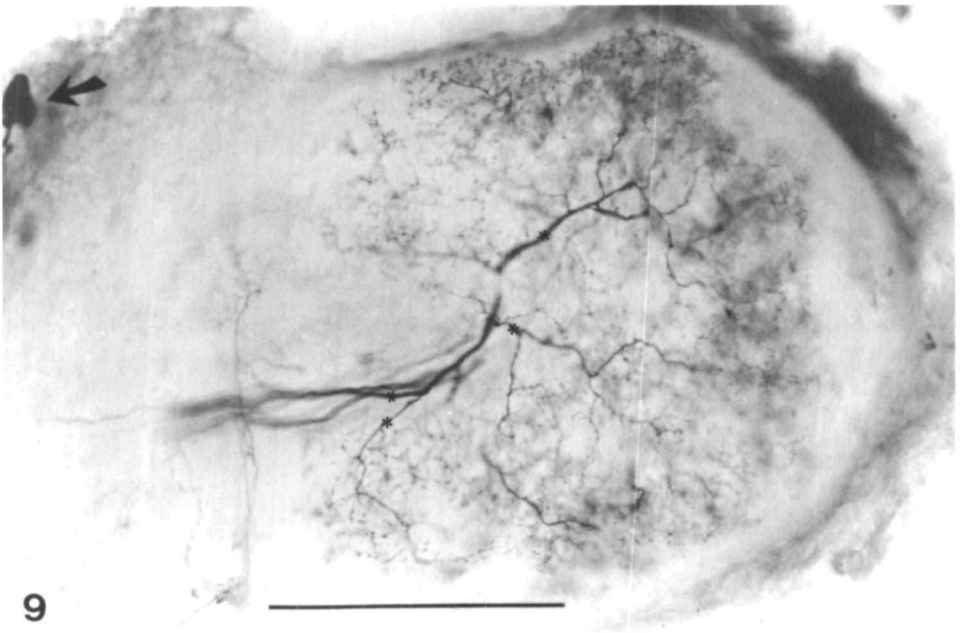

Fig. 9. Light micrograph of a section through a hemi-ellipsoid body containing several stained interneurones. Only a single neurone was penetrated and directly filled with Neurobiotin through the microelectrode; its soma (arrow) and major neurite branchings (small asterisks) are indicated. Staining of adjacent similar neurones may have been *via* gap junctions. Scale bar, 250 μm.

observed but were in the minority. They may represent a functionally separate class of cells, or they may be incompletely filled cells.

With only a few exceptions, intracellular dye-injection into impaled hemi-ellipsoid neurones produced multiple fills in which the neurone architectures of the filled cells were nearly identical with one another. Fig. 9 is a light micrograph of a 100 μm vibratome section through the hemi-ellipsoid body from a preparation in which a single neurone was penetrated with the dye-containing microelectrode. Processes of the impaled cell are probably those that are stained most intensely; other lightly stained major neuritic segments and dendritic arborizations can also be seen, however, and neighbouring sections reveal at least four additional Neurobiotin-filled cell bodies.

Discussion

The oscillatory interneurones in the hemi-ellipsoid neuropile that we have described here appear to be integral components of the olfactory pathway in crayfish. Axons of the OGT, the only known output of the olfactory neuropiles, have cell bodies in the lateral cluster of globuli cells and dendritic arborizations in both the olfactory and accessory neuropiles. Golgi studies show that the distal

branches of the OGT axons end in the hemi-ellipsoid neuropile in small knot-like terminals, which probably form the cores of the glomeruli seen in silver-impregnated preparations (Mellon *et al.* 1991).

The oscillatory behaviour that we describe for the hemi-ellipsoid neurones has parallels in other aspects of olfactory systems in general: evidence accumulated from preparations in both vertebrates and crustaceans suggests that olfactory stimuli are processed at the periphery in a phasic manner. Sniffing, olfactory irrigation and antennular flicking (which all amount to periodic flushing of the fluid volume surrounding the olfactory sensory neurones) are strategies used by animals to reset the sensitivity of their peripheral olfactory sense cells, which adapt very rapidly to continuously applied odorants (Schmitt and Ache, 1979; Nevitt, 1991). Thus, olfactory stimuli are presented as temporally short events. At the central nervous level, vertebrates, molluscs and now crayfish have been shown to exhibit periodic electrical activity in cells involved in processing olfactory input. The functional significance of central oscillatory activity and its possible relationship to the phasic acquisition of olfactory stimuli at the periphery are still highly conjectural, but several suppositions may be put forward. (i) Synchronization of bursting in coupled neurones might be one way of enlisting appropriate behaviour following exposure to certain categories of odorant stimuli, while other, inappropriate, stimuli would be ignored. (ii) Autogenic, repetitive activity in central neural circuits can extend the operating range of any responding system by permitting reduced as well as increased activity of the relevant pathways. (iii) The frequency of oscillatory activity in subsets of central olfactory neurones may represent an identification code for various odorants. Strong, brief patterns of input from olfactory receptors, perhaps strengthened or otherwise modified by events in the olfactory neuropiles, may transiently change the bursting frequency in some fraction – or perhaps all – of the oscillatory hemi-ellipsoid neurones, thereby providing subtle changes in the orchestration of hemi-ellipsoid neuronal activity. However, we have no evidence to suggest a link between the bursting of the hemi-ellipsoid neurones and antennal flicking. (iv) Finally, a change in the mode of firing of hemi-ellipsoid neurones may be the critical factor in odorant-triggered behaviour. For example, if all, or a significant fraction, of the hemi-ellipsoid neurones changed from a bursting mode to regular firing, this could have significant effects in other parts of the nervous system concerned with motor output. Our findings that experimentally imposed membrane potential changes modulate the burst frequency and firing mode of hemi-ellipsoid neurones are of some interest in this respect. It is important to note, furthermore, that excitation of axons in the OGT generates strong, long-lasting depolarisations that have an influence upon the state of excitation of hemi-ellipsoid neurones. Whatever mechanism may be operating, however, it would appear that the hope of correlating anatomically unique neurones with particular chemical specificities has to be abandoned for the neurones of the hemi-ellipsoid body.

The temporal regularity of the action potential bursts of hemi-ellipsoid neurones characterises these cells as oscillatory. The underlying events responsible for the

bursts are rhythmically occurring membrane depolarisations, BPPs, which can vary somewhat in amplitude, duration and waveform: the BPPs that underlie bursts recorded from some neurones, e.g. Fig. 1A,B, are quite uniform, while those in others, e.g. Figs 1C and 2, are variable. The frequency profile and duration of the respective impulse bursts reflect this constancy or variability. A possible cause of variability would be randomly occurring synaptic input that interacts with the mechanisms responsible for periodic membrane depolarisations. For example, fly neurosecretory cells are capable of very regular pacemaker activity if their synaptic input is blocked (Bruce and Wilkens, 1976). The data presented in Fig. 7 strongly suggest that endogenous and synaptic events interact in complex ways to determine the impulse output of hemi-ellipsoid neurones.

The origin of the BPPs is an even more important question. In theory they may be either intrinsic to the neurone under examination, or extrinsic. We believe that the individual hemi-ellipsoid neurones are all intrinsically oscillatory, because they respond to injected current in a manner that is similar to that of oscillatory cells of some other invertebrates. For example, intrinsic oscillations of cell L3 in the abdominal ganglion of *Aplysia californica* respond to moderately depolarising current by increasing burst frequencies and to intense depolarising currents by spiking continuously (Frazier *et al.* 1967). Hyperpolarising currents decrease bursting frequency in L3, and, if sufficiently strong, completely arrest burst generation. There may be at least six different ionic current channels in L3 which, through their collective actions, give rise to periodic impulse burst formation (e.g. Berridge and Rapp, 1979). Membrane instability is caused by a non-voltage-gated, non-inactivating slow inward membrane current (probably carried by sodium), which continuously drives the membrane potential in a depolarising direction. Although the effects of this current are temporally delayed by a voltage-gated, inactivating potassium current just prior to burst initiation, the slow inward current regains ascendancy during membrane potential relaxation following each burst and its hyperpolarising post-phase. If the oscillation in hemi-ellipsoid neurones is generated intrinsically it probably depends upon a slow-leak sodium current, which is neither voltage-gated nor inactivating. Experimentally injected currents will simply summate algebraically with such leak currents as a parallel source, consequently speeding up or slowing down the depolarising drift in the membrane potential between bursts. Thus, the time taken to reach threshold for the succeeding impulse bursts would be changed in a graded, polarity-dependent manner, modulating the burst frequency, as observed. Our incidental observations that depolarisation increases burst frequency and causes some cells to fire regularly are so similar to the findings of Frazier *et al.* (1967) that it is difficult not to presume that similar cellular mechanisms are responsible for the bursting behaviour in the neurones of *Aplysia* and of *Cherax*.

If, however, hemi-ellipsoid neurones were driven by an extrinsic oscillator, injecting current across the membrane would, depending its polarity, raise or lower the ambient membrane potential. In turn, this would modulate postsynaptic current density, thereby changing the amplitude of the periodic postsynaptic

potentials, the burst length and the intraburst impulse frequency. Although one consequence of this might indeed be to initiate impulse bursts earlier in each cycle, it would have no effect upon the frequency of the periodic synaptic input.

Various endogenously active crustaceans neurones have been studied in detail over the past 15 years. These studies have shown that network properties interact with bursting neurones in ways that are not intuitively obvious. In the pyloric network of the stomatogastric ganglion of *Homarus vulgaris*, for example, the individual neurones are apparently incapable of sustained burst generation without tonic input from other elements of the stomatogastric nervous system (Moulins and Cournil, 1981). Hence, these cells were termed conditional bursters. A possible explanation for their requirement for tonic input might be that the input hyperpolarises the membrane potential to a level where the cell's activity changes from regular firing to bursting. Final resolution of questions about bursting mechanisms, however, can be reached only after extensive physiological, biophysical and pharmacological studies have been made. Additionally, the development of slow membrane potential changes underlying impulse bursts may be complex; they may arise initially as pacemaker potentials and then, secondarily, as driver potentials. Moreover, each of these depolarising components may be confined to discrete regions of the neuronal membrane (Tazaki and Cooke, 1979*a–c*, 1983). Our observations that the oscillations of hemi-ellipsoid neurones can be reset by suprathreshold stimulation of the OGT and that the neurones exhibit a relatively long refractory period are consistent with driver potential involvement in bursting. In the absence of thorough analytical studies, however, predictions about burst-generating mechanisms must be made with great caution.

Several lines of evidence point to the olfactory–globular tract as the major presynaptic pathway to the hemi-ellipsoid neurones. First, we were able to elicit postsynaptic activity in the hemi-ellipsoid cells only when the focal stimulating electrode was positioned over the projection axons in the lateral protocerebral tracts, the lateral globuli cell clusters, or in the vicinity of the accessory and olfactory neuropiles. Since the olfactory–globular tract axons have their cell bodies in the lateral globuli cell clusters, their dendritic arborizations in the olfactory and accessory neuropiles, and since they bifurcate at a chiasm in the protocerebrum, their anatomical distribution is entirely consistent with our physiological observations. Furthermore, the measured conduction velocity of olfactory–globular tract axons of around $10\,cm\,s^{-1}$ at 20 °C in *Procambarus* is also consistent with the latency of synaptic events in hemi-ellipsoid neurones following shocks to the contralateral OGT in *Cherax*. Additionally, the broad dynamic range of the postsynaptic response to electrical stimulus intensity gradations indicates a presynaptic pathway with very large numbers of individual axons, again a corroborative feature of the OGT. One characteristic of the hemi-ellipsoid cell responses that cannot readily be attributed to properties of the OGT axons, however, is the rather long period of relative refractoriness, about 500 ms, which follows a single electrical shock to the lateral protocerebral tract. The duration of relative refractoriness of the *Procambarus* OGT compound action potential at

20°C is in the neighbourhood of 50–75 ms. Thus, some other refractory period – such as that associated with a driver potential – must be responsible. Relative refractoriness in the driver potential would produce impulse-generating membrane depolarizations of lower amplitude and with a slower rate of rise; they would therefore affect intraburst impulse frequency and impulse number, but they would not be expected to affect the amplitude of individual impulses.

Intracellular recordings from the hemi-ellipsoid neurones frequently revealed action potentials of different sizes, and intracellular injection of dyes more often than not led to multiple fills. Action potentials of different sizes in cells with extensive dendritic trees can be attributed to local spiking regions at the periphery of the synaptic field, the EPSPs from which would otherwise never reach the spike-initiating site of the main axon. Such an explanation assumes that, in neurones such as those found in the hemi-ellipsoid neuropile, the extensive branches are indeed the inputs to the cell. The indication that this is the case for the hemi-ellipsoid neurones comes from (1) finding Golgi-impregnated terminals of the olfactory–globular tract in this area (Mellon *et al.* 1991); (2) electron microscopical observations (Mellon *et al.* 1991) that these terminals are presynaptic to Neurobiotin-filled dendritic branches of hemi-ellipsoid oscillatory neurones; and (3) our electrophysiological findings that stimulating the lateral protocerebral tract will drive them synaptically. Multiple filling of neurones within a neuropile, in contrast, can indicate that the cells are dye-coupled (and therefore in all probability also electrically coupled). The most likely explanation of different-sized action potentials recorded in such a case is that the action potentials are from neighbouring cells. Multiple filling and the occurrence of multiple spike heights can be caused by electrode damage, particularly where branches of different cells lie bundled together at the point of electrode penetration. This is true for the hemi-ellipsoid cells, and so we are presently unable to rule out the possibility that injury caused the multiple fills.

An observation suggesting that electrical coupling may be a normal feature of these cells is the clear indication that impulse bursts in neighbouring cells are synchronized with one another. This can only happen consistently through either mutual entrainment, *via* electrical coupling, or if the cells are driven by the same common oscillator. The possibility that hemi-ellipsoid neurones are driven in common is weakened by our findings that the bursting frequency of individual neurones can be modulated by depolarizing or hyperpolarizing current passed into the cell from the recording electrode. This result is not what would be expected if the neurone were being driven rhythmically by conventional chemically mediated synaptic input. Given the evidence at hand, the most likely explanation of burst synchronization is that at least some hemi-ellipsoid neurones are electrically coupled.

Fig. 10 summarises the major crayfish olfactory pathway as we presently envisage it. The primary olfactory receptor cells are known to project to the olfactory neuropiles (Sandeman and Denburg, 1976; Mellon *et al.* 1989). Olfactory (and possibly taste) -driven neurones have been recorded in different regions of

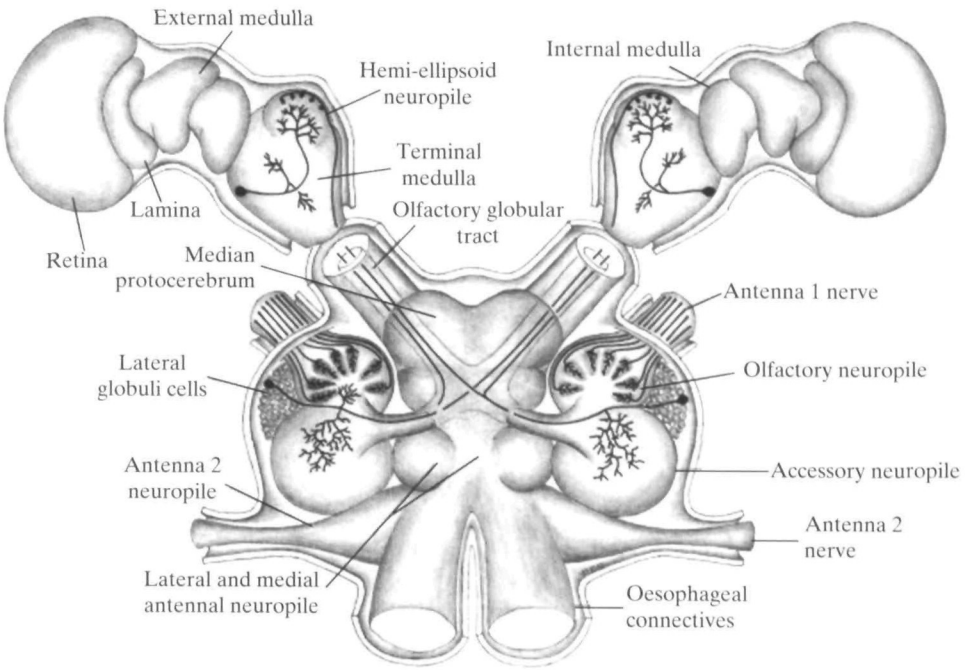

Fig. 10. Summary diagram of the crayfish olfactory pathways as far as they are presently known. Although single neurones are drawn in this diagram, it is not intended that they should reflect the true architecture of any single identified neurone. Instead, the diagram merely shows the paths taken by the populations of neurones and their regions of synaptic transfer. Large numbers of primary sensory neurones from each olfactory sensillum (aesthetasc) enter the brain ventrally and project to all neuropile columns in the olfactory neuropile. It is not known whether this distribution is brought about by the individual afferents branching at the surface of the neuropile or whether afferents from specific receptors collect within particular neuropile columns. Cells with their somata in the cluster lateral to the olfactory neuropile (projection neurones) have branches in both the accessory and olfactory neuropiles. Their axons project both ipsilaterally and contralaterally in the olfactory–globular tract and terminate upon the oscillatory interneurones in the hemi-ellipsoid neuropiles of the lateral protocerebrum (Hanstroem, 1925; Tsvilineva and Titova, 1985; Mellon and Alones, 1992). These cells may receive their input directly from the olfactory afferents, but this has not been examined critically.

the crayfish brain (Ache and Sandeman, 1980; Tautz *et al.* 1986; Arbas *et al.* 1988) but the pathway between the receptors on the antennule and these higher-order interneurones is not known and so they have been left out of the diagram. Projection neurones in the olfactory–globular tract have their cell bodies in the lateral cluster of globuli cells and end in the hemi-ellipsoid neuropile (Hanstroem, 1925; Mellon and Alones, 1992). Axons of projection neurones project either ipsi- or contralaterally, or branch and project to both ipsi- and contralateral hemi-ellipsoid neuropiles (Hanstroem, 1925; Mellon and Alones, 1992). The obvious direction for future investigations of this pathway is to determine how different

odorant stimuli applied to the antennular receptors modulate existing activity in the population of hemi-ellipsoid interneurones.

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