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FUNCTIONAL ROLES AND CIRCUITRY IN AN INHIBITORY PATHWAY TO FEEDING COMMAND NEURONES IN *PLEUROBRANCHAEA*

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SUMMARY

1. The paracerebral neurones (PCNs) of the brain of *Pleurobranchaea californica* serve a command role in the initiation of feeding behaviour (Gillette, Kovac & Davis, 1978). The PCNs are synaptically excited by food stimuli applied to the oral veil of hungry, naive animals. In food avoidance-conditioned animals, the PCNs are inhibited by a barrage of inhibitory postsynaptic potentials concomitant with the suppression of feeding (Davis & Gillette, 1978). In this paper, an interneuronal pathway is described which causes inhibition of the PCNs and potentially mediates the effects of learning.

2. The inhibitory pathway consists of three serially connected interneurones. One population, designated the Interneurone 1s (Int-1s), monosynaptically inhibits the PCNs. A second population, the Interneurone 2s (Int-2s), excites the Int-1 population. They also excite other neurones of the brain including the metacerebral giant neurones. A third population, the Interneurone 3s (Int-3s), monosynaptically excites the Interneurone 2 population.

3. Dual intracellular recordings and current injection show that ipsilateral members of the Int-2 population are electrically coupled *via* a non-rectifying connection. Contralateral members of the Int-2 population are excitatorily coupled *via* a polysynaptic pathway.

4. The Int-1 population is phasically active during the rhythmic motor activity that underlies feeding. In the isolated nervous system Int-1 activity is phase-locked with rhythmic PCN activity; Int-1 activity occurs maximally at the end of a PCN burst, during the retraction phase of the cycle. Int-2 activity also occurs during the retraction phase. During actual feeding in the whole animal preparation, the Int-2s are also phasically active; maximal excitation occurs during buccal mass retraction and maximal inhibition during protraction and the bite.

5. Stimulated activity in a single Int-2 can entirely suppress the rhythmic

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motor activity of the feeding network evoked by electrical stimulation of the stomatogastric nerve. The suppressant effects of Int-2 activity must be mediated widely within the feeding network because the rhythmic motor output so driven is not dependent on PCN spiking.

6. Application of an appetitive chemosensory stimulus to whole and semi-intact animal preparations initiated feeding and elicited excitation of the Int-1 and Int-2 populations. Noxious chemosensory stimuli, such as a dilute soap solution or ethanol, elicited oral veil withdrawal and inhibition of the Int-2s by multiple inhibitory postsynaptic potentials. When divalent ion concentration was raised in the saline surrounding the brain, to suppress polysynaptic activity, the Int-2s but not the PCNs were excited by appetitive chemosensory stimuli. This suggests that the Int-2s receive monosynaptic sensory input from the oral veil while the PCNs do not.

INTRODUCTION

Neurophysiological investigations of feeding behaviour in gastropod molluscs are providing useful models for understanding the neural mechanisms responsible for the generation and modulation of complex behaviour. Salient studies have been made upon the pulmonate snails *Lymnaea, Helisoma* and *Limax*, and the opisthobranchs *Tritonia, Aplysia* and *Pleurobranchaea*, including observations upon rhythmic pattern generation (Benjamin & Rose, 1980; Kaneko, Merickel & Kater, 1978; Willows, 1980), motivation and arousal (Kupfermann, 1974; Davis, Mpitsos, Pinneo & Ram, 1977), sensory processing (Audesirk & Audesirk, 1980; Reingold & Gelperin, 1980; Rosen, Weiss, Cohen & Kupfermann, 1982) and behavioural initiation (Granzow & Kater, 1977; Gillette, Kovac & Davis, 1978). *Lymnaea, Limax* and *Pleurobranchaea* are of additional interest because these animals' feeding behaviour may be modified through associative learning (Audesirk, Alexander, Audesirk & Moyer, 1982; Gelperin, 1975; Mpitsos & Collins, 1975). Here we document a pathway which potentially mediates such learning.

The feeding behaviour of the carnivorous marine snail, *Pleurobranchaea californica*, may be subdivided into an appetitive phase, in which the animal orientates to a prey object and bites at it, and a consummatory phase in which food is rapidly ingested (Davis & Mpitsos, 1971; Gillette & Gillette, 1983). The appetitive and initial consummatory phases are driven in part by a population of neurones in the brain (cerebropleural ganglion) which are called the paracerebral neurones (PCNs) (Gillette *et al.* 1978). The PCNs function in a command role and when active initiate and drive the oscillatory motor network that causes the rhythmic biting and swallowing movements of the buccal mass. The PCNs send output directly to many members of the feeding oscillator in the buccal ganglion *via* descending axons in the cerebrobuccal connectives (CBCs); in their turn, certain neurones of the oscillator send ascending axons to synapse mono- or polysynaptically on the PCNs. The result is a positive feedback loop of excitation between the buccal oscillator neurones and the PCNs, in which PCN activity is locked to the protraction phase of oscillator output. In effect, the PCNs are part of the oscillator which they drive and by virtue of their position they may exert a regulatory effect on oscillator output.

The PCNs mediate aspects of food-avoidance learning. *Pleurobranchaea* rapidl

learns to associate a specific food stimulus with an aversive electrical shock (Mpitsos & Davis, 1973; Mpitsos & Collins, 1975). Animals that receive paired food and shock exhibit avoidance behaviour and concomitant suppression of feeding behaviour, whereas animals that receive randomly paired food and shock still exhibit feeding behaviour. This learning is retained for periods of days to weeks, and trained animals exhibit extinction and savings (Mpitsos & Collins, 1975). The different behavioural response to appetitive stimuli in naive *versus* food avoidance-trained animals is reflected in the electrical activity recorded from the PCNs (Davis & Gillette, 1978). In a naive animal, feeding stimulated by food application to the oral veil and rhinophores is accompanied by strong synaptic excitation of the PCNs; during the ensuing rhythmic biting and swallowing, PCN activity is rhythmically punctuated by volleys of inhibitory postsynaptic potentials (IPSPs). In an animal trained to avoid food, the typical response to food stimulation recorded in the PCNs is a prolonged barrage of IPSPs that suppresses the PCN activity. The individual IPSPs that inhibit the PCNs in trained animals are indistinguishable in amplitude and form from those IPSPs that punctuate PCN firing in the feeding animal, suggesting that they arise in the same pathway (Davis & Gillette, 1978). The description of the pathways presynaptic to the PCNs that mediate inhibition is of interest because of the likelihood that these presynaptic pathways embody and express a mechanism for plasticity that underlies food avoidance conditioning.

This report describes an inhibitory pathway presynaptic to the PCNs. We document three serially connected interneurone populations in the brain, two of which are cyclically active during feeding and appear largely to mediate the cyclic inhibition of the PCNs during the feeding cycle. A preliminary report of these findings has been made (London & Gillette, 1981).

METHODS

Pleurobranchaea californica were obtained from Pacific Bio-Marine and maintained in a closed artificial sea water system (ASW, Instant Ocean) at 15 °C on a 12: 12 light/dark cycle. Specimens ranged in size from 200 to 800 g.

The isolated nervous system was used to study synaptic pathways and the role of cells in fictive feeding. The isolated nervous system consisted of the cerebropleural ganglion (brain) and the buccal ganglion attached *via* the cerebrobuccal connectives (CBCs). To facilitate microelectrode penetration, the ganglia were pre-treated with a 0·2 % pronase solution (CalBiochem) followed by a series of washes in ASW. The brain was desheathed and securely pinned to Sylgard in a water-jacketed recording chamber (12–14 °C) filled with saline (420 mM-NaCl, 10 mM-KCl, 10 mM-$CaCl_2$, 25 mM-$MgSO_4$, 25 mM-$MgCl_2$, 5 mM-Tris; pH 7·5) or with filtered, *Pleurobranchaea* blood (Gillette, Gillette & Davis, 1980, 1982*a*,*b*). When tests for mono- or polysynapticity were conducted, a high divalent ion solution (240 mM-NaCl, 10 mM-KCl, 30 mM-$CaCl_2$, 25 mM-$MgSO_4$, 125 mM-$MgCl_2$, 5 mM-Tris) was employed in order to elevate spike thresholds (Cohen, Weiss & Kupfermann, 1978).

The hemi-animal preparation was used to study the effects of sensory input on the activity of a cell and to study the role of a cell during feeding. The hemi-animal preparation consisted of the head of the animal, including the sensory apparatus (oral

veil, rhinophores and tentacles), the feeding apparatus (proboscis, mouth, buccal mass and oesophagus) and the nervous system (central ganglia and peripheral networks). The brain was slightly elevated and pinned to a wax-coated platform. The preparation was immersed in ASW (adjusted to pH 7·5). When the hemi-animal or whole animal preparation was used, the pronase solution was focally applied. A glass capillary tube (1 mm diameter) filled with the pronase solution was lowered by micromanipulator onto the area of the sheath that was to be softened. The solution was applied in this manner for 9 min, followed by a 20-min wash with ASW. This focal application allowed for gentle removal of the sheath overlying a restricted area of cells.

Using the hemi-animal preparation, the activity of a cell could be monitored during the application of stimuli and during elicited behaviour. Food and noxious chemical stimuli were presented by slowly pipetting 3 ml of the test substance across the oral veil. To determine whether sensory input to neurones in the brain was potentially poly- or monosynaptic, the brain was immersed in a high divalent ion solution (see above). For these experiments, the brain was pinned to a wax-covered platform that had Lucite walls on three sides; a grease bridge (Dow stopcock grease) was built on the fourth side. The brain was isolated from the surrounding fluid but the nerves were allowed to exit (Audesirk & Audesirk, 1980).

The 'whole animal' preparation was also used to study the behavioural role of specific neurones, similar to procedures used by Willows (1967) and Gillette & Davis (1977).

In all three types of preparations, standard electrophysiological recording techniques were employed: microelectrodes filled with 3 M-KCl (resistance 15–30 MΩ) connected to high impedance amplifiers (WPI M4-A) were used for intracellular recordings. Extracellular recordings were made using suction electrodes and amplified with differential amplifiers. All signals were displayed on an oscilloscope (Tektronix 5100) and permanent records were made using a chart recorder (Gould Brush 220).

Neurones were identified on the basis of size and position of the somata and electrophysiological criteria. The electrophysiological criterion used for the identification of an Int-1 was generation of short and constant latency IPSPs on the PCNs. The criteria used for the Int-2s were either driving of a long-latency volley of IPSPs on a PCN and/or increased activation of the metacerebral giant cell (MCG). MCG activity was easily monitored by recording extracellularly from the CBC the giant action potentials of the MCG axon (Gillette & Davis, 1977). This useful criterion arose from the observation that the Int-2s excite the MCG and were the only neurones found in the region to do so (see below). Activation of an Int-2 usually elicited increased spike activity in the MCGs. Int-3s were identified as cells whose activity generated EPSPs on the Int-2s with a short and constant latency.

Synaptic pathways were determined by simultaneous intracellular recordings from pre- and postsynaptic cells. Criteria used for determining monosynapticity were: (1) the occurrence of postsynaptic potentials one-for-one with the action potentials of the putative presynaptic cell: (2) a short (7–10 ms) and constant latency between pre- and postsynaptic events; and (3) the ability of the PSPs to occur in the presence of a high divalent ion saline (Kandel, Frazier, Waziri & Coggeshall, 1967; Cohen *et al.* 1978).

Cells were considered to be electrically coupled if polarization of one cell by injected

current caused polarization of the same sign in a second cell. An electrical synapse was considered to be non-rectifying if current passed equally well from either cell.

The criteria used to determine if a cell had an axon in a peripheral nerve were: (1) the recording of orthodromic spikes in peripheral nerves as a result of stimulated soma spikes and (2) the recording of antidromic spikes in the cell body as a result of nerve stimulation.

The morphology of the cells was studied using the intracellular fluorescent dye Lucifer Yellow (a gift of W. Stewart). Dye injection, histological procedures and photographic procedures were similar to those employed by Stewart (1978).

RESULTS

Three separate, serially-connected populations of neurones were identified, two of which were found to be involved in generating the phasic inhibition of the PCNs that occurs during feeding. One population, termed the Interneurone 1 (Int-1s), makes inhibitory and presumed monosynaptic synapses on the PCNs. A second population of neurones monosynaptically excites the Int-1s; these cells have been termed the Interneurone 2 group (Int-2s). The Int-2s receive inhibition from the Int-1s. The Int-2s also excite the serotonergic metacerebral giant neurones as well as other unidentified units in the brain and buccal ganglion. A third population of neurones synapse on the Int-2s in an excitatory manner and have been termed the Interneurone 3 group (Int-3s).

Interneurone morphology

The somata of all three interneurone populations are located in close proximity on the dorsal surface of the brain (Fig. 1); this area is exposed by reflecting the overlying rhinophore lobes posteriorly. The area is within and immediately adjacent to the region bounded medially by axon bundles leading to the oral veil nerves and laterally by axon bundles of the tentacle nerve ($N = 75$ preparations).

There are at least two Int-1s per hemi-ganglion. This is the maximum number found in any experiment, but there may have been more which were not found due to their small size and the relative difficulty in locating them. Intracellular Lucifer Yellow staining of two Int-1s from different preparations did not fill axons in peripheral nerves (Fig. 2A). Somata are relatively small, approximately 40 μm in diameter for large 600-g animals. There is some dendritic arborization from the axon near the soma. A single process leaves the cell body and terminates near the soma. Results from orthodromic and antidromic stimulation indicate that this cell does not have a peripheral axon.

The Int-2s are a bilaterally symmetrical population of at least two cells per hemi-ganglion. The typical cell morphology is illustrated in a line drawing of an Int-2 cell filled with Lucifer Yellow from the brain of a 600-g animal (Fig. 2B). The cell soma is approximately 60 μm in diameter, and there is extensive dendritic arborization surrounding the cell soma. A process of the cell passes through the commissure connecting the two halves of the brain and terminates in the contralaterally homologous area. Extensive branching occurs at this termination, which presumably

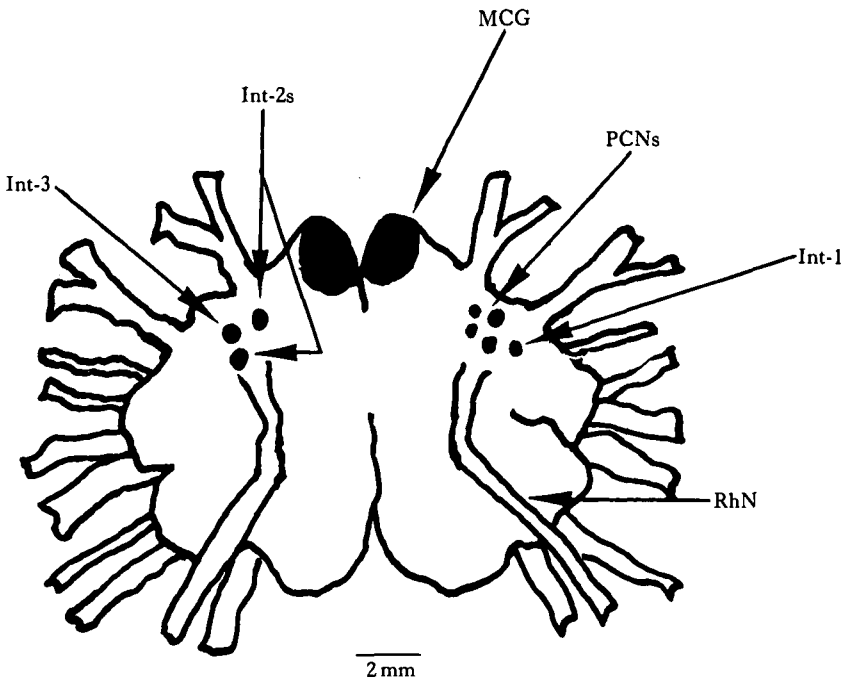

Fig. 1. Line drawing of the dorsal surface of the brain indicating the usual position of the inhibitory pathway cells and some of the cells they affect. The top part of the figure is anterior. The overlying rhinophore lobes have been reflected posteriorly by pinning the rhinophore nerves (RhN) posteriorly in order to achieve easy visualization of the cells studied. The cells are drawn in proportion to their cell soma size. For convenience, all populations except the MCGs are shown in either one or the other side of the ganglion, when they actually are bilaterally symmetrical. PCNs, paracerebral command neurones; MCG, metacerebral giant neurone; Int, Interneurone population.

mediates the contralateral synaptic output described below. The Int-2s do not appear to possess an axon in any peripheral nerves ($N = 4$ cells filled in four preparations). Orthodromic and antidromic stimulation failed to demonstrate the presence of an axon in peripheral nerves or connectives ($N = 4$ preparations).

No morphological data for Interneurone 3 was obtained. Electrophysiological data using orthodromic and antidromic stimulation indicated that the cells of the Int-3 group do not possess axons in the CBCs, nor in the oral veil and rhinophore nerves.

Synaptic connections

The IPSPs recorded in the PCNs were at least in part the result of the combined activity of the Int-1 group. Spike activity in an Int-1 caused discrete IPSPs of 1–2 mV amplitude on the PCN. All criteria for monosynapticity were met (Fig. 3; $N = 15$ dual recordings in 10 preparations). The IPSPs of several Int-1s could sum to generate a larger compound IPSP. Synaptic interaction among Int-1s was not observed, as stimulated spike activity in one Int-1 never caused IPSPs in the PCNs other than its own. The activity of a single Int-1 was not ordinarily sufficient entirely to prevent

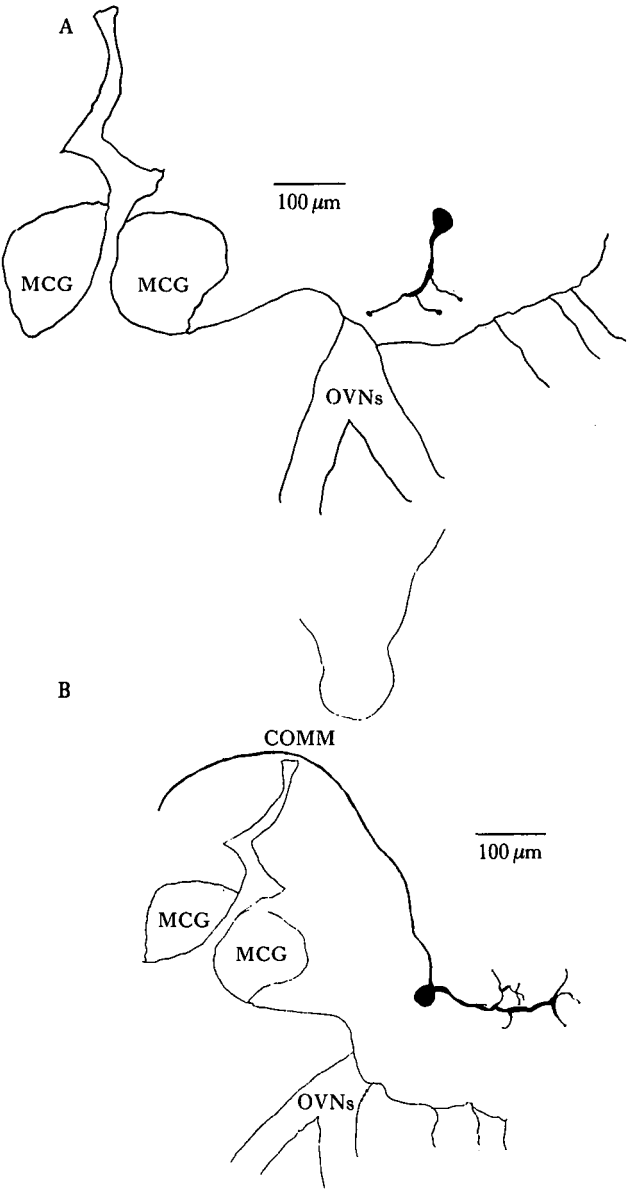

Fig. 2. Line drawings of Lucifer Yellow-filled Int-1 and Int-2 cells in whole mount. (A) The Int-1 cell soma shown is approximately 40 μm in diameter, with dendritic branching in the area close to the cell soma. No peripheral axons were visible, consistent with electrophysiological evidence that this cell is a purely local interneurone. (B) The Int-2 cell soma drawn is approximately 60 μm in diameter. There is extensive arborization of the dendritic tree. An axon passes through the commissure (COMM) and terminates in the contralateral Int-2 region of the brain. No axons exiting any peripheral nerves were found histologically or electrophysiologically supporting the suggestion that the Int-2 is an interneurone. OVNs, oral veil nerves; MCG, metacerebral giant neurone.

spontaneous spiking of a PCN, although it could decrease the frequency of PCN spiking.

In four separate experiments simultaneous intracellular records from Int-1s and

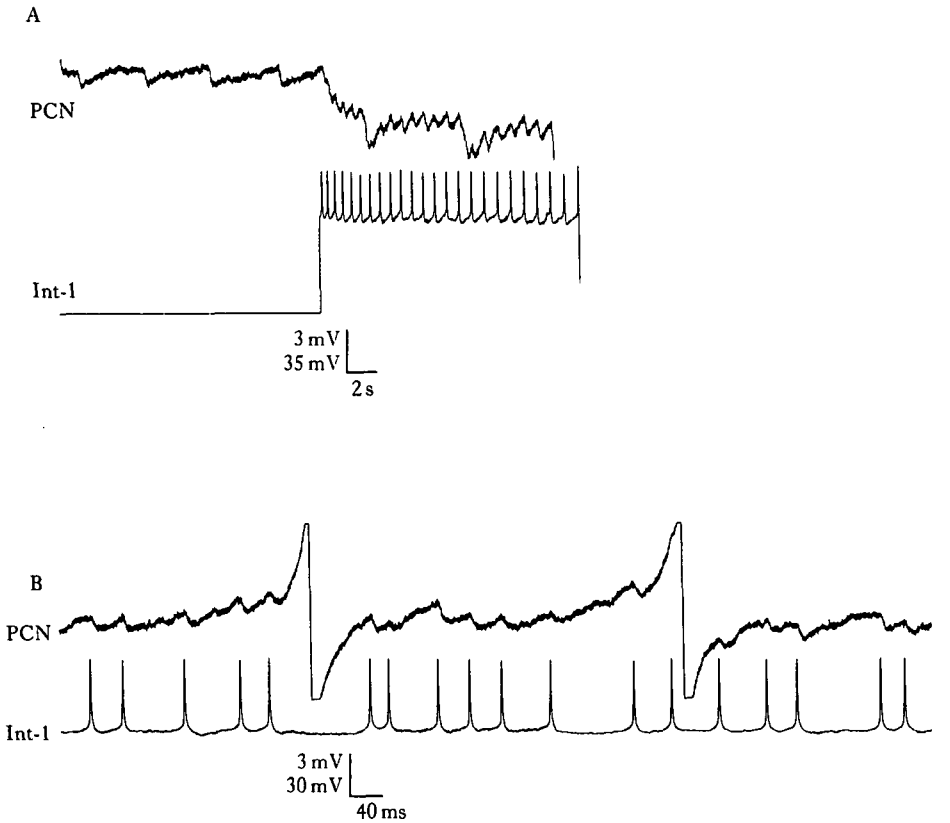

Fig. 3. Int-1 inhibition of PCNs in the isolated nervous system. (A) Depolarization of an Int-1 results in short latency, summating IPSPs in a PCN shown in the upper trace. The spontaneous occurrence of IPSPs which occur prior to activity of the Int-1 shown arise either from other members of the Int-1 group or from other unidentified cells. (B) Spontaneous activity of a single Int-1 is sufficient to decrease the frequency of firing of a PCN but not to prevent the PCN from firing.

Int-2s showed that the Int-2s directly excited Int-1s, and that the Int-1s in turn could exert negative feedback onto the Int-2s. The record of Fig. 4A(i) shows excitation of Int-1 activity caused by stimulation of Int-2 spiking. Conversely, Fig. 4A(ii) shows the suppression of spontaneous Int-2 activity by spiking in Int-1. The records of Fig. 4B show that excitation and inhibition between the two neurone populations arose from discrete EPSPs and IPSPs occurring one-for-one with presynaptic spikes. The PSPs were of short and constant latency and were presumably monosynaptic in origin.

Int-2 activity caused potent and polysynaptic inhibition of the PCNs which must have been mediated at least in part by the Int-1s. Activity in a single Int-2 resulted

Fig. 4. Activity of the Int-2–Int-1 pathway in an isolated nervous system. (Ai) Depolarization and action potentials of an Int-2 cause increased activity in the Int-1. (Aii) Depolarization of the same Int-1 causes inhibition of the same Int-2. (Bi) Records from a different experiment show that Int-2 action potentials occur one-for-one, with a constant latency, suggesting that this is a monosynaptic pathway. (Bii) Data taken from another animal shows that the Int-1 may make monosynaptic inhibitory connections with the Int-2s. Int-1 action potentials generate discrete IPSPs on the Int-2.

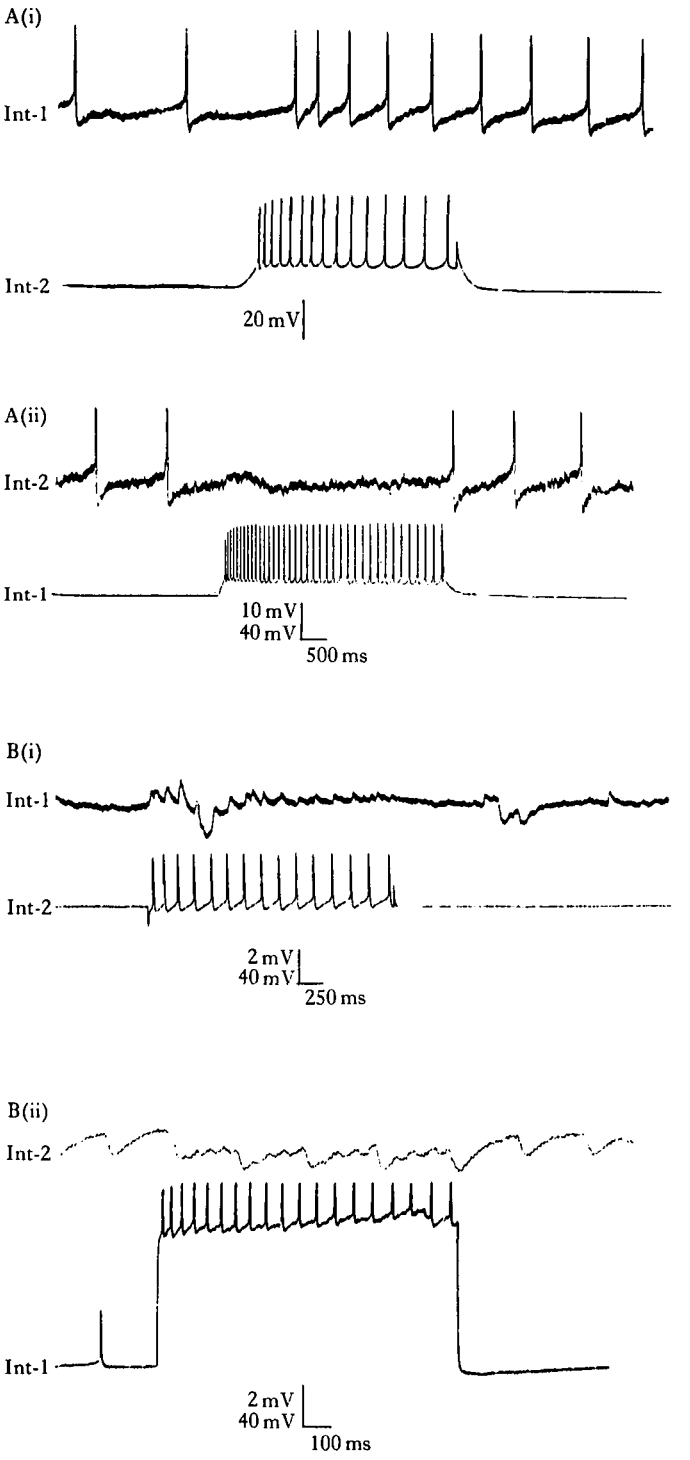
A(i)
Int-1
Int-2
20 mV
A(ii)
Int-2
Int-1
10 mV
40 mV
500 ms
B(i)
Int-1
Int-2
2 mV
40 mV
250 ms
B(ii)
Int-2
Int-1
2 mV
40 mV
100 ms

Fig. 4

in the occurrence of a multitude of IPSPs on the PCNs with latencies of 50–300 ms ($N = 50$ preparations, 70 cells) (Fig. 5). These IPSPs were of varying amplitude, indicating the simultaneous activation of several inhibitory cells as a result of Int-2 activity. The inhibitory barrage in the PCN could outlast Int-2 activity by several seconds, perhaps because of recruitment of the other Int-2s (see below). The inhibition generated by a single Int-2 was sufficient to suppress activity in a depolarized PCN (Fig. 5).

In addition to their connections to the Int-1 neurones, the Int-2s also made strong excitatory connections to a number of brain neurones with axons in the CBCs, among them the MCG neurones (Fig. 6). The records of Fig. 6A show that Int-2 activity increased the frequency of firing of the ipsilateral MCG axon as well as the firing frequency of several unidentified units recorded extracellularly from the CBC. This Int-2 driven activity occurred in the absence of the buccal ganglion, and therefore spiking in the unidentified units originated in the brain. Simultaneous intracellular recordings of an Int-2 and the ipsilateral MCG showed that Int-2 driven excitation of the MCG was a result of an excitatory monosynaptic connection. EPSPs occurred one-for-one with action potentials of the Int-2, 7–10 ms after the action potential regardless of the Int-2 frequency of firing (Fig. 6B). Such EPSPs continued to occur when the external concentration of divalent ions was raised, further indicating that

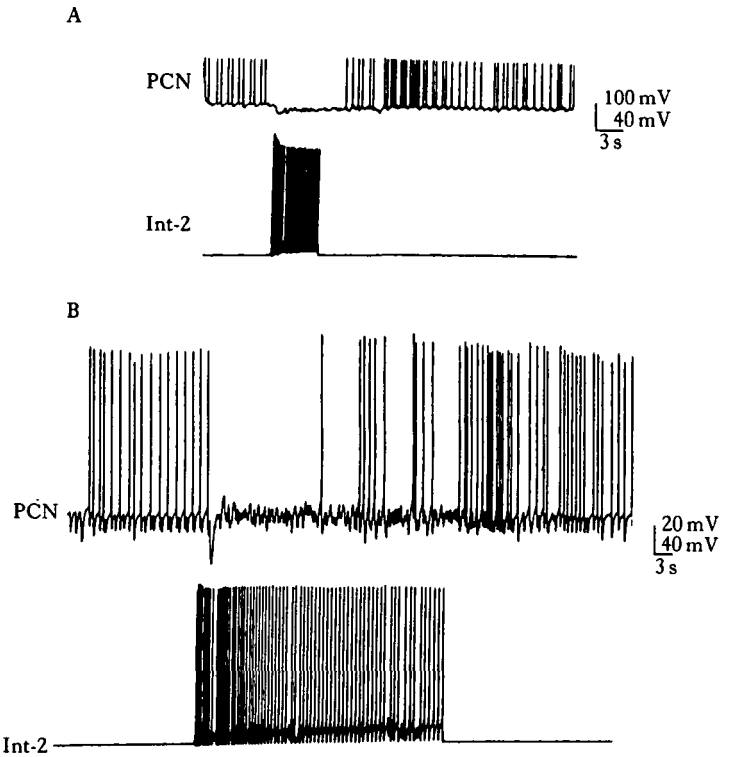

Fig. 5. Inhibition of PCN activity by an Int-2 in an isolated nervous system. (A) Depolarization and spiking in an Int-2 elicits IPSPs in a PCN and results in cessation of PCN activity. Onset of this inhibition is slow and may outlast the activity of the Int-2 by several seconds, indicating a polysynaptic pathway. (B) Action potentials in an Int-2 activate a population of inhibitory cells as reflected in the varied IPSP amplitudes in the PCN.

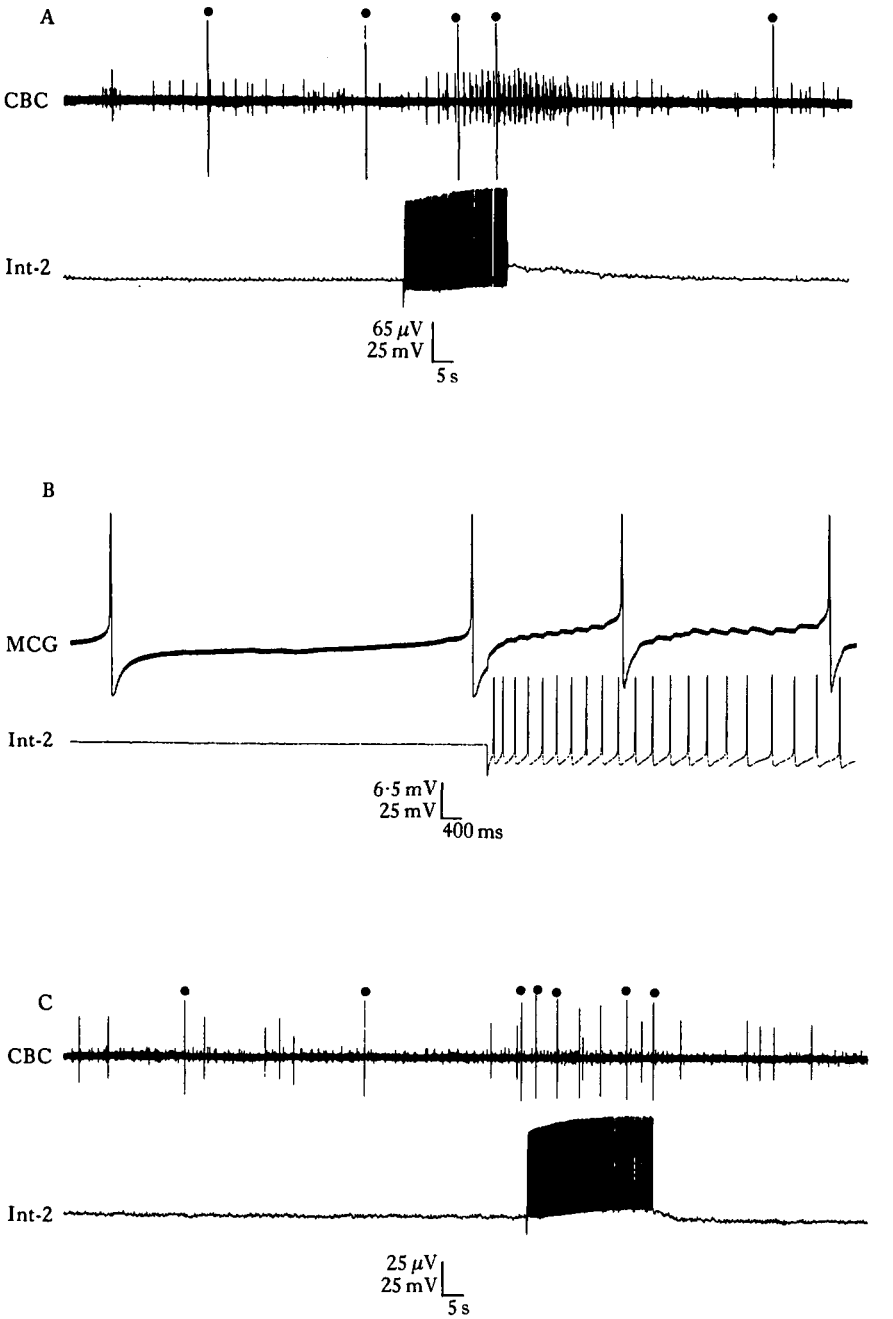

Fig. 6. Int-2 activity drives the metacerebral giant (MCG) and other neurones in the isolated brain. (A) Stimulated action potentials in an Int-2 cause an increase in the frequency of firing of the MCG and unidentified units recorded extracellularly from the cerebrobuccal connective (CBC). The giant axon spikes of the MCG are marked by filled circles. (B) Intracellular recordings of an Int-2 and the ipsilateral MCG. Action potentials in the Int-2 cause EPSPs one-for-one in the MCG and increase MCG spike activity. (C) An extracellular recording of the contralateral CBC and an intracellular recording of an Int-2. Depolarization of the Int-2 increases activity in several unidentified units and the MCG recorded extracellularly from the contralateral CBC.

this connection was monosynaptic. The contralateral MCG was also excited by Int-2 activity (Fig. 6C); however this activity did not persist in the high divalent ion solution, suggesting that activation is polysynaptic.

The Int-3s make presumed monosynaptic connections on the Int-2s ($N = 4$ preparations, five cells) (Fig. 7). Activity in an Int-3 resulted in discrete EPSPs on an Int-2 (Fig. 7A). While Int-3 activity could elicit Int-2 activity (Fig. 7B), activation of a single Int-3 neurone did not result in an increase in activity in the MCG ($N = 4$ preparations), indicating that the polysynaptic pathway from Int-3 to MCG by itself is not particularly effective.

Intrapopulation pathways

Simultaneous penetration of pairs of ipsilateral Int-2s revealed that these cells are electrically coupled in a non-rectifying fashion ($N = 4$ preparations) (Fig. 8). Injection of hyperpolarizing current in one Int-2 caused hyperpolarization in the other (Fig. 8A). The converse was also true (Fig. 8B). Action potentials in either cell of the

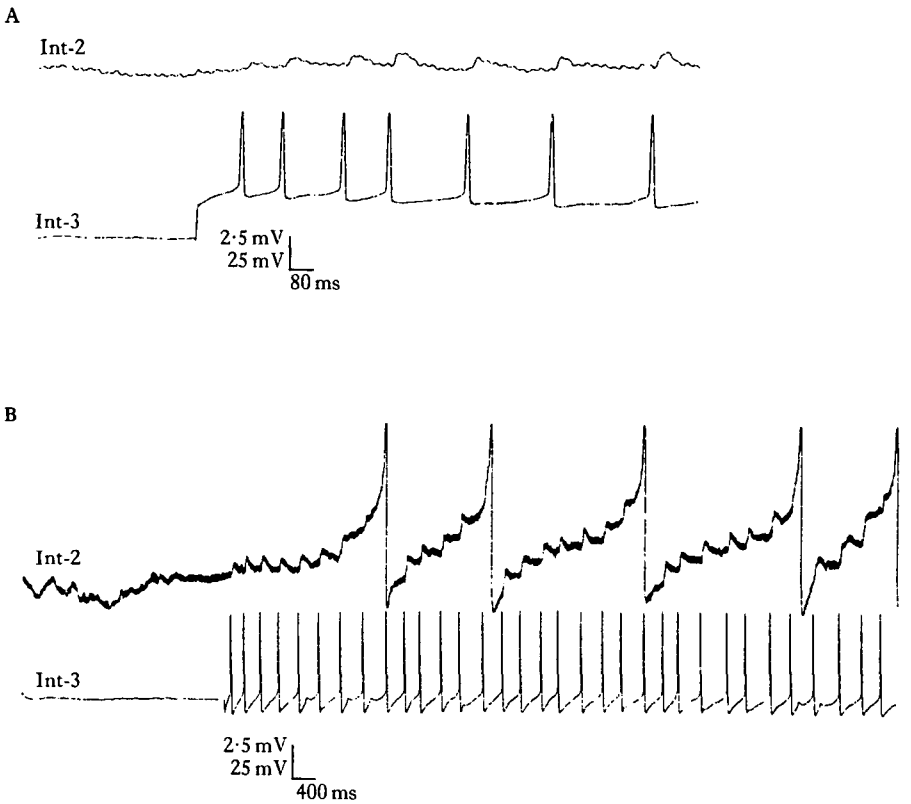

Fig. 7. Int-3s make excitatory connections onto Int-2s. Intracellular records from the isolated nervous system. (A) Spikes stimulated in an Int-3 cause facilitating EPSPs in an Int-2. (B) The Int-3 generated EPSPs can stimulate Int-2 spike activity.

pair resulted in small depolarizations in the other cell. The amplitude of these potentials was not affected by 10–20 mV hyperpolarization or depolarization of the postsynaptic cell.

Simultaneous penetration of two contralateral Int-2s showed that these cells make strong and reciprocal polysynaptic excitatory connections ($N = 3$ preparations) (Fig.

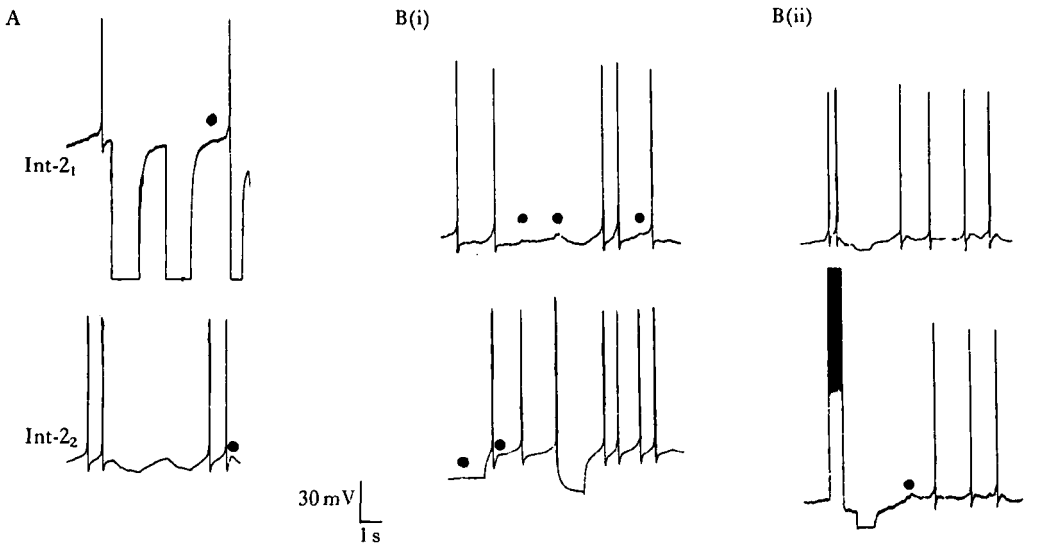

Fig. 8. Electrical coupling of ipsilateral Int-2s. (A) Hyperpolarizing current injected into Int-2$_1$ results in hyperpolarization of Int-2$_2$. An action potential in Int-2$_1$ causes a small depolarizing inflection in Int-2$_2$ (filled circle) and *vice versa*. (B) Reciprocally, hyperpolarization of Int-2$_2$ causes a like potential change in Int-2$_1$. The record of B(ii) also shows depolarization and action potentials elicited in one cell as a result of stimulated activity in the other. Spontaneous action potentials are relatively well synchronized in the two cells; when the spike in one cell fails, a depolarizing, presumably ephaptic PSP is usually evident (filled circles).

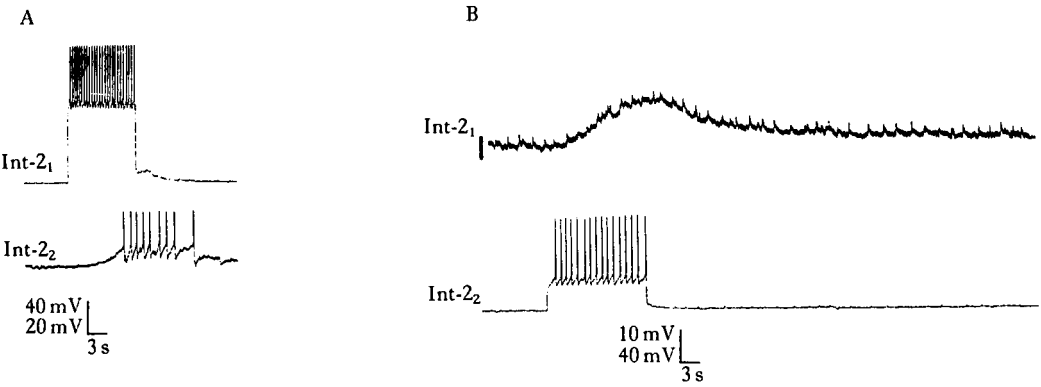

Fig. 9. Reciprocal excitation of contralateral Int-2s in the isolated nervous system. (A) Spiking activity stimulated in an Int-2 (top record) by depolarizing current injection causes delayed long-lasting excitation of a contralateral Int-2. (B) Using the same cells as in A, this data shows that excitation between contralateral Int-2s is two-way.

9). Activity in one Int-2 elicited a long enduring and compound EPSP, which could trigger multiple action potentials in a contralateral Int-2. Activity in the contralateral Int-2 elicited a large, 10 mV, compound EPSP that lasted for several seconds in the other Int-2 (Fig. 9B). The onset of excitation varied from 300 ms to 2 s, and the excitation outlasted activity of the presynaptic cell by several seconds.

Inhibitory neurone activity in rhythmic motor output and in feeding behaviour

Rhythmic motor output in the isolated nervous system may occur spontaneously. Otherwise it may be initiated by means of repetitive stimulation of the stomatogastric nerves of the buccal ganglion, or by stimulation of the PCNs with injected current (Davis & Mpitsos, 1971; Gillette *et al.* 1978). We have observed that the activity occurring in the Int-1 and Int-2 neurones under these conditions is phase-locked to the retraction phase of the feeding cycle; however, we have also observed that the activity phase sometimes varies from cycle to cycle. Such variability has not been observed in hemi-animal preparations, and might therefore be ascribed to the lack of sensory feedback from the cycling buccal mass, which is known to provide timing inputs to the feeding network (Siegler, 1977). When Int-1 activity was coordinated with network output it occurred during the retraction phase of the feeding cycle. This is shown in Fig. 10A as an intracellular record of an Int-1 firing in antiphase to cyclically active retraction units recorded extracellularly from the CBC. Fig. 10B shows looser coordination of Int-1 activity with that of a PCN, a protraction synergist, during cyclic motor activity.

The Int-2 population also fired during the retraction phase of the feeding cycle in the isolated nervous system (Fig. 11A); however in one of eight experiments, Int-2 activity peaks varied within the cycle. The records of Fig. 11B and C confirm this activity in the whole animal preparation induced to feed while an Int-2 was recorded intracellularly. These records compare favourably with similarly obtained records of PCN activity (see Fig. 1B of Gillette *et al.* 1978) in that Int-2 and PCN activity occur in antiphase in the feeding whole animal. Similar records of Int-2 activity were obtained in five hemi-animal preparations stimulated with squid homogenate (SH). Thus, cyclic inhibition of the PCNs by the Int-2s is likely to be a significant factor in timing the rhythmic PCN bursts in the feeding whole animal.

In a single experiment the Int-3s were not observed to be active nor to receive synaptic input during cyclic activity in the isolated nervous system. When the rhythmic pattern occurred and the Int-2 fired cyclically, the Int-3 was silent. Their function is presently not understood.

The potential role of the Int-2s in rhythmic pattern generation was further examined in the isolated nervous system. A rhythmic motor pattern was induced by SGN stimulation while Int-2 activity was intracellularly recorded. Depolarizing current was then injected into a single Int-2 to test the effects of sustained activity on motor output. Fig. 12 shows the results from one of three experiments in which sustained activity of a single Int-2 was sufficient entirely to interrupt the cyclic motor output of the feeding network. The bursting activity of root 1 was suppressed for the duration of Int-2 activity, which in this experiment was approximately 20 s. An increase in SGN stimulus voltage resulted in the resumption of the burst activity in root 1 and in the cessation of Int-2 activity. In view of the widespread output of the

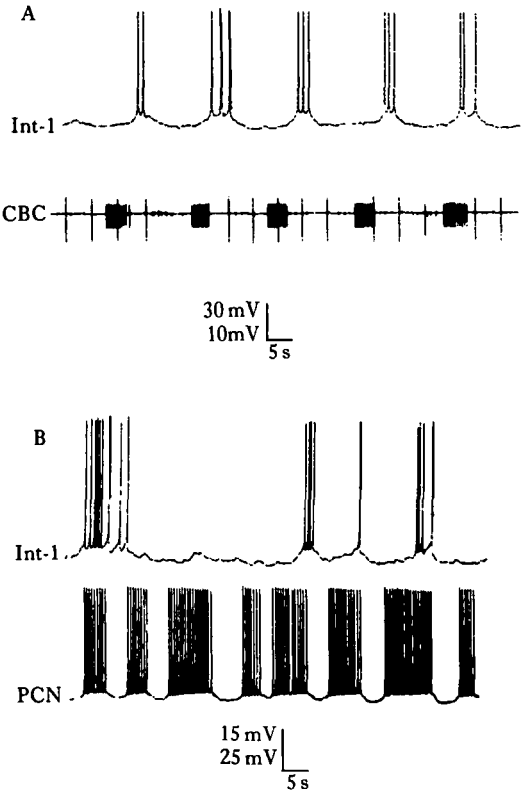

Fig. 10. Spontaneous cyclic activity in the isolated nervous system illustrating the relationship of PCN and Int-1 activity. (A) An intracellular recording of Int-1 activity locked in antiphase to units recorded in the cerebrobuccal connective (CBC). (B) Simultaneous intracellular records from a paracerebral command neurone (PCN) and an Int-1. Int-1 activity is rather loosely related to PCN activity in this isolated CNS, but there appears a tendency to fire towards the end of the PCN (protraction) burst.

Int-2 mentioned above, it is likely that its inhibitory effects in the feeding network are mediated at multiple loci.

Chemosensory inputs to the inhibitory pathway

The hemi-animal preparation was used to assess the effect of appetitive stimuli on the Int-1s, Int-2s and the PCNs. The whole animal preparation was also used to assess the effect of appetitive and noxious sensory stimuli applied to the oral veil on the Int-2s. In all observed preparations ($N = 4$) the Int-1s were excited by SH, and the magnitude of the Int-1 response was positively correlated with the strength of the dilution (Fig. 13). The excitation on the Int-1s was seen as discrete EPSPs, which summed to trigger Int-1 action potentials.

In all observed preparations ($N = 50$) SH elicited excitation of the Int-2s (Fig. 14). This excitation was seen as large EPSPs, up to 10 mV in amplitude, which summed and frequently drove action potentials, (Fig. 14A,C). SH effects were positively correlated with the strength of the dilution. For the cell whose records are shown in Fig. 14, a 100-fold dilution of SH elicited two to three action potentials (Fig. 14A),

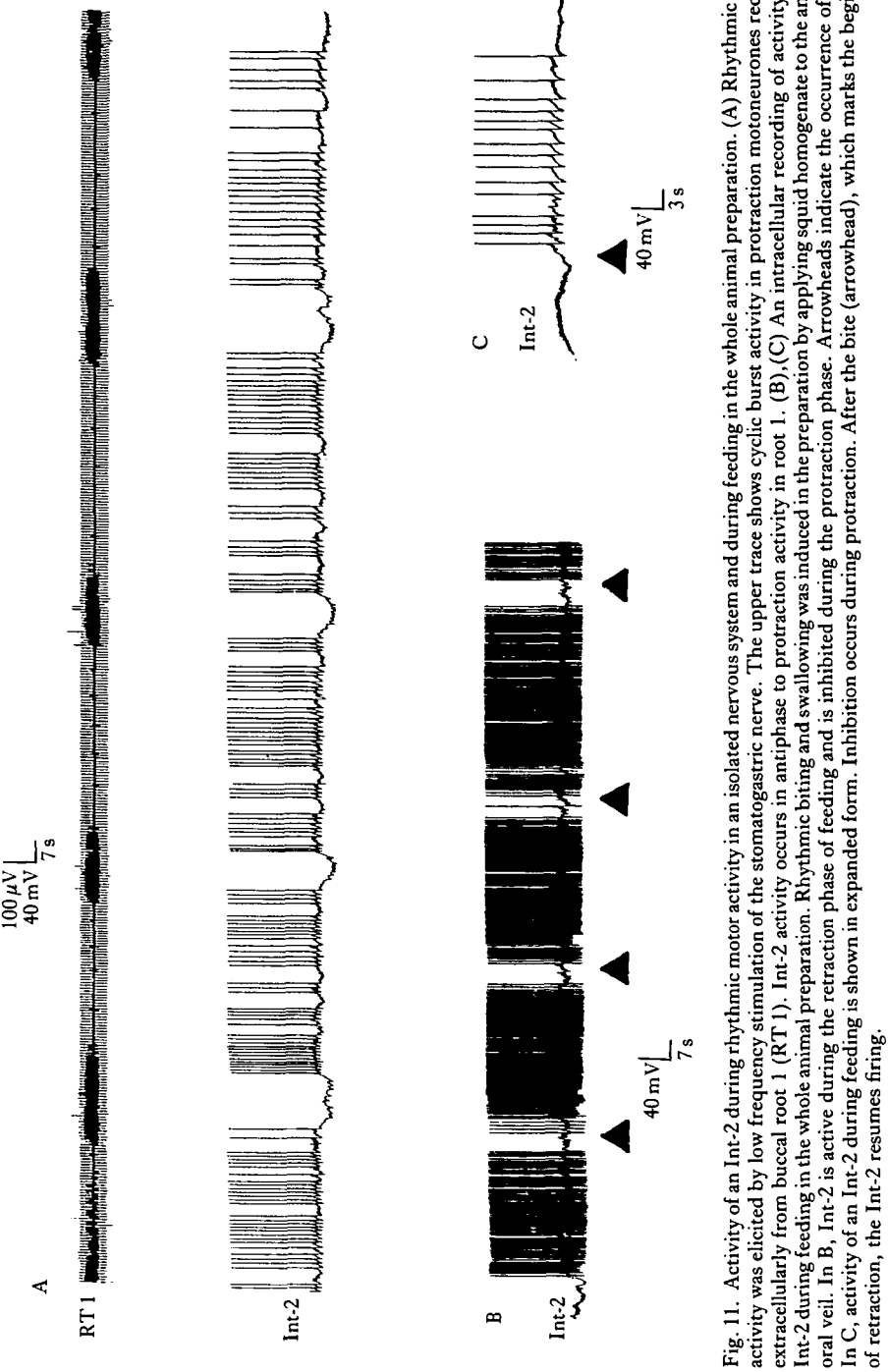

Fig. 11. Activity of an Int-2 during rhythmic motor activity in an isolated nervous system and during feeding in the whole animal preparation. (A) Rhythmic motor activity was elicited by low frequency stimulation of the stomatogastric nerve. The upper trace shows cyclic burst activity in protraction motoneurones recorded extracellularly from buccal root 1 (RT 1). Int-2 activity occurs in antiphase to protraction activity in root 1. (B),(C) An intracellular recording of activity of an Int-2 during feeding in the whole animal preparation. Rhythmic biting and swallowing was induced in the preparation by applying squid homogenate to the animal's oral veil. In B, Int-2 is active during the retraction phase of feeding and is inhibited during the protraction phase. Arrowheads indicate the occurrence of bites. In C, activity of an Int-2 during feeding is shown in expanded form. Inhibition occurs during protraction. After the bite (arrowhead), which marks the beginning of retraction, the Int-2 resumes firing.

Fig. 12. Suppression of cyclic motor output in the isolated nervous system by sustained Int-2 activity. Cyclic motor output was induced by stomatogastric nerve stimulation and recorded in buccal root 1 (RT 1) (upper trace). Stimulation artifacts are superimposed in the recording. The lower trace is an intracellular recording of an Int-2. Cyclic firing in the Int-2 occurs in antiphase with RT 1 bursts, while inhibitory PSP activity occurs in phase with RT 1 bursts. At the arrow the Int-2 was tonically depolarized by injected current; the resulting prolonged spike activity results in cessation of bursting activity in root 1. Increasing the stimulus intensity to the stomatogastric nerve, reflected in the RT 1 trace as an increase in artifact amplitude, silenced the Int-2 and re-established the motor rhythm of root 1.

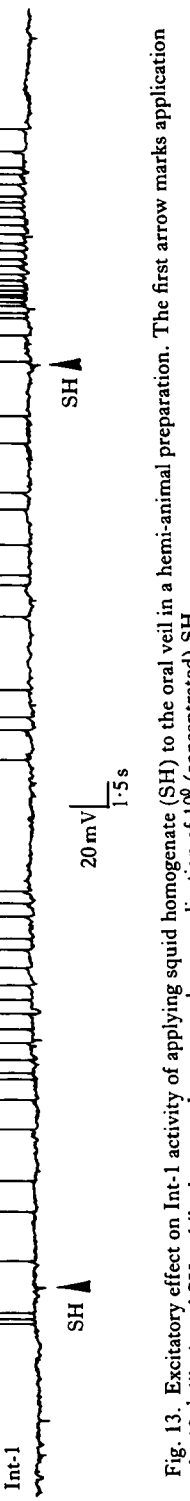

Fig. 13. Excitatory effect on Int-1 activity of applying squid homogenate (SH) to the oral veil in a hemi-animal preparation. The first arrow marks application of a 10^{-1} dilution of SH, while the second arrow marks an application of 10^{0} (concentrated) SH.

while undiluted SH elicited more than 20 action potentials (Fig. 14C). Application of a known aversive and emetic substance, 5 % ethanol in ASW (EtOH), caused slow and complex changes in membrane potential, predominantly inhibitory ($N = 10$, Fig. 14B,D). This inhibition resulted in cessation of Int-2 activity (not shown). Application of a different emetic stimulus, a 5 % Haemosol solution, elicited a response similar to that of the EtOH (not shown).

In both the whole animal and in the hemi-animal preparations, application of SH resulted in synaptic excitation of the Int-2s and PCNs and usually led to overt feeding behaviour. In order to determine whether the sensory input from the oral veil to the PCNs and to the Int-2s could be monosynaptic, we substituted the saline surrounding

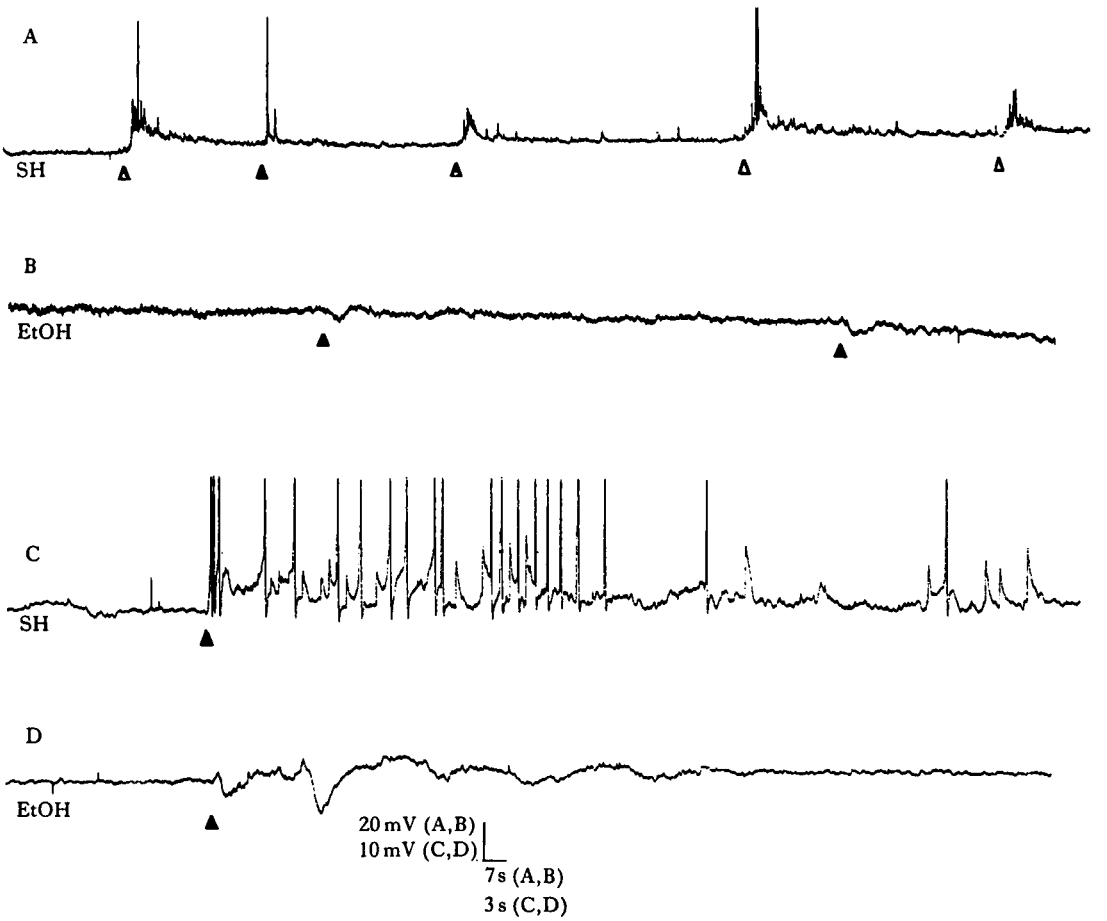

Fig. 14. The effects of the application of chemosensory stimuli to the oral veil on Int-2 activity in whole animal preparations. Arrowheads mark time of application. (A) A small application of a 10^{-2} dilution of squid homogenate (SH; arrowheads) caused a large compound EPSP. (B) Application of an aversive stimulus, 5 % solution of ethanol in sea water (EtOH), elicited inhibitory activity. (C) Intracellular record of an Int-2's response to application of 10^{0} SH (arrowhead). The long-lasting excitation and large EPSPs that occur as a result of the application of the food stimulus contrasts with the Int-2's response to the aversive stimulus, EtOH. (D) The response to EtOH is long lasting and predominantly inhibitory.

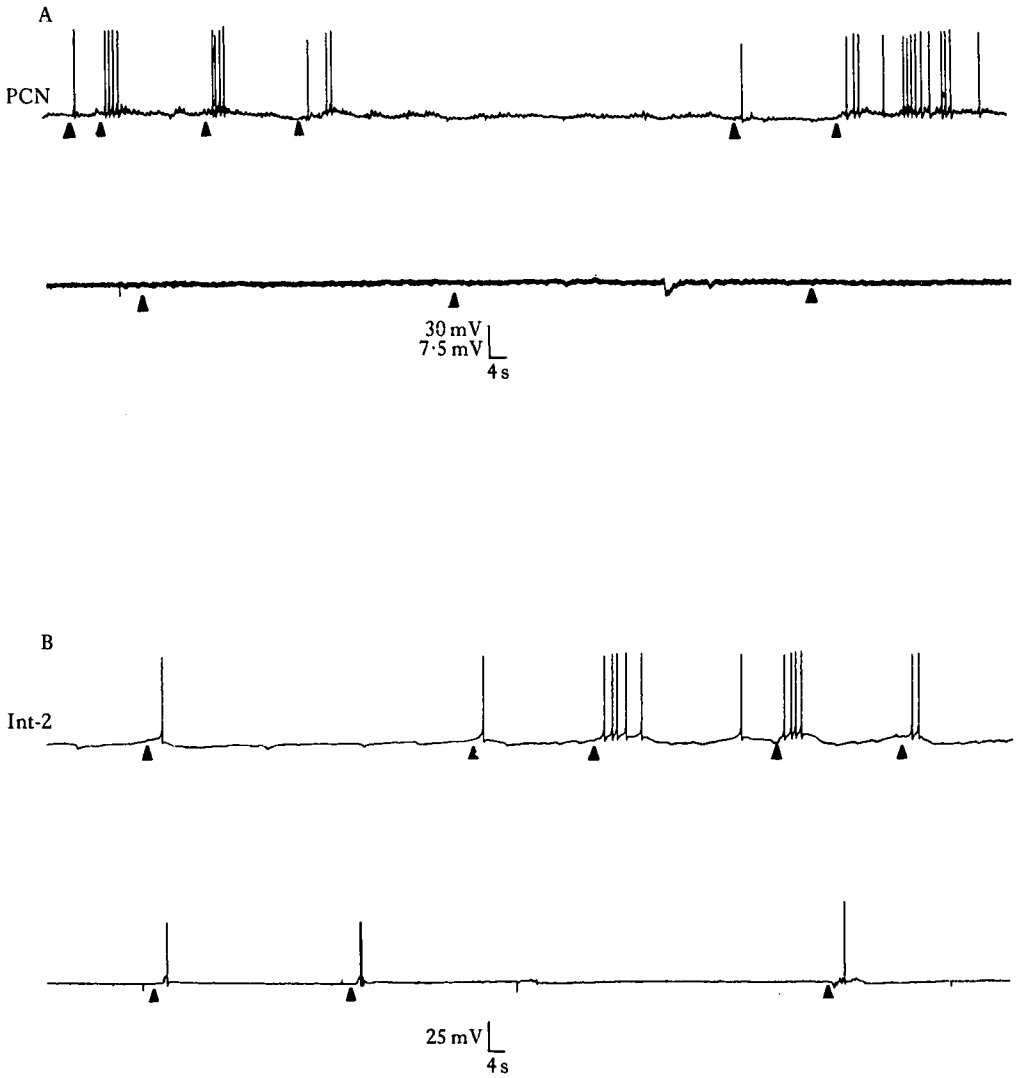

Fig. 15. Mono- and polysynaptic chemosensory pathways to Int-2s and paracerebral command neurones (PCNs). Food-stimulated EPSPs are suppressed in PCNs by a high divalent ion saline, but are not suppressed in Int-2s. (A) A PCN in normal saline (top record) is excited by SH applied to the oral veil (arrowheads). Exchanging the saline bathing the brain for one high in divalent ions suppresses the excitation of the PCN by food (lower record). The gain was increased in this record to emphasize the lack of synaptic input. (B) An Int-2 in normal saline is excited by food stimulation (top record); EPSPs and action potentials are still observed in response to SH stimulation in the high divalent ion saline (lower record). These data suggest that chemosensory excitation of the PCNs from the oral veil involves a polysynaptic pathway within the brain, while the Int-2s receive direct excitation.

the brain with a high divalent ion solution (Fig. 15). In high divalent ion saline PSP activity on the PCNs was not seen to result from application of SH ($N = 2$ preparations, three cells) (Fig. 15A). In contrast, the Int-2s under the same conditions still received excitation as a result of SH application ($N = 2$ preparations, two cells) (Fig. 15B). These data suggest that chemosensory input from the oral veil to the Int-2s is monosynaptic, while input to the PCNs is not monosynaptic.

DISCUSSION

We have described here an inhibitory pathway to the PCN neurones which has strong and pervasive effects within the nervous system, and potentially mediates the effects of food-avoidance training in the suppression of feeding behaviour. The pathway consists of three groups of serially connected interneurones named the Int-1s, Int-2s and Int-3s. The inhibitory Int-1s appear to synapse monosynaptically on the PCNs (Fig. 3) and are driven directly by the Int-2 interneurones (Fig. 4). Within the Int-2 group, connections reinforce excitation; ipsilateral Int-2s are electrically coupled (Fig. 8) and the contralateral Int-2s make reciprocal chemical excitatory connections (Fig. 9). While these chemical connections appear polysynaptic, they are also potent (Fig. 9). Of the three groups, the function of the Int-3 neurones remains unclear. The apparent absence of synaptic inputs to the Int-3s raises the possibility that these neurones might carry sensory information from the periphery and thus are not real interneurones.

Because of their coupling and extensive synaptic outputs, the Int-2s are singly the most potent neurones in the inhibitory pathway to the PCNs and have a strong and widespread influence within the feeding network. Aside from their excitatory connections to the Int-1s, cells in the Int-2 population make a monosynaptic excitatory synapse on the ipsilateral MCG (Fig. 6), a neurone which in *Pleurobranchaea* has been shown to exert influence on the feeding oscillator (Gillette & Davis, 1977). The Int-2s also excite numerous other unidentified units whose spikes could be recorded extracellularly from nerves and connectives. The Int-1s and some of the unidentified units are likely to be the agents through which the Int-2s are able to cause suppression of fictive feeding activity in the isolated neurone system; thus the Int-2s may occupy a crucial role in governing the expression of feeding behaviour. The pervasive effects of Int-2 action within the feeding network are illustrated by the observations that Int-2 activity can interrupt fictive feeding driven by repetitive stimulation of the SGN (Fig. 12). This indicates sites of Int-2 action in the buccal ganglion, because the motor rhythm driven by SGN stimulation originates largely in the buccal ganglion oscillator and is not dependent on the activity of the PCNs of the brain (Davis, Siegler & Mpitsos, 1973; Cohan & Mpitsos, 1983). Therefore the Int-2s act to suppress feeding behaviour both by inhibiting the PCN command neurones in the brain and also by way of units with axons descending to the buccal ganglion oscillator.

A potentially dominating role for the Int-2 neurones in feeding behaviour is also suggested by the observations that the Int-2 neurones may receive direct chemosensory input from the primary sensory apparatus, the oral veil, while the PCN command neurones do not (Fig. 15). The PCNs do receive excitation from the buccal oscillator. From these relationships it may be concluded that Int-2 inhibition of PCN activity

is accomplished in part indirectly, by suppressing excitation from the buccal oscillator, as well as by IPSPs from the Int-1s. It may also be concluded that 'command' neurones may sometimes not initiate decisions, but only mediate them.

Kovac, Davis, Matera & Croll (1983) have also described poly- and monosynaptic inhibitors of the PCNs and have studied their physiological properties in the isolated nervous system of *Pleurobranchaea*. These authors described two populations of polysynaptic inhibitors, one dorsally located on the brain and another ventral. Our laboratories are in agreement on the identity of the dorsal population with the Int-2s and the monosynaptic inhibitors as the Int-1s. Our observations on connections within the Int-2 population, and from the Int-2 to Int-1 to PCN are also in accord, as are our observations of activity patterns during fictive feeding. These authors have made the additional interesting observation that the three Int-2s of a side may be divided into three morphological classes, although the individual cells are physiologically indistinguishable.

A model for Int-2 function

These data provide for a neural model to explain the suppression of feeding behaviour in food-avoidance conditioned animals. In this model, pattern generation in the feeding network would be blocked by enhanced excitation of the Int-2s.

These relations are summarized in the model of Fig. 16. For the sake of simplicity the oscillator network is depicted as equivalent to a half-cell oscillator (Perkel & Mulloney, 1974), in which the populations of retractor neurones (R) and protractor neurones (P) are reciprocally and inhibitorily coupled. While the structure is undoubtedly more complex, the simplification is useful and based on the functional equivalency of the oscillator's output. That is, most feeding neurones fire either during retraction or protraction, there is significant positive coupling within retractor and protractor neurone populations, and there is demonstrable negative coupling between the populations (Siegler, Mpitsos & Davis, 1974,; Gillette & Davis, 1977; Gillette *et al.* 1978).

One major feature in the model is that chemosensory excitation from the oral veil is conveyed directly to the Int-2s, but only indirectly to the paracerebral command neurones. A second feature is that the Int-2 neurones cause widespread inhibition of protraction neurones and excitation of retractor neurones. These actions are consistent with their inhibitory effects on the PCNs (protractor neurones) and excitatory effects on the MCGs and Int-1s (retractors); the actions also fit well their abilities to phase-shift the feeding network during cyclic activity and even suppress feeding altogether. The inhibitory action of the Int-2s on protractor neurones is mediated by the Int-1s to the PCNs and possibly by unidentified brain neurones elsewhere in the network. The circuitry of the model is consistent with the notion that the suppression of feeding behaviour in the food avoidance-conditioned animals may be driven by prolonged Int-2 activity. This might result either from enhanced chemosensory inputs specifically to the Int-2s or from enhanced excitability of the Int-2s themselves. As drawn, the model does not show the excitatory chemical synaptic coupling between contralateral Int-2 populations which contributes to Int-2 excitation. The excitatory and inhibitory connections between the Int-2s and the oscillator neurones are reciprocal and are the synaptic context within which the Int-2s can

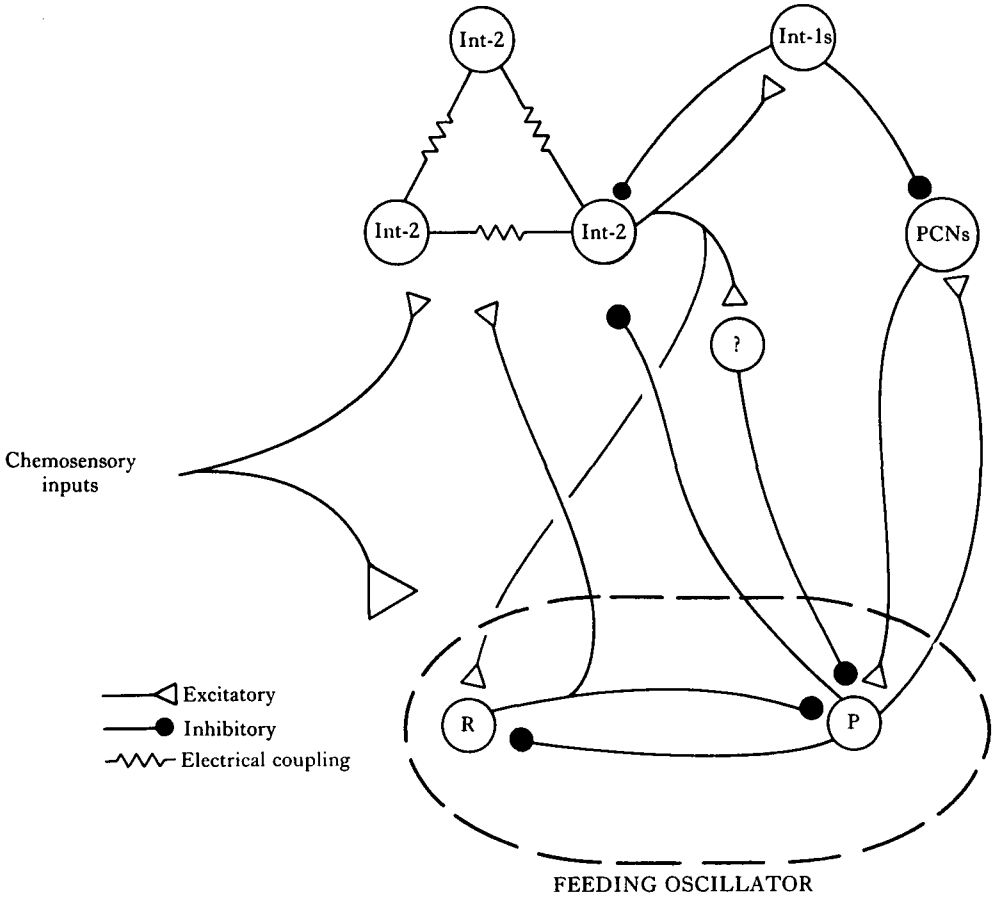

Fig. 16. A model for the inhibitory circuitry governing paracerebral command neurone (PCN) inhibition and effects on the feeding network. The function of the Int-2s within this context is discussed in the text. R, retractor neurones; P, protractor neurones.

regulate pattern generation; the Int-2s, Int-1s and PCNs are in fact components of the oscillator.

A critical role for the Int-2 neurones in the regulation of feeding behaviour is supported by our recent data, which indicates that these neurones are more excited by food stimuli in food-avoidance conditioned than in control animals (London & Gillette, 1983; J. A. London & R. Gillette, in preparation).

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