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SEROTONERGIC MODULATION OF PATTERNED MOTOR OUTPUT IN *LYMNAEA STAGNALIS*

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SUMMARY

Rhythmic feeding motor output from the buccal ganglia of *Lymnaea stagnalis* was evoked by tonic depolarization of the pattern-initiating interneurone SO in the isolated central nervous system.

Perfusion with 10^{-4} mol l^{-1} serotonin (5-HT) led to a reduction in frequency of the SO-driven rhythm, and in some cases rhythmic activity was completely blocked. The frequency reduction was predominantly due to an increase in duration of the 'inactive' phase of the rhythm. In a number of preparations, the normal buccal rhythm was replaced by an 'atypical' pattern of bursting in buccal motoneurones in the presence of 5-HT. This was characterized by the absence of one phase (N2) of interneuronal activity in the feeding pattern generator.

Stimulation of the serotonergic giant cerebral interneurones (CGCs), to increase the mean spike frequency from 1·0 to 2·5 Hz, mimicked some of the effects of 5-HT perfusion. However, the timing of onset of CGC stimulation in relation to depolarization of SO was critical; prolonged activation of a CGC led to an apparent decrease in its effectiveness in suppressing the buccal rhythm.

INTRODUCTION

The generation of rhythmic motor patterns by centrally located networks of neurones (central pattern generators; CPG) is well-documented (Delcomyn, 1980; Selverston, 1980). More recent work has demonstrated a putative role for a variety of neuroactive substances in the initiation and modification of behaviour patterns in both vertebrate and invertebrate preparations (Claassen & Kammer, 1986; Harris-Warrick & Cohen, 1985; Livingstone, Harris-Warrick & Kravitz, 1980; Murphy, Lukowiak & Stell, 1985; Trimble & Barker, 1984). In one study, endogenous biogenic amines have been proposed as mediators of 'plasticity' within a CPG, allowing for the generation of a number of different motor patterns by a single motor network (Flamm & Harris-Warrick, 1986*a*,*b*). However, the demonstration of an effect on motor output following gross application of a neuroactive substance does

Key words: *Lymnaea stagnalis*, gastropod feeding, central pattern generator, serotonin, rhythmic motor output.

not confirm a physiological role for that substance, particularly if its source within the nervous system has not been located. It is therefore important to identify both the source of a putative modulator and its activity during the generation of patterned output. One step towards this goal would be, for example, to show that application of a biogenic amine can, at least in part, mimic the actions of identified aminergic cells on the activity of the CPG. This would then permit more useful studies to be carried out on the precise effects of neuromodulators on elements of the CPG and thus on behavioural output.

The neural circuitry controlling feeding motor output in the snail *Lymnaea stagnalis* has been studied in depth (Benjamin, 1983), including a detailed description of the interneuronal elements of the CPG, and their interconnections (Elliott & Benjamin, 1985*a*). The CPG, located in the buccal ganglia, comprises three main subnetworks of interneurones, N1, N2 and N3(phasic) interneurones, which burst consecutively to produce the three phases of each feeding cycle, radula protraction, radula retraction and swallowing (Benjamin, 1983; Elliott & Benjamin, 1985*a*). Synaptic connections made by these interneurones with buccal motoneurones lead to a three-phase cycle of coordinated bursting activity. The activity of the elements of the CPG can thus be monitored by recording synaptic and spiking activity in identified buccal motoneurones. For example, the 4-cell, a retractor motoneurone, receives successive compound inhibitory inputs from N1 and N2 interneurones and then fires a burst by postinhibitory rebound (Benjamin, 1983). This burst is often fractionated into sub-bursts by a series of brief inhibitory inputs from N3(phasic) interneurones. The buccal 1-cell receives a compound excitatory input from N1 interneurones, causing it to fire a burst of spikes during the radula protraction phase. Rhythmic feeding motor output can occur spontaneously in the isolated CNS; however, such activity is often quite variable. It can also be driven by tonic depolarization of the buccal slow oscillator (SO) interneurone (Rose & Benjamin, 1981; Elliott & Benjamin, 1985*b*), which produces a much more consistent pattern of activity than that occurring spontaneously. During steady depolarization of SO to activate the CPG, SO itself receives synaptic feedback from CPG interneurones (Elliott & Benjamin, 1985*b*). In particular, N2 activity produces a strong compound inhibition of SO, and N3 inhibitory inputs are also seen.

The paired serotonergic cerebral giant cells (CGCs; McCrohan & Benjamin, 1980*a*) of *Lymnaea* are known to modify feeding motor output from the buccal ganglia. Tonic excitation of the CGCs increases the intensity of motor bursts occurring in buccal motoneurones, but has little or no effect on their frequency (McCrohan & Benjamin, 1980*b*). A brief (1–4 s) high-frequency burst of spikes in a CGC often leads to a much longer-lasting (up to 40 s) alteration in the frequency and intensity of feeding motor output (McCrohan & Audesirk, 1987), indicating prolonged effects on the CPG. In a quiescent preparation, long-term depolarization of a CGC can lead to initiation of rhythmic motor output, but only after a delay of more than 10 s (McCrohan & Audesirk, 1987). The CGCs are therefore clear candidates for modifiers of patterned motor output, using the biogenic amine, serotonin (5-HT).

In this paper we compare the effects of bath perfusion of 5-HT with stimulation of CGCs on feeding motor output in *Lymnaea*. The phases of interneuronal activity in each feeding cycle were monitored by recording rhythmical synaptic inputs to buccal motoneurones (types 1 and 4) and to SO. Patterned buccal activity was driven in repeatable bouts by activation of SO. This avoided as far as possible the large variations in frequency, intensity and occurrence of the rhythm that occur over periods of only a few minutes of spontaneous buccal activity. It also enabled us to assess the modulatory effects of 5-HT and CGC stimulation on an activated rhythm. The importance of this point was stressed by Gelperin (1981) when he stated that 'to understand the role of serotonin it is absolutely *essential* to be able to interact serotonergic stimulation with activation of other pathways to a neuron or effector'.

MATERIALS AND METHODS

Specimens of *Lymnaea stagnalis* weighing between 3 and 4 g were collected from ponds in Stockport, Cheshire. They were kept in tap water at room temperature and fed on lettuce.

The central nervous system (CNS) preparation was pinned onto a Sylgard-lined platform and mounted in the recording chamber of a perfusion system. Prior to recording, the outer connective tissue sheath of the areas to be recorded was stripped using fine forceps. The inner sheath was left intact. Intracellular recordings were made from one or two identified feeding motoneurones in the buccal ganglia using conventional electrophysiological techniques (McCrohan & Audesirk, 1987). This enabled us to monitor the occurrence and phase of rhythmic feeding motor output (Benjamin, 1983). A further electrode penetrated the buccal slow oscillator (SO) interneurone. This was used both for recording, and for current injection. Feeding motor output was driven periodically by a series of tonic 45-s constant-current depolarizations (up to 4 nA) of SO. Each 45-s depolarization was followed by a 30-s rest period. This produced highly repeatable 45-s periods of rhythmic bursting activity in both the motoneurones and SO.

The perfusion apparatus was based on the design of Glaizner (1973). This allows for a continuous flow of saline over the preparation at a rate of $2{\cdot}5\,\mathrm{ml\,s^{-1}}$. For the 5-HT perfusion experiments, the preparation was first perfused with normal Hepes-buffered saline (McCrohan & Benjamin, 1980*a*) for 5 min. Next the perfusate was switched remotely to a solution of $10^{-4}\,\mathrm{mol\,l^{-1}}$ 5-HT (5-hydroxytryptamine creatinine sulphate; Sigma) in saline for 10 min, before switching back again to normal saline for a further 15 min. The dead-space time of the perfusion apparatus was estimated by observing the time for removal of a dye solution during perfusion. The solution was completely replaced within 30 s.

In the experiments to assess the effect of CGC activity on driven feeding motor output, a single CGC was penetrated with a microelectrode and stimulated phasically using 1-s, 3- to 4-nA depolarizing pulses at 0·5 Hz. This led to an increase in mean spike frequency from approximately 1·0 to 2·5 Hz. Phasic stimulation avoided the

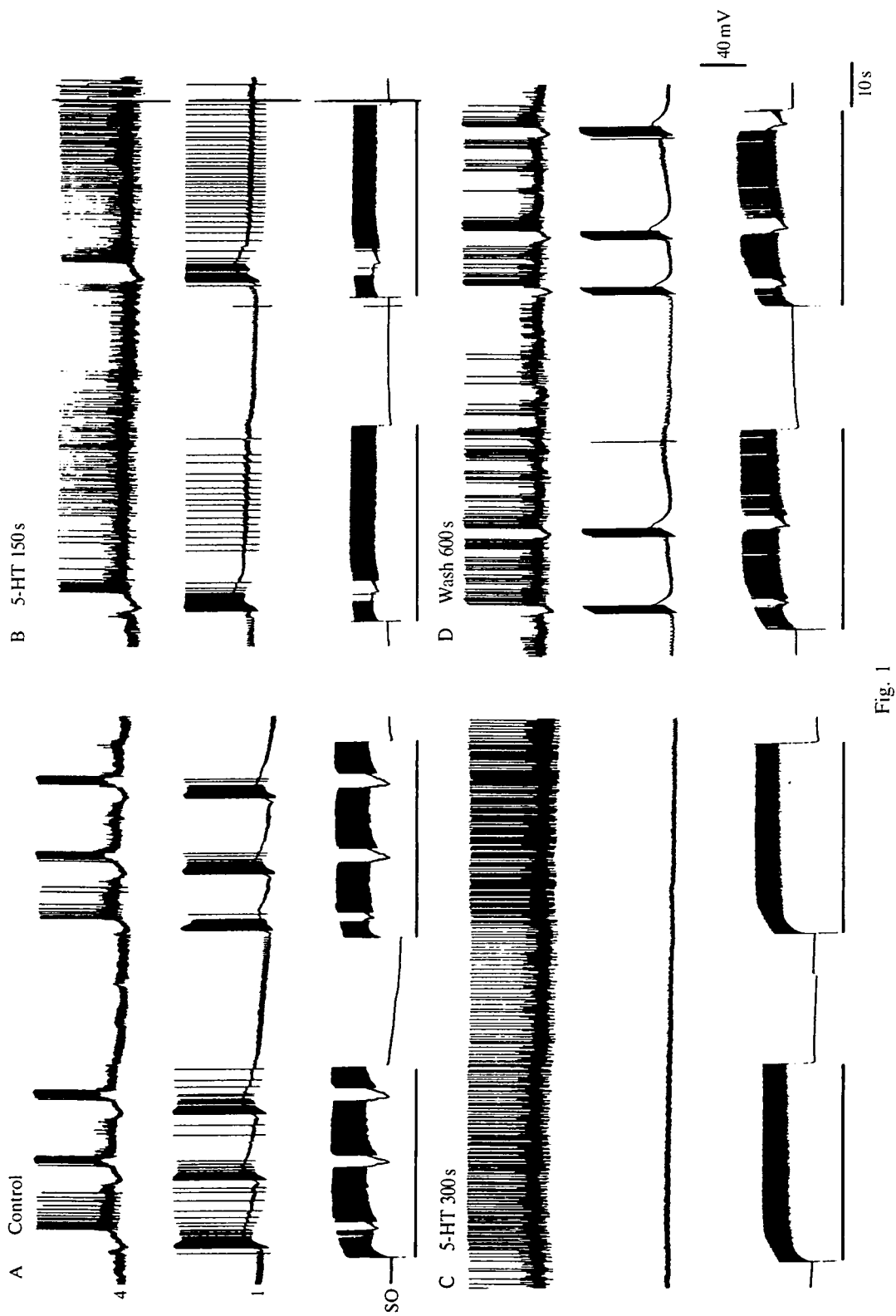

Fig. 1

accommodation of spike frequency seen in these cells during tonic depolarization (McCrohan & Audesirk, 1987).

RESULTS

Effect of 5-HT on driven feeding motor output

Eleven preparations were perfused with 10^{-4} mol l^{-1} 5-HT whilst being subjected to the 45 s:30 s depolarization:rest procedure for stimulation of SO. Prior to perfusion with 5-HT, SO stimulation induced highly reproducible 45-s bouts of rhythmic motor output, which were identifiable as rhythmic synaptic inputs and bursts of spikes in buccal feeding motoneurones and in the SO itself (Figs 1A, 3A). During the 30-s rest period no feeding cycles occurred.

5-HT perfusion produced two clear effects on the pattern of the rhythm. First, it caused a decrease in the frequency of normal feeding cycles in all 11 preparations. This led to a reduction in the capacity of SO to elicit rhythmic motor output. For example, for the preparation illustrated in Fig. 1, a progressive decline in frequency of cycles occurred after 5-HT application until, after 150 s, the SO could drive no more than a single feeding cycle per 45 s (Fig. 1B). Ultimately, 5-HT blocked SO-driven feeding motor output altogether (Fig. 1C). This complete block of rhythmicity was seen in seven of the 11 preparations. In the presence of 5-HT, tonic firing activity in the absence of rhythmical inputs was increased for buccal retractor motoneurones (4-cells). In addition, depolarization of SO led to an increase in firing rate in these cells (Fig. 1B,C). All these effects of 5-HT were reversible after 5–10 min of washing in normal saline (Fig. 1D). When examined in more detail, the effects of 5-HT on feeding cycle frequency were seen to be mainly due to an increase in the duration of the period between feeding cycles (I; Fig. 2). There was a slight increase in the duration of the first, N1, phase of the cycle. This is best seen as an increase in the duration of the protraction-phase burst of spikes in the 1-cell of Fig. 2. There was also some evidence for a reduction in amplitude of the second, N2, phase of synaptic input in the presence of 5-HT, especially when compared with the second and third cycles evoked per 45 s bout in normal saline (Fig. 2). N2 phase input is clearly seen as a compound inhibitory input on the SO.

A second effect of 5-HT on the pattern of SO-driven rhythmic activity was observed in eight of 11 preparations. In the presence of 5-HT, normal feeding cycles were reduced in frequency, but were largely replaced by a higher-frequency atypical rhythm (Fig. 3). After about 5 min in 5-HT, this rhythm dominated the activity of the motoneurones during SO depolarization (Fig. 3C), reaching a maximum

Fig. 1. Effect of perfusion with 10^{-4} mol l^{-1} 5-HT on SO-driven rhythmic motor output, monitored from buccal motoneurones 1 and 4. SO was depolarized by constant current injection during the periods indicated by bars. (A) Normal saline: 3 cycles of feeding motor output are generated for each 45-s period of SO depolarization. (B) 150 s after onset of 5-HT perfusion: SO drives only 1 cycle per 45 s. (C) 300 s after onset of perfusion: generation of rhythmic activity is blocked. (D) 600 s after onset of washing with normal saline: buccal neurones generate 2 or 3 cycles per 45 s of SO depolarization.

frequency of between 12 and 17 cycles per 45 s. After a period of washing in saline, normal feeding cycles began to reappear and the atypical rhythm declined (Fig. 3D). The atypical rhythm was reflected in the activity of the motoneurones and SO (Fig. 4B). When compared with the normal rhythm, it was characterized by the

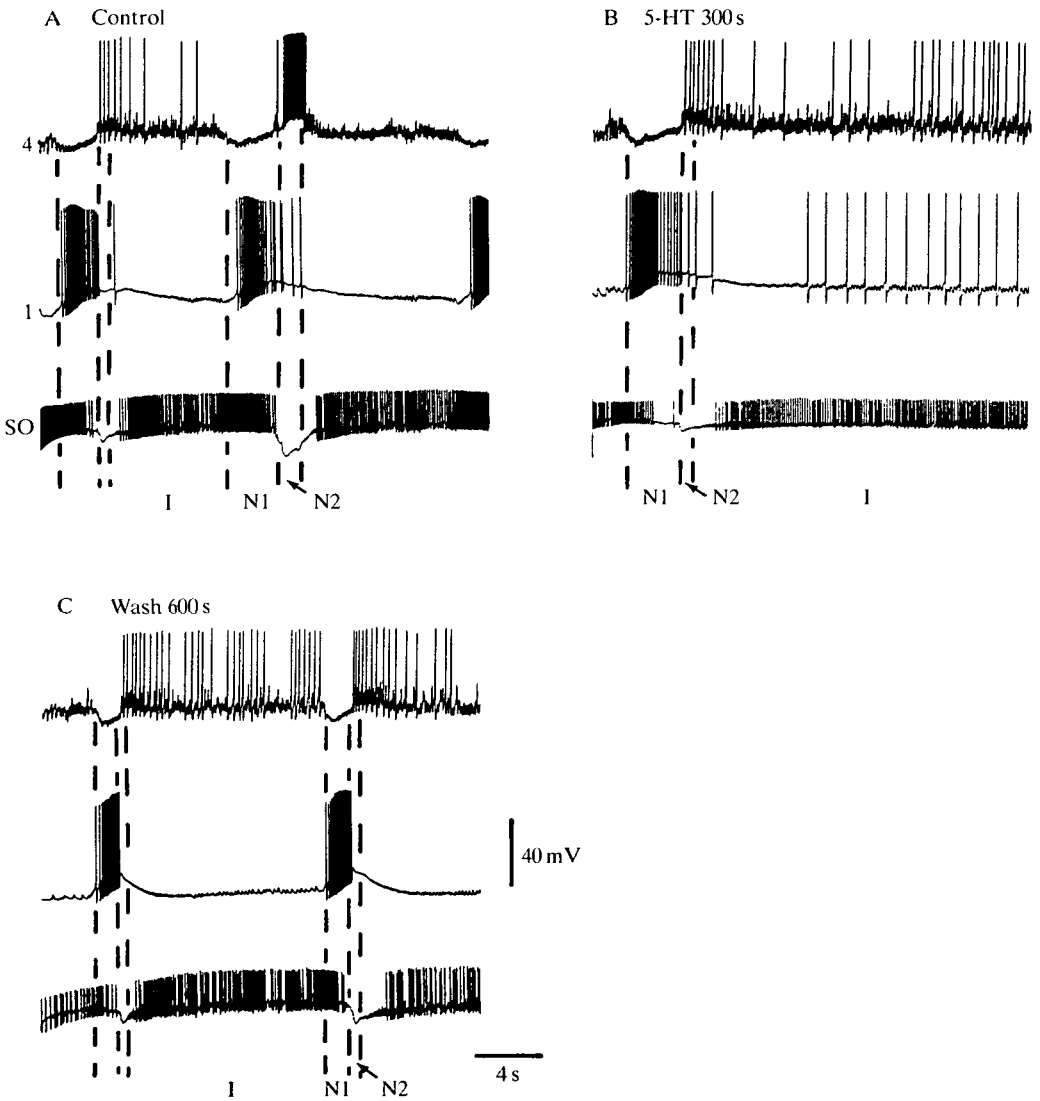

Fig. 2. Effect of perfusion with 10^{-4} mol l^{-1} 5-HT on SO-driven rhythmic motor output. Detail from experiment shown in Fig. 1. Phases of buccal interneuronal activity during the feeding cycle (N1 and N2) are shown. I indicates inactive period between cycles. (A) Normal saline: first two cycles from an SO-driven bout. (B) 150 s after onset of 5-HT perfusion: single feeding cycle generated at onset of SO depolarization. (C) 600 s after onset of washing with normal saline: first two cycles from an SO-driven bout of rhythmic activity. The main effect of 5-HT on CPG output is an increase in the duration of I.

absence of N2 phase inhibitory inputs in both 4-cell retractor motoneurones and SO, and by more obvious phasic N3 inputs which fractionated the 4-cell bursts and were also visible as oscillatory inhibitory inputs to SO (Fig. 4). The atypical rhythm can thus best be interpreted as an alternation of activity in the N1 and N3 networks of the feeding rhythm generator with little or no contribution from N2.

Effect of CGC stimulation on driven feeding motor output

Seven trials were carried out on four animals. In each trial, 45-s bouts of SO-driven activity were initiated in the absence of CGC stimulation, and then at different times (30, 15, 0 s) after the onset of phasic CGC stimulation. The effect of CGC stimulation on the rhythm depended on the time for which the CGC had been activated prior to depolarization of SO. Fig. 5 shows the results for one trial, which were representative of those obtained for all seven trials.

When CGC stimulation started 30 s before the next bout of evoked activity, cycle frequency was reduced compared with the control (from 11 to 9 cycles per 45 s; Fig. 5A,B). When examined in more detail (Fig. 6), this effect was seen to be due to an increase in the interval (I) between cycles rather than any significant alteration in amplitude or duration of N1 or N2 inputs to motoneurones. After only 15 s of CGC stimulation the reduction in cycle frequency was more marked; for the preparation in Fig. 5 only 3 cycles were generated per 45 s, compared with 11 in the control (Fig. 5A,C). When the onset of CGC and SO stimulation occurred at the same time, rhythm generation was completely blocked (Fig. 5D). Fig. 5E shows the rhythm restored in the absence of CGC stimulation after a recovery period of 30 s.

The results obtained with CGC stimulation can be compared with those of the 5-HT perfusion experiments. A reduction in frequency of feeding motor output is seen during the early stages of 5-HT application and when the onset of CGC stimulation occurs some time before depolarization of SO. After about 5 min in 5-HT, rhythm generation could be blocked; this also occurred when CGC and SO stimulation were initiated simultaneously. The atypical rhythm was never seen in the CGC stimulation experiments.

DISCUSSION

Perfusion of the isolated CNS of *Lymnaea* with 10^{-4} mol l^{-1} 5-HT led to a progressive decline in the frequency of driven feeding motor output and, in a number of preparations, to eventual block of rhythmicity. Stimulation of the CGCs also led to slowing or complete block of the driven rhythm, depending on the timing of onset of stimulation. At first sight these observations appear to contradict experiments on the related pond snail *Helisoma trivolvis*, in which perfusion with 5-HT has been found to cause an increase in the frequency of the buccal rhythm and was also able to initiate a rhythm in a quiescent preparation (Granzow & Kater, 1977; Trimble & Barker, 1984). However, the present study examined effects on SO-driven activity, as opposed to the spontaneous rhythmicity examined in *Helisoma*. Modulatory effects of 5-HT or giant cerebral neurones might be substantially different for a SO-

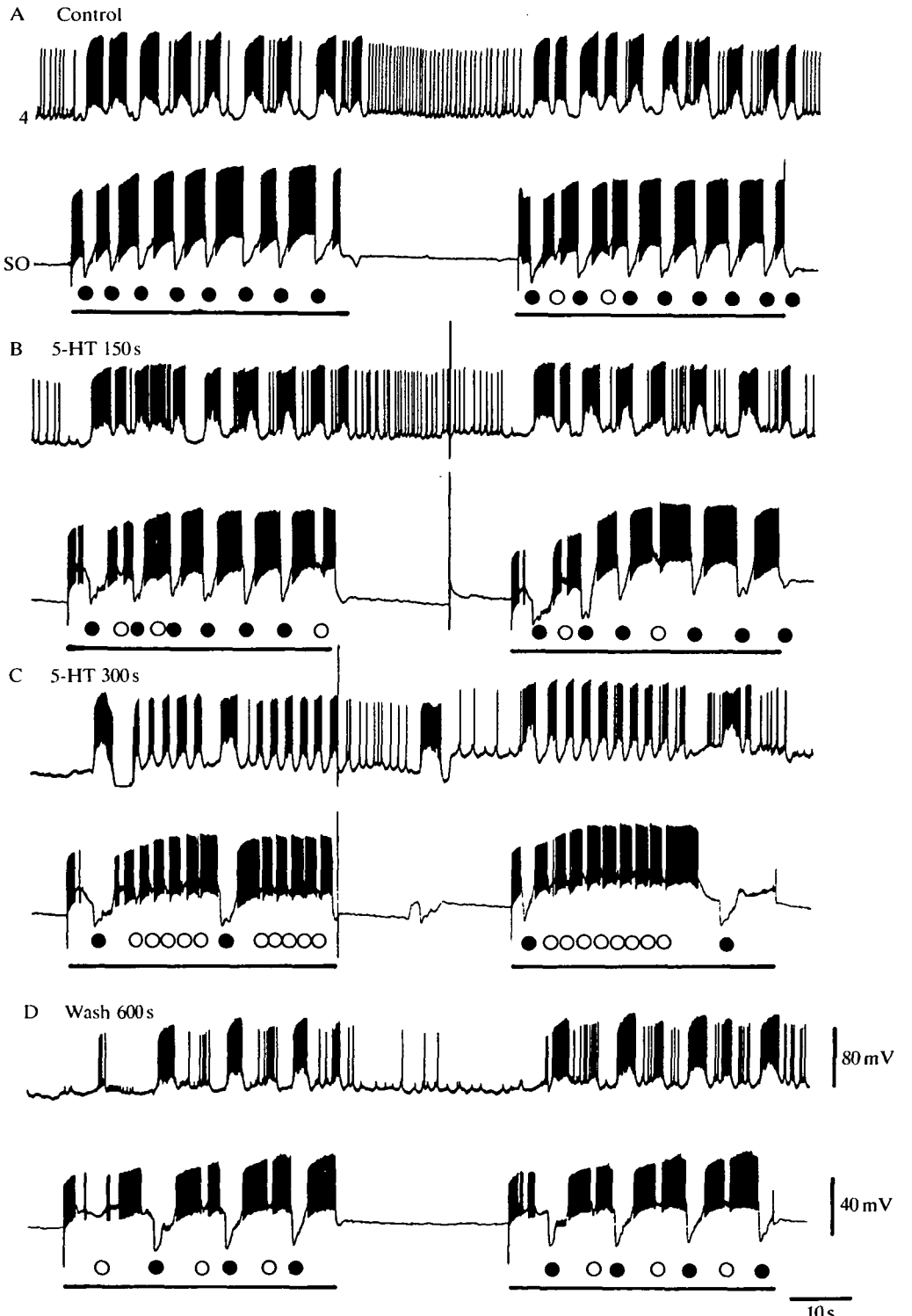

Fig. 3

driven rhythm. Indeed McCrohan & Audesirk (1987) showed that high-frequency bursts in a CGC can have apparently opposite effects on patterned motor output depending on the original level of activity in the feeding circuitry. Perfusion of the CNS of *Lymnaea* with 10^{-5} mol l^{-1} 5-HT has been shown to initiate rhythmic

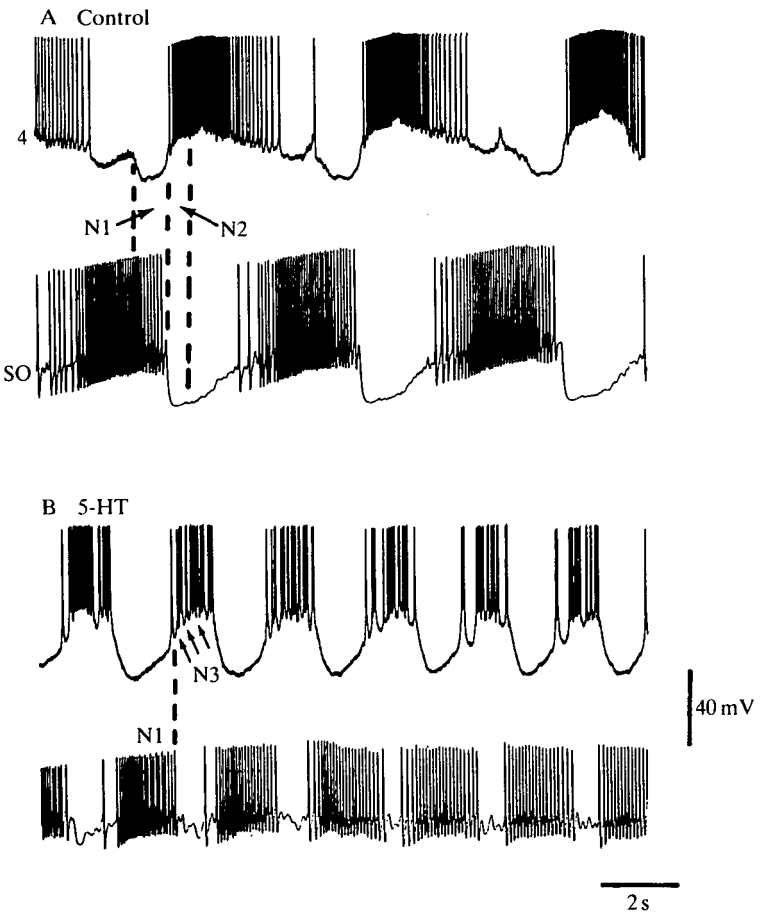

Fig. 4. Effect of perfusion with 10^{-4} mol l^{-1} 5-HT on SO-driven rhythmic motor output. Detail from Fig. 3 to show normal and atypical rhythms. N1, N2 and N3 interneuronal inputs to buccal motoneurone 4 and SO are indicated. (A) Normal saline. (B) 300 s after onset of perfusion with 5-HT: N2-phase inputs are absent and N3 (phasic) inputs are more predominant.

Fig. 3. Effect of perfusion with 10^{-4} mol l^{-1} 5-HT on SO-driven rhythmic motor output, monitored from buccal motoneurone 4. SO depolarization is indicated by bars. ● shows normal feeding cycles as described by Benjamin (1983); ○ shows atypical cycles of activity. (A) Normal saline. (B) 150 s after onset of 5-HT perfusion: frequency of normal cycles is reduced. (C) 300 s after onset of perfusion: atypical cycles predominate. (D) 600 s after onset of washing with normal saline: atypical cycles have declined in frequency, whilst normal cycles are increasing.

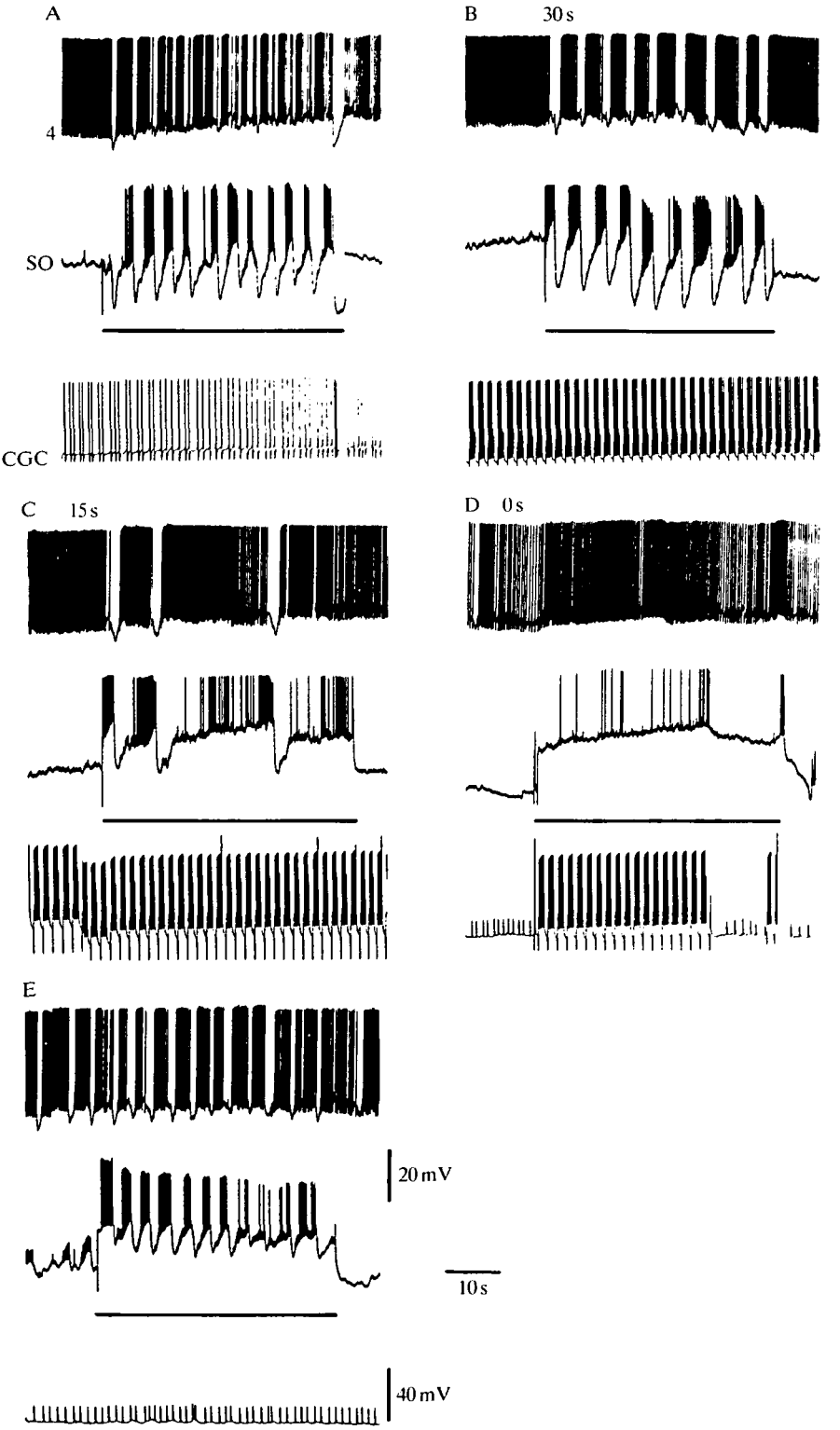

Fig. 5

buccal activity in a previously quiescent preparation (Tuersley & McCrohan, 1986), though this activity may not represent normal feeding motor output.

The progressive effect of 5-HT perfusion, first to slow the rhythm and then to block it, was presumably due to a gradual increase in tissue concentration of 5-HT. The effects of CGC stimulation mimicked those of 5-HT. However, slowing of the rhythm was obtained when CGC stimulation was initiated 15 or 30 s prior to SO

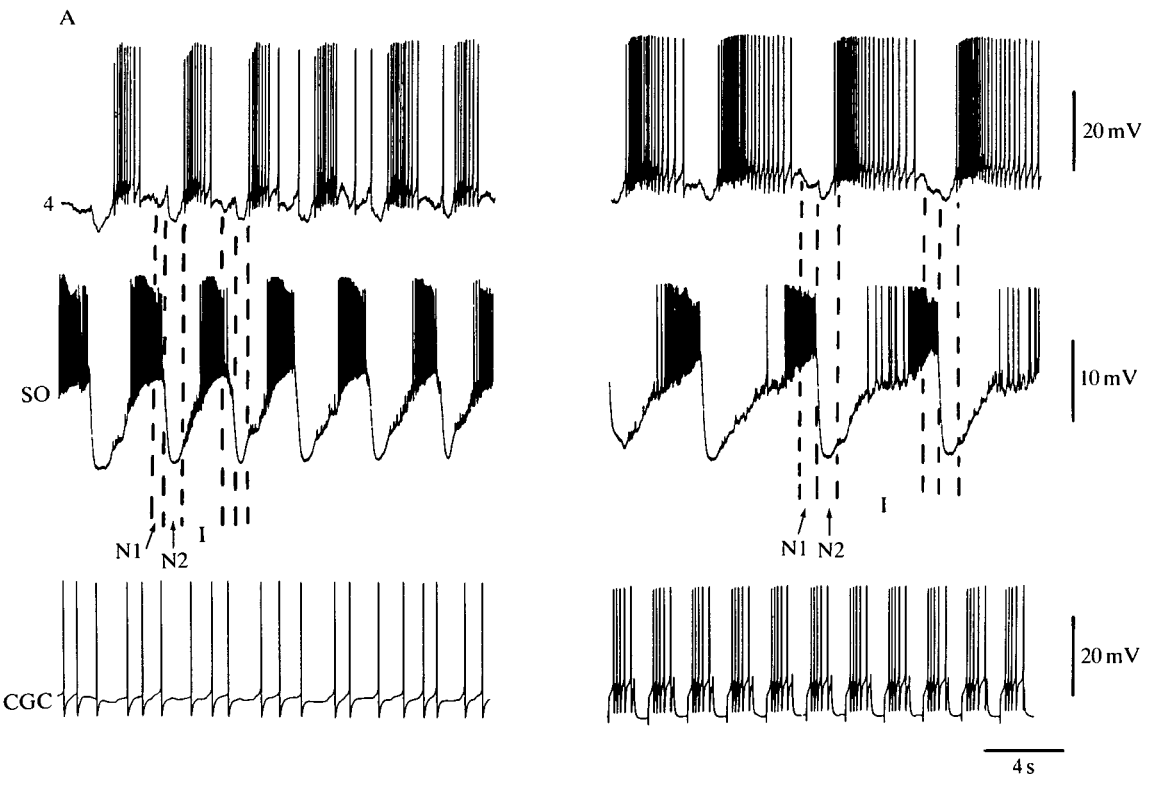

Fig. 6. Effect of stimulation of CGC on SO-driven rhythmic motor output. Detail from Fig. 5. N1, N2 and I phases of the buccal rhythm are marked. (A) No CGC stimulation. (B) CGC stimulation started 30 s prior to onset of SO depolarization: feeding cycle frequency is reduced and this is mainly due to an increase in the duration of I.

Fig. 5. Effect of stimulation of CGC on SO-driven rhythmic motor output monitored from buccal motoneurone 4 and SO itself. SO depolarization with constant current is marked with bars. CGC was stimulated using 1-s, 3-nA depolarizing pulses at 0·5 Hz. (A) No CGC stimulation: buccal CPG generates 11 feeding cycles during 45 s of SO depolarization. (B) Onset of CGC stimulation 30 s prior to onset of SO depolarization: nine feeding cycles are generated in 45 s. (C) Onset of CGC stimulation 15 s prior to SO depolarization: three feeding cycles are generated. (D) Onset of CGC stimulation simultaneous with SO depolarization: rhythm generation is blocked. (E) No CGC stimulation, after a rest period of 30 s: 11 cycles generated in 45 s. Note: in D and E, CGC does not generate full soma spikes in the absence of stimulation. However, axon spikes are clearly seen, which reflect the level of spike activity at the terminals in the buccal ganglia (see McCrohan & Benjamin, 1980*a* for discussion).

stimulation; that is, when the CGCs had already been activated for some time. Complete block of SO-driven rhythmicity was obtained only at the very start of CGC activation. This suggests that, during a period of prolonged CGC activation, 5-HT levels are at their greatest at the start and then decline despite maintained CGC activity. This is probably due to depletion of 5-HT at the CGC endings in the buccal ganglia during a period of high activity. It may be significant that spontaneous high-frequency firing in CGCs rarely lasts more than a few seconds (McCrohan & Audesirk, 1987). After this time such activity may be ineffective owing to depression of transmitter release. An alternative hypothesis, that of receptor desensitization in the buccal ganglia, is less likely. The perfusion experiments produced no evidence that the buccal circuitry became desensitized to 5-HT. Also, ionophoretic application of 5-HT onto postsynaptic somata in the buccal ganglia shows no decline in response with repeated injections (M. D. Tuersley, unpublished results). In conclusion, it appears that 5-HT causes a decrease in frequency of a driven motor output from the buccal CPG, and at higher concentrations inhibits the output altogether. These effects are mimicked by appropriate stimulation of the serotonin-containing CGCs, and therefore the effect of 5-HT is likely to be mediated, at least in part, by the CGCs. This finding enables us to be more confident about interpreting the results of gross perfusion experiments in terms of the activities of higher-order elements in a motor hierarchy. It should be noted, however, that the precise timing of the effects of 5-HT perfusion and CGC stimulation cannot be directly compared, from the present study. Neither is it possible to estimate the absolute concentration of 5-HT which reaches its site of action in either case, or the rate at which it is removed.

The effect of 5-HT on the frequency of feeding motor output was achieved mainly by lengthening intervals between cycles rather than greatly altering phase durations and relationships within feeding cycles. This suggests an action of serotonin on the CPG as a whole. However, in a significant number of cases 5-HT produced differential effects on components of the CPG, causing a switch to the atypical rhythm. This was characterized by the absence of the N2 phase of the rhythm. The atypical rhythm was only seen in perfusion experiments and not following CGC stimulation, raising the possibility that it is a non-physiological artefact. However, it is still interesting to discuss its possible function since it was so clear cut and reproducible. In the marine gastropod *Pleurobranchaea*, at least three and possibly as many as seven different functional rhythms have been identified as arising from the buccal CPG (Croll, Davis & Kovac, 1985; McClellan, 1982*a*,*b*). These control activities such as ingestion, egestion, swallowing and writhing, and are characterized by subtle differences in output from different elements of the feeding circuitry. Since the atypical rhythm of *Lymnaea* lacks the N2 phase, during which radula rasping occurs (Elliott & Benjamin, 1985*a*), it may represent either egestion or swallowing. Using the lobster, Flamm & Harris-Warrick (1986*a*,*b*) showed that different amines (5-HT, dopamine, octopamine) produced different motor outputs from the pyloric CPG. The rhythms were distinguished by alterations in cycle frequency, phase relationships and spiking activity of the various elements of the network. It was

found that each amine, though affecting most neurones in the circuit, exerted a unique combination of effects on the different elements, probably acting both on endogenous neuronal properties and on synaptic efficacy (Flamm & Harris-Warrick, 1986*b*). It is likely that 5-HT, at the concentrations used in the present study, acts in a similar way, leading to suppression of activity in the N2 network, whilst allowing for alternating activity in N1 and N3. A candidate monoamine for eliciting true feeding motor output in *Lymnaea* is dopamine. Application of dopamine to the *Limax* nervous system triggers and sustains a rhythmical output apparently identical to the normal feeding motor programme, whereas 5-HT elicits a rhythm which is unlike the feeding rhythm (Wieland & Gelperin, 1983). Our own preliminary work, currently being extended, yields similar results (Tuersley & McCrohan, 1986), supporting the hypothesis that two amines, 5-HT and dopamine, act to select different types of rhythmic activity in a CPG.

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