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Research Article

A Comparison of the Electrophysiological Characteristics of Identified Neurons of the Feeding System of *Lymnaea stagnalis* (L.) *In Situ* and in Culture

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Abstract

Using electrophysiological techniques, the firing patterns and action potential shapes of the cerebral giant cells (CGCs) and identified buccal feeding motor neurons, 1, 2 and 4 of *Lymnaea stagnalis* (L.) were compared in the intact brain and in isolated cell culture. Action potential shapes, amplitudes and half-widths of neurons in culture were similar to those *in situ*. The CGCs retained their usual tonic regular pattern of firing *in vitro* as *in vivo*. The synaptically modulated bursting discharges of the buccal 1- and 2- neurons *in vivo* were lost in culture. The buccal 4-cells sometimes retained their characteristic patterned discharge when isolated in culture. This appears to be due to seasonal variations. 50% of the CGCs, the buccal 1-, 2- and 4-cells, which were spontaneously active *in vivo*, were silent in culture.

Keywords: *Feeding System; Central Pattern Generator; Cell Culture; Electrophysiology; Lymnaea*

Introduction

Many of the identified neurons in the buccal ganglia of *Lymnaea stagnalis* are motor neurons that control the feeding system in this animal. These neurons may be identified by their different patterns of discharge, in addition to size, location and pigmentation [1,2]. As in motor systems controlling such rhythmic behaviours as respiration in mammals [3], swimming in leech [4], locomotion [5], respiration [6,7] and feeding in *Lymnaea*, [1,2,8,9], the pattern generating mechanism is driven by a "central pattern generator" (CPG) consisting of a network of interneurons. However, feeding behaviour is complex in *Lymnaea* and the CPG appears to be modulated by a distributed neural network rather than a linear hierarchical system [9]. The interneurons of CPGs are often small cells, believed to impose their activity on the follower motor neurons to produce the different motor patterns. In *Lymnaea*, the CPG is located in the buccal ganglia and it is made up of three subnetworks; N1, N2 and N3 (phasic) interneurons [1,10-16]. These interneurons are known to be themselves interconnected by inhibitory synapses, and they burst consecutively to produce three phases in each feeding cycle; radula protraction (R1), radula retraction [R2 (rasping)] and swallowing (R3) [10,13]. The N1, N2 and N3 interneurons make synaptic connections with the buccal motor neurons leading to a three phase cycle of bursting activity. For example, the paired 4-group neurons have similar morphology and electrophysiology to one another. They are retractor motor neurons which receive double inhibitory inputs from N1 and N2 interneurons in succession and then fire a burst of action potentials by post-inhibitory rebound excitation, an endogenous property of this group of neurons [1]. This burst is often fractionated into sub-bursts by brief inhibitory inputs from the N3 interneurons. Amongst the 4-group neurons, the largest, the 4-cell, is situated in the middle of the group, while the smaller cells grouped around this large cell are the 4-cluster cells [1].

The paired 1-cells, which are salivary gland motor neurons, are the largest cells in the buccal ganglia (80-120 µm) and receive a compound excitatory input from the N1 interneuron which causes them to fire a burst of spikes during the radula protraction phase [1]. They have a tendency to fire only a few spikes at the peak of the rising phase of the excitatory post synaptic potentials (EPSPs), but their spike generating mechanism adapts to subsequent maintained depolarisation [1].

The paired 2-cells are the gut motor neurons and are the second largest cells in the buccal ganglia. They receive double excitatory inputs from the N1 and N2 interneurons. These cells generate long bursts of action potentials lasting throughout the two phases of synaptic inputs. and short bursts due to release from inhibitory synaptic input, through a mechanism of post-inhibitory hyperpolarisation [1].

The rhythmic feeding motor output which is often variable can occur spontaneously in the isolated brain preparation or can be driven artificially by depolarisation of the buccal slow oscillator (SO) interneuron, which is a higher order interneuron with a modulatory influence on the feeding system. The SO can initiate activity in the CPG from which it receives synaptic feedback [10-13]. The SO interneuron is believed to produce a much more consistent pattern of activity than the spontaneously occurring one, and it also causes an increase in the intensity of bursting of the buccal motor neurons. However, the cerebral giant cells (CGCs) also have synaptic connections with the CPG circuitry as well as the SO [17,18].

Despite in depth knowledge about the activities of the feeding motor neurons and their various synaptic inputs from interneurons *in vivo* in *Lymnaea stagnalis*, little is known about the behaviour of these neurons in culture conditions. No attempt has yet been made to characterise them in the absence of the influence of the CPG. This would be very difficult *in situ* since the CPG has very powerful inputs to the neurons. Here, the firing patterns and the action potential shapes of the CGCs and some of the identified buccal neurons (1-, 2- and 4-cells) are described, using intracellular recordings from these neurons both *in vivo* and isolated in culture.

Materials and Methods

Experiments were carried out on isolated brain preparations of *Lymnaea stagnalis* consisting of the paired buccal ganglia with the cerebro-buccal connectives intact. Part of the anterior oesophagus and the salivary gland ducts were left attached to the buccal ganglia by the dorsobuccal nerves to facilitate pinning to a Sylgard-lined dish containing normal 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (HBS) [19] for *in situ* experiments. In these experiments, the brains were treated for a short period with pronase (4 mg/ml; Sigma) to facilitate electrode penetration. Intracellular recordings were made from identified cell bodies in the cerebral and buccal ganglia using conventional glass microelectrodes and bridge balance amplifiers (Neurolog, NL 102G).

Data analysis

Results are expressed as means ± standard deviation (S.D) and statistical tests were performed using the Student's t-test.

Culture techniques

Brains were dissected from juvenile snails and identified neurons were isolated as described previously [20,21]. Neurons were cultured in defined medium (DM) for 24 hours. During experiments, both isolated brain preparations and cultured neurons were continuously superfused with HBS, adjusted to pH 7.9.

Electrophysiology

Using standard electrophysiological techniques, intracellular recordings were made using glass microelectrodes filled with the supernatant from a saturated solution of K_2SO_4 [19]. Electrode resistances varied from 20-40 MΩ. Signals were displayed on a Gould 2-channel storage oscilloscope after preamplification and bridge balancing. Permanent recordings were made on either a Gould channel pen recorder or an Elonex 486 computer via a CED interface. A CED Spike 2 program, developed in our laboratory by Yar., *et al.* [22] was used for the determination of action potential half-width (AP½). Furthermore, because it has been established that increased firing rates in a neurone caused action potential broadening [23,24], only spikes of similar firing frequencies were compared in each group (See table 1).

Results

Viability of the CGCs and buccal motor neurons in culture

Neurons were classified as viable if they retained the same morphology and pigmentation *in vitro* as *in vivo*, in addition to their ability to spontaneously initiate action potentials after microelectrode penetration, or in the case of silent neurons, when depolarised, or after release from hyperpolarisation. In defined medium, most cells remained as rounded cells, although a few showed signs of neurite outgrowth after 24 hours of plating. However, in the experiments reported here, recordings were made from only rounded cells during the months of June and July (summer) and also from November to January (winter). The action potential characteristics of the different cell types, both *in situ* and in culture, are summarised in table 1.

	Action potential shape (mV)		Action potential amplitude (mV)		Action potential half-width (msec) at frequencies stated	
Types of neurons	*In situ*	In culture	*In situ*	In culture	*In situ*	In culture
Cerebral giant cells (CGCs)	Type 2 (n = 11)	Type 2 (n = 8)	102.48 ± 7.86	93.28 ± 5.49 (NS)	18.51 ± 4.85 Freq = 0.89 ± 0.45 spikes/s	20.48 ± 8.38 (NS) Freq = 0.86 ± 0.55 spikes/sec (NS)
Buccal 1-cells	Broad type 1 (n = 10)	Broad type 1 (n = 7)	88.41 ± 5.23	76.09 ± 5.07 (NS)	11.92 ± 7.48 Freq = 1.56 ± 2.01 spikes/s	14.21 ± 9.00 (NS) Freq = 1.49 ± 2.06 spikes/s (NS)
Buccal 2-cells	Type 2 (n = 8)	Type 2 (n = 6)	95.19 ± 6.92	88.92 ± 5.86 (NS)	15.70 ± 5.39 Freq = 2.00 ± 0.68 spikes/s	19.35 ± 8.91 (NS) Freq = 1.90 ± 0.40 spikes/s (NS)
Buccal 4-cells	Narrow type 1 (n = 15)	Narrow type 1 (n = 20)	73.67 ± 5.79	75.35 ± 4.62 (NS)	5.43 ± 7.58 Freq = 2.62 ± 1.13 spikes/s	7.52 ± 4.88 (NS) Freq = 2.64 ± 1.20 spikes/s (NS)

Table 1: *A comparison of the action potential shape, amplitude and half-width between Lymnaea neurons in situ and in culture. The data shows the mean ± S.D. (Standard deviation) of the parameters mentioned above in only cells that either spontaneously fired action potentials or were stimulated to fire. Statistical significance was determined as P < 0.05 were considered as significant. There are no significant differences in the parameters mentioned above between neurons in culture and those in situ. Action potential amplitude was measured from the peak of the depolarisation to the peak of the hyperpolarisation. Half-widths were determined at appropriate frequencies as shown above in the table.*

In all cases, cultured neurons (n = 41) retained their typical action potential shapes when compared with neurons *in situ* (n = 44). No significant differences in spike amplitude or width were detected, although the mean values for spike width increased in cultured neurons as also demonstrated in neurons of the parietal and visceral ganglia and the giant dopamine containing neuron, RPeD1 of the right pedal ganglion [25,26].

Electrophysiological characteristics of the CGCs *in situ* and in culture

In these experiments, recordings were made from 11 CGCs in the isolated brain preparation and 8 CGCs in culture. In all the neurons studied, the resting membrane potential varied between -40 and -80mV both in cultured neurons and in neurons in isolated brain preparations. *In situ*, the CGCs are usually spontaneously active cells that fire regular action potentials at the rate of 0.5 to 2.0 spikes/sec (Figure 1A). The action potentials of these cells were of the type 2, which is characterized by the presence of a pseudoplateau on the repolarisation phase (Insert) [19]. In the whole brain experiments, this feature alone, apart from the position and size of the CGCs in the cerebral ganglia, was used as an additional criterion in characterizing these neurons. Silent neurons or neurons with little spiking activity were rarely encountered. In some preparations, action potentials occurred in bursts (n = 2) (Figure 1B).

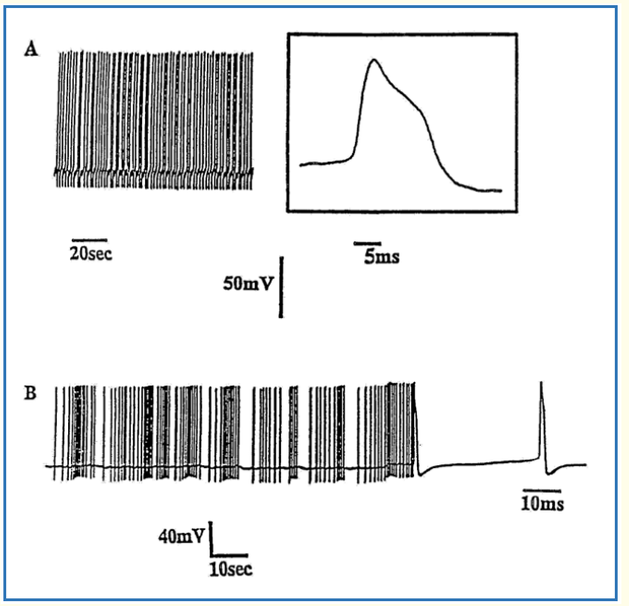

Figure 1: *Patterns of firing activity of cerebral giant cells (CGCs) in situ recorded from two different preparations. (A) Regular spiking activity, showing the shape of a single spike on a faster time base (Insert). (B) An irregular discharge pattern may also occur in the CGCs, with bursts of spikes. The action potential shape is also shown on a faster time base at the right.*

In culture, 5 out of 8 CGCs maintained their characteristic spontaneous, regular, single-spiking pattern of activity (Figure 2A) and also the type 2 action potential shape, (insert). This is in agreement with previous work by Yar., *et al.* [25,27] confirming that *Lymnaea* neurons maintain their action potential types in culture. The remaining CGCs were silent neurons (n = 3), with resting potentials of about -60mV. In these neurons, the type 2 action potentials could only be initiated following depolarizing current pulses or after release from hyperpolarisation (Figure 2B).

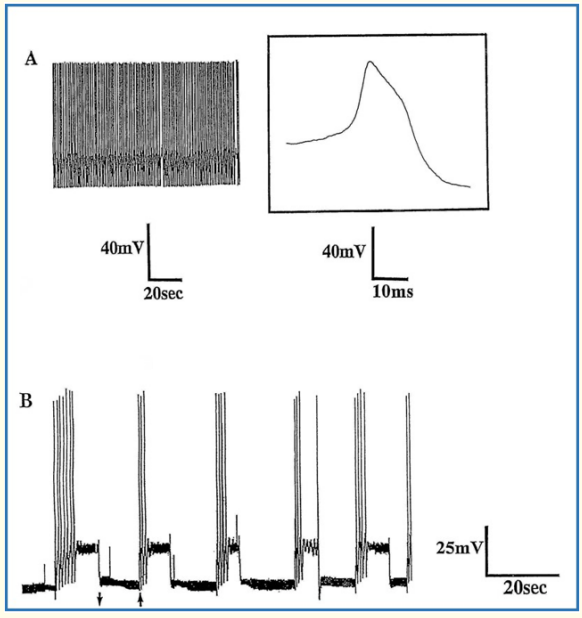

Figure 2: *Patterns of firing activity of cultured CGCs. (A) Regular spikes similar to those in situ. The action potential shown on a faster time base (insert) is also similar. (B) In this silent neurone, action potentials could only be evoked after release from hyperpolarisation.*

Electrophysiological characteristics of the buccal motor neurons *in situ* and in culture

Each buccal motor neuron exhibits a characteristic burst pattern that forms a basis for its identification. In these experiments, recordings were made from identified buccal neurons 1-, 2- and 4-cells in the isolated central nervous system (CNS) preparations and from cultured neurons.

1-cell characteristics

The 1-cells receive a single excitatory synaptic input from the CPG and coupled with their inherent spike adaptive properties, this results in specific burst patterns. Due to this spike adaptation mechanism, the 1-cells rarely fire more than a few spikes in a burst and these, during or at the peak of the rising phase of the EPSPs, but not during maintained suprathreshold depolarisation. Ten 1-cells were investigated *in situ* and these exhibited spontaneous firing as illustrated in figure 3A and 3B. In addition, these neurons exhibited a broad type 1 action potential (Insert) which was also used as a criterion for identification of these neurons. In 8 out of 10 cases these neurons fired fewer numbers of spikes per burst (5-10) than the other buccal cells investigated (Figure 3A), and these only during or at the peak of the rising phase of the EPSPs preceding the bursts. Occasionally, cells which fired more spikes per burst (Figure 3B) were also observed (n = 2).

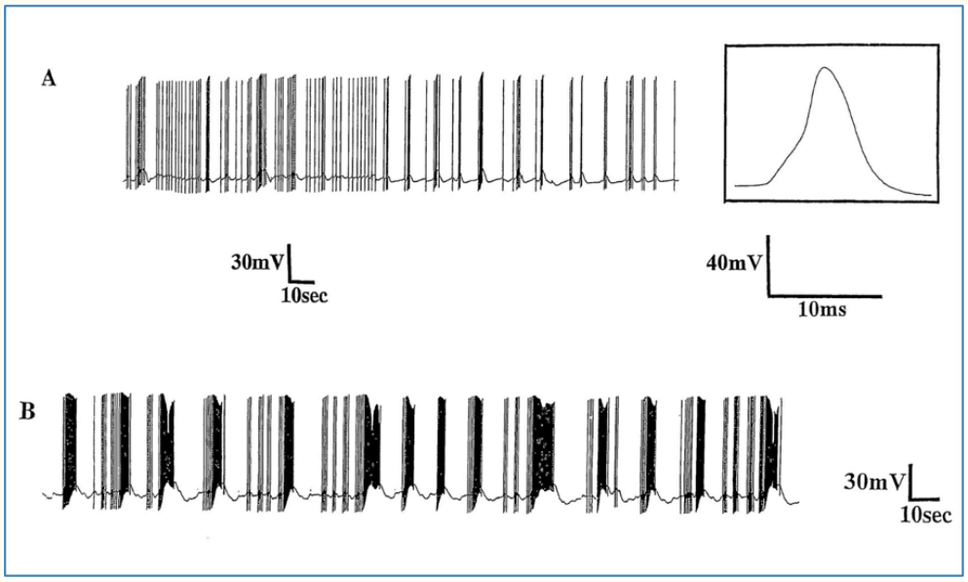

Figure 3: *Intracellular recordings from the buccal 1-cell in situ showing the different patterns of firing activities. (A) and (B) illustrate examples of bursting activities observed in these groups of neurons. On a faster time base, the action potential shape is as shown (insert).*

Seven 1-cells were investigated in culture and were found to have either lost the usual pattern of burst firing and instead showed spontaneous regular firing at a steady rate (n = 2) (Figure 4A) or were silent on electrode penetration (n = 4). However, these silent neurons could be stimulated to fire action potentials following depolarisation or after release from hyperpolarisation (Figure 4C and 4D). In figure 4D, the highest discharge frequency could be seen at the peak of the rising phase of depolarisation, implying that although the 1-cells might have lost their characteristic burst patterns in culture, they still maintained their adaptation to spike generation at maintained depolarisation and also their action potential shapes. The remaining 1-cell actually displayed this characteristic 1-cell spike adaptation during spontaneous plateau potentials (Figure 4B) with the absence of spikes during the plateau phase. However, in one case, a pattern of bursting was observed that was different from that seen in cells in the isolated brain preparation, although its frequency was similar (Figure 4E). In all these neurons, the broad type 1 action potential was also maintained in culture (Figures 4A, C and D, Inserts). Both *in situ* and cell culture preparations, the resting membrane potential varied between -40 and -60mV in different cells.

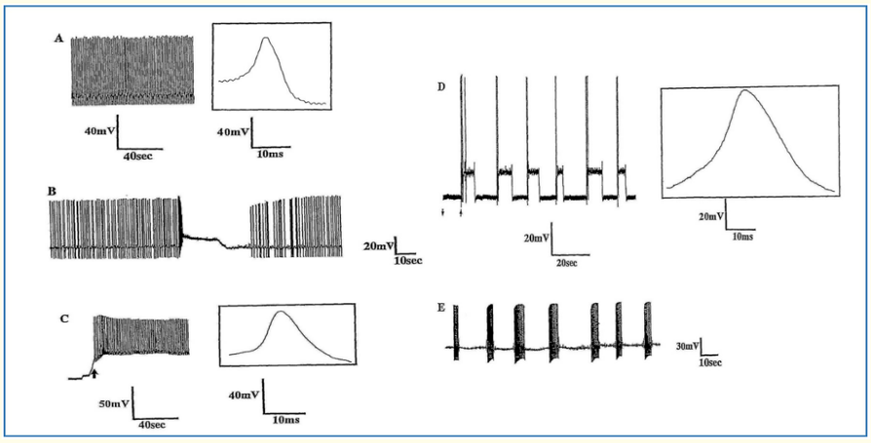

Figure 4: *Intracellular recordings from cultured buccal 1-cells demonstrating their different activity patterns. (A) Spontaneous, regular firing showing the action potential shape (Insert). (B) A spontaneously firing buccal 1-cell, showing a spontaneously occurring plateau potential and spike adaptation. (C) and (D) In these silent neurons, action potentials could only be evoked either following a depolarisation (C), or after release from hyperpolarisation (D). (E) Spontaneous patterns of burst activity at a frequency similar to those observed in this neurone in situ.*

2-cell characteristics

The 2-cells are the only buccal cells that receive two phases of excitatory synaptic input per burst from the CPGs and are known to fire long bursts of spikes throughout these two periods [1]. Shorter bursts of spikes also occur between the long bursts. Eight cells were investigated *in situ*, and figure 5 shows a typical 2-cell discharge observed in these experiments. These neurons exhibited type 2 action potentials with a pseudoplateau on the repolarizing phase of the action potential (Insert). In these cells, a few long bursts of spikes usually occurred together with several short bursts as previously observed [1].

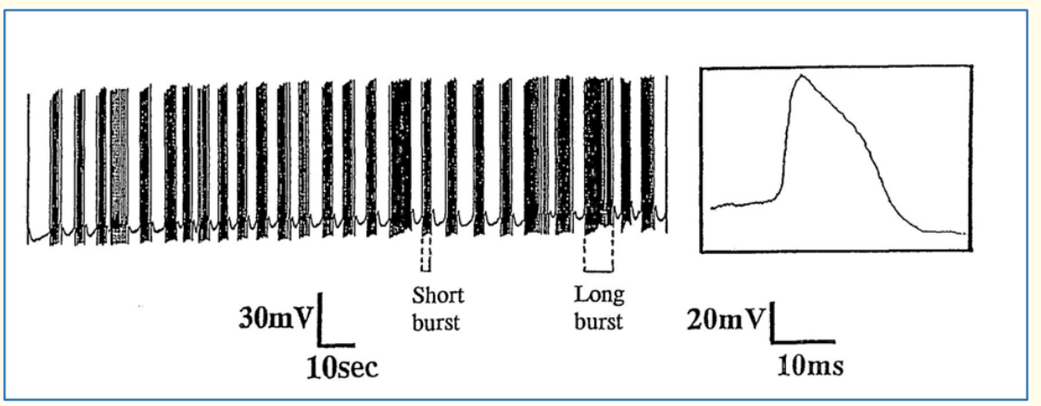

Figure 5: *An intracellular recording of a buccal 2-cell in situ showing the characteristic burst activity. Notice the occurrence of several short bursts interspersed with long bursts. The action potential of this neurone is a characteristic type 2 (Insert).*

Six cells were investigated in culture. The 2-cells failed to maintain the characteristic 2-cell burst, but rather, fired regular spikes, whose frequency gradually declined, either during depolarisation (n = 2), or after release from hyperpolarisation (n = 3) (Figure 6A). In these neurons in culture, the type 2 action potential characteristic was also found to be maintained (Figure 6B, Insert) but the 2-cell burst was lost. In one case however, the 2-cell fired in bursts interspersed by periods of deep hyperpolarisations as seen in figure 6B. The resting membrane potential in these cells varied between -40 and -80mV in both cultured neurons and in neurons of the isolated CNS preparation.

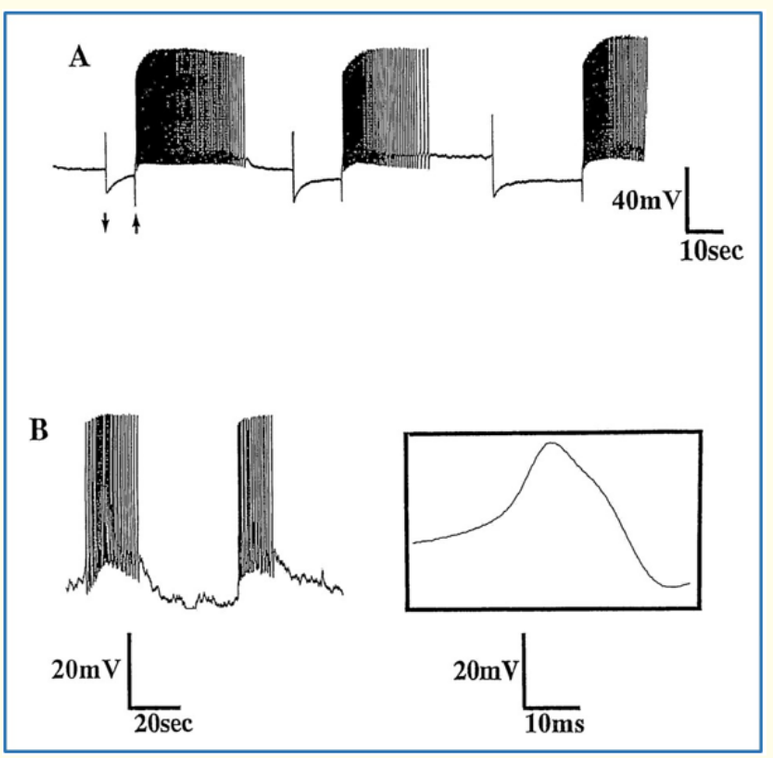

Figure 6: *Intracellular recordings from buccal 2-cells in culture. (A) In a silent 2-cell, action potentials could only be evoked after release from hyperpolarisation. (B) Spontaneous activity from another neurone showing burst activities similar to those observed in these neurons in situ. On a faster time base, the type 2 action potential is maintained (Insert).*

4-cell characteristics

In the 4-cells, the resting membrane potentials varied between -40 and -60mV both *in situ* and in cultured neurons. *In vivo*, these cells have been shown to receive double inhibitory inputs from the CPGs, at the cessation of which the cells fire synchronized bursts. The synchronized bursting is believed to involve a rebound from inhibition which is due to the endogenous membrane properties of these buccal neurons and also due to the electronic connections within this group of neurons [1]. Figures 7A, B and C illustrate typical examples of the 4-cell bursts *in situ* (n = 15). Of all the buccal neurons investigated, the 4-group cells were found to be the most excitable. The action potential shape is a narrow type 1 (Figures 7A and 7B Inserts).

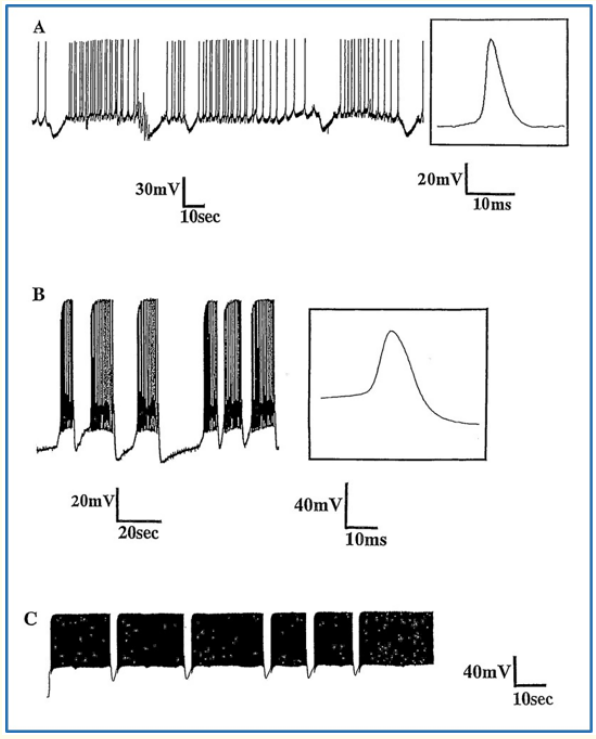

Figure 7: *Patterns of firing activity observed in the buccal 4-cells in situ. (A), (B) and (C) demonstrate examples of the different burst patterns seen in these neurons. The characteristic action potential shape is as illustrated on a faster time base (Inserts).*

Twenty cells were investigated in culture. In this case also, the 4-cells were found to be the most excitable, in terms of spontaneous action potential discharges, of all the buccal neurons investigated in culture (Figure 8A) (n = 9). However, some silent neurons were also observed (n = 5), in which action potentials could be elicited following the injection of depolarizing current pulses (0.2 to 0.8nA) (Figure 8C) or after release from hyperpolarisation (Figure 8B). In these, the characteristic narrow type 1 action potential (Figure 8B Insert) was maintained. In the remaining cells (n = 6); (30%), we were surprised to observe that the 4-cells retained bursts resembling the characteristic 4-cell bursts *in situ*. Examples of these bursts are illustrated in figures 9A, B, C, D and E. These types of bursts were only observed during the months of June-July. This may be due to the actions of increasing 5-HT concentrations on these neurons at this time of the year, since seasonal variations in the level of tissue and CNS 5-HT has been reported in some molluscan species, with levels increasing during summer and decreasing during winter (in *Lymnaea stagnalis* [28,29] and in *Helix pomatia* [30]).

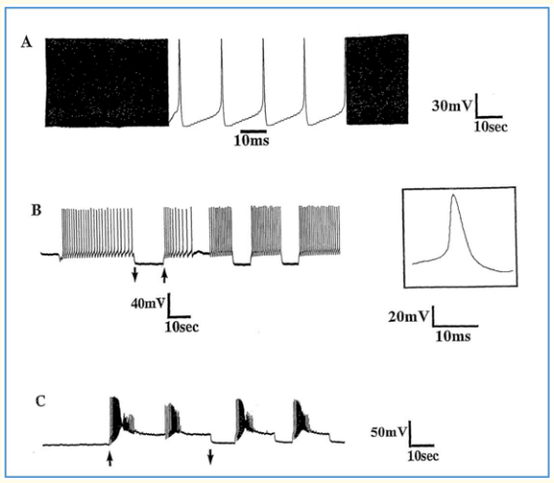

Figure 8: *Various activity patterns observed in the buccal 4-cells in culture. (A) A 4-cell firing rapidly and regularly. In the middle of the trace, the action potentials are as seen on a faster time base. (B) In this silent 4-cell, action potentials were evoked after release from hyperpolarisation. The action potential shape as shown on a faster time base (insert) is similar to those observed in this neurone in situ. (C) Bursts of spikes were observed in this silent neurone after release from hyperpolarisation.*

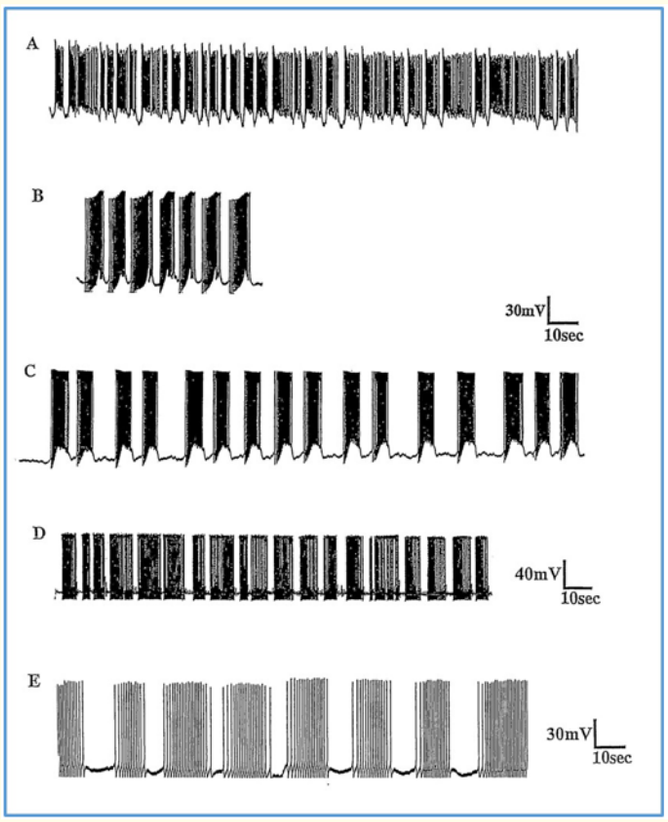

Figure 9: *(A)-(E) illustrate different spontaneous burst activities seen in five different buccal 4-cells in culture, similar to those observed in these neurons in situ. Their membrane potentials varied by 2 - 4mV in comparison with those of cells recorded in situ (-60mV).*

Discussion and Conclusion

A comparison of electrophysiological parameters such as the action potential shape, the half-width, and the amplitude in the present experiments show that cultured neurons largely retained similar characteristics as those in the intact CNS, as is evidenced in table 1.

The activity patterns of *Lymnaea* buccal feeding motor neurons have been well documented *in vivo* [10,13-16], but not yet *in vitro*. The present results show that although much of the characteristic burst pattern which forms one of the bases for the identification of these neurons is lost *in vitro*, some of the B4 neurons still maintained burst patterning resembling that observed *in vivo*. Although such characteristic patterned activity is believed to be imposed on the buccal neurons from inputs from the buccal CPGs, these inputs were also believed to interact with the endogenous properties of the membranes of the buccal neurons, e.g. a tendency to post-inhibitory rebound, which is a characteristic of the 4-cell bursts, and spike adaptation, a characteristic of the 1-cells [1]. Further, the electronic connections between these cells (the 4-group in particular) were believed to reinforce post-inhibitory rebound in this group, producing mutual synchronization of individual bursters, resulting in the whole group being considered to show synchronized bursting. While the 1- or 2- buccal neurons did not appear to maintain burst firing in cultured neurons, up to 30% of the 4-cells retained bursting properties in culture. This strongly suggests that 4-cell bursting during the summer months may not be entirely due to the imposition of synaptic input from the buccal CPGs but are an endogenous property of the cells.

It is important to consider the origin of the 4-cell burst since a proportion of cells maintained their bursting discharge in culture. Such bursting activity may be due in part to the intrinsic membrane properties of these cells. The fact that 30% of these cells exhibited bursting discharges in June and July, compared with 0% at other times of the year, suggests that a seasonal alteration in their membrane properties may occur as has previously been demonstrated in other *Lymnaea* neurons [31-33]. Furthermore, 5-HT, the neurotransmitter involved in synaptic transmission between the CGC and the buccal neurons was demonstrated to show a steady rise between March/April to June in *Lymnaea stagnalis* [28,29]. This may partly explain why these cells exhibit bursting properties during that time of year. In addition, the endogenous properties of the 4-cells unlike other buccal cells, has also been suggested to be important in burst termination [1]. However, by some unknown mechanism, the 4-cells show an inherent property to burst sometimes, but not at other times, thus implying that in addition to receiving external rhythmic inputs, the 4-cell is also capable, periodically, of generating bursts of spikes, making them endogenous busters. Similar endogenous bursting activities have also been demonstrated in the protractor and retractor buccal neurons of the Pteropod mollusc *Clione limacina* [34] and in the buccal neurons of the freshwater snail *Planorbis corneus* [35] after microelectrode isolation of these neurons from external rhythmic inputs. These authors also demonstrated the endogenous bursting activity in both types of neurons, as they continued bursting activity for several hours after isolation. The 4-cells were also shown to maintain their characteristic type-1 action potential shapes *in vitro* as *in vivo*.

Even though the characteristic 1-cell burst pattern was lost *in vitro*, some of these cells still maintained their endogenous characteristic of spontaneous spike adaptation in culture. In other 1-cells, spike adaptation could be observed under maintained depolarisation, suggesting that the 1-cells retained their inherent membrane properties in culture. However, the fact that bursting activity was still observed in some of these neurons in culture (June and July), implies that these cells may still retain some degree of bursting activity.

Alterations in the electrical activity seen in some neurons could be due to the artificial conditions under which they were isolated and cultured. However, since no significant differences were observed in the electrophysiological characteristics between neurons *in situ* and those in culture, the implication is that the method of culturing did not grossly affect the electrophysiology of these neurons. The 2-cells, while preserving some of their electrophysiological characteristics in culture, lost their characteristic burst pattern. This suggests that for the 1- and 2-cells, inputs from the CPG are necessary for the generation of rhythmic activity. Finally, even though the buccal 1- and 2-cells may need interactions from the buccal CPGs in *Lymnaea stagnalis* for their characteristic bursting activities, such connections may not be the only factor in determining the pattern of activity for the 4-cells. These cells may on their own be capable of acting as endogenous busters, with activity being modulated by other neurons in the CNS.

Acknowledgements

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