ELSEVIER

Neurobiology of Aging 27 (2006) 1892–1901

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Changes in the properties of the modulatory cerebral giant cells contribute to aging in the feeding system of *Lymnaea*

B.A. Patel [a,b], M. Arundell [a,b], M.C. Allen [b], P. Gard [b], D. O'Hare [a], K. Parker [a], M.S. Yeoman [b,*]

[a] *Physiological Flow Studies Group, School of Bioengineering, Imperial College, Prince Consort Road, London, SW7 2AZ, UK*
[b] *School of Pharmacy and Biomolecular Sciences, Cockcroft Building, University of Brighton, Moulsecoomb, Brighton, BN2 4GJ, East Sussex, UK*

Received 1 December 2004; received in revised form 14 September 2005; accepted 29 September 2005
Available online 9 November 2005

Abstract

This study examined whether electrophysiological changes in the endogenous properties and connectivity of the modulatory serotonergic cerebral giant cells (CGCs) contributed to the age-related changes in feeding behavior of the pond snail, *Lymnaea*. With increasing age there was a decrease in spontaneous CGC firing rates and decreased excitability of the CGCs to both chemosensory stimulation (0.05 M sucrose applied to the lips) and direct intracellular current injection. These changes could be accounted for by a decrease in the input resistance of the neuron and an increase in the amplitude and the duration of the after-hyperpolarization. Decreases were also seen in the % of CGC pairs that were electrically coupled causing asynchronous firing. Together these changes would tend to reduce the ability of the CGCs to gate and control the frequency of the feeding behavior. Part of the ability of the CGCs to gate and frequency control the feeding network is to provide a background level of excitation to the feeding motor neurons. Recordings from B1 and B4 motor neurons showed an age-related hyperpolarization of the resting membrane potential consistent with a deficit in CGC function. Increases were seen in the strength of the evoked CGC → B1 connection, however, this increase failed to compensate for the deficits in CGC excitability. In summary, age-related changes in the properties of the CGCs were consistent with them contributing to the age-related changes in feeding behavior seen in *Lymnaea*.

Keywords: Neuronal aging; Serotonin; Feeding system; Modulation; Mollusk

1. Introduction

In the previous paper (Arundell et al., 2005) we examined how the feeding behavior of the pond snail *Lymnaea stagnalis*, changed as the animal aged. Increasing age was associated with an increase in the % non-responding animals, an increase in the duration of the swallow phase of each feeding cycle and a decrease in the number of sucrose-evoked bites. These changes were seen with both the 0.01 and 0.05 M sucrose stimuli but were absent when the sucrose concentration was increased to 0.1 M. Electrophysiological analysis of the chemosensory pathway demonstrated no age-related change in the information passing from the primary sensory neurons in the lips to the cerebral ganglia, but a significant change in the information passing from the cerebral ganglia to the feeding circuitry in the buccal ganglia. These age-related changes were also seen when 0.01 and 0.05 M sucrose solutions were used to activate feeding in the intact animal, but were absent when 0.1 M sucrose was used. The parallel changes seen in both the electrophysiological and behavioral studies suggested that alterations in the central processing of the chemosensory information may underlie the age-related changes in feeding behavior. One pair of neurons known to be excited following the application of sucrose to the lips are the giant serotonin cells that form a pair of electrically coupled neurons located bilaterally in the cerebral ganglia of all mollusks. The electrical coupling ensures that when one cell fires the other is rapidly excited so that the pair of neurons fire as a single unit providing a coordinated release of serotonin on to the neurons of the feeding circuitry located

DOI of original article:10.1016/j.neurobiolaging.2005.09.040.
* Corresponding author. Tel.: +44 1273 642078; fax: +44 1273 679333.
E-mail address: m.s.yeoman@brighton.ac.uk (M.S. Yeoman).

0197-4580/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2005.09.041

in the buccal ganglia. These cells have extensive inputs to all levels of the feeding network regulating the activity of the central pattern generator (CPG) that generates the rhythmic behavior [35], the motor neurons [8,17,18,28] and the muscles [30]. They are, therefore, ideally placed to regulate the output of the feeding network and deficits in the functioning of these neurons would have major effects on the feeding behavior of the animal. In *Lymnaea*, these cells are known as the cerebral giant cells (CGCs) and work has shown that application of sucrose to the lips of the animal induces a burst of action potentials in the CGCs in semi-intact preparations where the isolated CNS is attached to the lips of the animal via the paired lip nerves and the tentacular nerve [12,21]. Similar increases in neuronal activity were seen following the application of lettuce to the lips of freely moving *Lymnaea* [33]. In a closely related mollusk, *Aplysia* [9,15], application of a feeding stimulus, seaweed, to the lips of the animal also induced a burst of action potentials in homologs of the CGCs, the metacerebral giant cells (MCCs).

The giant serotonin cells have a role in modulating the feeding behavior of the animal [30,32,33]. In *Lymnaea*, the CGCs have previously been shown to be the sole serotonergic input to the buccal ganglia [11]. They have a gating role where a minimum level of activity in these neurons is required to allow the animal to respond to a food stimulus [32,33]. Further increases in the firing frequency of these neurons have been shown to regulate the frequency of fictive feeding cycles, recorded as cyclical bursts of motor neuron action potentials in the isolated CNS [32,33]. In part these functions are achieved by the CGCs providing a background level of excitation to the feeding network, and thus lowering the threshold for activation of the feeding system [18,35]. Lesioning of the CGCs with 5,6-dihydroxytryptamine also reduced the frequency of fictive feeding movements and in addition slowed the swallow phase of the feeding cycle [13]. Similar work in *Aplysia*, has demonstrated that lesioning the MCCs also caused a reduction in the frequency of repetitive biting movements and a slowing of the swallow phase of each feeding cycle [25], data that are consistent with the age-related changes in feeding behavior seen in *Lymnaea*.

These data infer that deficits in the functioning of aged CGCs could contribute to the age-related deficits in feeding behavior described in the previous paper [Arundell et al., 2005]. This paper examines how the electrophysiological properties of the CGCs changes with increasing age, and discusses the putative role these cells play in the aging of the feeding system.

2. Materials and methods

2.1. Experimental animals

All animals were bred in house at the University of Brighton. Animals were kept in large tanks at 18–20 °C on a 12 h light/dark cycle in copper-free tap water. They were fed on alternate days with either lettuce or fish flakes (Tetra U.K. Ltd.). Animals were kept in groups of up to 600 in large circulating tanks at a stocking density of approximately one snail per litre. Three groups were examined, 3–4 month (young), 6–7 month (middle age) and 11–12 month (old). Three to four months was chosen for the young animals as it represented the earliest age when the animals were sexually mature. Six to seven months was chosen for the middle-aged at it represented the time at which 40–50% of the population had died. Eleven to twelve months was chosen to represent old age, as this was the period when typically 85% of animals had died.

2.2. Intracellular recordings from the CGCs in the semi-intact lip-nerve preparation

In a series of electrophysiological experiments the response of the CGCs to the application of sucrose to the lips was examined in a semi-intact lip-CNS preparation consisting of the lips attached to the CNS by means of the three lips nerves (Fig. 1A; [29]).

The head of the snail plus the central nervous system was carefully dissected from the rest of the animal making sure that the superior and median lip nerves and tentacle nerves were left intact. All remaining peripheral nerves were cut. The semi-intact preparation was pinned in a Sylgard (Corning, U.K.)-lined recording chamber and the outlet of a superfusion pipette was placed in front of the mouth region for constant superfusion of the preparation with fresh *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES)-buffered saline, composition in mM: NaCl 50, KCl 1.5, $CaCl_2$ 4, $MgCl_2$ 2, HEPES 10, pH 7.8 with 5 M NaOH at a rate of 1 ml min^{-1}. Excess saline was removed from the opposite end of the recording chamber via a suction line. HEPES-buffered saline was continually perfused over the lips via superfusion pipette. At 10 min intervals a sucrose (Sigma, U.K.) solution (0.05 M) made up in HEPES-buffered saline was perfused through the same superfusion pipette for 30 s. The pipette had a dead space of <50 μl, which minimized mixing of the solutions. A combination of dye perfusion experiments and a series of preliminary experiments involving the application of 0.1 M sucrose to the cell body of the CGC via a superfusion pipette confirmed that sucrose was not capable of directly affecting the firing properties of the CNS neurons.

The ganglia of interest were de-sheathed using fine forceps and the remaining connective tissue was enzymatically treated with 0.1% pronase (Sigma Type XIV; Sigma, U.K.). Intracellular recordings from the CGCs were made using glass microelectrodes (5–15 MΩ) filled with 4 M potassium acetate in distilled water (Sigma, U.K.). Signals from the intracellular electrodes were amplified using an Axoclamp 2B amplifier (Axon Instruments, U.S.A.). The amplified signals were digitised at 2 KHz using a CED micro 1401 and stored on a PC for subsequent analysis. Off-line analysis of

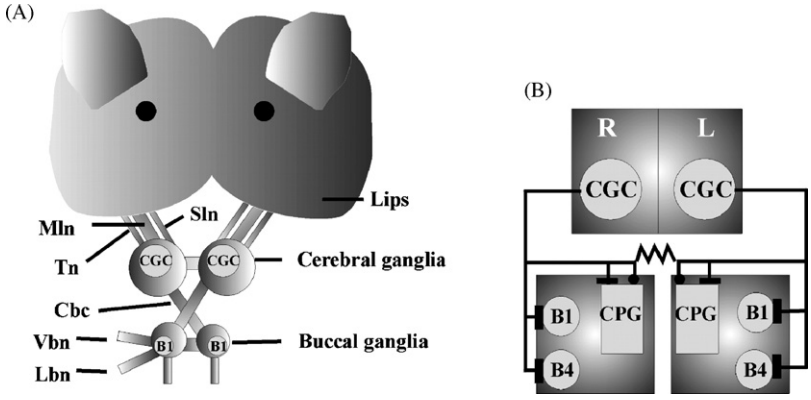

Fig. 1. Diagrammatic representation of the feeding circuitry of the pond snail, *Lymnaea stagnalis*. (A) Diagram to illustrate the positions of the CGCs and B1 and B4 motor neurons in the CNS. Also shown are the three lip nerves, the superior lip nerve (sln), median lip nerve (mln) and tentacular nerve (tn) which allow information to pass between the lips and the CNS and the cerebrobuccal connective (cbc) that joins the cerebral and buccal ganglia. (B) Cartoon of the cerebral and buccal ganglia. The modulatory cerebral giant cells (CGCs) are a pair of cells located in the paired cerebral ganglia. They send their main processes to the buccal ganglia where the two cells are electrically coupled. The CGCs also make synaptic connections with the neurons that form the central pattern generator (CPG) and with the motor neurons, two of which (B1 and B4) are illustrated on the diagram. N.B. Bars represent excitatory chemical synapses and circles inhibitory chemical synapses.

the records was performed using Spike 2 software (CED). CGC firing rates were recorded for periods of 30 s prior to the application of sucrose, during the 30 s sucrose application and during the first 30 s of the wash with HEPES-buffered saline. Both the sucrose and HEPES-buffered saline were applied through a single superfusion pipe with a measured dead space of 50 μl allowing rapid switching and minimal mixing of the solutions. Successive sucrose solutions were applied at 10 min intervals to prevent adaptation of the response (Yeoman, unpubl. obs.).

2.3. Intracellular recordings from the CGCs and motor neurons in the isolated CNS preparation

The CNS was removed from *Lymnaea* and simultaneous intracellular recordings were made from identified neurons in the isolated CNS with the use of previously described techniques (e.g. [1]). The isolated CNS preparation consisting of the main ganglionic ring, including the cerebral ganglia and a pair of buccal ganglia with a small piece of the pro-esophagus still attached was then mounted in a Sylgard-lined chamber that was continuously perfused with HEPES-buffered saline at a rate of 1 ml min^{-1}. Intracellular recording was carried using techniques described in Section 2.2.

Simultaneous recordings were made from the paired CGCs or a CGC and motor neurons B1 and B4 with which the CGC makes a monosynaptic connection ([17]; see Fig. 1B). Correct identification of the neurons relied on previously described methods [34,35]. CGCs fire spontaneously in the isolated CNS and so recordings were made of the endogenous properties of these neurons in animals of different ages. Recordings were made 60 s after electrode impalement to allow the cells time to recover. Specifically, the firing rates (determined over a 60 s period), resting membrane potential (RMP), action potential threshold, half width of the action potential and the amplitude and duration of the after-hyperpolarization (AHP) were recorded from spontaneously active CGCs. The amplitude of the after-hyperpolarization was determined by measuring the voltage difference between the RMP and the peak of the after-hyperpolarization. The duration of the after-hyperpolarization was the time between the first point at which the potential of the cell fell below the RMP and the time it took to return to the RMP. CGCs were artificially depolarized by injection of a 2 s, 5 nA square wave current pulses and the number of action potentials evoked by the current pulse examined. The input resistance of the CGCs was also determined by passing 2 s square wave hyperpolarizing current pulses into the cells to acquire an *I–V* curve and using the slope of this curve to calculate the input resistance.

To examine whether the coupling between the paired CGCs was altered by increasing age simultaneous intracellular recordings were made from the paired CGCs and the proportion of animals from each age group that demonstrated clear synchronous activity (measured as 1:1 firing over a 30 s time period) was determined.

In experiments designed to determine the efficacy of the monosynaptic chemical CGC → B1 connection, simultaneous intracellular recordings were made from the two neurons. The motor neuron was impaled with two electrodes, one to inject current to adjust the membrane potential and the other to measure voltage. The membrane potential of the motor neuron was held at −80 mV by current injection and its voltage response to spontaneous or current-evoked action potentials in the CGCs examined. The amplitude of the observed excitatory postsynaptic potentials (EPSPs) were determined in CNSs bathed with HEPES-buffered saline or with a saline high in divalent cations ("Hi-Di" saline) composition in mM: NaCl 26, KCl 1.5, $CaCl_2$ 14, $MgCl_2$ 8, HEPES 5 mM, pH 7.8 with 5 M NaOH. This saline is high in both

Ca^{2+} and Mg^{2+} ions, and it has previously been used to successfully block polysynaptic connections in *Lymnaea* [6] and other mollusks [7]. It was used here to ensure that only direct monosynaptic connections between the CGC and B1 motor neuron were recorded and to potentiate the amplitude of the evoked EPSP. In this series of experiments both the HEPES and Hi-Di ringers were bath perfused and electrophysiological recordings were made after 1 min of switching between the different ringers. Dye perfusion experiments suggested that 1 min was sufficient to completely exchange the ringer. These observations were supported by experiments, which showed that any changes in the amplitude of the evoked EPSP occurred within 1 min of the switch from HEPES to Hi-Di ringer inferring that 1 min was sufficient to exchange the bath solution.

2.4. Data analysis

All data was analysed by performing a one-way analysis of variance. If this demonstrated a significant difference between the data then a post hoc Tukey test was performed to determine the location of the significant change. Values presented in the paper are the mean ± S.E.M.

3. Results

3.1. Responses of the CGCs following application of sucrose to the lips

Semi-intact preparations were used to examine age-related changes in the electrical responses of the CGCs following the application of sucrose to the lips. Application of 0.05 M sucrose to the lips increased the spontaneous CGC firing rate in young animals (Fig. 2Ai). Rates increased from 34.6 ± 2.8 to 47.4 ± 3.0 spikes min^{-1} following the application of sucrose ($p < 0.01$) and returned to control levels during the wash (Fig. 2B). Sucrose also increased CGC firing rates in the middle-aged group (Fig. 2Aii). Values increased from 30.4 ± 2.6 to 40.8 ± 2.8 spikes min^{-1} following the application of sucrose ($p < 0.05$) and returned to baseline during the wash (Fig. 2B). In the old group CGC firing rates did not change significantly following the application of 0.05 M sucrose to the lips (Fig. 2Aiii and B).

3.2. Responses of the CGCs to an artificial depolarising current pulse

The effects of age on the general level of excitability of the CGCs was examined by determining the number of action potentials that could be evoked by a depolarizing current pulse. Depolarization of young CGCs generated a high frequency burst of action potentials (Fig. 3Ai). Quantitative analysis of the data showed that this standard current pulse

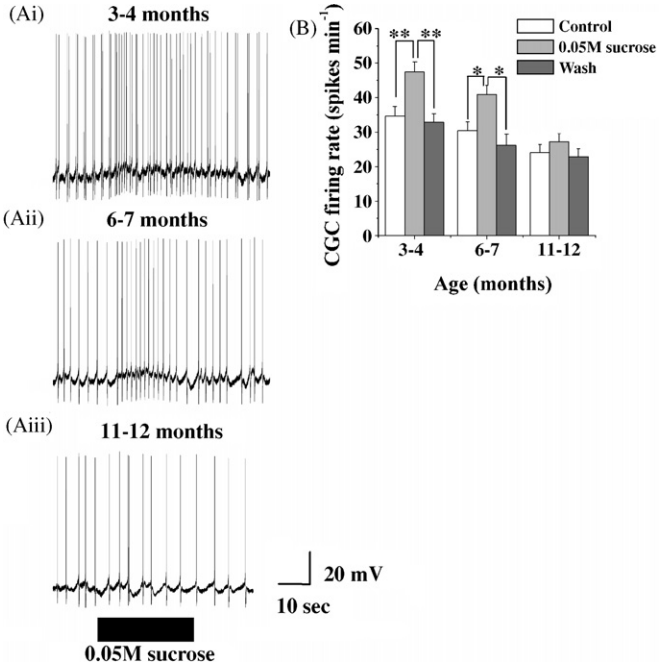

Fig. 2. Aging affects the ability of sucrose to excite the CGCs in a semi-intact lip nerve preparation. (A) Intracellular recordings from a single CGC from a (Ai) 3–4 month, (Aii) 6–7 month and (Aiii) 11–12 month animal. In all three cases the CGCs fired spontaneously prior to the application of sucrose to the lips. A 30 s application of sucrose (0.05 M; black bar) to the lips caused an increase in the firing rate of both the 3–4 month and 6–7 month cells. Sucrose failed to have a consistent effect on the firing rate of the CGC in the 11–12 month animal. (B) Bar graph to show mean changes in CGC firing rates following a 0.05 M sucrose stimulus. Sucrose caused significant increases in the firing rate of the 3–4 month and 6–7 month CGCs that were reversed on washing. No significant change was seen in the 11–12 month group. $*p < 0.05$, $**p < 0.01$, $n = 10$ for each group.

was capable of evoking an average of 14.4 ± 0.9 action potentials during the 2 s pulse (Fig. 3B). The excitability of the middle-aged CGCs was significantly reduced compared to the young cells (Fig. 3Aii), with standard current pulses only evoking 11.2 ± 1.1 spikes. Old CGCs were the least excitable (Fig. 3Aiii). Cells from this group only fired 6.7 ± 0.41 spikes during the 2 s depolarising pulse values that were significantly lower than both the young and middle-aged groups (Fig. 3B).

3.3. Changes in spontaneous CGC firing rates with increasing age

In both semi-intact and isolated CNS preparations increasing age was seen to cause a reduction in spontaneous CGC firing rate (Fig. 4). In semi-intact preparations, firing rates were 34.6 ± 2.8 spikes min^{-1} in young animals. These values decreased in the middle-aged group to 30.4 ± 2.6 spikes min^{-1} and were further reduced in the old group to 24 ± 2.4 spikes min^{-1} ($p < 0.01$; Fig. 4A). In the isolated CNS preparations decreases in CGC firing rates were also seen with increasing age ($p < 0.01$) although basal firing

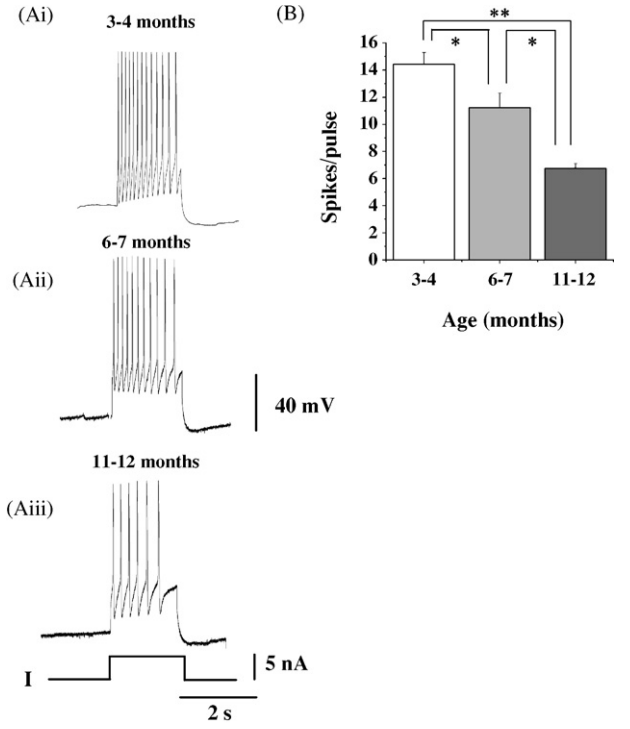

Fig. 3. Age-related decreases in the excitability of the CGC. (A) Sample traces of a CGC taken from a (Ai) 3–4 month, (Aii) 6–7 month and (Aiii) 11–12 month animal. Bursts of action potentials were evoked by the application of a 2 s, 5 nA square wave depolarising current pulse (*I*). (B) Graph showing the mean number of spikes evoked during the depolarising current pulse. Clear reductions in the numbers of evoked spikes are seen with increasing age. $*p<0.05$, $**p<0.01$, $n=20$ for each group.

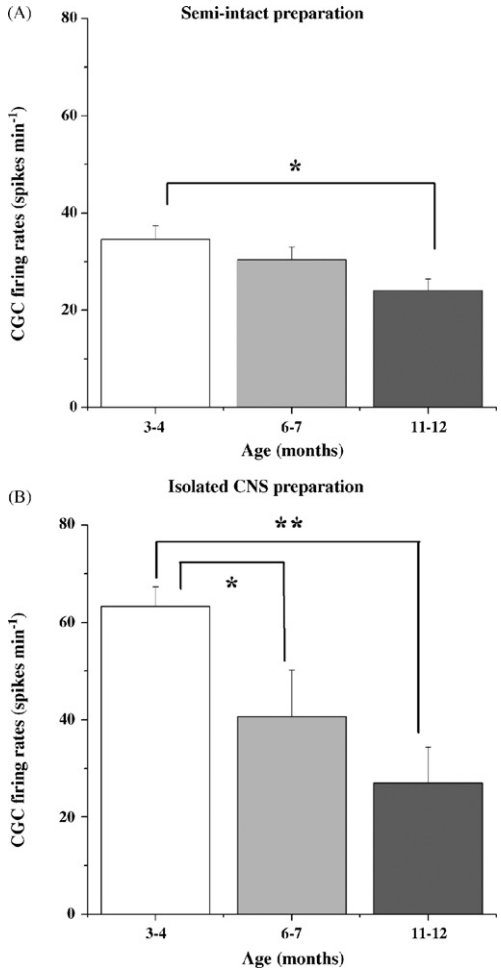

Fig. 4. Age-related changes in spontaneous CGC firing rates. Bar graphs in (A) and (B) illustrate the changes in CGC firing rate with increasing age from a (A) semi-intact preparation and (B) isolated CNS preparation. In both cases increasing age was associated with a decrease in the spontaneous firing rate of the CGCs. $*p<0.05$, $**p<0.01$, $n=10$ for each group.

rates were higher than those seen in the semi-intact preparation (Fig. 4B). Rates in young animals were 63.4 ± 3.9 spikes min^{-1}, which decreased in middle-aged animals to 40.6 ± 9.6 spikes min^{-1} and 27 ± 7.3 spikes min^{-1} in the old group.

3.4. Changes in the properties of the CGC action potential with increasing age

Fig. 5Ai shows sample CGC action potentials from young, middle-aged and old animals demonstrating a clear age-related increase in the amplitude of the AHP. AHP amplitude increased from 21.3 ± 1.1 mV in the young to 22.8 ± 1.4 mV in the middle-aged group to 25.7 ± 1.4 mV in the old animals ($p<0.05$; Fig. 5Aii). Similarly, the duration of the AHP also increased with increasing age ($p<0.001$; Fig. 5Bi). Durations increased from 168.1 ± 8.8 ms in the young to 205.1 ± 10.2 ms in the middle-aged to 276.3 ± 21.3 ms in the old group (Fig. 5Bii).

Significant decreases in the half widths of the CGC action potential were seen with increasing age (Fig. 5Ai). The mean half width for young CGC spikes was 19.3 ± 1.8 ms. This value decreased non-significantly in the middle-age group to 18.1 ± 1.6 ms and was further reduced in the old CGCs to 14.6 ± 0.75 ms ($p<0.05$ compared to young group; Fig. 5Ci). Examination of the spike shapes (Fig. 5Ai), showed that while the action potential of the young CGC had a characteristic plateau phase (see arrow), this was markedly reduced in the middle-aged action potential and almost completely absent in the old CGC spikes. In Fig. 5Cii the width of a typical action potential from each of the three age groups has been scaled so that the rapid repolarization phase seen following the peak of the action potentials is overlaid, demonstrating that the plateau phase is clearly reduced as the animal age (arrow, Fig. 5Cii).

The input resistance of the CGCs decreased with increasing age. Values decreased significantly from 21.5 ± 1.22 MΩ in the young group to 15.6 ± 1.11 in the middle-aged group and 11.8 ± 0.85 MΩ in the old group ($p<0.001$). No changes were seen in the RMP, threshold for action potential generation or the amplitude of the CGC action potentials with increasing age ($p>0.05$).

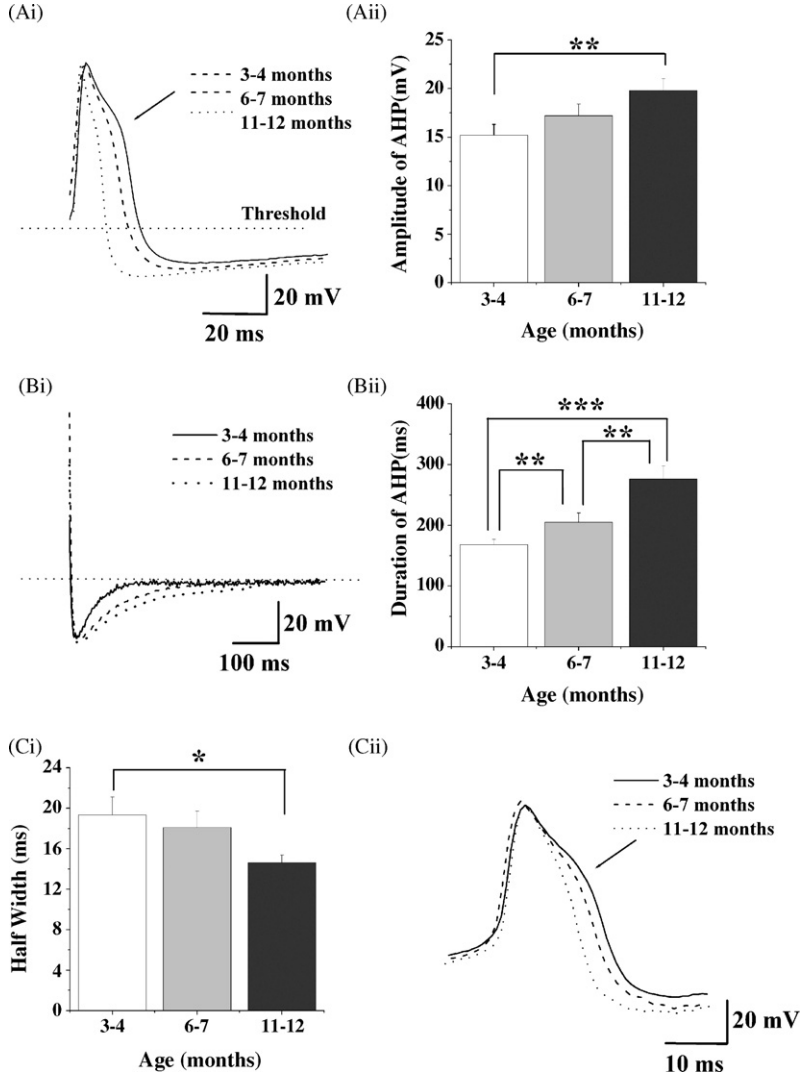

Fig. 5. Increasing age alters the properties of CGC action potentials. (Ai) Representative CGC action potentials from a 3–4, 6–7 and 11–12-month old animal showing an increase in the amplitude of the after-hyperpolarization (AHP). (Aii) Bar graph illustrating the age-related increase in the mean amplitude of the AHP. (Bi) Representative CGC action potentials from a 3–4, 6–7 and 11–12-month old animal demonstrating an age-related increase in the duration of the AHP. (Bii) Bar graph illustrating the age-related increase in the duration of the AHP. (Ci) Bar graph showing the age-related decreases in the half width of spontaneous CGC action potentials. (Cii) Scaled CGC action potentials from a 3–4, 6–7 and 11–12-month old animal, showing a clear age-related decrease in the plateau phase of the action potential. Arrows in (Ai) and (Cii) mark the plateau phase of the CGC action potential. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. its control unless indicated; $n = 15$ for all groups.

3.5. Changes in the synchronous CGC firing with increasing age

In young animals, intracellular recordings of the paired CGCs show clear synchronous 1:1 action potentials in both cells in 100% of preparations (Fig. 6Ai and B). In middle-aged animals the number of preparations in which synchronous CGC activity was recorded was reduced to 70% (Fig. 6B). In 58% of the old animals tested there was a lack of synchronous firing between the paired CGCs (Fig. 6Aii and B) a value that was significantly different from both the young and middle-aged groups.

3.6. Effects of increasing age on the resting membrane potential of the feeding motor neurons B1 and B4

The feeding motor neurons B1 and B4 receive monosynaptic inputs from the paired CGCs. Part of the CGCs gating and frequency control functions relies on its ability to provide a background level of excitation to the feeding motor neurons. We therefore examined whether the RMP of B1 and B4 was affected by increasing age. Increasing age was associated with a significant hyperpolarization of the RMP of the B1 motor neuron ($p < 0.001$; Fig. 7A). In young animals the RMP for B1 was -47.9 ± 1.1 mV. This became significantly more hyperpolarized in the middle-

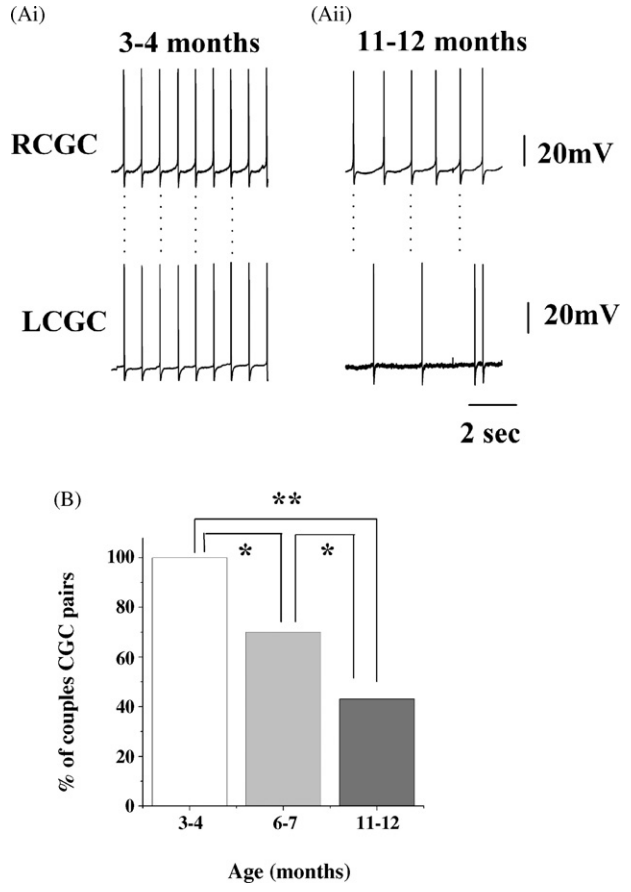

Fig. 6. CGC coupling is reduced with increasing age. (A) Sample traces showing simultaneous recordings from the paired CGCs in (Ai) a 3–4 month and (Aii) an 11–12 month animal. The paired CGCs fire synchronous action potentials in the young animal (see dotted lines), but fire asynchronously in the old animal. (B) Bar graph showing a decrease in the % of animals with coupled CGC pairs with increasing age. *$p < 0.05$, **$p < 0.01$, $n = 20$ for each.

aged group (-54.5 ± 1.0 mV) and further hyperpolarized in the old group (-61.0 ± 1.29 mV). Similar increases in the level of hyperpolarization were seen for the B4 motor neuron ($p < 0.001$; Fig. 7B). The RMP of B4 in the young animals was -53.9 ± 1.47 mV. This became more negative in both the middle-aged (-56.1 ± 1.5 mV) and old groups (-61.8 ± 1.3 mV).

3.7. Age-related changes in CGC → B1 monosynaptic connection

In a series of experiments the efficacy of the synaptic connection between the CGCs and the B1 motor neuron was examined by examining the amplitude of the evoked monosynaptic EPSP. While it was possible to record compound EPSPs in the B1 motor neuron following depolarization-evoked bursts of action potentials in the CGCs, the observation that the number of spikes evoked per burst was age dependent (see Fig. 3) meant that any age-related change in synaptic efficacy would be difficult to interpret. On the basis of this result all recordings of synaptic efficacy were determined by examining unitary evoked EPSPs from B1 motor neurons held at -80 mV. Intracellular recordings of B1 motor neurons from young animals in normal HEPES-buffered saline failed to demonstrate consistent unitary EPSPs (Fig. 8Ai, grey trace). EPSPs were recorded in a minority of preparations under these conditions with a mean amplitude of 0.2 ± 0.15 mV (Fig. 8B). Perfusion with Hi-Di saline increased the amplitude of the unitary EPSP to 3.07 ± 0.36 mV ($p < 0.001$; black trace). In middle-aged animals the mean amplitude of the evoked-EPSP in normal HEPES ringer was 1.1 ± 0.2 mV. This increased significantly to 2.9 ± 0.4 mV in the Hi-Di ringer ($p < 0.01$). In old animals unitary EPSPs could be recorded from B1 motor neurons in normal HEPES-buffered saline (mean amplitude 2.49 ± 0.4 mV). These EPSPs were significantly larger than those recorded in normal HEPES-buffered saline in the young group ($p < 0.001$), but not significantly different to the EPSPS recorded from B1 motor neurons from the young CNSs perfused with Hi-Di saline (Fig. 8B). Interestingly, perfusion of CGCs from old animals with Hi-Di saline failed to significantly enhance the amplitude of the evoked EPSP (Fig. 8Aii and B).

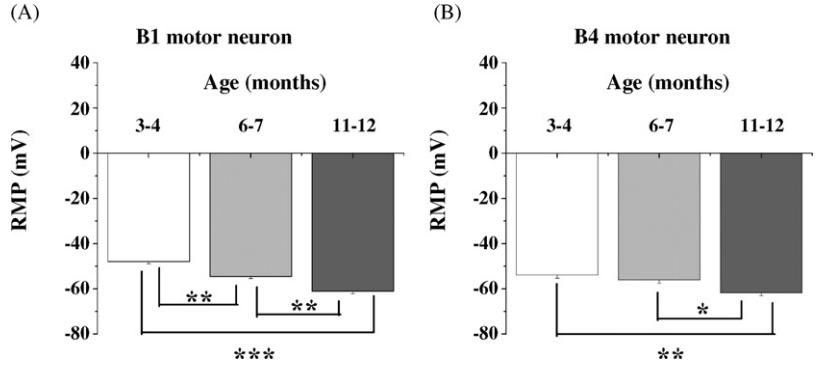

Fig. 7. Age-related changes in the resting membrane potential (RMP) of buccal motor neurons. Bar graphs showing the increase in RMP with increasing age for the (A) B1 and (B) B4 motor neurons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n > 20$ for all groups.

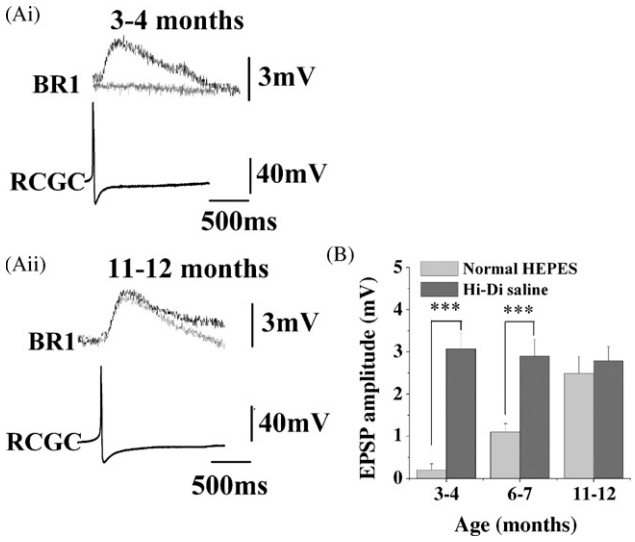

Fig. 8. Age-related changes in the CGC → B1 chemical connection. (A) Intracellular recordings from a CGC and a B1 motor neuron from a (Ai) 3–4 month and (Aii) 11–12-month old animal. In the CNS of young animals perfused with normal HEPES-buffered saline depolarization-induced CGC action potentials failed to consistently evoke a measurable EPSP in the B1 motor neuron (grey trace). Large unitary EPSPs could be recorded in the B1 neuron following perfusion with a Hi-Di ringer (black trace). In the old CNS unitary EPSPs could be seen in response to action potentials in the CGCs perfused with a normal HEPES-buffered ringer (grey trace). Perfusion with a Hi-Di saline failed to increase the amplitude of the evoked EPSP (black trace). (B) Bar graph demonstrating an age-related increase in the mean amplitude of the recorded EPSP in normal HEPES-buffered saline. In a Hi-Di saline CGC-evoked EPSPs in B1 were significantly increased in both young and middle-aged groups compared to EPSPs in normal HEPES-buffered saline. EPSPs recorded in old B1 neurons bathed in two different salines did not differ significantly. N.B. B1 neurons were held at −80 mV by current injection in all preparations. $^{***}p < 0.001$, $n = 10$ for each group.

4. Discussion

In the previous paper we demonstrated age-related changes in feeding behavior. Based on previous work, this could be due to a malfunctioning of a pair of modulatory serotonergic cells known as the CGCs. In this paper we have examined how the endogenous properties and synaptic connectivity of this pair of cells changes with increasing age and discuss how the observed changes in their properties relate to the age-related changes in feeding behavior.

Previous work has shown that application of sucrose to the lips is capable of exciting the CGCs causing them to fire more action potentials [12,21]. Work performed in this paper has confirmed these findings but also demonstrated that the magnitude of the CGC response is reduced with increasing age. In addition, spontaneous CGC firing rates were seen to decrease with increasing age. Previous work by Yeoman et al. [32,33] showed that the feeding responses in the freely moving intact animal were associated with increases in the firing frequency of the CGCs. This increase was important in allowing the animal to respond to food but was also capable of regulating the frequency of feeding movements. Therefore a combination of the decreased spontaneous firing rates and the reduced ability for sucrose to consistently excite the CGCs in the old animals could explain the age-related increase in the number of non-responding animals and a decrease in the number of sucrose-evoked feeding movements described in the previous paper. Given that the level of chemosensory information entering the cerebral ganglia from the lips was unaffected by increasing age and that the reduced ability for sucrose to activate a feeding behavior most likely represented a deficit in central processing, the findings in this paper suggest that a possible site of this deficit is the lip sensory neuron → CGC connection. Injection of standard depolarising current pulses into the CGC showed that a reduction in the endogenous excitability was at least partially responsible for the reduced ability of the CGCs to respond following sucrose application to the lips. However, reductions in the ability of the sensory neurons to release their transmitter or for the CGCs to respond to the released transmitter can not be excluded, as currently little is known about the transmitters used by the sensory neurons or indeed whether the sensory neuron → CGC connection is mono- or polysynaptic.

The observed changes in the excitability of the CGCs were shown not to be due to an age-related change in the resting membrane potential of the CGCs or alterations in the threshold for CGC action potential generation both of which remained unchanged with increasing age. However, decreases were recorded in the CGC's input resistance that would reduce the excitability of the CGCs as shown previously in *Lymnaea* [14]. Increases were also seen in the amplitude and duration of the AHP with increasing age. Increases in the slow AHP have been consistently recorded from a wide variety of aged neurons (e.g. [4,16]) and have been shown to regulate neuronal firing. Indeed, many neurotransmitters act to alter the firing frequency of a target neuron by modulating the currents responsible for generating the AHP. A variety of currents have been proposed to be involved in generating the AHP including small and large conductance Ca^{2+}-activated K^+ currents and the slowly inactivating delayed rectifier type currents, although to date only the small conductance Ca^{2+}-activated K^+ channel has been shown to be involved in age-related increases in the AHP of hippocampal neurons [24].

Associated with the age-related increase in the amplitude and duration of the AHP was a decrease in the half width of the spontaneously generated action potential that would tend to decrease transmitter release from the CGCs and reduce their ability to gate and regulate the frequency of the feeding rhythm. The CGC action potential is conspicuous by its characteristic plateau, which has been shown to be due a persistent L-type Ca^{2+} current [26]. Recordings from action potentials of old CGCs showed that this plateau phase was greatly reduced compared to young animals, suggesting that as the animals age there is either a decrease in the size of the L-type Ca^{2+} current or an increase in the amplitude of the

K^+ currents that act to increase the rate of repolarization the neuron [26].

Both the increase in the amplitude and duration of the AHP and the decrease in half width of the action potential could be explained by an increase in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ would tend to reduce the amplitude of the L-type Ca^{2+} current either by reducing the driving force for Ca^{2+} entry or by a mechanism known as Ca^{2+} dependent inactivation [5]. Additionally, increases in $[Ca^{2+}]_i$ would tend to increase the activity of Ca^{2+}-dependent K^+ channels and so increase the rate of repolarization and increase the amplitude and duration of the AHP. Ca^{2+}-dependent K^+ channels were first described in molluskan neurons by Meech [20]. Recent work by Staras et al. [26] on young *Lymnaea* demonstrated that a small conductance Ca^{2+}-activated K^+ current could be isolated from the current complement present in the CGC soma, providing a potential mechanism for changes in the AHP in this species.

4.1. Alterations in CGC → CGC connectivity with increasing age

With increasing age significant decreases were seen in the number of CGC pairs that fired synchronously. This synchronicity is believed to be due to an electrotonic connection between the paired CGCs that occurs in the buccal-buccal commisure. While we can only speculate that the uncoupling of these cells is due to a decrease in the strength of the electrical connection between the two cells, similar age-related decreases in electrotonic coupling have been reported previously in the pond snail *Lymnaea* between a pair of neurons located in the parietal ganglia [31] and in a variety of mammalian cell types including heart muscle cells [3], astrocytes [2] and fibroblasts [27]. The decrease observed *Lymnaea* would tend to reduce the synchronicity of CGC inputs to the buccal feeding circuitry and would therefore contribute to the change in feeding behavior seen with increasing age.

4.2. Changes in the excitability of the feeding motor neurons

The experiments performed in this paper provide clear evidence that the excitability of the CGCs is compromised by increasing age and based on previous work these changes could account for the observed effects of age on feeding behavior [32,33,35]. Part of the ability of the CGCs and its neurotransmitter 5-HT to gate and frequency control the feeding network is due to their ability to provide a background level of excitation to both the CPG [35] and the feeding motor neurons (e.g. B1 and B4; [18,32,33]). Examination of the RMP of both B1 and B4 motor neurons showed them both to be hyperpolarized with increasing age. These changes were consistent with a deficit in CGC function and would make the motor neurons less excitable to inputs from the CPG.

However, the evoked monosynaptic EPSP increased with increasing age, data that appeared to contradict the RMP findings detailed above. The effect of the CGCs/5-HT on RMP has been shown to be long-lasting (>1 min) and are presumably due to 5-HT acting on a G-protein-coupled receptor [18,28]. One such conductance shown to be present in both the B1 and B4 motor neurons that could be activated by 5-HT and would contribute to the RMP is cAMP-dependent persistent Na^+ current [19]. The monosynaptic EPSP recorded in B1 in this study is much shorter in duration (≈2 s) and will therefore not contribute to the CGCs long-term regulation of RMP. It would however, increase the short-term excitability of the B1 neuron and could compensate for some of the deficits seen in the CGC excitability. As the peak amplitude of the unitary B1 EPSP was only 2.49 ± 0.4 mV and the age-related hyperpolarization in RMP was ≈ 10 mV, the unitary EPSPs could not completely compensate for the change in RMP. Given the mean duration of the B1 EPSP the CGCs would have to be firing at greater than 30 spikes min^{-1} in order for summation to occur. Recordings from both the semi-intact and isolated CNS preparations showed that the rates of CGC firing in old animals never exceeded 30 spikes min^{-1}, even when evoked by a food stimulus such as sucrose. This suggested that summation of the unitary B1 EPSPs was unlikely. It should be noted that the firing rates of the CGCs in the intact old animals are currently unknown. However, based on results obtained in young animals [33] firing rates are likely to be lower than those seen in the preparations used in this study.

4.3. Do changes in the properties of the CGCs explain the observed age-related changes in short-term feeding behavior?

Our model of aging described in the first of these two papers proposes that the observed effects of age on feeding behavior could be explained by deficits in the functioning of a pair of serotonergic neurons. In this paper we have detailed a variety of changes in the functioning of these cells that are consistent with this hypothesis. Is it therefore possible that the observed deficits in the functioning of this neural network seen with increasing age are due to the effects of age on a specific pair of neurons? Certainly these two neurons are ideally placed to affect the functioning of the feeding system as they have widespread synaptic connections with the CPG neurons, motor neurons and muscles that regulate feeding. Therefore, deficits in the functioning of these cells are likely to affect the functioning of the majority of the components of this system.

Acknowledgements

We would like to thank the Engineering and Physical Sciences Research Council (EPSRC) and the Royal Pharmaceutical Society of Great Britain for supporting this work.

References

[1] Benjamin PR, Rose RM. Central generation of bursting in the feeding system of the snail, *Lymnaea stagnalis*. J Exp Biol 1979;80:93–118.

[2] Cotrina ML, Gao Q, Lin JH, Nedergaard M. Expression and function of astrocytic gap junctions and aging. Brain Res 2001; 901(1–2):55–61.

[3] Dhein S, Hammerath SB. Aspects of the intercellular communications in aged hearts: effects of the gap junction uncoupler palmitoleic acid. Nanyun Schmeidesbergs Arch Pharmacol 2001;364(5):397–408.

[4] Disterhoft JF, Thompson LT, Moyer Jr JR, Mogul DJ. Ca^{2+}-dependent after-hyperpolarization and learning in young and aged hippocampus. Life Sci 1996;59(5–6):413–20.

[5] Eckert R, Chad JE. Inactivation of calcium channels. Prog Biophys Mol Biol 1984;44(3):215–67.

[6] Elliott CJH, Benjamin PR. Interactions of pattern generating interneurones controlling feeding in *Lymnaea stagnalis*. J Neurophysiol 1985;54:1396–411.

[7] Getting PA. Mechanisms of pattern generation underlying swimming in *Tritonia*. I. neuronal network formed by monosynaptic connections. J Neurophysiol 1981;46:65–79.

[8] Goldschmeding JT, Van Duivenboden YA, Lodder JC. Axonal branching pattern and coupling mechanisms of the cerebral giant neurons in the snail, *Lymnaea stagnalis*. J Neurobiol 1981;12: 405–24.

[9] Horn CC, Geizhals CR, Kupfermann I. Further studies of bulk and orosensory decrement in producing satiation of feeding in *Aplysia*. Brain Res 2001;918(1–2):51–9.

[11] Kemenes G, Elekes K, Hiripi L, Benjamin PR. A comparison of four techniques for mapping the distribution of serotonin and serotonin-containing neurons in fixed and living ganglia of the snail, *Lymnaea*. J Neurocytol 1989;18(2):193–208.

[12] Kemenes G, Elliott CJH, Benjamin PR. Chemical and tactile inputs to the *Lymnaea* feeding system: effects on behaviour and neural circuitry. J Exp Biol 1986;122:113–38.

[13] Kemenes G, Hiripi L, Benjamin PR. Behavioural and biochemical changes in the feeding system of *Lymnaea* induced by the dopamine and serotonin neurotoxins 6-hydroxydopamine and 5,6-dihydroxytryptamine. Philos Trans R Soc Lond B Biol Sci 1990;329:243–55.

[14] Klassen LJ, Janse C, Van der Roest M. Multiple synaptic connections of a single neuron change differentially with age. Neurobiol Aging 1998;19(4):341–9.

[15] Kupfermann I, Weiss KR. Activity of an identified serotonergic neuron in free moving *Aplysia*, correlates with behaviour. Brain Res 1982;241(2):334–7.

[16] Landfield PW, Pitler TA. Prolonged Ca^{2+} dependent after-hyperpolarizations in hippocampal neurons of aged rats. Science 1984; 226(4678):1089–92.

[17] McCrohan CR, Benjamin PR. Patterns of activity and axonal projections of the cerebral giant cells of the snail, *Lymnaea stagnalis*. J Exp Biol 1980;85:149–68.

[18] McCrohan CR, Benjamin PR. Synaptic relationships of the cerebral giant cells with motor neurons in the feeding system of *Lymnaea stagnalis*. J Exp Biol 1980;85:169–86.

[19] McCrohan CR, Gillette R. Enhancement of cyclic AMP-dependent sodium current by the convulsant drug pentylenetetrazol. Brain Res 1988;452(1–2):21–7.

[20] Meech RW. The sensitivity of *Helix aspersa* neurones to injected Ca^{2+} ions. J Physiol 1974;237(2):259–77.

[21] Nakamura H, Kojima S, Kobayashi S, Ito I, Fujito Y, Suzuki H, et al. Physiological characterisation of the lip and tentacle nerves in *Lymnaea stagnalis*. Neurosci Res 1999;33(4):291–8.

[24] Power JM, Wu WW, Sametsky E, Oh MM, Disterhoft JF. Age-related enhancement of the slow outward calcium activated potassium current in the hippocampal CA1 pyramidal neurons in vitro. J Neurosci 2002;22(16):7234–43.

[25] Rosen SC, Weiss KR, Goldstein RS, Kupfermann I. The role of a modulatory neuron in feeding and satiation in *Aplysia*: effects of lesioning the serotonergic metacerebral cells. J Neurosci 1989;9:1562–78.

[26] Staras K, Gyori J, Kemenes G. Voltage-gated ionic currents in an identified modulatory cell type controlling molluscan feeding. Eur J Neurosci 2002;15(1):109–19.

[27] Statuto M, Bianchi C, Perego R, Del Monte U. Drop of connexin 43 in replicative senescence of human fibroblasts HEL-299 as a possible biomarker of senescence. Exp Gerontol 2002;37(8–9):1113–20.

[28] Straub VA, Benjamin PR. Extrinsic modulation and motor pattern generation in a feeding network: a cellular study. J Neurosci 2001;21(5):1767–78.

[29] Straub VA, Styles BJ, Ireland JS, O'Shea M, Benjamin PR. Central localisation of plasticity involved in appetitive conditioning in *Lymnaea*. Learn Memory 2004;11(6):787–93.

[30] Weiss KR, Cohen JL, Kupfermann I. Modulatory control of the buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. J Neurophysiol 1978;41:181–203.

[31] Wildering WC, Van der Roest M, de Vlieger TA, Janse C. Age-related changes in junctional and non-junctional conductances in two electrically coupled peptidergic neurons of the mollusc, *Lymnaea stagnalis*. Brain Res 1991;547(1):89–98.

[32] Yeoman MS, Kemenes G, Benjamin PR, Elliott CJH. Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail, Lymnaea. II. Photoinactivation. J Neurophysiol 1994;72:1372–82.

[33] Yeoman MS, Pieneman AW, Ferguson GP, ter Maat A, Benjamin PR. Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail *Lymnaea*. I. fine wire recording in the intact animal and pharmacology. J Neurophysiol 1994;72:1357–71.

[34] Yeoman MS, Vehovszky A, Kemenes G, Elliott CJH, Benjamin PR. A novel interneuron having hybrid modulatory-central pattern generator properties in the feeding system of the pond snail, *Lymnaea stagnalis*. J Neurophysiol 1995;73:112–24.

[35] Yeoman MS, Brierley MJ, Benjamin PR. Central pattern generating interneurones are targets for the modulatory serotonergic cerebral giant cells in the feeding system of *Lymnaea*. J Neurophysiol 1996;75(1):11–25.