Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2008 | 106 | 1699–1709 doi: 10.1111/j.1471-4159.2008.05528.x

Synapse-specific changes in serotonin signalling contribute to age-related changes in the feeding behaviour of the pond snail, *Lymnaea*

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Abstract

This study utilised the pond snail, *Lymnaea* to examine the contribution that alterations in serotonergic signalling make to age-related changes in feeding. Age-related decreases in 5-HIAA levels in feeding ganglia were positively correlated with a decrease in the number of sucrose-evoked bites and negatively correlated with an increase in inter-bite interval, implicating alterations in serotonergic signalling in the aged phenotype. Analysis of the serotonergic cerebral giant cell (CGC) input to the protraction motor neurone (B1) demonstrated that fluoxetine (10–100 nM) increased the amplitude/duration of the evoked EPSP in both young and middle aged but not in old neurones, suggesting an age-related attenuation of the serotonin transporter. 5-HT evoked a concentration-dependent increase in the amplitude/duration of B1 EPSP, which was greater in old neurones compared to both young and middle aged. Conversely, the 5-HT-evoked depolarisation and conditional bursting of the swallow motor neurone (B4) were attenuated in old neurones, functions critical for a full feeding rhythm. The CGCs' ability to excite B1 was blocked by cinanserin but not by methysergide. Conversely, the CGC to B4 connection was completely blocked by methysergide and only partially by cinanserin suggesting that age-related changes may be receptor-specific. In summary, synapse-specific attenuation of the CGC-B4 connection and enhancement of the CGC-B1 connection would slow the swallow phase and maintain protraction, consistent with behavioural observations.

Keywords: feeding, *Lymnaea*, neuronal ageing, serotonin transporter.

J. Neurochem. (2008) **106**, 1699–1709.

In a wide variety of animals, ageing is associated with a marked reduction in both the quantity and quality of motor activity (Peng *et al.* 1980; Bennett *et al.* 1996; Ingram 2000 and Larsson and Ramamurthy 2000). Specifically, deficits can be seen in rhythmic motor behaviours such as locomotion (Bennett *et al.* 1996), ventilation (Hiss *et al.* 2001) and feeding movements (e.g. Baum and Bodner 1983; Fucile *et al.* 1998; Stanford *et al.* 2003; Arundell *et al.* 2006). However, in many cases, the precise neurophysiological correlates underlying these changes are so far unknown. Recently, it has been shown in the pond snail, *Lymnaea* that ageing is associated with a decrease in the frequency of rhythmic feeding movements, due mainly to a prolongation of the swallow phase of each feeding cycle (Arundell *et al.* 2006). Feeding in the pond snail consists of a series of rhythmic feeding cycles. Each cycle consists of three active phases known as protraction, rasp and swallow. In protraction, the mouth is opened and the radula is forced out of the mouth. During rasp the radula is rotated forwards and scraped along the substrate to collect food. Finally, during the swallow phase, the radula is rotated backwards and the food forced into the oesophagus (for review, see Benjamin and Elliott 1989; Elliott and Susswein 2002). This basic rhythm is driven by a central pattern-generating circuit (CPG) located in a region of the CNS known as the buccal ganglia

Received March 10, 2008; revised manuscript received June 2, 2008; accepted June 9, 2008.

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Abbreviations used: 5-HIAA, 5, hydroxyindole acetic acid; 5-HT, 5, hydroxytryptamine or serotonin; CGCs, cerebral giant cells; CPG, central pattern generator; EPSP, excitatory post-synaptic potential; HPLC, high performance liquid chromatography; MAO, monoamine oxidase; SERT, serotonin transporter.

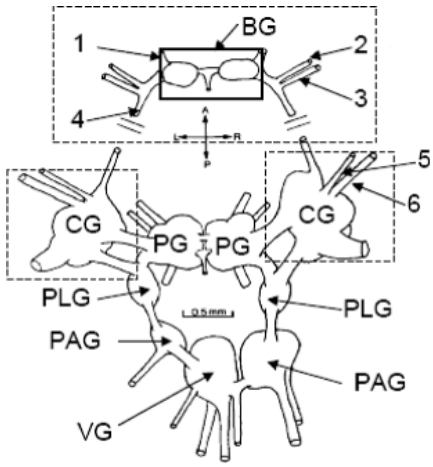

Fig. 1 Diagrammatic representation of the CNS from *Lymnaea stagnalis*. The diagram illustrates a dorsal view of the main ganglia and nerves associated with the CNS. Details of the regions taken for HPLC analysis are shown by the dotted boxes. The cerebral ganglia (CG) are shown with the cerebral commisure cut and the left and right ganglia folded out to give a flattened, two-dimensional view of the CNS. Ganglia: buccal (BG); cerebral (CG); pedal (PG); pleural (PLG); parietal (PAG) and visceral (VG). L and R indicate left and right and A and P anterior and posterior. Major nerves and connectives: (1) dorso-buccal nerve; (2) latero-buccal nerve; (3) ventrobuccal nerve; (4) cerebro-buccal connective; (5) superior lip nerve; (6) median lip nerve.

(see Fig. 1). The CPG comprises of three populations of interneurones termed N1, N2 and N3. The N1 interneurones fire during the protraction phase, N2 during rasp and the N3 neurones during the swallow phase. Through their connections with the motor neurones the interneurones ensure the coordinated contraction of the buccal muscles that are responsible for producing the three active phases of feeding. In addition to understanding a great deal about the connectivity of the feeding circuitry, the neurotransmitters utilised by some of these neurones are also known. The N1 interneurones utilise acetylcholine as their main neurotransmitter (Elliott and Kemenes 1992; Yeoman *et al.* 1993) while the N2 neurones are glutamatergic (Brierley *et al.* 1997a,b).

This basic rhythm can be fine tuned by a variety of modulatory neurones (Kyriakides and McCrohan 1989; Yeoman *et al.* 1994a,b, 1996; Hernadi *et al.* 2004) that are distinct from the CPG. The paired serotonergic cerebral giant cells (CGCs) are a specific type of modulatory neurone, located in the cerebral ganglia of the CNS (see Fig. 1). The CGCs send their axons down the cerebro-buccal connective to the buccal ganglia, providing the sole serotonergic input to the feeding circuitry (Kemenes *et al.* 1989). Previous studies have shown that they have both, a gating and a frequency control function, allowing the feeding system to both respond to a food stimulus as well as being able to regulate the frequency of feeding movements (Yeoman *et al.* 1994a, b, Yeoman *et al.* 1996). Specifically, *in vivo* recordings of the CGCs activity showed that a minimum level of firing (6 spikes min^{-1}) was necessary to allow the animals to respond to a feeding stimulus (gating function), while increases in CGC firing rates between 6-15 spikes min^{-1} were capable of increasing the frequency of feeding movements (Yeoman *et al.* 1994a). The CGCs have their actions by altering the excitability and endogenous properties of the N1, N2 and N3 interneurones (Yeoman *et al.* 1996) and also through their ability to regulate the excitability and endogenous properties of identifiable motor neurones (B1; protraction and B4; swallow) with which they make monosynaptic connections (McCrohan and Benjamin 1980a,b,; Straub and Benjamin 2001). The ability of serotonin to regulate motor function is not limited to *Lymnaea* but has been demonstrated in other molluscs (e.g. Rosen *et al.* 1989) and in mammals (Hultborne and Kiehn 1992). The fact that these CPGs play important roles in generating fundamental life sustaining behaviours (e.g. feeding and ventilation) suggests that their ability to generate a basic rhythm is unlikely to be altered dramatically during the ageing process. However, the decreases observed in quantity and quality of rhythmic motor activity may represent changes in the functioning of modulatory neurons that act to shape this basic rhythm. In support of this hypothesis we have recently shown that with increasing age there are marked decreases in the spontaneous firing rate and the excitability of the CGCs (Patel *et al.* 2006), which could underlie the observed changes in feeding behaviour seen with increasing age. As the CGCs form the sole serotonergic input to the feeding circuitry in the buccal ganglia, this simple system allows a unique opportunity to examine the effects of age on serotonergic signalling and feeding behaviour.

This paper utilises biochemical, pharmacological and electrophysiological techniques to detail age-related molecular changes in the ability of the serotonergic CGCs to signal to key target motor neurones. This paper demonstrates a functional attenuation in the serotonin transporter (SERT) and changes in the sensitivity of identifiable protraction (B1) and swallow (B4) phase motor neurones to exogenously applied 5, hydroxytryptamine (5-HT) as the animal's age. These changes strengthen the CGC $\rightarrow$ B1 synapse, but attenuate the CGC $\rightarrow$ B4 synapse. The potential contributions these age-related changes make to feeding behaviour are discussed.

Methods

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 food (Tetrapond fish flakes; Tetrapond UK Ltd., Southampton, UK). Animals were kept in groups of up to

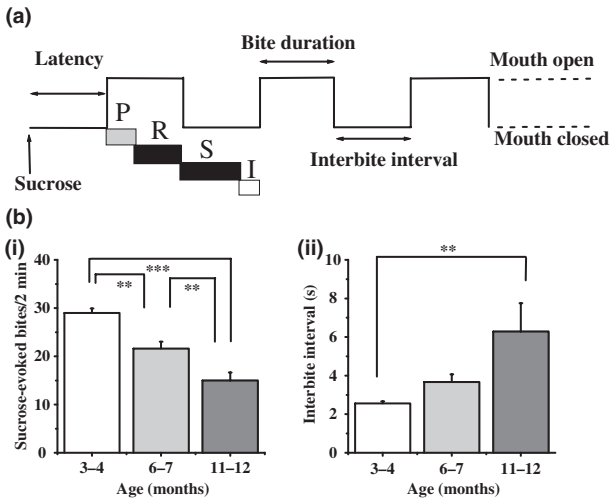

Fig. 2 Age-related changes in feeding behaviour. (a) Diagrammatic representation of a typical feeding trace illustrating the main feeding parameters examined. The shaded bars show typical timings of the 3 active phases (protraction (P); rasp (R), swallow (S)) and 1 inactive phase (I) that comprises each feeding cycle. (b) Bar graphs showing age-related changes in the number of sucrose-evoked bites (b-i) and inter-bite interval (b-ii). Values plotted are the mean ± SEM $n \geq 9$ for each bar. $^{**}p < 0.01$, $^{***}p < 0.001$.

600 in large circulating tanks at a stocking density of approximately one snail per litre.

Measurement of changes in short-term feeding behaviour

The effects of age on short-term feeding were examined using a previously described method (Staras *et al.* 1998; Arundell *et al.* 2006). The feeding behaviour was recorded using a software package produced by Staras *et al.* (1998) yielding a typical feeding trace (Fig. 2a). Three age groups were examined, 3–4 months (young), 6–7 months (middle-aged) and 11–12 months (old). The choice of these three age groups has previously been discussed in Arundell *et al.* (2006). The CNSs from the animals used in this part of the study were then removed for HPLC analysis.

HPLC sample preparation

The CNS was removed from *Lymnaea stagnalis* and pinned out in a silicone elastomer (Sylgard; Corning, UK) – lined dish, filled with ice-cold 4-(2-hydroxylethyl)piperazine-1-ethanesulphonic acid (HEPES)-buffered saline (consisting of 10 mM HEPES, 50 mM NaCl, 1.7 mM KCl, 2 mM $MgCl_2.6H_2O$ and 4.0 mM $CaCl_2.2H_2O$, buffered to pH 7.9). The CNS was dissected into two regions. These were (i) buccal ganglia + lateral and ventral buccal nerves and cerebro-buccal connectives (BG); (ii) cerebral ganglia (CG; Fig. 1). These two regions were chosen as both are intimately involved in the initiation and regulation of feeding. Each of these tissue samples was homogenised in 200 μL of ice cold 0.1 M perchloric acid (BDH Ltd., Poole, UK) and centrifuged at 20 000 *g* at 4°C. All samples were run within 20 min of preparation, and were stored on ice prior to analysis.

HPLC-EC determination of levels of 5-HT and 5-HIAA

The methods used have been described previously by Patel *et al.* (2005). Standard solutions were prepared from a 100 μg dm^{-3} stock standard of each analyte and were made up in freshly prepared ice-cold 0.1 M perchloric acid (BDH). Each of the standard solutions was prepared on the day of analysis and stored on ice between injections.

Spike and recovery data were obtained to account for errors during sample preparation. Recovery factors were calculated using standard IUPAC procedures (Patel *et al.* 2005).

Electrophysiology

Previous work had shown that the main age-related changes in feeding behaviour were due to consistent increases in the duration of swallow (N3 phase) of the feeding cycle, with only occasional, batch-specific changes in the bite duration (N1/N2 phases). Of the limited number of batches that showed a change in bite duration closer examination showed that changes were specifically because of the increases in the duration of protraction (N1 phase) with no significant change in rasp (N2 phase; Arundell *et al.* 2006). Therefore, our studies of the effects of age on serotonergic signalling concentrated on an examination of the monosynaptic connection between the CGC and either the protraction phase motor neurone (B1) or the swallow phase motor neurone (B4).

In an initial series of experiments the strength of the CGC → B1 and CGC → B4 connections were examined in the presence and absence of different concentrations of fluoxetine (10–100 nM; Sigma-Aldrich, Gillingham, UK). The CNS was dissected in HEPES-buffered saline and pinned in a Sylgard-lined dish. The outer connective tissue overlying the cerebral and buccal ganglia was removed with fine forceps and the inner connective tissue sheath softened with protease (Type XIV, Sigma-Aldrich) to facilitate electrode impalement. The bath containing the CNS was perfused constantly with HEPES-buffered ringer, and fluoxetine (Sigma-Aldrich) was applied to the motor neurone via a local superfusion pipette. Intracellular recordings were made from both the CGC and the motor neurone using glass microelectrodes that had resistances ranging from 10–15 MΩ when filled with 4 M potassium acetate. The membrane potential of the motor neurone was held at −70 mV by a second current injection electrode and the CGCs firing rate maintained at 0.5 Hz by constant current injection. The strength of the CGC → B1 and CGC → B4 connections were determined by recording the amplitude of the excitatory postsynaptic potentials (EPSPs) that were evoked in the motor neurone following spontaneous action potentials in the CGCs. In experiments designed to examine the effects of fluoxetine (10–100 nM) on the strength of the CGC → motor neurone connection, fluoxetine was applied locally to the motor neurones for 20 s via the superfusion pipette. These concentrations were chosen as they significantly reduced uptake of [^{3}H] 5-HT in isolated CNS preparations (data not shown).

In a separate series of experiments designed to examine the sensitivity of B1 and B4 to 5-HT, the CNS was first perfused with a high Mg^{2+}/zero Ca^{2+} ringer, containing 50 mM NaCl, 1.7 mM KCl, 6 mM $MgCl_2.6H_2O$, 10 mM HEPES, pH 7.9 in order to chemically isolate the motor neurone. Serotonin was then applied in a concentration range of 10^{-8} to 10^{-4} M for 5 s via the superfusion pipette and the amplitude of the evoked depolarisation was determined in both B1 and B4 cells. In addition, the ability of 5-HT to induce conditional bursting in the B4 motor neurone was also determined (Straub and Benjamin 2001). Mean burst frequency

was calculated by averaging the number of bursts evoked during the first 100 s after 5-HT application.

Determination of the pharmacology of the CGC → B1 and CGC → B4 synapses

The ability of methysergide (5-$HT_{1/2}$ receptors antagonist; 50 mM), tropisetron (1 μM) and MDL 72222 (10 μM; 5-HT_3 antagonists) and cinanserin (5-HT_2 antagonist; 10 μM) to block a significant portion of monosynaptic CGC → B1 or CGC → B4 connections was examined in isolated CNSs perfused with a ringer high in Ca^{2+} and Mg^{2+} containing 1 mM hexamethonium (Hi-Di/Hex) to ensure we were looking at monosynaptic excitatory serotonergic connections (see Straub *et al.* 2007). Both the CGC and B1 or B4 motor neurones were impaled with microelectrodes and continually perfused with the HiDi/Hex ringer. B1 or B4 were also locally perfused with the HiDi/Hex ringer, or a 100 s application of the serotonergic antagonists. The effectiveness of the antagonists was determined by examining the amplitude of the large compound EPSPs evoked by 2 s bursts of action potentials generated in the CGCs by constant current injection.

Data analysis

The number of bites per 2 min, were compared using a one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test (Minitab Vs 13). Values for the inter-bite interval and bite duration were analysed by calculating the mean values of all the bites evoked by sucrose for each animal. Values were then averaged to yield a group average for the two parameters. Values were statistically compared using a one-way ANOVA. Statistical differences in the levels of 5-HT and 5-HIAA, with respect to both CNS region and the age of the animal, were compared using a two-way ANOVA followed by a *post hoc* Tukey test (Excel). Correlations between changes in the CNS levels of the analytes and alterations in the feeding parameters were determined using a standard regression (Excel). A two-way analysis of variance was used to examine the effects of fluoxetine and serotonin (concentration × age) on the amplitude of the post-synaptic potential recorded in the B1 and B4 motor neurones. All values plotted are the mean ± SEM, $p < 0.05$ taken as significant.

Results

Alterations in short-term feeding behaviour

The effects of increasing age were examined on short-term sucrose-evoked feeding responses. A significant decrease in the number of sucrose-evoked bites per minute was seen with increasing age ($p < 0.001$; Fig. 2b-i). This decrease was associated with an increase in the duration of the inter-bite interval (swallow phase; $p < 0.01$; Fig. 2b-ii). There were no significant changes in the bite duration (protraction/rasp phases) of each feeding cycle, or any other feeding parameter examined.

Changes in CNS serotonergic systems

ANOVA showed no significant changes in the levels of 5-HT with increasing age in either the buccal or cerebral ganglia. However, age-related changes in the levels of 5HIAA showed consistent decreases with increasing age across both CNS regions examined ($F_{(2,\ 65)} = 32.34$, $p < 0.001$). Correlational analyses were used to examine the relationship between each of the feeding parameters and the levels of its metabolite 5-HIAA in both CNS regions examined. Analyses using pooled data from all three age groups demonstrated that the number of sucrose-evoked bites was positively correlated with levels of 5HIAA in the buccal ganglia ($p < 0.01$, Fig 3a) and the cerebral ganglia ($p < 0.05$, Fig 3b). Analysis of the inter-bite interval showed that it was negatively correlated with levels of 5HIAA in the buccal ganglia ($p < 0.05$, Fig 3c).

Correlations performed between the biochemical data and corresponding behavioural data within a particular age group were all non-significant.

Age-related changes in the sensitivity of the CGC → motor neurone connections to fluoxetine

We have previously shown that amplitude of the CGC-evoked EPSP in the B1 protraction phase motor neurone was increased with increasing age (Fig. 6a in Arundell *et al.* 2006). One possible mechanism to increase the amplitude of the-evoked EPSP would be to reduce removal of the released 5-HT from the CGC → B1 synapse via inhibition of the SERT. In order to test this hypothesis, the amplitude and duration of the CGC-evoked EPSP were examined in the presence and absence of fluoxetine. Figure 4a-i shows a CGC-evoked EPSP in a 3–4 months old, B1 protraction phase motor neurone recorded in normal HEPES-buffered ringer. Following the application of 10 nM fluoxetine both the amplitude and the duration of the EPSP increased ($p < 0.01$ for both measures Fig. 4a-ii). Application of 100 nM fluoxetine failed to further enhance the amplitude of the CGC-evoked EPSP but did cause a further significant increase in the duration ($p < 0.01$; Fig 4a-iii). Similar results were obtained for the 6–7 month group (Fig. 4c and d). For both the 3–4 months and 6–7 months old groups the effects of fluoxetine could be completely reversed by washing for 5 min in normal HEPES-buffered saline. Fig. 4b shows the effect of fluoxetine on the amplitude of a CGC-evoked B1 EPSPs in an 11–12 months old animal. Addition of either 10 nM (Fig. 4b-ii) or 100 nM fluoxetine (Fig. 4b-iii) failed to significantly enhance the amplitude or the duration of the evoked EPSP and in a number of cases actually reduced EPSP amplitude, particularly in CNSs perfused with 100 nM fluoxetine. two-way ANOVA (age, concentration) showed that the effects of fluoxetine on the change in amplitude of the CGC-evoked B1 EPSP were age-dependent ($F_{(2,52)} = 9.42$ $p < 0.001$), but showed no significant effect of concentration. *Post hoc* analysis showed that while there was no difference in the response of the young and middle-aged animals to fluoxetine, both these groups were significantly more sensi-

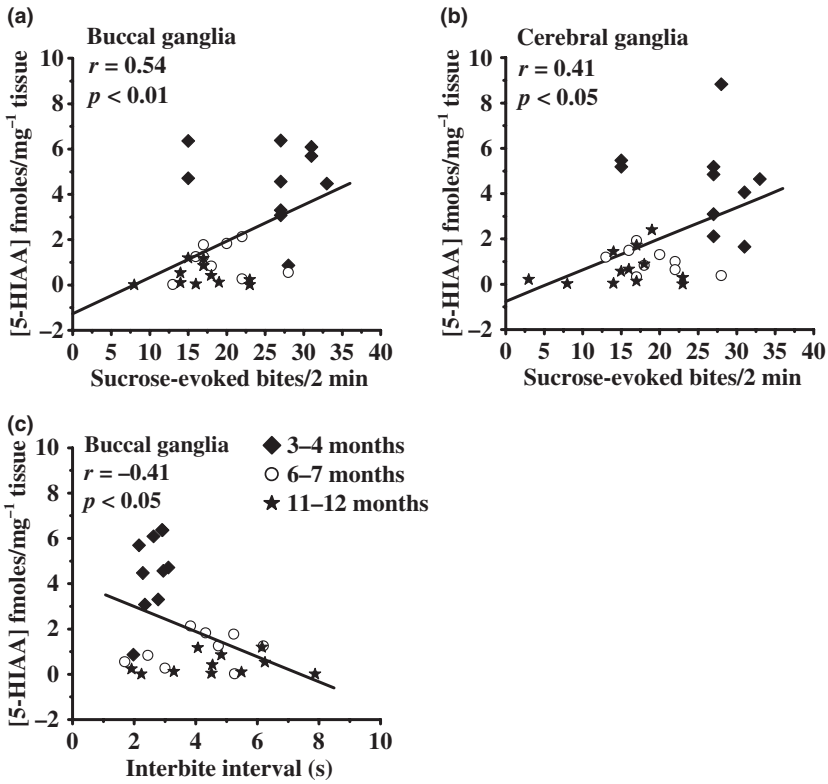

Fig. 3 Relationship between various feeding parameters and CNS levels of 5-HIAA. The number of sucrose-evoked bites was positively correlated with 5-HIAA levels in the buccal (a) and cerebral ganglia (b). The inter-bite interval was negatively correlated with the 5-HIAA content of the buccal ganglia (c). Symbols represent individual animals for each of the three age groups.

tive to fluoxetine than the old group ($p < 0.001$ and $p < 0.05$ respectively; Fig. 4c). Two-way analysis of variance of the EPSP duration data showed a significant concentration effect ($F_{(2,52)} = p < 0.001$), but no age effect. Analysis of the population data demonstrated that fluoxetine was capable of causing a concentration-dependent increase in EPSPs evoked in the young or middle-aged groups. However, in the old group fluoxetine was unable to alter the duration of the evoked EPSP (Fig. 4d). Attempts to record EPSPs in the B4 motor neurone evoked by single CGC action potentials in normal HEPES-buffered ringer were unsuccessful because of the small amplitude of the evoked EPSPs. Previous work has shown that it is possible to visualise CGC → B4 EPSPs using a combination of a saline high in divalent ions (Hi-Di saline) and hexamethonium to block the resulting large inhibitory cholinergic inputs from other pattern generating interneurones (Straub *et al.* 2007). However, we have previously shown that the effects of Hi-Di saline negate age-related changes in the CGC-evoked EPSP amplitude (Patel *et al.* 2006) and this differential effect precluded its use in this study.

Age-related changes in the sensitivity of B1 and B4 to exogenously applied 5-HT

An alternative explanation for the observed increase in CGC → B1 EPSP with increasing age would be an increase in the sensitivity of the motor neurone to 5-HT. In order to test this, motor neurones were chemically isolated from other neurones in the intact but isolated CNS by bathing the preparation in a high Mg^{2+}/zero Ca^{2+} ringers. Successful isolation was confirmed by the disappearance of the classical CPG inputs that are seen in the B1 (protraction phase) motor neurone (see arrows Fig. 5a). Application of 5-HT to the B1 neurone caused a concentration-dependent increase in the amplitude of the evoked depolarisation in both the 3–4 months (Fig. 5b) and 11–12 months old (Fig. 5c) groups ($F_{(2,80)} = 21.75$; $p < 0.001$; Fig. 5b and c). Two-way analysis of variance indicated that while both the 3–4 months and 6–7 months old neurones responded similarly to 5-HT the 11–12 months old neurones responded more strongly to a given concentration of 5-HT ($F_{(2,84)}$ =3.75; $p < 0.05$; Fig. 5b–d).

Intracellular recordings from the B4 (swallow phase) motor neurone in normal HEPES-buffered ringer typically resulted in regular bursts of activity that occur following a series of characteristic inhibitory inputs from both the N1 and N2 interneurones (Fig. 6a-i). Following perfusion with a high Mg^{2+}/zero Ca^{2+} ringer, bursting became irregular and there was a complete loss of N1 and N2 inhibitory inputs (Fig. 6a-ii). The remaining spontaneous depolarisations and consequential burst of action potentials recorded in the B4 motor neurone are an endogenous property of these neurones that is activated by 5-HT (Straub and Benjamin 2001). Application of 5-HT to the B4 motor neurone caused a concentration-dependent increase in the amplitude of the evoked depolarisation in both the 3–4 months (Fig. 6b) and

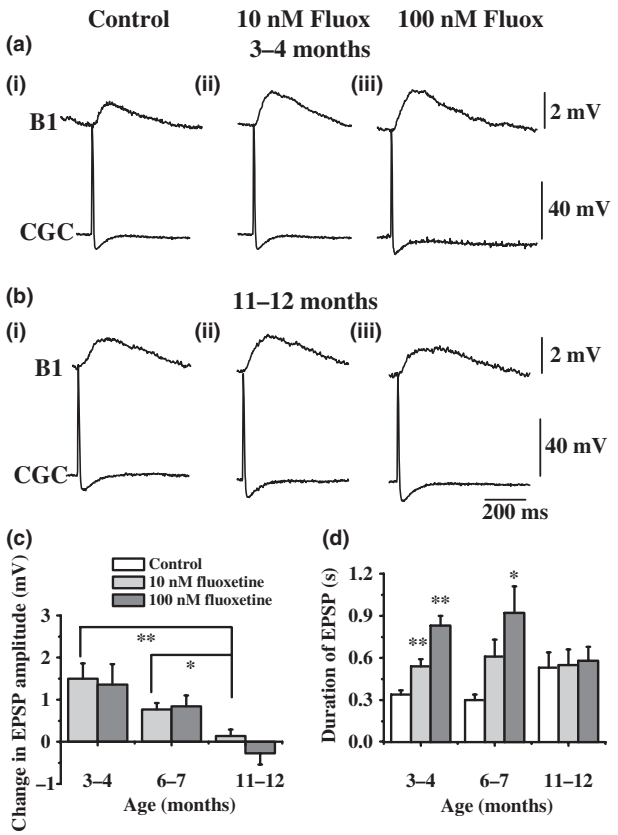

Fig. 4 Fluoxetine sensitivity of the CGC → B1 synapse. Action potentials in the CGCs-evoked unitary EPSPs in the B1 motor neurone (a-i/b-i). In 3–4-month-old CNSs application of either 10 nM flluoxetine (a-ii) or 100 nM fluoxetine (a-iii) increased the amplitude of the EPSP. In 11–12-month-old animals fluoxetine failed to significantly increase EPSP amplitude (bi-iii). (c) Bar graph showing the change in EPSP amplitude from control following fluoxetine application. Fluoxetine significantly increases EPSP amplitude in the 3–4-month and 6–7-month groups but fails to significantly alter EPSP amplitude in the 11–12 month old group. (d) Fluoxetine increases the duration of the CGC-evoked EPSP in both 3–4 month and 6–7-month old groups, but failed to significantly alter EPSP duration in the 11–12-month group. $^*p < 0.05$; $^{**}p < 0.01$, values are the mean ± SEM, $n = 8$ for all groups.

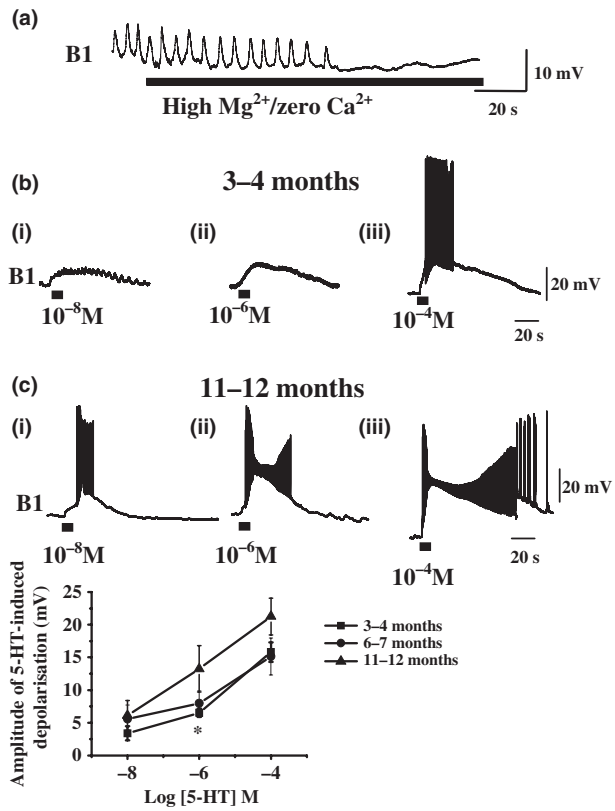

Fig. 5 5-HT sensitivity of the B1 protraction phase motor neurone increases with age. (a) Application of a High Mg^{2+}/zero Ca^{2+} ringer solution to the isolated CNS leads to the chemical isolation of the B1 motor neurone (N.B. the disappearance of spontaneous synaptic inputs). Application of 5-HT to chemically isolated B1 motor neurones from 3–4 month (b) and 11–12-month-old animals (c) causes a concentration-dependent increase in the evoked depolarisation. (d) Graph showing the increase in the amplitude of the 5-HT-evoked depolarisation is greater in 11–12-month-old group compared to either the 3–4 month and 6–7 month groups. $^*p < 0.05$, values are mean ± SEM, $n = 8$ for all points.

11–12 months (Fig. 6c) groups ($F_{(2,88)} = 32.19$; $p < 0.001$; Fig 6b and c). This depolarisation far out-lasted the duration of application of 5-HT (Fig. 6b and c). Unlike the B1 motor neurone, there was a clear decrease in the ability of the B4 motor neurone to respond to 5-HT with increasing age ($F_{(2,80)} = 7.44$; $p < 0.01$; Fig. 6b–d). *Post hoc* analysis showed there to be no difference between the responsiveness of the young and middle-aged groups to applied 5-HT, however, both groups responded significantly better to 5-HT than the old group ($p < 0.001$ and $p < 0.05$ respectively). The 5-HT-induced depolarisation was also accompanied by conditional bursting in the B4 motor neurone (Fig. 7a and b). The bursting again far outlasted the duration of application of 5-HT typically lasting for periods in excess of 100 s, for a 5 s application of 5-HT. With increasing age the bursting frequency in response to a 5 s application of 10^{-6} M 5-HT decreased significantly (compare Figs. 7a-ii and b-ii and see Fig. 7c). A *post hoc* Tukey test showed that bursting in the young age group was significantly different from the old but did not differ significantly from the middle-aged cells (Fig. 7d). This differential effect could be negated by applying higher concentrations of 5-HT (10^{-4} M) to the B4 neurones (data not shown).

Pharmacology of the CGC → B1 and CGC → B4 connection

Previous work has shown that a burst of action potentials in the CGC evoked an excitatory compound potential in the B4 motor neurone, consisting of a mixture of fast and long-

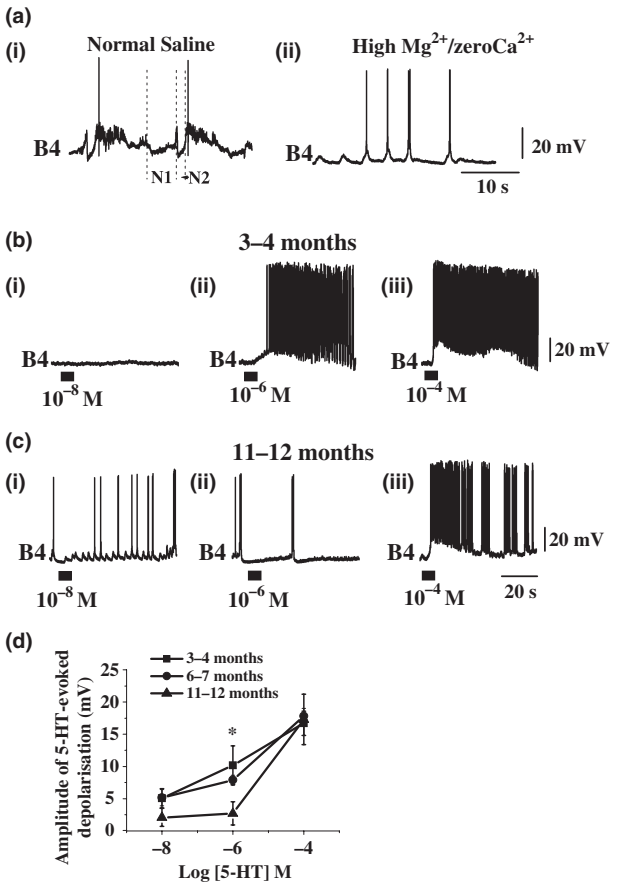

Fig. 6 5-HT sensitivity of the B4 swallow phase motor neurone decreases with age. (a-i) B4 motor neuron recorded in normal HEPES-buffered ringer showing typical chemical inputs from N1 and N2 interneurones. (a-ii) Chemical isolation of B4 with a high Mg^{2+}/zero Ca^{2+} ringer removes rhythmic N1 and N2 inputs leaving smaller irregular bursts that are endogenously generated. Application of 5-HT to chemically isolated B4 motor neurones from 3 to 4 month (b) and 11–12-month-old animals (c) causes a concentration-dependent increase in the evoked depolarisation. N.B. the lack of response of 11–12-month-old cells to both 10^{-8} and 10^{-6} M 5-HT. (d) Graph showing the increase in the amplitude of the 5-HT-evoked depolarisation in 3–4, 6–7 and 11–12-month old animals. *$p < 0.05$, values are mean ± SEM, $n = 8$ for all points.

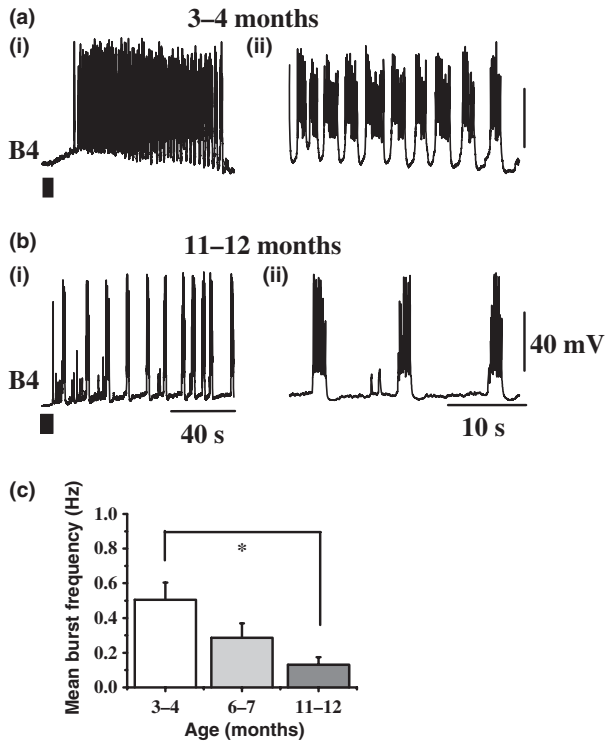

Fig. 7 Increasing age reduces 5-HT-induced bursting in B4 motor neurones. Application of a 5 s pulse of 10^{-6} M 5-HT induced bursting in chemically isolated B4 motor neurones from (a) 3-4 month and (b) 11–12 month animals. A 30 s sample of each of these traces is shown on a faster time base in a-ii and b-ii. (c) Graph showing the decrease in the mean frequency of 5-HT-induced bursts with increasing age (averaged over the first 100 s following 5-HT application). $n = 5$ for each bar. $p < 0.05$.

lasting components both of which could be blocked with 50 mM methysergide a mixed 5-$HT_{1/2}$ receptor antagonist (Fig. 8b; Straub *et al.* 2007). However, application of 50 mM methysergide to the B1 motor neurone failed to significantly reduce the amplitude of CGC-evoked EPSPs (Fig. 8a). Similarly, MDL 7222 and tropisetron, 5-HT_3 antagonists, also failed to significantly reduce the CGC-evoked EPSP in B1 neurones (data not shown). However, application of cinanserin, a 5-HT_2 receptor antagonist, caused a significant reduction in both the amplitude ($p < 0.001$) and duration ($p < 0.001$) of the CGC-evoked B1 EPSP that was not reversed on washing (Fig. 9b and c). Interestingly, cinanserin reduced the duration ($p < 0.001$) of the CGC evoked B4 compound EPSP and the amplitude of the early component ($p < 0.05$; Fig 9a and c). These data suggest that the two synapses utilise different types of 5-HT receptor to transduce the effects of 5-HT.

Discussion

The aim of the current study was to examine whether changes in 5-HT signalling contributed to age-related changes in feeding behaviour in the pond snail, *Lymnaea*.

Age-related changes in feeding behaviour and its relationship to serotonin metabolism

The behavioural data presented in this paper confirms previous work that demonstrated age-related decreases in the number of sucrose-evoked bites and a consistent increase in the duration of the inter-bite interval (Arundell *et al.* 2006). The far less consistent, batch-specific increase in the bite duration was not observed in this study.

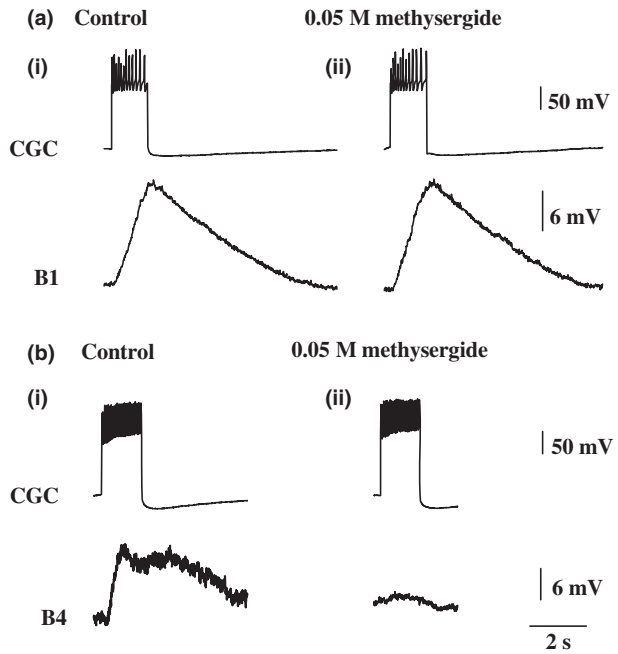

Fig. 8 Methysergide selectively blocks the CGC → B4 connection. Bursts of CGC action potentials evoked compound EPSPs in the B1 (a) and B4 (b) motor neurones. Application of 0.05 M methysergide caused a marked block of the CGC → B4 motor neurone (b-ii) but failed to attenuate the CGC → B1 synapse (a-ii). $N = 6$ for all groups.

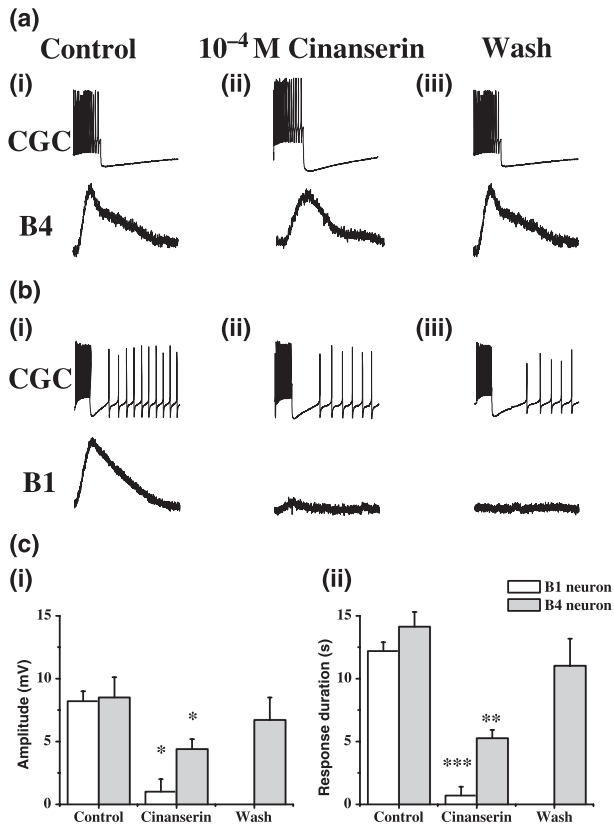

Fig. 9 Cinanserin preferentially blocks the CGC → B1 connection. Bursts of CGC action potentials evoked compound EPSPs in the B4 (swallow phase) motor neurone (a-i) and the B1 (protraction phase) motor neurone (b-i) perfused with a normal HEPES-buffered ringer containing 1 mM hexamethonium to block cholinergic inputs. CGC-evoked biphasic compound EPSP in the B4 swallow phase motor neuron consisted of a fast large amplitude component and a long-lasting, lower amplitude component (a-i). Application of cinanserin predominantly blocked the fast longer-lasting component (a-ii) an effect that was reversed on washing (a-iii). The CGC → B1 connection was completely blocked by cinanserin (b-ii) and could not be reversed by washing (b-iii). Cinanserin blocks the amplitude (c-i) and duration (c-ii) of the CGC → B1 and CGC → B4 connections. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to respective controls; $n = 6$ for all groups.

Neurones in the buccal and cerebral ganglia have been shown to be intimately involved with the regulation of feeding behaviour (for reviews, see Benjamin and Elliott 1989; Elliott and Susswein 2002). Specifically, the CGCs provide the sole serotonergic input to the buccal ganglia meaning that any changes observed in serotonergic metabolism in these ganglia are due to alterations in CGC signalling (Kemenes *et al.* 1989). In both the BG and CG increasing age was associated with a decrease in 5-HIAA levels. 5-HT is primarily metabolised to 5-HIAA by the enzyme monoamine oxidase A (MAO-A) following its release and re-uptake into the nerve terminal. Supply of 5-HT for metabolism could be altered by a number of different mechanisms. Previously, we have shown that CGC firing rate decreases with increasing age, potentially reducing the availability of 5-HT for metabolism (Patel *et al.* 2006). In this paper, we determined whether alterations in re-uptake could also contribute to the observed decrease in 5-HIAA levels in aged CNSs, by examining the amplitude of the evoked CGC → B1 EPSP in the presence of different concentrations of fluoxetine a SERT inhibitor. In the young and middle-aged animals fluoxetine increased the amplitude and duration of the CGC-evoked EPSP consistent with its ability to block SERT. However, in the old animals, fluoxetine failed to significantly alter the amplitude of the EPSP inferring a lack of functional SERT or an age-related change in the sensitivity of SERT to fluoxetine. The ability of fluoxetine to cause a concentration-dependent increase in the duration of the CGC-evoked EPSP is also consistent with its ability to block re-uptake. The lack of a similar concentration-dependent effect on the amplitude of the evoked EPSP suggests that levels of 5-HT in the synapse in the presence of 10 nM fluoxetine are saturating the post-synaptic receptors. While these data are consistent with fluoxetine blocking 5-HT re-uptake, we cannot discount that fluoxetine may have other non-specific effects on the *Lymnaea* CNS that could contribute to the observed changes in EPSP amplitude/

duration. Although we consider this unlikely, further work is required to resolve this issue.

In humans, binding studies have demonstrated a decrease in SERT levels with increasing age (Van Dyck *et al.* 2000; Kakiuchi *et al.* 2001), inferring an age-related impairment of re-uptake. However, we believe this current study is the first to suggest an age-related functional decrease in SERT activity. Surprisingly, in rats increasing age appears to be associated with an increase in SERT (Meister *et al.*1995; Duncan *et al.* 2000; Krajnak *et al.* 2003), indicating species-specific regulation of SERT expression.

Changes in the levels of 5HIAA in the BG were positively correlated with the number of sucrose-evoked bites and negatively correlated with the duration of the inter-bite interval suggesting a link between 5-HT and feeding behaviour. Previous work by Yeoman *et al.* (1994a,b) showed that increases in CGC firing rates above six spikes min^{-1} can regulate the frequency of fictive feeding movements recorded in the isolated CNS preparation and that CGC firing rate was proportional to the frequency of feeding movements in the intact animal. This change in CGC firing rate may therefore provide an explanation for the age-related changes in the frequency of feeding movements. However, the CGCs have the ability to alter the excitability of a wide range of motor neurones that are active in all three phases of the feeding rhythm (McCrohan and Benjamin 1980b) and also have effects on the endogenous properties and excitability of the N1, N2 and N3 CPG interneurones (Yeoman *et al.* 1996). Why therefore, does this study only show significant changes in the duration of the inter-bite interval (N3 phase) and not the bite duration (N1, protraction/ N2, rasp phases)? First, reductions in re-uptake at the CGC → B1 synapse would help to compensate for the age-related decreases in CGC firing rate and may help to preserve the functioning of this synapse and the protraction phase of the feeding cycle. Second, the sensitivity of the B1 protraction phase motor neurone to exogenously applied 5-HT was increased with increasing age further helping to maintain the function of this synapse.

While this study was unable to examine the function of the SERT protein at the CGC → B4 connection it did record a marked age-related decrease in the sensitivity of the B4 (swallow phase) motor neurone to exogenously applied 5-HT. This would decrease the ability of the CGC to depolarise B4, reduce B4 firing frequency and cause an increase the duration of the swallow phase. In addition to the attenuation in 5-HT-induced depolarisation, there was also an age-related decrease in the frequency of 5-HT-induced bursting in B4 cells. Previous work has shown that the ability of 5-HT to cause a prolonged depolarisation and also to induce conditional bursting in the B4 motor neurones are a necessary requirement for these neurones to be activated during a full feeding rhythm (Straub and Benjamin 2001). These data are therefore consistent with an attenuation of the CGC → B4 synapse and an increase in the inter-bite interval and a consequential slowing of the feeding rhythm. The observation that there were no significant age effects in either 5-HT-induced depolarisation or conditional bursting in B4 cells perfused with the highest concentration of 5-HT (10^{-4} M), suggests that the mechanisms involved in regulating these processes are still intact but less sensitive in the old animals.

Differences in the post-synaptic 5-HT receptor complement may explain the selective changes in the feeding rhythm

Examination of the pharmacology of the CGC → B1 and CGC → B4 connections showed some interesting differences. Previous work by Straub *et al.* (2007) showed that the monosynaptic excitatory component of the CGC → B4 connection consisted of two components; a large amplitude fast component and a much smaller amplitude slower component. The authors also showed that methysergide, a mixed 5-$HT_{1/2}$ receptor antagonist, was capable of blocking both the major fast component and the smaller long-lasting component, data that was confirmed in this study. However, in our hands the same concentrations of methysergide were unable to significantly reduce the amplitude of the CGC → B1 connection suggesting that 5-HT is acting on B1 via a methysergide-insensitive receptor. Similarly, attempts to block the effects of the CGC on B1 using two 5-HT_3 receptor antagonists, MDL 72222 and tropisetron were also unsuccessful. However, CGC-evoked B1 responses were completely blocked by cinanserin a selective 5-HT_2 receptor antagonist. Interestingly, at the concentrations used, cinanserin was only capable of blocking the smaller long-lasting component of the CGC → B4 connection, inferring that this component was mediated via 5-HT_2 receptors while the faster component was due to activation of 5-HT_1 receptors. Previous work has cloned two 5-HT receptors in *Lymnaea*, termed 5-HT_{1Lym} (Sugamori *et al.* 1993) and 5-HT_{2Lym} (Gerhardt *et al.* 1996). The 5-HT_{2Lym} receptor has previously been shown to couple to inositol-trisphosphate (IP_3, Gerhardt *et al.* 1996), and while the transduction mechanism of the 5-HT_{1Lym} receptor is not currently known the structure of the receptor is consistent with it being coupled to cyclic adenosine monophosphate (cAMP). The reduced sensitivity of the B4 motor neurone to exogenous 5-HT infers a problem with signalling via the 5-HT_1 receptor and cAMP signalling pathways. Previous work in *Lymnaea* has linked age-related changes in neuronal function to deficits in cAMP signalling (Janse and van der Roest 2001), suggesting that the cAMP signalling pathway maybe a target for age-related changes in the nervous system. Additionally, in higher organisms application of cAMP-specific phosphodiesterase inhibitors to aged rats has been shown to reverse some of the markers of cholinergic dysfunction in the nervous system (Asanuma *et al.* 1993), further supporting this hypothesis.

Related work in invertebrates

Previous work by Klassen *et al.* (1998) has demonstrated that multiple synaptic connections of a single dopaminergic interneurone (RPeD1) change differentially with age, however, the precise reasons for these differences were not determined. Work in a related mollusc, *Aplysia*, demonstrated age-related changes in two pathways involved in regulating the gill of the animal. The first the gill withdrawal pathway was sensitive to the effects of age, becoming weaker with increasing age due to a reduction in facilitation of the motor neurone. The other, the respiratory pathway appeared unaffected by the ageing process (Peretz *et al.* 1984; Peretz and Srivatsan 1992). Peretz and colleagues showed that the decrease in facilitation was directly correlated with an age-related increase in the proportion of motor neurone terminal in contact with the gill muscle and an increase in the width of the synaptic cleft. These latter data point to alterations in the presynaptic terminal affecting age-sensitivity providing the possibility for similar changes in our model system.

Summary

We have shown for the first time that synapse-specific changes in 5-HT signalling contribute to age-related changes in the feeding behaviour of the pond snail, *Lymnaea*. Specifically, we have shown that decreasing levels of 5-HIAA in the BG are well correlated with age-related decreases in feeding behaviour. At a neuronal level these behavioural changes involve decreases in CGC firing rates, compensatory decreases in the functioning of SERT and selective alterations in the sensitivity of the key feeding motor neurones to 5-HT. These changes help maintain the function of protraction phase motor neurones and reduce the function at swallow phase motor neurones and most likely contribute to the effects of age on feeding behaviour. Specifically these changes may infer a problem with signalling through 5-HT_1 receptors and the cAMP signalling cascade.

Acknowledgements

We would like to thank the EPSRC for funding this research and Glaxo-Smith Kline for donating the HPLC system to BAP.

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