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

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Abbreviations:

5-HT, 5, hydroxytryptamine or serotonin

5-HIAA, 5, hydroxyindole acetic acid

MAO, monoamine oxidase

SERT, serotonin transporter

CGCs, Cerebral Giant cells

Abstract

This study utilised the feeding system of the pond snail, *Lymnaea* to examine the contribution that alterations in serotonergic signalling make to age-related changes in motor function. Behavioural experiments demonstrated that age-related decreases frequency of feeding movements were due to a specific increases in the duration of the inter-bite interval (swallow phase), with no change in the bite duration (protraction/rasp phases) of each feeding cycle. The modulatory Cerebral Giant cells (CGCs) provide the sole serotonergic input to feeding neurones in the buccal ganglia of the CNS and are important in allowing the feeding system to respond to a food stimulus. HPLC analysis of the buccal ganglia, demonstrated an age-related decrease in 5-HIAA levels that was positively correlated with the feeding frequency and negatively correlated with the duration of the inter-bite interval. A combination of electrophysiology and antibody labelling experiments were used to examine whether the decrease in 5-HIAA was due to a decrease in 5-HT re-uptake. Antibody labelling experiments demonstrated a qualitative decrease in the levels of the serotonin transporter (SERT) in the buccal ganglia of 11-12 month snails compared to either the 3-4 month or 6-7 month group. Analysis of the monosynaptic connection between the CGC and B1 (protraction phase) motoneurone demonstrated that while fluoxetine (10-100 nM) was capable of increasing the amplitude of the CGC-evoked B1 EPSP in both the young and middle aged group it had no significant effect in the old group. Both lines of evidence suggest a functional attenuation of SERT in the old age group.

Age-related changes in the postsynaptic actions of 5-HT were examined using two key motoneurones that are monosynaptically excited by the CGCs. Application of 5-HT to B1 (protraction phase) motoneurones caused a concentration dependent increase in the amplitude of the evoked depolarisation, which was significantly greater in the old neurones compared to both young or middle aged. Conversely, the amplitude of the 5-HT-induced depolarisation in

the B4 (swallow phase) motoneurone, and the ability of 5-HT to induce conditional bursting in B4 cells were attenuated in the old neurones. The ability of the CGCs and its main transmitter 5-HT to depolarise key feeding motoneurones and also to induce conditional bursting in the B4 motoneurone are both necessary to allow their appropriate activation during feeding. We have previously shown that the CGCs firing rate is decreased with increasing age. The observed changes in sensitivity to 5-HT and the decreases in SERT activity would act to strengthen the CGC→B1 connection and compensate for the decreases in the spontaneous CGC firing allowing the function of this protraction phase motoneurone to be maintained. However, changes in the sensitivity of B4 cells to 5-HT would weaken the CGC→B4 synapse and attenuate the functioning of the swallow phase motoneurone and would slow the feeding rhythm.

Application of the selective serotonin re-uptake inhibitor, fluoxetine (0.1 nmoles – 3 nmoles) to freely moving young animals was capable of mimicking the effects of age by significantly reducing the frequency of feeding and increasing the duration of the inter-bite interval.

In summary these data show that a functional attenuation of SERT and differential changes in sensitivity of key feeding motoneurones to 5-HT contribute to the age-related changes in feeding behaviour.

Running Title: Serotonergic signalling and feeding in ageing *Lymnaea.*

Key words: Serotonin transporter, feeding, neuronal ageing, *Lymnaea*.

1. Introduction

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 (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 motoneurones 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

neurontransmitter (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 neurons (Hernadi et al. 2004; Kyriakides and McCrohan 1989; Yeoman et al. 1994a, b, 1996) that are distinct from the CPG. The paired serotonergic cerebral giant cells (CGCs) are a specific type of modulatory neurone, located in the cerebral ganglion of the CNS (see Fig. 1). The CGCs send their axons down the cerebro-buccal connective to the buccal ganglia, providing the sole serotonergic imput 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, 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. 1994b). The CGCs have their actions via alterations in 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 key motoneurones (B1; protraction and B4; swallow) with which they make monosynaptic connections (McCrohan et al 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 motoneurones. The paper demonstrates a functional attenuation in the serotonin transporter and changes in the sensitivity of key protraction (B1) and swallow (B4) phase motoneurones to exogenously applied 5-HT as the animal's age. These changes strengthen the CGC→B1 synapse, but attenuate the CGC→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 12hr 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.). Animals were kept in groups of up to 600 in large circulating tanks at a stocking density of approximately 1 snail per litre.

Measurement of changes in short-term feeding behaviour

The effects of age on short-term feeding were examined using a method previously described by Staras et al. (1998). Briefly, animals were removed from their home tank and maintained in smaller tanks in copper-free tap water for 7 days with free access to lettuce. 12 hours before the experiment, the lettuce was removed, and the animals starved overnight prior to experimentation. Animals were tested by placing them in a petri dish filled with 90ml of copper-free tap water. The time taken for them to emerge from their shells (both tentacles visible) was recorded after which 5 ml of copper-free tap water was pipetted around the lips of the animal and feeding movements recorded over the next 2 minutes. At the end of the 2 min period 5ml of sucrose (final concentration 0.01M) was added to the dish and feeding movements recorded for a further 2 minutes. 0.01 M sucrose was chosen as previous work by Kemenes et al. (1986) demonstrated that this concentration was capable of evoking feeding responses in 100% of young animals and this stimulus, unlike lettuce was not prone to seasonal variations in quality. The feeding behaviour was recorded using a software package produced by Staras (1998) yielding a typical feeding trace (Fig. 2A). Several feeding parameters could be measured from the trace including the latency to first bite, bite duration,

inter-bite interval and the number of sucrose-evoked bites in 2 minutes. Three age groups were examined, 3-4 month (young), 6-7 month (middle aged) and 11-12 month (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 emoved for HPLC analysis (see below).

In a further series of experiments the effects of different doses of fluoxetine (0.1 nmoles- 3 nmoles) on short-term feeding behaviour were examined. Animals were injected into the haemoceol with either fluoxetine or HEPES-buffered saline 10 minutes prior to the measurement of short-term feeding (see details above).

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 2 regions. These were i) buccal ganglia + lateral and ventral buccal nerves and cerebrobuccal connectives (BG); ii) cerebral ganglia (CG; Fig. 1). Each of these tissue samples was homogenised in 200 µl of ice cold 0.1 M perchloric acid (BDH) and centrifuged at 20000 g at 4 °C. All samples were run within 20 minutes 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). Briefly, samples were injected into a LUNA® ODS 3 µm 150 × 1.0 mm I.D. analytical column with a

4.0 × 2.0 mm I.D. 5 μm guard column (Phenomenex®, Macclesfield, UK). Epilson® LC amperometric detector (Bioanalytical systems, West Lafayette, IN, USA) was used to control detector voltage and record the current. A 6 mm glassy carbon electrode (Unijet, BAS) served as the working electrode and was used with a Ag|AgCl reference electrode and a stainless steel auxiliary block as the counter electrode. The working electrode was set at a potential of +750 mV vs. Ag|AgCl reference electrode. The sensitivity of the detector was maintained at 50 nA full scale deflection. Control and data collection/processing were handled through BAS ChromGraph™ software. The mobile phase, containing 25 mM sodium dihydrogenorthophosphate, 27 μM disodium ethylene-diamine-tetra-acetate (EDTA), 50 mM sodium citrate, 10 mM of diethylamine, 10 mM sodium chloride and 2 mM of decane-sulfonic acid sodium salt was made up in deionized, distilled water and buffered to pH 3.2 using concentrated phosphoric acid. This was then mixed with UV- grade acetonitrile (CHROMSOLV® for HLPC, Riedel de Haën) in a ratio of 82.5: 17.5 v/v and filtered through a 0.20 μm membrane filter and degassed under vacuum after mixing.

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).

Whole mount immunohistochemistry

Lymnaea CNSs were dissected in HEPES-buffered saline, the intact CNS removed and the cerebral-cerebral commisure severed in order to pin the preparation flat in a Sylgard-lined dish. The preparation was then incubated in 0.5% protease (Sigma, type XIV) in

HEPES-buffered saline for 30 minutes at room temperature and subsequently fixed overnight in 1% paraformaldehyde/ 1% acetic acid solution at 4°C. After fixation, the CNSs were washed hourly for 8 hours at 4°C in Supermix containing 50mM Tris (hydroxymethylaminomethane), 150 mM NaCl and 2% (v/v) Triton X-100, and then incubated overnight at 4°C in an anti-serotonin transporter (SERT) primary antibody (Abcam, developed in rabbit) diluted 1 in 600 in Supermix containing 2% Triton X-100. The tissue was then rinsed hourly for 6 hours at 4°C in Supermix without Triton X-100 and then incubated overnight at 4°C in fluorescein-labelled goat anti-rabbit secondary antibody (Sigma) diluted 1 in 50 in Supermix containing Triton X-100. Finally the tissue was rinsed hourly for 4 hours at 4°C in Supermix without Triton X-100 and then mounted in 1% ethylenediamine/ 75% glycerol and viewed under an inverted microscope (Zeiss, Axiovert 25). Levels of fluorescence were compared qualitatively from photomicrographs taken under standard exposure conditions.

Electrophysiology

Previous work had shown that the main age-related changes in feeding behaviour were due to increases the duration of swallow (N3 phase) of the feeding cycle, with occasional, batch specific, changes in the bite duration (N1/N2 phases; Arundell et al. 2006). Closer examination of the bite duration showed that changes were specifically due to increases in the duration of protraction (N1 phase) with no significant change in rasp (N2 phase). Therefore in a series of experiments the strength of the monosynaptic connection between the CGC and either the protraction phase motoneurone (B1) or the swallow phase motoneurone (B4) were examined in the presence and absence of different concentrations of fluoxetine (10 -100 nM; Sigma Chemical Co., U.K.). 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 Co. U.K.) to facilitate electrode impalement. The bath containing the CNS was perfused constantly with HEPES-buffered ringer and drugs (fluoxetine, serotonin, Sigma Chemical Co., U.K.) were added to the motoneurone via a local superfusion pipette. Intracellular recordings were made from both the CGC and the motoneurone using glass micorelectrodes that had resistances ranging from 10-15 MΩ when filled with 4 M potassium acetate. The membrane potential of the motoneurone was held at -70 mV and the CGCs spontaneous firing rate maintained at 0.5Hz by constant current injection. The strength of the CGC $\rightarrow$ B1 and CGC $\rightarrow$ B4 connections were determined by recording the amplitude of the excitatory post-synaptic potentials (EPSPs) that were evoked in the motoneurone 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 $\rightarrow$ motoneurone connection, fluoxetine was applied locally to the motoneurones for 20 s via the superfusion pipette.

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 motoneurone. Serotonin was then applied in a concentration range of 10^{-8} M 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 motoneurone was also determined (Straub and Benjamin, 2001). Mean burst frequency was calculated by averaging the number of bursts evoked during the 1^{st} 100 s after 5-HT application.

Data Analysis

The feeding data were analysed as follows. For emergence, latency to first bite and number of bites per minute, values were taken for each animal within a particular age group, the mean calculated and compared using a 1-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 anlaysed by calculating the mean values of all the bites evoked by sucrose for each animal. These values were then averaged to yield a group average for the two parameters. These values were statistically compared using a 1-way ANOVA (see above). 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 2-way ANOVA (age x CNS region) 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 also used to examine the effects of either fluoxetine or serotonin (concentration x age) on the amplitude of the postsynaptic potential recorded in the B1 and B4 motoneurones. All values plotted are the mean $\pm$ 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. 2Bi). This decrease was associated with an increase in the duration of the inter-bite interval ($p<0.01$; Fig. 2Bii). 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

A 2-way ANOVA of the combined 5-HT data obtained from both the buccal and cerebral ganglia showed a clear age effect ($F_{(2, 65)} = 6.26$; $p<0.01$) with the 6-7 month group having higher 5-HT levels than either the 3-4 month ($p<0.05$) or 11-12 month group ($p<0.01$). Although the increase in 5-HT was apparent in both the buccal and cerebral ganglia samples analysis of changes in either one of the ganglia using a post-hoc Tukey test failed to reach significance (Fig. 3A).

Age-related changes in the levels of 5HIAA showed consistent decreases with increasing age across both CNS regions examined ($F_{(2, 65)} = 32.34$, $p<0.001$). In the BG levels of 5HIAA in the 3-4 month group were significantly higher than the 6-7 month group ($p<0.001$) and the 11-12 month group ($p<0.001$), although there was no significant difference between the 6-7 and 11-12 month groups (Fig. 3B). Values for the cerebral ganglia showed similar changes to those seen in the BG (Fig. 3B).

Examination of the ratio of 5HIAA:5-HT again showed consistent decreases with increasing age across all CNS regions examined ($F_{(2, 65)} = 13.43$, $p<0.0001$). In the buccal ganglia the 5HIAA:5-HT ratio was higher in the young group compared to both the middle

aged ($p<0.001$) and old groups ($p<0.001$, Fig 3C). Although qualitatively similar changes were seen in the cerebral ganglia these changes did not reach significance ($p>0.05$, Fig. 3C).

Correlations between age-related changes in the HPLC data and feeding behaviour

The data from all three age groups was combined and correlational analyses used to examine the relationship between each of the feeding parameters and the levels of 5-HT and its metabolite 5-HIAA in both CNS regions examined. Specifically, the age-related change in the number of sucrose-evoked bites was positively correlated with levels of 5HIAA in the buccal ganglia ($p<0.01$, Fig 4A) and the cerebral ganglia ($p<0.05$, Fig 4B). Analysis of the inter-bite interval showed that it was negatively correlated with levels of 5HIAA in the buccal ganglia ($p<0.05$, Fig 5).

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

Age-related changes in SERT labelling

Analysis of the 3-4 month and 6-7 month old animals showed fluorescent labelling in the buccal ganglia of both age groups. Labelling could be clearly seen in the neuropile, extending across the buccal-buccal commisure and exiting the buccal ganglia via the paired dorsal buccal nerves towards the oesophagus. Fluorescent processes were also seen in the oesophagus that were particularly evident in the 3-4 month CNSs (Fig 6A), less evident in the 6-7 month group (Fig. 6B) and appeared to originate from labelled processes in the dorsal buccal nerve. In the majority of old animals (5 out of 6 preparations) there was no clear labelling in the buccal ganglia or oesophagus (Fig. 6C), although labelling could be seen in other areas of the CNS not involved in the regulation of feeding (Fig. 6D). Occasionally (1 out of 6 preparations), SERT labelling could be seen in the buccal ganglia of an old animal;

although this was noticeably fainter than the labelling seen in either the young or middle-aged groups (Fig. 6E). No labelling was seen in the absence of the primary antibody (Fig. 6F).

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

We have previously shown that amplitude of the CGC evoked EPSP in the B1 protraction phase motoneurone 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 the serotonin transporter (SERT). In order to test this hypothesis, the amplitude of the evoked EPSPs was examined in the presence and absence of fluoxetine. Fig. 7Ai shows a CGC evoked EPSP in a 3-4 month old, B1 protraction phase motoneurone recorded in normal HEPES-buffered ringer. Following the application of 10 nM fluoxetine the amplitude of the EPSP increased ($p<0.01$; Fig. 7Aii). Application of 100 nM fluoxetine failed to further enhance the amplitude of the CGC-evoked EPSP (Fig 7Aiii). Similar results were obtained for the 6-7 month group (data not shown). For both the 3-4 month and 6-7 month old groups the effects of fluoxetine could be completely reversed by washing for 5 minutes in normal HEPES-buffered saline. Fig. 6B shows the effect of fluoxetine on the amplitude of a CGC-evoked B1 EPSPs in an 11-12 month old animal. Addition of either 10nM (Fig. 7Bii) or 100 nM fluoxetine (Fig. 7Biii) failed to significantly enhance the amplitude of the evoked EPSP and in a number of cases actually reduced EPSP amplitude, particularly in CNSs perfused with 100 nM fluoxetine. 2-way ANOVA showed that the effects of fluoxetine on the change in amplitude of the CGC evoked B1 EPSP were age-dependent ($F_{(2,52)} =9.42$ $p< 0.001$.). 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 sensitive to fluoxetine than the old

group ($p<0.001$ and $p<0.05$ respectively), which showed no consistent change in EPSP amplitude with fluoxetine (Fig 7C). Attempts to record EPSPs in the B4 motoneurone evoked by single CGC action potentials in normal HEPES-buffered ringer were unsuccessful due to the small amplitude of the evoked EPSPs. Previous work has shown that it is possible to visualise CGC $\rightarrow$ 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 pers. comm.). However, we have previously shown that the effects of Hi-Di saline are age-dependent (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 $\rightarrow$ B1 EPSP with increasing age would be an increase in the sensitivity of the motoneurone to 5-HT. In order to test this motoneurones 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) motoneurone (see arrows Fig 8A). 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 month (Fig. 8B) and 11-12 month old (Fig. 8C) groups ($F_{(2,80)} = 21.75$; $p<0.001$; Fig. 8B/C). Two-way analysis of variance showed that while both the 3-4 month and 6-7 month old neurones responded similarly to 5-HT the 11-12 month old neurones responded more strongly to a given concentration of 5-HT ($F_{(2,84)} = 3.75$; $p<0.05$; Fig. 8B/C/D). This stronger depolarisation caused the old motoneurones to fire action potentials following the application of all three concentrations of 5-HT, while the young motoneurones only fired action potentials at the highest concentration of 5-HT.

Intracellular recordings from the B4 (swallow phase) motoneurone 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. 9Ai). 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. 9Aii). The spontaneous depolarisations and consequential burst of action potentials recorded in the B4 motoneurone are an endogenous property of these neurones that is activated by 5-HT and has previously been described by Straub and Benjamin (2001). Continued perfusion with high Mg^{2+}/zero Ca^{2+} ringers caused the motoneurones to become silent. Application of 5-HT to the B4 motoneurone caused a concentration-dependent increase in the amplitude of the evoked depolarisation in both the 3-4 month (Fig. 9B) and 11-12 month (Fig. 9C) groups ($F_{(2,88)}$ = 32.19; $p<0.001$; Fig 9B/C). This depolarisation far out-lasted the duration of application of 5-HT (Fig. 9B/C). Unlike the B1 motoneurone, there was a clear decrease in the ability of the B4 motoneurone to respond to 5-HT with increasing age ($F_{(2,80)}$ =7.44; $p<0.01$; Fig. 9B/C/D). Post-hoc analysis showed there to be no difference between the responsiveness of the young and middle aged groups to applied 5-HT but a 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 motoneurones (Fig. 10A/B). The bursting again far outlasted the duration of application of 5-HT typically lasting for periods in excess of 100s, 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. 10Aii and Bii and see Fig. 10C). 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. 10D). This differential effect could be negated by perfusing the B4 cells with 10^{-4} M 5-HT (data not shown).

Effects of fluoxetine on feeding behaviour

Although we have recorded post-synaptic changes in 5-HT sensitivity for two key motoneurones in the feeding system of *Lymnaea*, trying to mimic these changes in the intact animal was not possible. We therefore decided to test whether changes in SERT could account for the observed changes in feeding behaviour seen in aged *Lymnaea*. To test this hypothesis 3-4 month old animals were injected with fluoxetine, a 5-HT transporter antagonist to see if it was possible to mimic the age-related changes in feeding behaviour. These data were also compared with the effects of fluoxetine on the feeding behaviour of 11-12 month old animals. Using a 2-way ANOVA injection of animals with increasing doses of fluoxetine caused a significant dose-dependent decrease in the number of sucrose-evoked bites ($F_{(4,53)}$ =25.9; $p<0.001$; Fig 11A) and an increase in the duration of the inter-bite interval ($F_{(4,53)}$ = 5.61; $p<0.05$; Fig 11B). These changes were seen consistently in both the young and old groups of animals although the ability of the fluoxetine to decrease feeding rate and the increase in the duration of the inter-bite interval were much greater in the 3-4 month group than the 11-12 month group ($F_{(4,53)}$ = 7.7 ; $p<0.01$ and $F_{(4,53)}$ =2.68; $p< 0.05$ respectively; Fig 11A/B). Interestingly, at the highest dose of fluoxetine there was no significant difference between the feeding behaviour of the 3-4 month and 11-12 month old group (Fig 11A/B).

Discussion

The aim of the current study was to examine whether changes in 5-HT signalling could explain the 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 increases in the duration of the inter-bite interval (Arundell et al 2006).

Neurones within the buccal and cerebral ganglia have previously been shown to be intimately involved with the regulation of feeding behaviour (for reviews see Benjamin and Elliott, 1989; Elliott and Suswein 2002). In particular 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 the properties of the paired CGCs (Kemenes et al. 1989). In both the BG and CG increasing age was associated with a decrease in the 5HIAA:5-HT ratio that could be explained by a significant decrease in 5-HIAA levels over the same time course. 5-HT is primarily metabolised to 5-HIAA by the enzyme monoamine oxidase A (MAO-A) following its re-uptake into the nerve terminal after vesicular release. Alterations in serotonin re-uptake via SERT could regulate 5-HT metabolism. Two approaches were taken to test the possibility that SERT was impaired in old animals. The first used immunohistochemical techniques to label the SERT protein in the buccal ganglia of the CNS. Clear labelling was seen in the 3-4 and 6-7 month old animals but was absent in the majority of 11-12 month old animals indicating a reduction in the levels of SERT with increasing age. However, using this method alone it was impossible to ascertain whether the observed reduction in labelling was functionally significant. To test this we examined the amplitude of the evoked CGC→B1 EPSP in the presence of differing concentrations of

fluoxetine a SERT inhibitor. In the young and middle aged animals fluoxetine was capable of increasing the amplitude of the evoked EPSP inferring the presence of functional SERT proteins. However, in the old animals, fluoxetine failed to significantly alter the amplitude of the EPSP suggesting a lack of functional proteins. Previous work by Patel et al. (2006) demonstrated an age related increase in the amplitude of the CGC→B1 connection. The decreases in SERT function detailed in this study would increase synaptic 5-HT and could contribute to the observed increase in CGC-evoked EPSP.

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 describe 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, 2003), indicating that the regulation of SERT expression with increasing age may differ between species.

Decreases in 5-HIAA may also represent decreases in the release of 5-HT. Patel et al (2006) previously demonstrated an age-related decrease in the firing rate of the paired CGCs. This decrease in firing would reduce 5-HT release, the availability of 5-HT for re-uptake and therefore metabolism and could explain the age-related decrease in the 5-HIAA/5-HT ratio in these ganglia. Changes in the levels of 5HIAA in the BG were positively correlated with the number of sucrose-evoked bites per minute and negatively correlated with the duration of the inter-bite interval suggesting a link between CGC firing rate, 5-HT release and feeding behaviour. Previous work by Yeoman et al. (1994a, b) showed that increases in CGC firing rates above 6 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. The fact that cerebral ganglia levels of 5-HIAA were also correlated with the same two feeding parameters may reflect the release of 5-HT from processes of the CGCs which remain within the cerebral ganglia or it maybe reflective of the activity of other serotonergic neurons whose cell bodies and processes also lie within this pair of ganglia, but whose function is unknown (Kemenes et al. 1989). .

The observed reductions in re-uptake would help to compensate for the observed age-related decreases in CGC firing rate and may help to preserve the functioning of the CGC→B1 synapse. However, the CGCs have the ability to alter the excitability of a wide range of motoneurones 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. 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)?

Changes in the postsynaptic 5-HT sensitivity can explain age-related changes in specific phases of the feeding rhythm

While a decrease in SERT activity could explain the observed age-related increase in the amplitude of the CGC-evoked EPSP in the B1, protraction phase motoneurone, it was possible that the motoneurone's sensitivity to exogenously applied 5-HT may also increase with increasing age. Indeed, while application of 5-HT caused a concentration dependent increase in the amplitude of the B1 depolarisation, this increase was age-dependent with older animals being more sensitive to the applied 5-HT than the young or middle-aged groups. Thus despite an age-related decrease in the firing rate of the CGCs (Patel et al. 2006), which would act to decrease 5-HT signalling at this synapse, the CGC→B1 connection attempts to

compensate by decreasing re-uptake and increasing the sensitivity of the B1 motoneurone to 5-HT. These compensatory changes presumably contribute to the lack of a change in the bite duration observed in the behavioural experiments in this group of animals.

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 motoneurone 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 inter-bite interval. 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 motoneurones are a necessary requirement for these neurones to be activated during a 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 down-regulated in the old animals.

Can a down-regulation of SERT explain age-related changes in feeding behaviour?

In order to examine whether an inhibition of SERT function could explain the age-related changes in feeding behaviour, animals were injected with increasing doses of fluoxetine and the effects on feeding behaviour examined. Application of fluoxetine to intact 3-4 month old animals caused a concentration-dependent decrease in the feeding frequency and an increase in the duration of the inter-bite interval, data that were consistent with changes observed during ageing. While these data are suggestive that inhibition of SERT

contributes strongly to the changes in feeding behaviour observed during ageing in *Lymnaea,* it is important to realise that in young animals the inhibition of SERT function is occurring in the presence of relatively fast CGC firing rates that would not be seen in the old animals (Patel et al. 2006). This makes the data difficult to interpret, but at the very least suggests that disruptions in 5-HT signalling can reduce feeding behaviour. Fluoxetine was also capable of decreasing feeding response in the 11-12 month old animals albeit to a lesser extent than the 3-4 month old animals. This reduction is interesting because it appears to contradict the effects of fluoxetine on the CGC→B1 synapses and suggests that functional SERT proteins are present in old animals and can influence feeding. However, antibody labelling experiments in this study were limited toan examination of the CNS and did not examine putative changes in the expression of SERT on serotonergic terminals in the buccal muscles that drive feeding. At the highest concentrations there were no significant differences in the behaviour of the young or old animals suggesting this dose was capable of blocking all functional SERT proteins.

While a down-regulation of SERT may contribute strongly to the differences in feeding behaviour between young and old animals, the electrophysiological data suggest that these changes occur sometime between 7-11 months and therefore can not explain the reduction in 5HIAA:5-HT ratio and the reduction in feeding frequency seen in the 6-7 month compared to the 3-4 month group. It is possible therefore, that these changes are due to reductions in the CGC firing frequency seen in this age group (Arundell et al. 2006) or maybe due to temporal differences in SERT function at synapses distinct from those examined in this study.

What are the possible causes of the changes in serotonergic signalling?

While very little is known about the molecular mechanisms by which 5-HT excites both the B1 and B4 motoneurones there are clear published differences in both the kinetics of these responses and their pharmacology. Specifically, the latency of the CGC→B1 connection is much shorter than the CGC→B4 connection, despite both neurones receiving monosynaptic connections from the CGCs (McCrohan and Benjamin, 1980). In *Helix* these slower responses, recorded in homologous neurones, have been shown to be due to the closing of a K^+ channel (Cottrell 1982), while the faster responses presumably represent either opening of Na^+ or Ca^{2+} channels (Gershenfeld and Paupardin-Tritsch, 1974). Additionally, an examination of the pharmacology of the CGC-motoneurone synapses showed the B1 synapse to be sensitive to d-tubocurarine, while the B4 synapse was insensitive (Tuersley and McCrohan 1989). A fuller understanding of these differences may help explain the differential sensitivity of the CGC→B1 and CGC→B4 synapses to the effects of increasing age.

Regulation of SERT expression in the membrane has been fairly well studied. SERT levels have been shown to be down-regulated by activation of PKC (Qian et al. 1997), a loss of basic fibroblast growth factor (Kubota et al. 2001) and by a loss of oestrogen (McQueen et al. 1999; Lu et al., 2003, Krajnak et al. 2003). *Lymnaea* are hermaphrodites and although egg-laying behaviour has been studied in great detail in *Lymnaea* (ter Maat, 1992), to date there is no known role for oestrogen in this process. However the timing of the down-regulation in SERT (7-11 months) is consistent with an onset of menopause in these animals which has been shown to occur from 8 months of age (Janse et al.1989). In addition, changes in Ca^{2+} homeostasis (eg. Verkhratsky and Toescu, 1998) and a loss of responsiveness to growth factors (e.g. Rylett and Williams, 1994) have been suggested as possible explanations for changes in the ageing CNS and these possibilities are currently being investigated. It is

interesting to note that the loss of SERT-labelling in the buccal ganglia was not accompanied by a similar loss in the main ring ganglia (see Fig. 6D). This is suggestive that regulation of SERT in these two regions of the CNS is either under differential control or is differentially sensitive to the same regulator.

Summary

We have shown for the first time that changes in 5-HT signalling can account for 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 and CG 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 motoneurones to 5-HT. These changes help maintain the function of protraction phase motoneurones and reduce the function at swallow phase motoneurones and most likely contribute to the effects of age on feeding behaviour. Interestingly, at the behavioural level the effects of age could be mimicked by the application of fluoxetine a SERT inhibitor providing the possibility that all the changes described above are linked to an inhibition of SERT.

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Figure Legends

Figure 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) dorsobuccal nerve; (2) laterobuccal nerve; (3) ventrobuccal nerve; (4) cerebrobuccal connective; (5) superior lip nerve; (6) median lip nerve.

Figure 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 (Bi) and inter-bite interval (Bii). Values plotted are the mean $\pm$ SEM $n \geq 9$ for each bar. ** $p<0.01$, *** $p<0.001$.

Figure 3: Age-related changes in CNS levels of 5-HT and 5-HIAA. Bar graphs showing age-related differences in (A) 5-HT; (B) 5-HIAA and (C) 5HIAA: 5-HT ratio in the buccal and cerebral ganglia. Values represent the mean $\pm$ SEM, $n=\geq 9$ for each group. Analysis performed using a 2 way ANOVA and post-hoc Tukey test. **$p<0.01$, *** $p<0.001$.

Figure 4: Relationship between number of sucrose-evoked bites per minute 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). Symbols represent individual animals for each of the 3 age groups.

Figure 5: Relationship between inter-bite interval and CNS levels of 5-HIAA. Correlational analysis of animals in all three age groups showed that inter-bite interval was negatively correlated with the 5-HIAA content of the buccal ganglia. Symbols represent individual animals for each of the 3 age groups.

Figure 6: Whole mount SERT immunohistochemistry in the *Lymnaea* CNS. Anti-SERT labelling is visualised in the buccal ganglia (Bg) as light grey processes extending between the paired Bg and travelling via the dorsal buccal nerves to the oesophagus (Oes) of 3-4 month (A) and 6-7 month old animals (B). In the majority of 11-12 month old animals anti-SERT labelling is absent from the buccal ganglia (C), but can be visualised in other CNS ganglia (D). Occasionally (1 out of 6 preparations), faint anti-SERT labelling can be seen in the buccal ganglia of 11-12 month old animals (E). Controls performed in the absence of the primary antibody failed to show distinctive SERT labelling (F). A, B, C, E, F are x100, D x 50. All scale bars 500 μm.

Figure 7: Fluoxetine sensitivity of the CGC→B1 synapse. Action potentials in the CGCs evoked unitary EPSPs in the B1 motoneurone (Ai/Bi). In 3-4 month old CNSs application of either 10 nM flluoxetine (Aii) or 100 nM fluoxetine (Aiii) increased the amplitude of the EPSP (Bi-iii). In 11-12 month old animals fluoxetine failed to significantly increase EPSP amplitude. C) Bar graph showing the change in EPSP amplitude 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. * $p<0.05$; ** $p<0.01$, values are the mean $\pm$ SEM, n=8 for all groups.

Figure 8: Sensitivity of the B1 protraction phase motoneurone 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 motoneurone (N.B. the disappearance of spontaneous synaptic imputs). Application of 5-HT to chemically isolated B1 motoneurones 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 $\pm$ SEM, n=8 for all points.

Figure 9: Sensitivity of the B4 swallow phase motoneurone decreases with age. Ai) B4 motoneurone recorded in normal HEPES-buffered ringer showing typical chemical inputs from N1 and N2 interneurones. (Aii) 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 by B4. Application of 5-HT to chemically isolated B4 motoneurones from 3-4 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 $\pm$ SEM, n=8 for all points.

Figure 10: Increasing age reduces 5-HT-induced bursting in B4 motoneurones. Application of a 5 sec pulse of 10^{-6}M 5-HT induced bursting in chemically isolated B4 motoneurones

from (A) 3-4 month, (B) 11-12 month animals. A 30 second sample of each of these traces is shown on a faster time base in Aii and Bii. C) Graph showing the decrease in the mean frequency of 5-HT-induced bursts with increasing age (averaged over the 1st 100 s following 5-HT application). n=5 for each bar. $p<0.05$.

Figure 11: Application of fluoxetine can mimic age-related changes in feeding behaviour. Ai) Fluoxetine causes a dose-dependent decrease in the number of sucrose-evoked bites and an increase in the duration of the inter-bite interval (Aii). Values are mean $\pm$ SEM, n=8 for all points, * $p<0.05$, *** $p<0.001$.

Figure 1 Yeoman et al. J. Neurochem

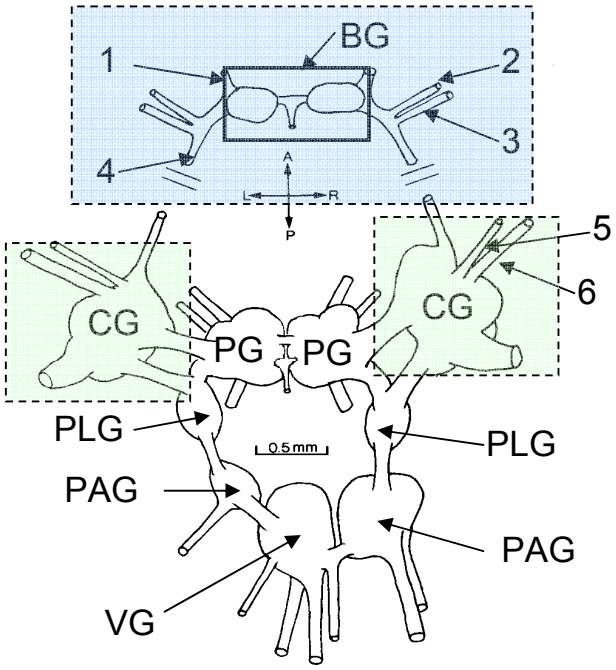
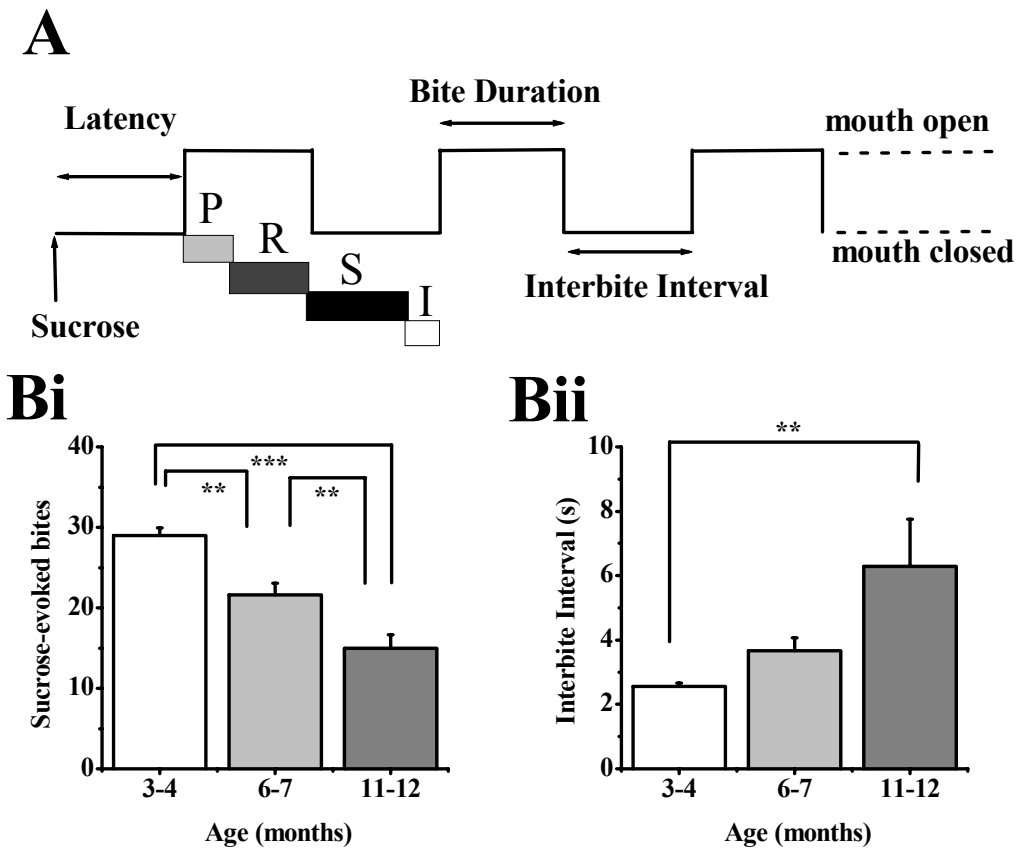


Figure 2 Yeoman et al. J. Neurochem



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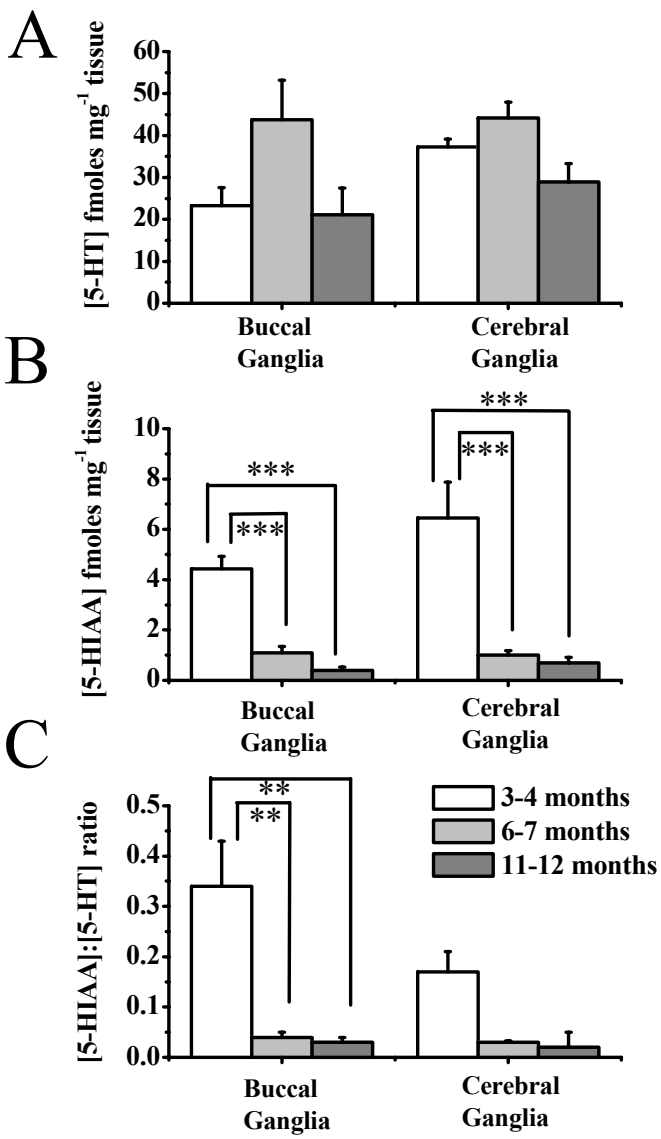

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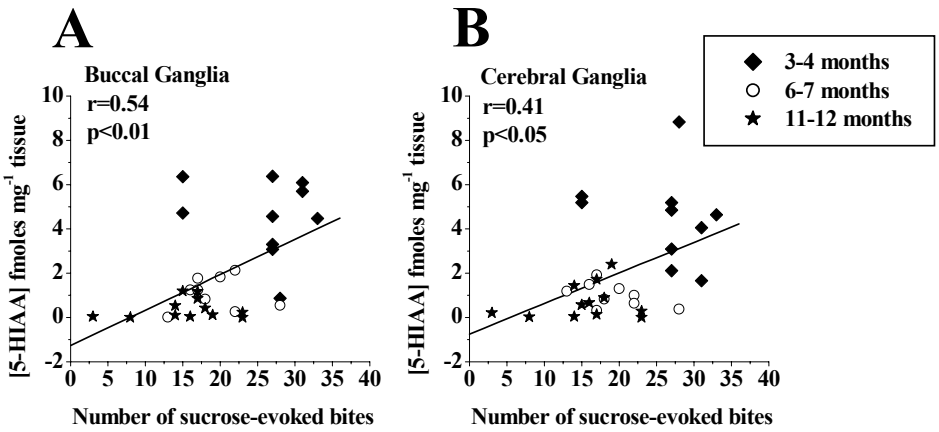

Figure 5 Yeoman et al. J. Neurochem

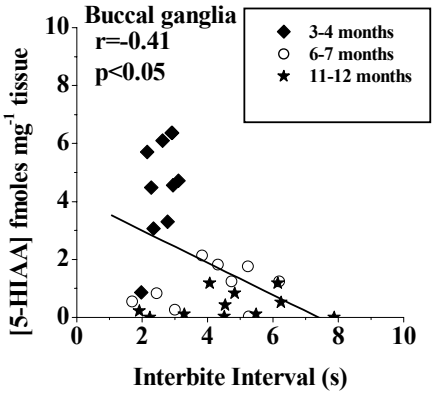

Figure 6 Yeoman et al. J. Neurochem

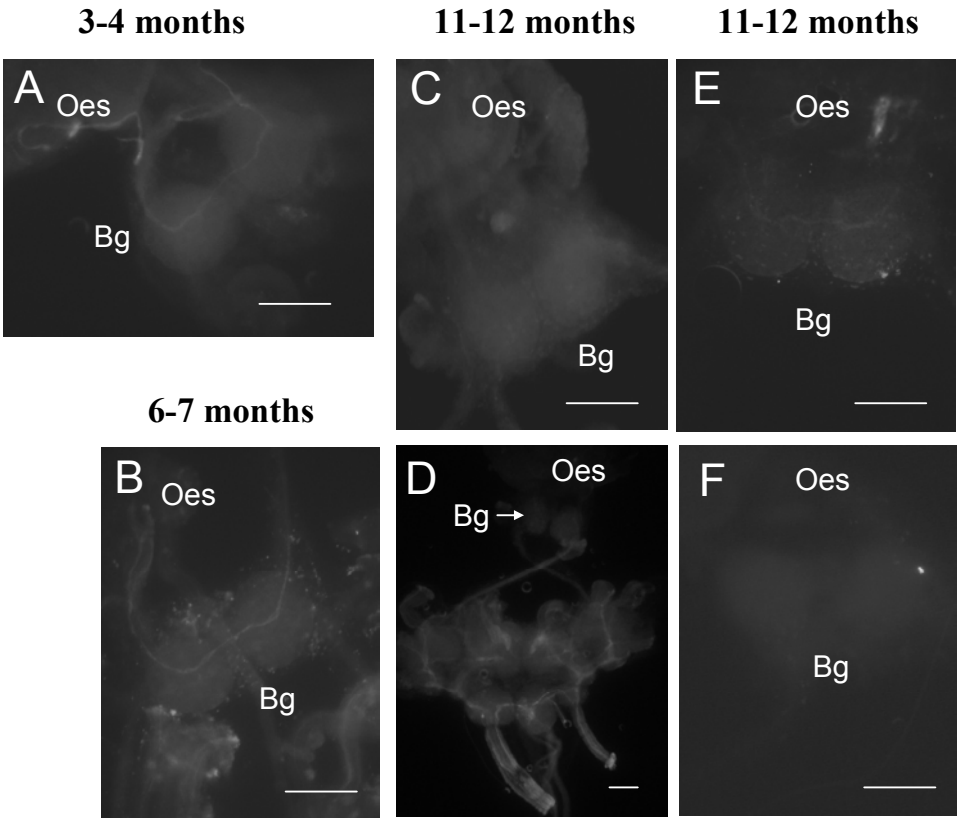

Figure 7 Yeoman et al. J. Neurochem

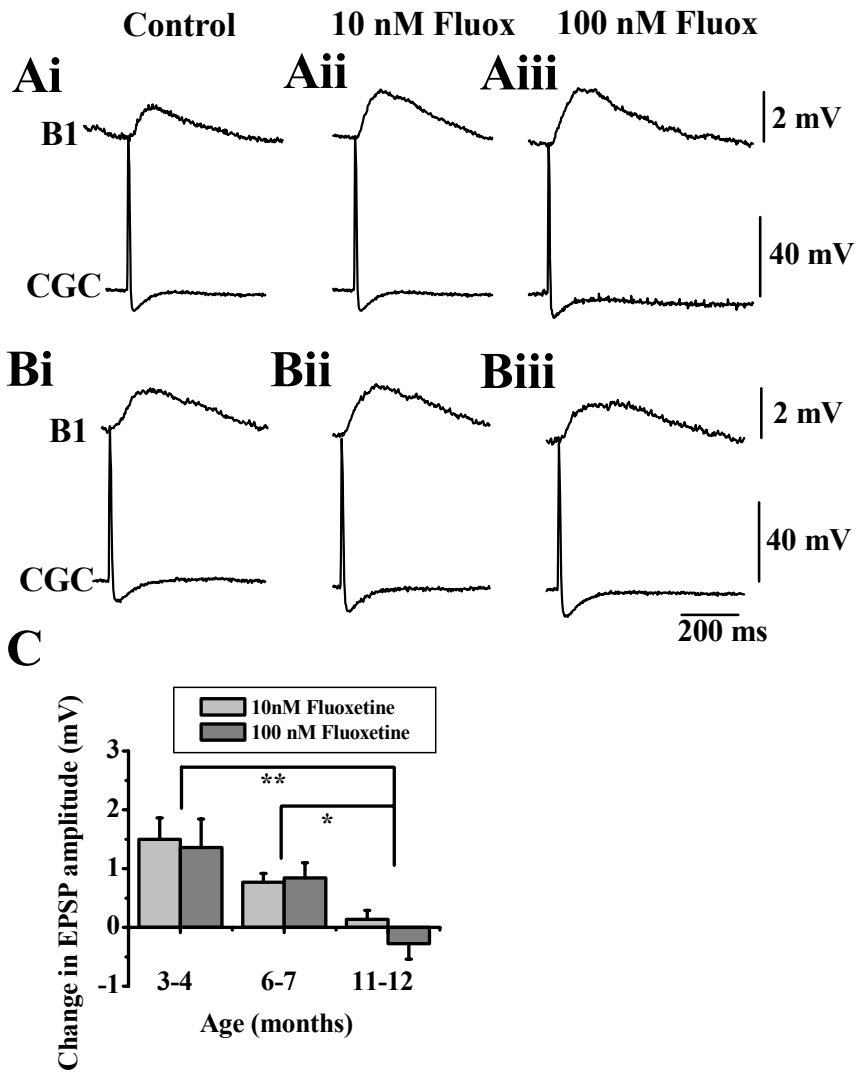

Figure 8 Yeoman et al. J. Neurochem

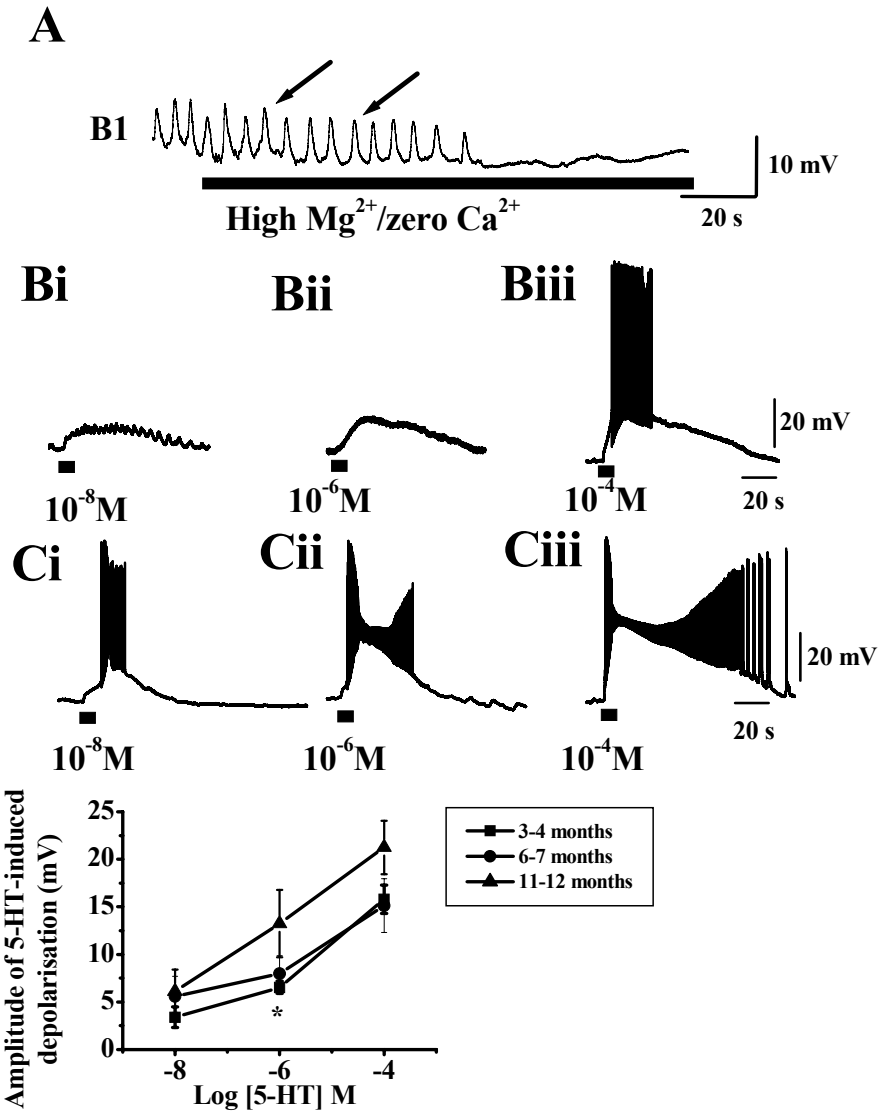

Figure 9 Yeoman et al. J. Neurochem

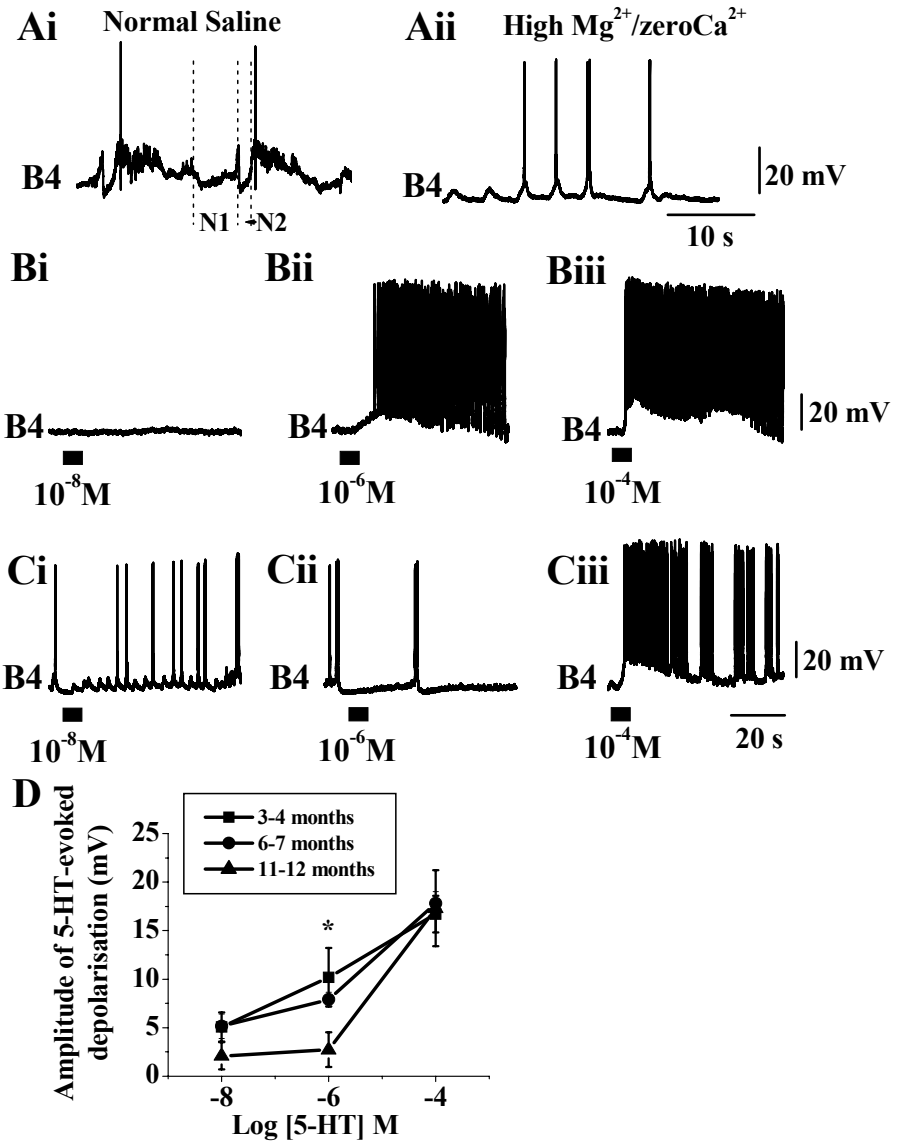

Figure 10 Yeoman et al. J. Neurochem

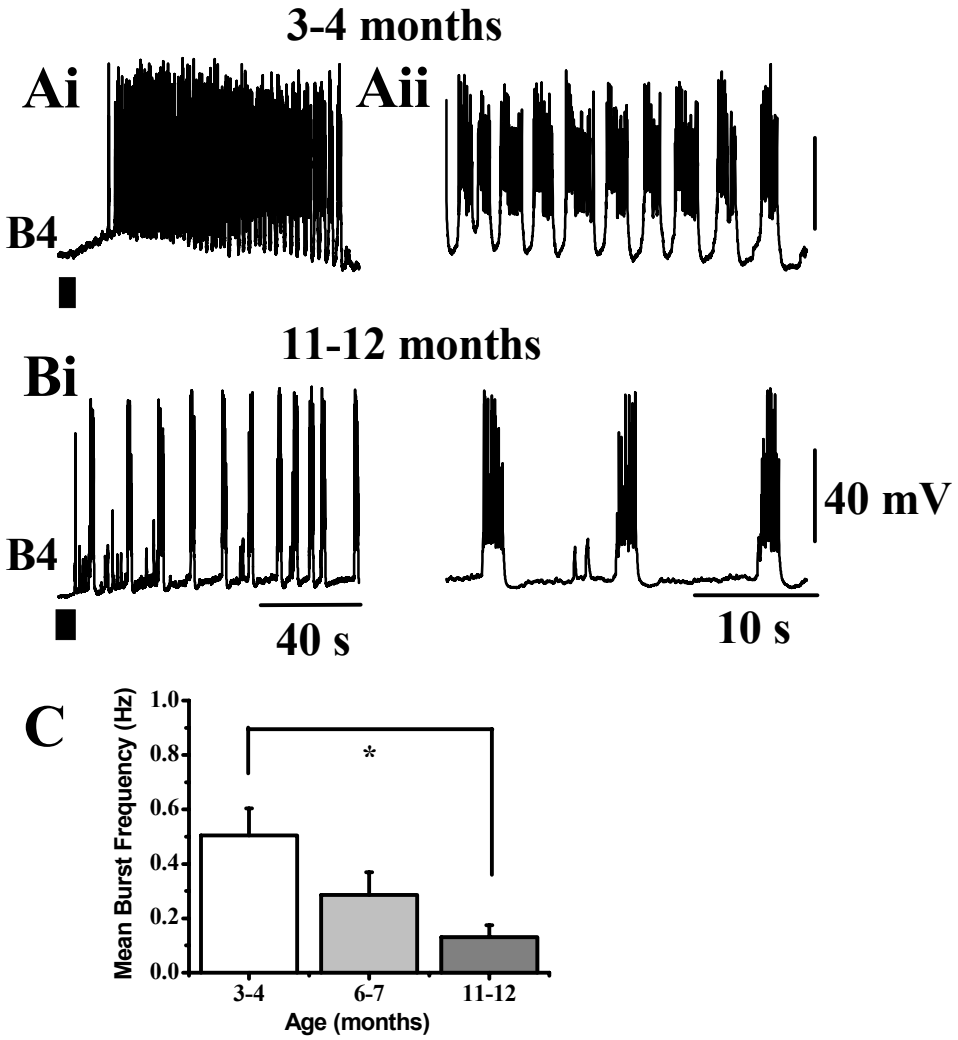

Figure 11 Yeoman et al. J. Neurochem