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RESEARCH REPORT

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Decreased 14-3-3 expression correlates with age-related regional reductions in CNS dopamine and motor function in the pond snail, *Lymnaea*

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Abstract

Ageing is associated in many organisms with a reduction in motor movements. We have previously shown that the rate of feeding movements of the pond snail, *Lymnaea,* decreased with age but the underlying cause is not fully understood. Here, we show that dopamine in the cerebro-buccal complex is an important signalling molecule regulating feeding frequency in *Lymnaea* and that ageing is associated with a decrease in CNS dopamine. A proteomic screen of young and old CNSs highlighted a group of proteins that regulate stress responses. One of the proteins identified was 14-3-3, which can enhance the synthesis of dopamine. We show that the *Lymnaea* 14-3-3 family exists as three distinct isoforms. The expression of the 29 kDa isoform (14-3-3Lym3) in the cerebro-buccal complex decreased with age and correlated with feeding rate. Using a 14-3-3 antagonist (R18) we were able to reduce the synthesis of L-DOPA and dopamine in ex vivo cerebro-buccal complexes. Together these data suggest that an age-related reduction in 14-3-3 can decrease CNS dopamine leading to a consequential reduction in feeding rate.

KEYWORDS

14-3-3, ageing, dopamine, feeding, *Lymnaea*, oxidative stress

1 | INTRODUCTION

The effects of age on the central nervous system (CNS) are to cause a progressive decline in both biochemical and physiological function (Lister & Barnes, 2009). These effects are primarily due to alterations in the efficacy of cell-cell signalling processes, although some neuronal loss may occur in some discrete regions of the brain (Morrison & Hof, 1997; Yeoman et al., 2012). Increasing age is associated with a reduction in the quality and quantity of motor functions. Dopamine (DA) is an important regulator of motor function and changes in DA signalling pathways contribute to age-related changes in motor function in a range of organisms (Maasz et al., 2017; Noda et al., 2020; O'Reilly et al., 1965; Stanford et al., 2003; Teyke

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et al., 1993; Vidal-Gadea & Pierce-Shimomura, 2012; Yamamoto & Seto, 2014). The mechanisms underlying age-related changes in CNS DA signalling are incompletely understood. However, there is a consensus that alterations in the rate of synthesis of DA or alterations in DA uptake and transporter function in a relatively stable neuronal pool may explain the aged behavioural phenotype (Allard & Marcusson, 1989; Bannon et al., 1992; Cruz-Muros et al., 2009; Yurek et al., 1998; Zelnik et al., 1986).

The rate-limiting enzyme in the synthesis of DA is tyrosine hydroxylase (TH). Previous work on rat TH has shown that phosphorylation of TH at a range of serine residues can increase its activity. In vitro studies have shown that phosphorylation of both serines at positions 19 and 40 can increase TH activity by 1.5- to 2-fold (Dunkley et al., 2004; Fitzpatrick, 1999). 14-3-3 and α-synuclein can stabilise the phosphorylated form of TH by preventing access to phosphatases and other kinases, thereby maintaining the increase in TH activity (Kleppe et al., 2001; Perez et al., 2002; Sachs & Vaillancourt, 2004). The effects of age on the regulation of TH activity are incompletely understood. Several observations have shown that the activity of TH is sensitive to oxidative stress, a factor believed to drive ageing in the CNS (Di Giovanni et al., 2012). Others have suggested that an age-related decrease in protein kinase A (PKA) reduces the degree of phosphorylation of TH thereby reducing its activity (Aguilamansilla et al., 1993; Miyajima et al., 2013).

The study of molluscan model systems has contributed to our understanding of the functioning of the CNS and neuronal signalling mechanisms and allowed us to start to understand the causes and consequences of the natural ageing process (Arundell et al., 2006; Fodor et al., 2020; Hermann et al., 2014; Patel et al., 2006, 2010; Pirger et al., 2014; Scutt et al., 2015; Watson et al., 2012, 2014; Yeoman et al., 2008, 2012). We have previously demonstrated that ageing is associated with a decrease in the rate of feeding movements in the pond snail, *Lymnaea* (Arundell et al., 2006). This reduction was linked to alterations in 5-hydroxytryptamine (5-HT) signalling due to changes in the firing frequency of key serotonergic neurones, the cerebral giant cells (CGCs) and alterations in the strength of the connection of the CGCs with key motor neurons in the feeding circuitry (Patel et al., 2006; Yeoman et al., 2008). In molluscs, DA can regulate motor function. It can initiate feeding behaviour and regulate the frequency of feeding movements and fictive feeding cycles in the isolated CNS in both *Lymnaea* (Kemenes et al., 1990; Kyriakides & McCrohan, 1989; Vehovszky et al., 2007) and *Aplysia* (Baxter & Byrne, 2006; Bédécarrats et al., 2013; Elliott & Susswein, 2002). In *Lymnaea*, the CNS localisation of dopaminergic neurons has been well characterised (Elekes et al., 1991; Voronezhskaya et al., 1999). Given the relative simplicity of the *Lymnaea* CNS (<25,000 neurons) and the large size of their neuronal cell bodies, individually identifiable neurons can be reproducibly recorded in different preparations, allowing simple neuronal networks to be characterised and their properties linked to readily quantifiable behaviours, such as feeding (Benjamin et al., 2000; Elliott & Susswein, 2002). DA has been shown to localise to populations of neurons in both the cerebral and buccal ganglia that contain the main neural circuitry for controlling feeding (Elekes et al., 1991). This includes a cluster of neurons on the ventral surface of the cerebral ganglia that are consistent with the neurons in the CV1 cluster (Elekes et al., 1991). These neurons are capable of driving the feeding rhythm (McCrohan, 1984; McCrohan & Kyriakedes, 1989). The CGCs also express D1 receptors (Hernádi et al., 2012), providing a possible mechanism by which DA can influence 5-HT signalling to gate and regulate the frequency of feeding (Yeoman et al., 1994; Yeoman et al., 1994).

In the pond snail, *Lymnaea*, studies have shown that dopaminergic receptor expression in molluscs decreases with age (Chandhoke et al., 2001; Stefano et al., 1982), although currently the effects of age on CNS DA levels and their potential link to age-related reductions in the frequency of feeding have not been examined.

This study explores the contribution that age-related changes in DA make to the previously described reduction in feeding frequency and by utilising a proteomics approach determines a novel age-dependent regulatory mechanism for DA synthesis.

2 | METHODS

2.1 | Animals

Lymnaea stagnalis were bred in-house at the University of Brighton and maintained in groups of up to 600 in copper-free water, at 18–20°C, 12 hr light/dark cycle and fed lettuce or fish flakes (Tetra UK Ltd) ad libitum as described previously (Yeoman, Pieneman, et al., 1994). We examined three age groups of snails; 3–4 months, young; 6–7 months, middle aged; and 10–11 months, old. These age groups were chosen based on criteria outlined in our previous studies (Arundell et al., 2006; Patel et al., 2006). Animals were behaviourally tested to quantify their feeding behaviour (Arundell et al., 2006). Briefly, animals were starved for 24 hr and then placed in a large 15-cm petri dish containing 90 ml of copper-free water. Once the animal emerged from its shell 5 ml of water was applied gently to the lips of the animal and the number of bites the animal made in a 2 min period recorded. Five mL of 0.1 M sucrose (final concentration 0.02 M) was then applied to the lips and the number of bites made in the following 2 min recorded. Feeding rates were calculated by subtracting the number of bites in water from those made in sucrose.

2.2 | Effects of DA on feeding behaviour

To explore the role that DA has on feeding behaviour, animals were injected with either 2×10^{-5} M flupenthixol (D1/D2 DA receptor antagonist, 30 min before experimentation), or 6-hydroxy DA (6-OH DA; a DA neurotoxin, 24 hr prior to experimentation) before examining sucrose-evoked feeding in young snails (Section 2.1). As 6-OH DA works by lowering the DA content of the CNS, we analysed DA concentrations in the CNSs of 6-OH DA treated snails using high-performance liquid chromatography (HPLC).

2.3 | Tissue dissection

CNSs were removed in 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES)-buffered saline (53 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, and adjusted to pH to 7.9 (NaOH), as described previously (Benjamin & Rose, 1979).

2.4 | HPLC determination of monoamines and TH and tryptophan hydroxylase (TpH) activity

The methods for determining CNS concentrations of DA and 5-HT and TH or TpH activity have previously been described (Morgan et al., 2012; Patel et al., 2005). Briefly, CNSs were dissected and divided into two pieces. The first contained the cerebro-buccal ganglia (CB complex) and the second the visceral, parietal, pedal and pleural ganglia (VP complex). We chose to divide the CNS into these two regions as the main neurons responsible for regulating feeding are in the CB complex, allowing the remaining VP complex to act as an internal control. For analysis of 5-HT, DA and its metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), CB and VP complexes were homogenised separately in 250 µl of ice cold 0.1 M perchloric acid, centrifuged, passed through a 0.2 µm filter and stored at −80°C until analysis. To measure TH/TpH activity, we examined the time-dependent conversion of tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA) and tryptophan into 5-hydroxytryptophan (5-HTP). CB and VP complexes were homogenised in a reaction mixture consisting of HEPES enriched with phosphatase and protease inhibitors and 10 µM of tyrosine, 10 µM tryptophan and 50 µM NSD-1015 (L-DOPA/5-HTP decarboxylase inhibitor) before being split into two aliquots. Ice-cold 0.1 M perchloric acid was added to one aliquot to precipitate out protein in the sample, thereby halting the reaction. This sample constituted time T_0. The second sample was left at room temperature for 30 min before the addition of perchloric acid to give the second time point, T_{30}. This allowed each individual CNS to act as its own control. In experiments that tested the ability of the selective 14-3-3 antagonist R18 to reduce TH or TpH activity, 1.1 nM R18 (Tocris Bioscience, UK) was dissolved in water and added to the reaction mix. Immediately after the addition of perchloric acid, samples were centrifuged, filtered and stored at −80°C until required. The supernatant was assayed using HPLC with electrochemical detection and the remaining pellet assayed for protein content. Chromatogram peaks were analysed using Chi instruments software and the concentrations of analytes normalised to protein content.

2.5 | Lucifer Yellow (LY) filling of CV1 neurons and TH immunohistochemistry

The circumoesophageal ganglia were dissected and fixed (4% paraformaldehyde buffered with 0.1 M phosphate buffer (PBS), pH 7.4) for 6 hr at 40°C. They were washed in phosphate-buffered saline overnight at 4°C. The connective tissue sheath surrounding the ganglia was removed with fine forceps and the CNS incubated in phosphate buffered saline-Triton X-100 and bovine serum albumin (PBS-TX-BSA) for 4 hr to block non-specific binding sites. TH immunohistochemistry was carried out on both whole mount preparations and 40-µm-thick cryostat sections applying a monoclonal TH antibody (Immunostar anti-mouse, 1 in 2,000 in PBS-TX-BSA) known to be specific for labelling DA-containing neurons in snails (Hernádi et al., 1993) using the avidin–biotin peroxidase method (Hsu & Raine, 1981). Images were viewed under a light microscope. For experiments examining whether the CV1 cluster are TH positive, CV1 cluster neurons were identified by backfilling the cerebro-buccal connective with LY (1 mg/ml), and locating the fluorescent cluster of neurons which characteristically lies between origins of the lip nerves. CNSs were fixed, labelled and imaged as described above.

2.6 | 14-3-3 immunohistochemistry

CNSs were dissected and fixed overnight at 4°C in 1% paraformaldehyde/1% acetic acid solution made up in PBS. Preparations were dehydrated, embedded in wax and 7 µm sections taken. Following dewaxing sections were rehydrated in PBS and then incubated in blocking reagent (4% goat serum in PBS) for 1 hr. Sections were then incubated in either the isoform-specific 14-3-3ε (SC-102) antibody or the 14-3-3β antibody which detects all vertebrate 14-3-3 isoforms (SC-629; Santa Cruz Biotechnology). Both antibodies were diluted 1 in 1,000 in blocking reagent overnight at 4°C. Sections were washed three times with PBS and then incubated with the secondary antibody (goat anti-rabbit alkaline phosphatase

conjugate 1 in 50 in PBS; Sigma Aldrich A3687) for 1 hr at room temperature. 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) reagent (Sigma Aldrich) was then added to the sections and the coloured end product allowed to develop. Sections were then viewed under a light microscope.

2.7 | Analysis of age-related changes in CNS protein expression

A total of 80 young and 80 old animals were behaviourally characterised, and the CB complex removed. CB complexes from 20 different animals were dried, pooled and snap frozen in liquid nitrogen and stored at −80°C until required, yielding four young and four old samples. Two-dimensional (2D-PAGE) gel electrophoresis was carried out according to previously described methods (Perlson et al., 2004).

2.8 | Two dimensional difference gel electrophoresis (2D DIGE) experimental design

A minimal labelling Cydye™ kit obtained from Amersham Biosciences identified differentially expressed proteins within our two sample sets. Fifty μg of total protein was labelled with dyes dissolved in dimethylformamide (DMF; 400 pmol/μl) for 30 min in the dark and the reaction stopped by the addition of 1 μl of 10 mM lysine. The Cy2 fluorophore acted as an internal pooled standard containing a 50:50 mixture of young and aged samples. The Cy3 and Cy5 dyes were used to label the young and old CB complex homogenates respectively. Cy3 and Cy5 dyes were cross-labelled to reduce intra-gel variability and false-positive matches during DeCyder analyses. We analysed four biological replicates per age group. Samples were run twice as a technical replicate.

2.9 | Digitisation and analysis of 2D DIGE image

The resulting DIGE gels were digitised using a Typhoon Scanner (GE Healthcare life sciences). Protein expression and statistical analyses were performed within DeCyder 6.0 (GE Healthcare Life Sciences). Density of protein spots was considered altered if they showed >10% change.

2.10 | Tandem mass spectrometry (MS/MS)

Selected 2D gel plugs were excised and subjected to tryptic digestion (Shevchenko et al., 2007). Tryptic peptides were analysed on a Thermo Scientific LTQ Orbitrap hybrid Fourier Transform mass spectrometer (FT-MS). Samples were fractionated on a reverse phase column (PepMap 100 C18, Dionex) using an ultimate nano-LC system (Dionex). The tryptic peptide fragments were separated using a linear gradient of 100% solvent A (water, acetonitrile, formic acid; 97.9:2.0:0.1 vol:vol) and 0% solvent B (acetonitrile, water, formic acid; 90:9.9:0.1 vol:vol) to 60% Solvent B and 40% Solvent A at a flow rate of 350 nl/min and introduced directly into the FT-MS for analysis. Data were collected in the Orbitrap FT detector by sequential acquisitions of one full MS scan followed by MS/MS spectra of the top five most abundant precursor ions/scan (*m/z* range for both: 350–1,800) over the duration of the eluting gradient. Following the use of Sequest v5 to identify the putative peptide sequences from each mass spectrum, peptides were then characterised by de novo sequencing using PEAKS Studio software v 4.5 (Bioinformatics Solutions Inc.). The following parameters were used for de novo sequencing: parent and fragment mass error tolerance: 10 ppm and 0.1 Da, respectively; enzyme: trypsin; variable modifications assumed: carboxyamidomethylation (Cys), deamidation (Asn and Gln) and oxidation (Met). For each MS/MS spectrum derived, the de novo sequences were submitted to BLAST homology search (Altschul et al., 1997) against the NCBI non-redundant database for identification.

2.11 | Identifying 14-3-3-like expressed sequence tag sequences from the cDNA library

A sequenced cDNA library (Feng et al., 2009) was searched for putative 14-3-3 sequences and these sequences were translated into their likely protein sequences using CLC sequence viewer (v6.3). The protein sequences were uploaded to the ExPASy proteomics server, maintained by the Swiss Institute of Bioinformatics (SBI; Gasteiger et al., 2003) and were analysed using the ProtParam tool to determine the physicochemical properties of the identified sequences. To determine the origin of the 14-3-3 sequences, the *Lymnaea* protein constructs were compared to established database entries using a combination of the NCBI tBLASTx and BLASTx tools (Altschul et al., 1997). The sequences were transferred to the CLC sequence viewer (v6.3) and expressed sequence tags (ESTs) and nucleotides were translated into peptides. Where translated ESTs, translated nucleotides and proteins within a species were found to be identical, the verified protein sequence was saved and the other two dropped from the analysis. The likely evolutionary history of each of the 14-3-3 proteins was estimated using computational phylogenetics, culminating in the construction of a phylogenetic tree.

2.12 | Western Blot analysis

Briefly, CNSs were removed from *Lymnaea* in ice-cold HEPES-buffered saline and dissected into the CB and VP complexes. Samples (10 μg of total protein) were boiled for 5 min in denaturing sodium dodecyl sulphate (SDS) loading buffer, separated on 12% 7.5 cm polyacrylamide SDS gels (Protean II, Biorad, UK). Gels were transferred to a polyvinylidene fluoride (PVDF) membrane and the membranes blocked for 1 hr in PBS with 0.1% Tween 20 and 10% skimmed milk powder at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. The antibodies used were anti-14-3-3β (SC-629; Santa Cruz Biotechnology) which can detect all 14-3-3 isoforms, anti-14-3-3ε (SC-1020; Santa Cruz Biotechnology), which is selective for the ε isoform. Both 14-3-3 antibodies were diluted 1 in 2,000 in PBS + 0.1% tween-20 v/v (PBS-T). The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; SC-25778) which acted as our housekeeping gene was diluted 1 in 500 in PBS-T. All antibodies were sourced from Santa Cruz Biotechnology. Antibody labelling was visualised using a horseradish peroxidase-conjugated mouse-anti-rabbit secondary antibody diluted 1:4,000 (Dako). Membrane blots were detected using ECL™ plus kit (GE Healthcare) and exposed on Amersham Hyperfilm™ ECL, as per manufacturer's instruction. The developed immuno-reactive bands were scanned and digitised into ImageQuant software (GE Healthcare) for densitometric analysis. Proteins of interest were normalised to GAPDH.

2.13 | Statistical analysis of data

The effects of flupenthixol and 6-OH DA on feeding rate and the effects of 6-OH DA on CB complex DA were analysed using an unpaired *t* test with Welch's correction. Age-related changes in the numbers of water or sucrose evoked bites were analysed using a one-way ANOVA followed by a post hoc Tukey test. Comparisons of CNS DA levels and the HVA + DOPAC:DA ratio in the different brain regions with age were tested for normality and then analysed using a two-way ANOVA followed by a post hoc Tukey test. Correlations between feeding rates and CNS analyte concentrations were performed using a Pearson's correlation. Age-related differences in expression spots were determined using a Mann–Whitney *t* test assuming unequal variance. Age-related differences in protein expression obtained using Western blots for three sets of data (young, middle aged and old) were compared using a Kruskal–Wallis one-way ANOVA followed by a Dunn's multiple-comparison test. Those Western experiments comparing two sets of data were compared using a Mann–Whitney test. Correlations between feeding rates and 14-3-3 expression were performed using a Spearman correlation. The normalised HPLC responses at T_0 and T_{30} and between T_0 and T_{30} in the presence of R18 were compared using a Wilcoxon signed-rank test. $p < 0.05$ was taken as significant, all values are mean ± *SEM*. All data were analysed using GraphPad Prism Vs 6. For those data analysed using a one- or two-way ANOVA, the data presented in the text are the *p*-value for the ANOVA with post hoc statistical analysis presented in the figures. Individual *p*-values are presented as equalities and to four decimal places except where the *p*-value is less than 0.0001 where $p < 0.0001$ is reported.

3 | RESULTS

3.1 | DA regulates feeding rate in Lymnaea

To explore the role that altered dopaminergic signalling has on feeding behaviour, *Lymnaea* were injected with either the mixed DA receptor antagonist flupenthixol or the DA neurotoxin 6, OH DA. Both flupenthixol and 6, OH DA reduced the number of sucrose evoked bites in young snails ($p = 0.029$ and $p = 0.0002$, respectively; Figure 1a,bi). Analysis of CB complex DA expression showed a significant decrease following 6, OH DA injection consistent with it being a DA neurotoxin ($p = 0.041$; Figure 1bii).

3.2 | CNS DA decreases with age and correlates with reduced feeding rates

Increasing age reduced both the numbers of water ($p = 0.0151$) and sucrose evoked bites ($p = 0.0005$) and the feeding rate ($p = 0.0130$; Figure 2ai). A two-way ANOVA showed a significant effect of age on CNS DA concentrations ($F_{2,44} = 98.78$; $p < 0.0001$) and a significant interaction ($F_{2,44} = 9.864$; $p = 0.0003$; Figure 2aii). DA concentrations in the CB complex were positively correlated with feeding rates ($p = 0.0010$; Figure 2aiii), but there was no correlation between VP DA levels and feeding rates ($p = 0.1395$; Figure 2aiv). DA turnover (HVA + DOPAC:DA ratio) increased significantly in both the CB and VP complexes with age ($p < 0.0001$ and $p = 0.0003$, respectively; Figure 2av), although these changes did not show a significant correlation with feeding rate ($p = 0.1735$; Figure 2avi).

Increases in DA turnover have been shown to be a compensatory mechanism designed to maintain dopaminergic function and have been observed to immediately precede neurodegeneration (Barrio et al., 1990; Rodriguez & Castro, 1991; Sossi et al., 2002). We therefore examined whether the observed increase in DA turnover reflected dopaminergic neuronal degeneration. TH immunohistochemistry performed on both whole mount and frozen sections

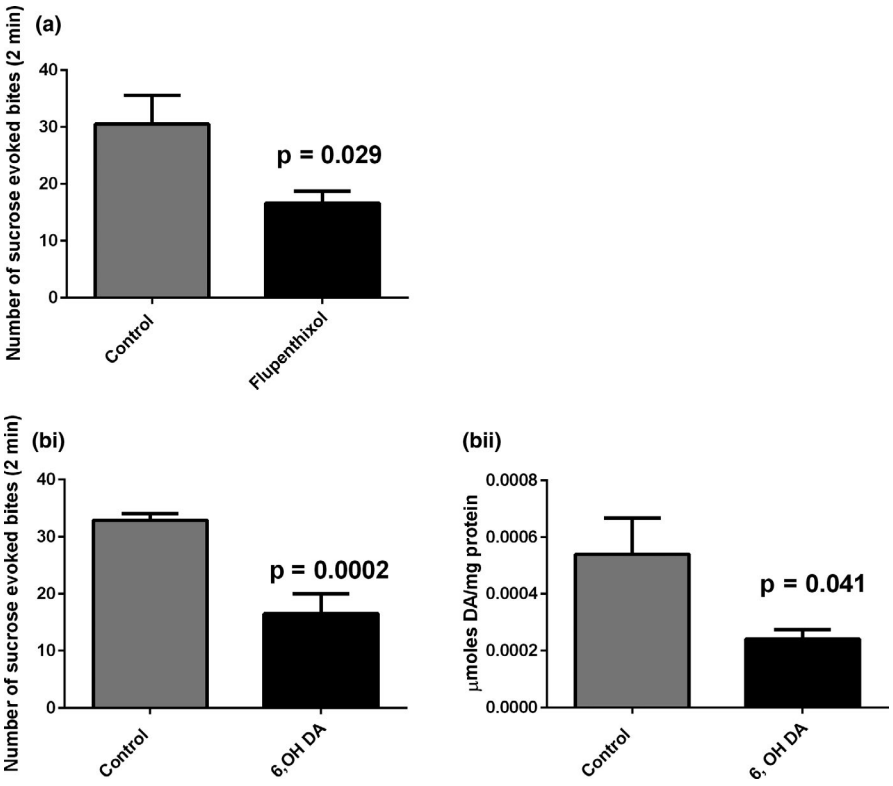


FIGURE 1 Dopamine regulates feeding frequency in Lymnaea. (a) The mixed D1/D2 receptor antagonist, flupenthixol reduced sucrose-evoked feeding rate. (bi) The dopaminergic neurotoxin 6-OH DA reduced sucrose-evoked feeding rate. (bii) The dopaminergic neurotoxin 6-OH DA reduced DA expression in the cerebro-buccal complex. $N = 6$ for all groups

of CNS tissue demonstrated a positively labelled group of 5–6 cell bodies and their axons in the cerebral ganglia of all age groups (Figure 3ai–iii). These neurons belong to the group of cerebro-buccal interneurons known as the CV cells (McCrohan & Croll, 1997) whose neurons evoke robust effects on the buccal feeding network (Figure S1; Kemenes et al., 2001; McCrohan & Croll, 1997). The visually identifiable giant DA cell RPeD1 in the right pedal ganglia was also visible in all age groups along with its axon (Figure 3bi–iii). The positive TH labelling in the soma and primary axons of both the CV neurons and the RPeD1 cell strongly suggest that neurodegeneration is not a major cause of the loss of DA with age (Figure 3a,b), although we cannot exclude that more distant terminals of the labelled neurons are not lost with age.

3.3 | Age-related changes in protein expression

To explore the potential causes of the age-related decrease in DA, we examined the changes in protein expression between young and old snails by only accepting young snails with bite rates between 25 and 35 bites per 2 min and old snails between 12 and 17 bites per minute to ensure our old animals were also biologically old. Following application of sucrose young animals averaged 32.14 ± 0.58 bites per 2 min, while the old group averaged 14.2 ± 0.97 bites per 2 min ($p < 0.0001$) consistent with previous studies (Arundell et al., 2006).

Multi-gel analysis of the CB complexes from young and old CNSs, performed using DeCyder 6.0 software, enabled detection of 1,834 proteins with an assignable protein-spot boundary. A total of 47 protein spots were identified as significantly different between the two groups (15 up-regulated and 32 down-regulated). Only 30/47 protein spots were visualised using either Coomassie Blue stain or Silver stain. Analysis of the 30 different protein spots using MS/MS followed by de novo sequencing identified 26/30 proteins as highly abundant proteins, showing a high degree of conservation to protein sequences already in the non-redundant database. A total of 17 of the 26 proteins were independent proteins. The remaining 9 of the 26 proteins were possible modifications of the proteins identified, and were therefore identified more than once, or were proteins for which no assignable homology was available.

From the 17 proteins that showed a significant change in their expression, the majority were categorised as either stress response proteins or cytoskeletal proteins. Stress response proteins included several hits for 14-3-3 proteins which were down-regulated with increasing age. Glutathione-S-transferase (GST), ribosomal protein SA (RPSA) and heat shock protein 90 (HSP90) were all significantly up-regulated. The cytoskeletal proteins retrograde binding protein 51 (RGBP51), an intermediate filament protein and alpha tubulin (α-Tub) were up-regulated while tropomyosin-2 (TM-2), beta actin (β-actin) and cytoskeletal actin (cyt-actin) were all significantly down-regulated. A total of 15 of the 17 proteins are shown in Table 1. Two of the proteins, namely, lysozyme (gil2781273)

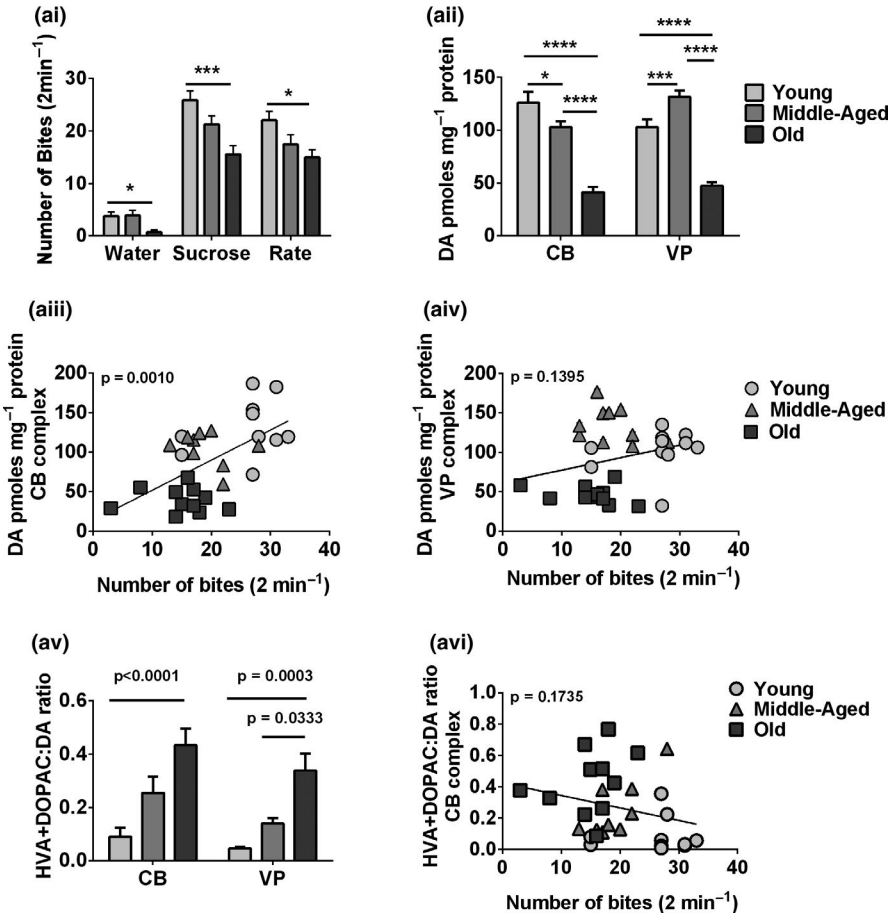


FIGURE 2 Effects of age on CNS dopamine and its link to sucrose-evoked feeding rate. (ai) Effects of age on water and sucrose evoked bites and feeding rate. (aii) CNS DA decreases with increasing age. (aiii) Positive correlation between CB complex DA and feeding rate. (aiv) No correlation is observed between feeding rate and VP complex DA. (av) Age-related increase in DA turnover in both the CB and VP complexes. (avi) Lack of a significant correlation between CNS DA turnover and feeding rate. $n = 10$ (young), $n = 9$ (middle-aged) and $n = 11$ (old)

which was increased 1.34-fold in the old, and Leishmania major strain Friedlin hypothetical protein (gi|72546785) which was decreased 1.33-fold in the old have been omitted from the table due to poor confidence in their identification.

3.4 | Characterisation of 14-3-3 isoforms

Given that 14-3-3 proteins have previously been shown to regulate the activity of TH and therefore the synthesis of DA (Ichimura et al., 1987), we characterised the family of 14-3-3 proteins in *Lymnaea* and examined how their expression was altered with increasing age. Most species examined to date have multiple 14-3-3 isoforms (Aitken et al., 1995). By screening a sequenced cDNA library, we identified 17 EST sequences with homology to 14-3-3 in the *Lymnaea* CNS. The first five clones translated to give the same predicted protein, highly similar to the human 14-3-3ε isoform (14-3-3Lym1). The next four sequences all identified a second 14-3-3-like protein with high similarity to the *C. elegans* 14-3-3-like protein 2 (14-3-3Lym2). The next seven sequences aligned best with *G. gallus* 14-3-3 protein β/α (14-3-3Lym3). However, four of the seven sequences diverged strongly in the C-terminus as a result of a base insertion/deletion, causing a frame shift in the reading of the remaining sequence (14-3-3Lym3var). The final clone sequence showed weak similarity to the 14-3-3 protein β/α-1 of *Salmo salar* (14-3-3Lym4; Table 2).

Figure 4a shows an alignment of the *Lymnaea* 14-3-3-like isoforms, illustrating homology in the five core regions exhibited by all 14-3-3 family members across evolution (thick black bars) but also illustrating the extension of the 14-3-3Lym4 sequence between 280 and 300 amino acids, and the extensive C terminus. We determined the physicochemical properties of the *Lymnaea* 14-3-3-like proteins to see how similar they were to those from other species. All predicted proteins were acidic with a pI of 4. 5.5 and are approximately 30 kDa in mass (Figure 4b), except for the clone 14-3-3Lym4 which had a molecular weight of 38 kDa and a pI of 9.5.

Using a neighbour-joining algorithm, we demonstrated the likely evolutionary relationships of the *Lymnaea* 14-3-3-like proteins with other species (Figure S2).

3.5 | Changes in 14-3-3 isoform expression with age and their correlation with feeding rates

Western blot analysis with the 14-3-3β antibody detected two distinct bands with approximate molecular weights of 29 and

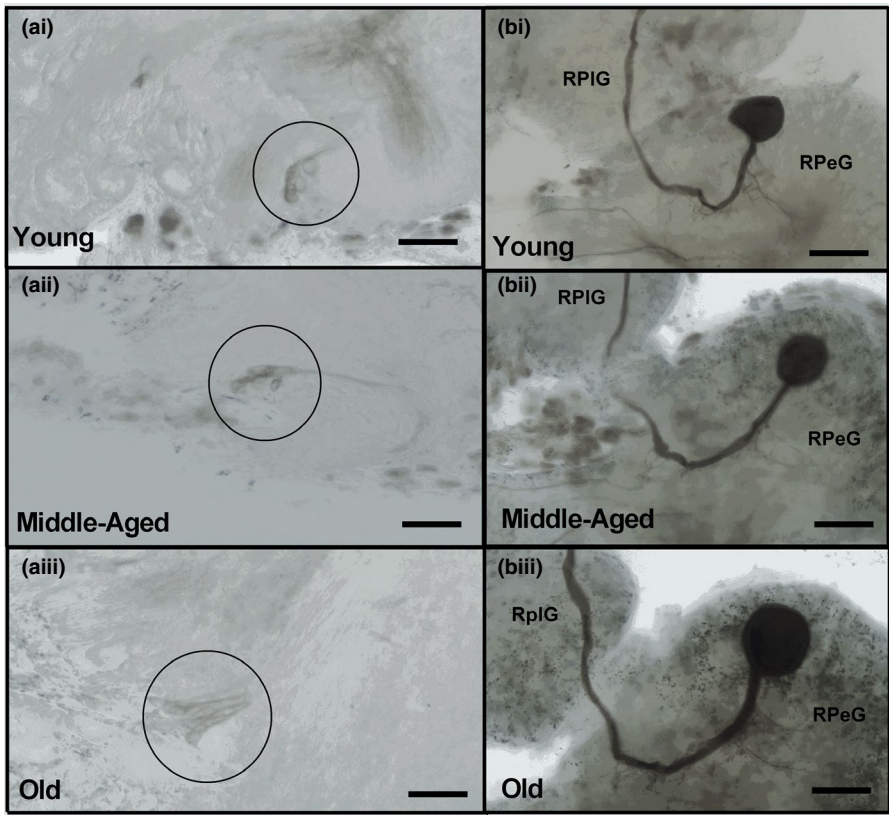


FIGURE 3 Dopaminergic neuronal cell bodies and proximal axons do not degenerate with age. Immunohistochemical images of sections of the Lymnaea CNS labelled with an anti-TH antibody. (a) Section through the cerebral ganglia illustrating the continued expression of an identified cluster of TH positive neurones (see black circles) in young (ai), middle-aged (aii) and old (aiii) CNS's. (b) Sections illustrating the soma, axons and proximal neurites of a giant dopaminergic neuron RPeD1 in the right pedal ganglia of young (bi), middle-aged (bii) and old (biii) CNS. Scale bar is 100 μm

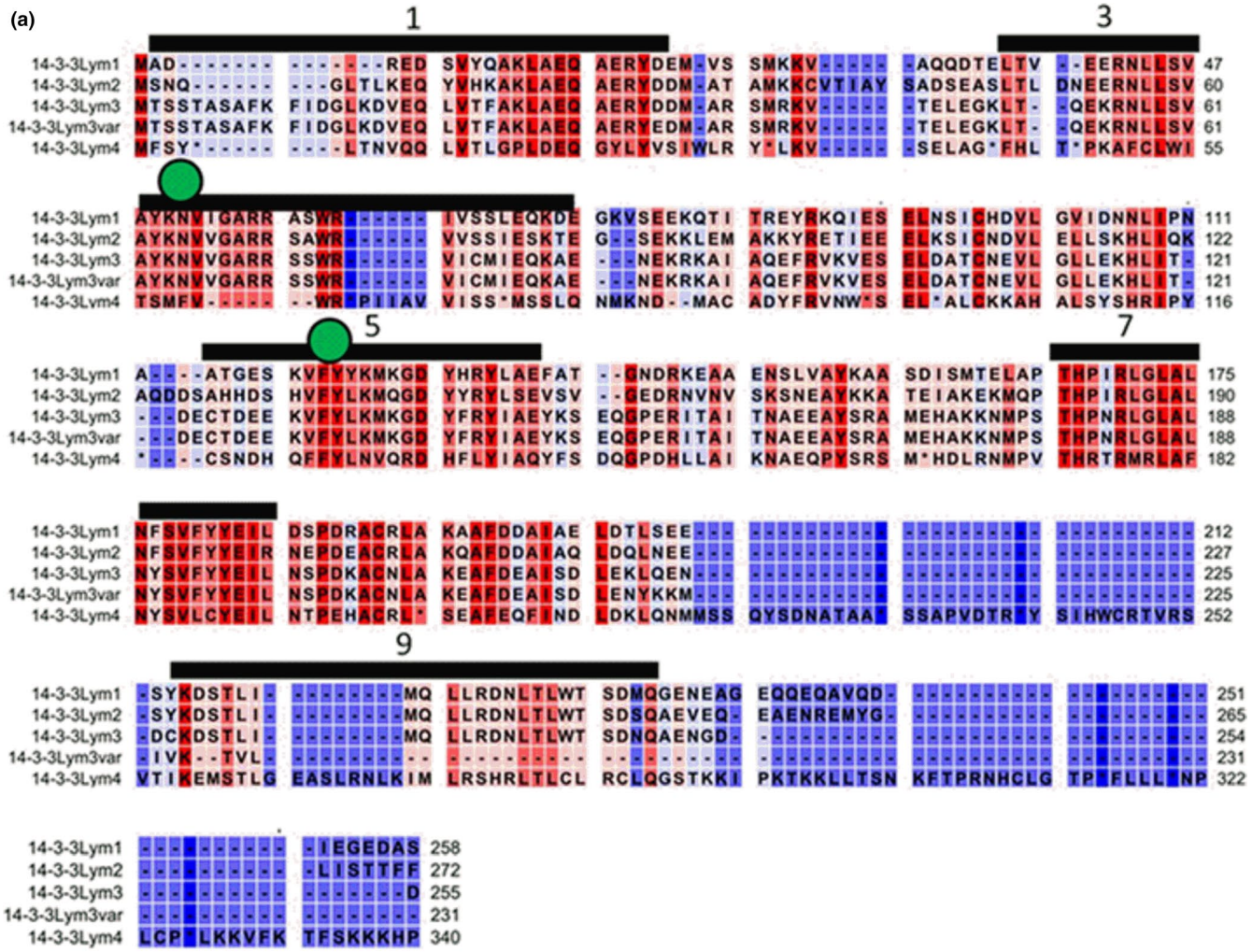
TABLE 1 Effects of age on cerebro-buccal ganglia protein expression. Details are given for each protein ID including the old:young (O/Y) ratio of expression, the number of peptides sequenced within each protein (Pep count), how much of the protein is covered by the sequenced peptides and the degree of certainty that the PEAKS software has in allocating the ID. Protein changes could be classified into groups, including stress response proteins, cytoskeletal proteins, metabolic proteins and signalling proteins

Function	Accession number	Protein designation	Protein description	O/Y ratio	Pep count	% Protein coverage	% Peaks score
Stress response	gi/112687	14-3-3	14-3-3	−1.3	5	22	99
	gi/66534655	GST	Glutathione-S-transferase	1.26	4	16	99
	gi/28897595	Hsp90	Heat shock protein 90-	1.14	19	19	90
	gi/10908171	RPSA	40S ribosomal protein sa	1.57	4	4	95
	gi/71652882	Reduc	Reductase	−1.53	14	5	97
	gi/51105058	Reduc	Reductase	−1.26	14	4	99
Cytoskeletal Proteins	gi/468226	RGBP51	Retrograde binding protein 51	2.72	75	69	99
	gi/2724046	β-actin	Beta actin	−1.10	8	26	99
	gi/174755	Tmy-2	Tropomyosin-2	−1.31	81	80	99
	gi32967406	α-Tub	A-tubulin	1.13	19	9	99
	gi/47551035	Cyt-actin	Cytoskeletal actin	−1.68	4	14	99
Metabolism	gi/13647103	argk	Arginine kinase	1.27	6	15	
	gi/78696405	glyox	Glycolate oxidase	1.22	3	4	90
Signalling	gi/110773414	Kinesin	Kinesin-like protein	−1.31	4	16	90
	gi/47169297	AChBP	Acetylchoine binding protein	−1.71	35	15	99

TABLE 2 Identification of putative 14-3-3 isoforms in the *Lymnaea* CNS. Putative primary amino acid sequences of the five distinct peptides encoded by the 17 ESTs identified from the *Lymnaea* CNS cDNA library, and their sequence nomenclature. Bold letters indicate the variant C-terminus on the 14-3-3Lym3 and 14-3-3Lym3var putative proteins

EST sequence identifier	Translation of full sequence	Best hit (nomenclature hereafter)
FPS013.CR_A15 FPS013.CR_E13 PS013.CR_O23 FPS011.CR_B02 FPS0110.CR_D23	MADREDSVYQAKLAEQAERYDEMVSSMKRVAQQDTELTVEERNLLSVAYKNVIGARRASWRIV SSLEQKDEGKVSEEKQTITREYRKQIESELNSICHDV LGVIDNNLIPNAATGESKVFYYKMKGDYHRYLAEFATGNDRKEAAENSLVAYKAASDISMTELA PTHPIRLGLALNFSVFYY EILNSPDRACRLAKAAFDDAIAELDTLSEESYKDSTLIQLLRDNLTLWTSDMQGENEGEQQEQ AVQDIEGEDAS	H. sapiens 14-3-3 ε protein **(14-3-3Lym1)**
FPS0110.CR_D23 FPS018.CR_A20 FPS013.CR_E23 FPS019.CR_P19	MSNQGLTLKEQYVHKAKLAEQAERYDDMATAMKKCVTIAY SADSEASLTLDN EERNLLSVAYKNVVGARRSAWRVVSSIESKTEGSEKK LEMAKKYRETIEEELKSICNDVLELLSKHLIQKAQDD SAHHDSHVFYLKMQGDYYRYLSEVSVGEDRNVNVSKSNEAYKKATEIAKEK MQPTHPIRLGLALNFSVFYYEIRNEPDEACRLAK QAFDDAIAQLDQLNEESYKDSTLIMQLLRDNLTLWTSDSQAEVEQEAENFF	C. Elegans 14-3-3-like protein 2 **(14-3-3Lym2)**
FPS016.CR_L07 FPS017.CR.1_G18FPS019.CR_H14 FPS0113.CR_E16	MTSSTASAFKFIDGLKDVEQLVTFAKLAEQAERYDDMARSMRKVTELEGKLTQEKRNLLSVA YKNVVGARRSSWRVICMIEQKAE NEKRKAIAQEFRVKVESELDATCNEVLGLLEKHLITDECTDEEKVFYLKMKGDYFRYIAEYKSEQGPERITAITNAEEAYSRAMEH AKKNMPSTHP NRLDLALNYSVFYYEILNSPDKACNLAKEAFDEAISDL***EKLQENDCKDSTLIMQL*** ***LRDNLTLWTSDNQAENGDD***	G. Gallus 14-3-3 protein β/α **(14-3-3Lym3)**
FPS011.CR_I09 FPS014.CR_J13 FPS011.CR_A15	MTSSTASAFKFIDGLKDVEQLVTFAKLAEQAERYEDMARSMRKVTELEGKLTQEKRNLLSVAYKNVVGARRSSWRVIC MIEQKAENEKRKAIAQEFRVKVESELDATCNEVLGLLEKH LITDECTDEEKFYLKMKGDYFRYIAEYKSEQGPERITAITNAEEAYSRAMEHAKKNMPSTHPNRLGLALNYSVFYYE ILNSPDKACNLAKEAFDEAISDL***ENYKKMIVKTVL***	G. Gallus 14-3-3 protein β/α **(14-3-3Lym3var)**
FPS011.CR_H12 translation of frame + 2 with 5′UTR (aa 1–91) and 3′UTR (aa 432–454) removed	MFSY*LTNVQQLVTLGPLDEQGYLYVSIWLRY*LKVSELAG*FHLT*PKAFCLWITSMFVWR*PIIAVVISS*MSSL QNMKNDMACADYFRVNW*SEL*ALCKK AHALSYSHRIPY*CSNDHQFFYLNVQRDHFLYIAQYFSDQGPDHLLAIKNAEQPYSRSM*HDLRNMPVTHRTRMRLAF NYSVLCYEILN TPEHACRL*SEAFEQFINDLDKLQNMMSSQYSDNATAA*SSAPVDTR*YSIHWCRTVRSVTIKEMSTLGEASLRNLKIMLRSHRLT LCLRCL QGSTKKIPKTKKLLTSNKFTPRNHCLGTP*FLLL*NPLCP*LKKVFKTFSKKKHP	S. Salar 14-3-3 protein β/α-1 **(14-3-3Lym4)**

(a)



(b)

	Length (number of amino acids)	Approximate Molecular Weight (kDa)	Theoretical pI
14-3-3Lym1	256	29	4.61
14-3-3Lym2	272	31	5.11
14-3-3Lym3	255	29	5.07
14-3-3Lym3Var	230	26	6.07

FIGURE 4 Characterization of 14-3-3 isoforms expressed in the Lymnaea CNS. (a) Sequence alignment comparing the primary putative sequences of the Lymnaea 14-3-3-like proteins. Green circles indicate the residues that form the phospho-peptide binding pocket. Black lines mark the relative positions of five of the nine α helices of which the protein consists in its natural configuration. Red indicates 100% residue conservation, blue indicates 0%, - indicates a gap and * indicates a stop codon. Note the extensions of 14-3-3Lym4 between the seventh and ninth helix. (b) The predicted physiochemical properties of the Lymnaea 14-3-3–like proteins determined in silico according to ProtParam (hosted by ExPASy)

32 kDa consistent with the predicted molecular weights of 14-3-3Lym1-3 (Figure 5a). No 26 kDa band corresponding to 14-3-3Lym3Var was observed (Figure 5a) indicating that this isoform was either not expressed in our population of *Lymnaea* or levels were below the resolution of the antibody. *Lymnaea* has two 14-3-3 isoforms with a predicted molecular

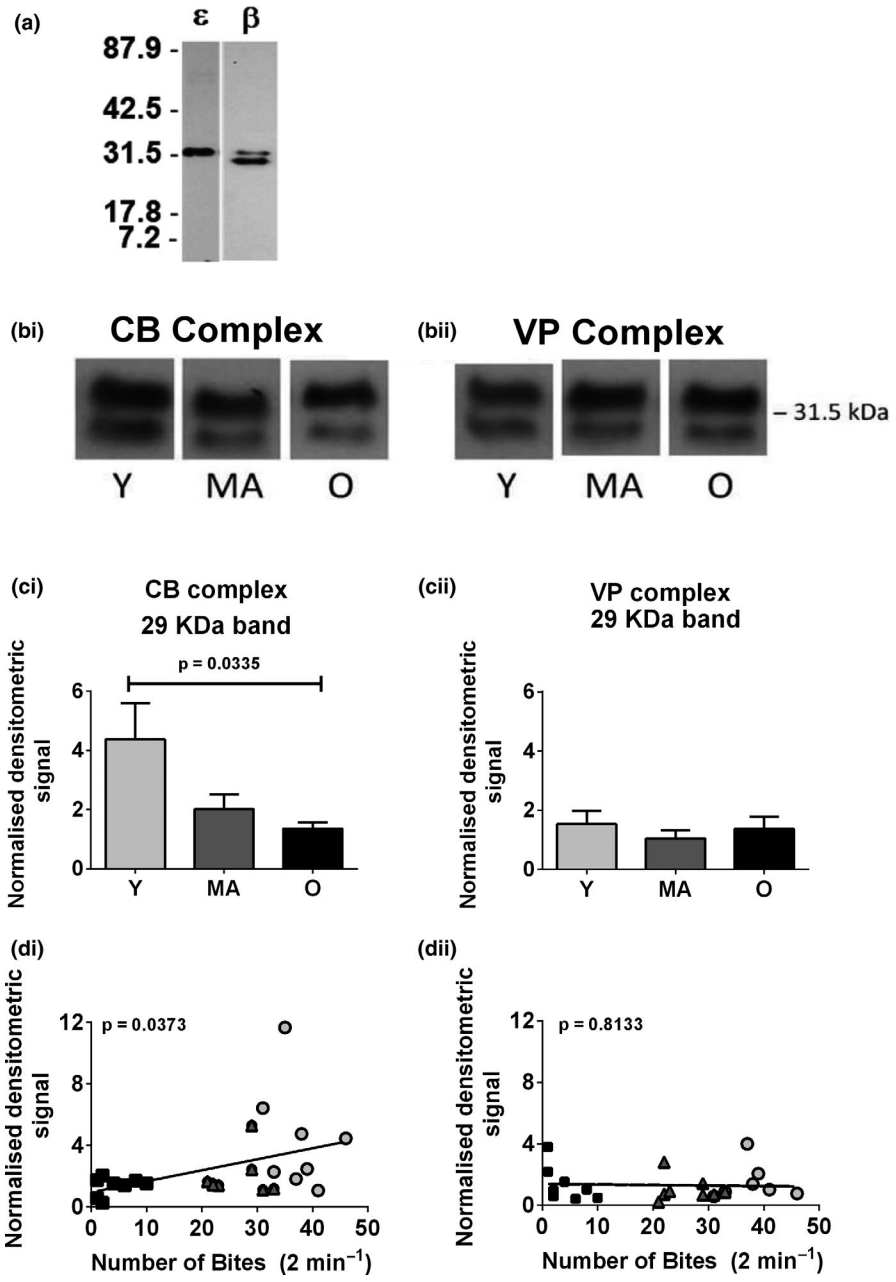


FIGURE 5 Age reduces 14-3-3 expression in the CB complex. (a) Western blots labelled with the 14-3-3β antibody or 14-3-3ε antibody. (b) Western blot labelled with a 14-3-3β antibody illustrating the presence of two distinct bands estimated at 32 kDa and 29 kDa in young, middle aged and old CB (bi) and VP (bii) complexes. (c) Mean densitometric signal from the 29 kDa band from the same samples shown in part b. (di/dii) Scatter plots of the density of the 29 kDa band versus number of sucrose-evoked bites per 2 min. Bite rate was positively correlated with expression of the 29 kDa band in the CB complex (di) but not in the VP complex (dii). N.B. 14-3-3 bands on Western blot were taken from the same snails that generated the feeding rate. $N = 8$ for each age group except young VP complex, where $n = 7$

weight of 29 kDa, 14-3-3Lym1 (ε isoform) and 14-3-3Lym3. However, labelling for the evolutionarily conserved 14-3-3ε isoform detected a single band in all age groups with an approximate molecular weight of 32 kDa indicating a significant post-translational modification (Figure 5a).

In Western blots labelled with the 14-3-3β antibody, densitometric analysis of the lower 29 kDa band demonstrated a significant decrease in expression of the 14-3-3Lym 3 isoform with increasing age in the CB complex ($p = 0.0335$; Figure 5bi,ci). No significant difference in expression with age was apparent in the VP complex ($p = 0.6094$, Figure 5bii,cii). A significant correlation was observed between the expression of the 29 kDa protein in the CB complex and feeding rates (Figure 5di; $p = 0.0373$). No such correlation was seen with the VP complex (Figure 5bii, $p = 0.8113$).

Analysis of the 32 kDa band labelled with the 14-3-3β antibody showed no significant effect of age on the expression of 14-3-3 in either the CB complex ($p = 0.1568$, Figure S3aii,bi) or the VP complex ($p = 0.9190$, Figure S3aiv,biii) or their correlation with feeding rates (Figure S3bii,iv, $p = 0.2127$ and $p = 0.9874$, respectively). The 32 kDa band was also probed with the 14-3-3ε antibody (Figure S3ai,iii). Once again no significant changes in expression were observed with increasing age in either the CB complex (Figure S3ci, $p = 0.6856$) or the VP complex (Figure S3ciii, $p = 0.9741$). There were also no significant correlations of the 14-3-3ε labelled band with feeding rates for either the CB complex (Figure S3cii, $p = 0.1805$) or the VP complex (Figure S3civ, $p = 0.8514$).

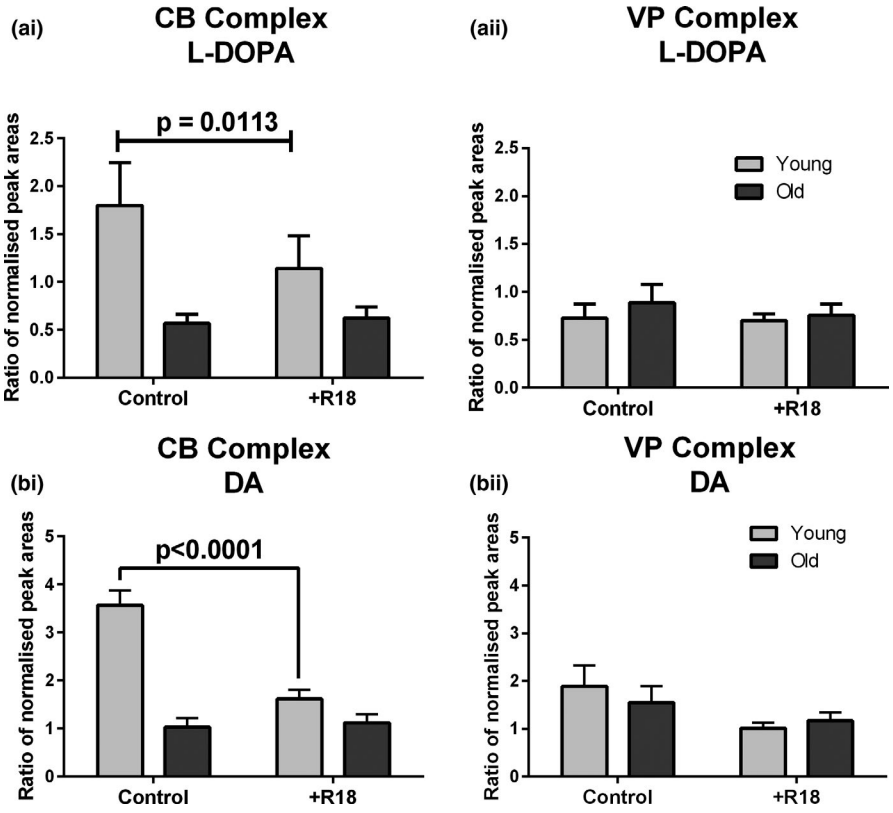


FIGURE 6 Effects of 14-3-3 and age on L-DOPA and DA synthesis. (ai) The 14-3-3 antagonist R18 reduced the production of L-DOPA in the CB complex of young animals but not in the CB from old snails. (aii) R18 had no effect on L-DOPA synthesis in the VP complex of either age group. (bi) R18 reduced the synthesis of DA in the young CB complex but not in the old. (bii) R18 had no effect on DA synthesis in the young or old VP complexes. $N = 6$ for all groups except the young DA group where $n = 5$

3.6 | 14-3-3 regulates DA synthesis

14-3-3 has previously been shown to regulate the activity of TH and the synthesis of L-DOPA (Ichimura et al., 1987). We therefore used HPLC analysis to examine whether 14-3-3 can regulate TH activity and the production of L-DOPA in *Lymnaea* CB and VP complexes. TH activity, determined by measuring the rate of production of L-DOPA, showed a significant age x R18 interaction ($F_{1,10} = 7.205$; $p = 0.02$; Figure 6ai). There were no significant effects of age ($p = 0.5433$), R18 ($p = 0.3899$) or an interaction ($p = 0.5430$) in the VP complex (Figure 6aii). Post hoc tests confirmed that R18 decreased the production of L-DOPA in the young CB complex ($p = 0.0113$; Figure 6ai), but was without effect on the old CB complex ($p > 0.9999$; Figure 6ai) or on the young or old VP complexes (Figure 6aii, $p = 0.9784$ and $p = 0.5782$ respectively). Importantly, the production of DA also showed significant interaction (age x R18, $p = 0.0005$), with post hoc tests showing that R18 reduced DA production in the young CB complex ($p < 0.0001$). Although there was a weak effect of R18 on VP DA concentrations ($F_{1,9} = 5.564$, $p = 0.0427$), post hoc tests showed that these effects were not significant in the young ($p = 0.1031$) or old VP complexes (Figure 6bii, $p = 0.5336$).

Given that 14-3-3 has previously been shown to regulate TpH activity (Ichimura et al., 1987), the rate-limiting enzyme in the production of 5-HT and as 5-HT has previously been shown to regulate feeding frequency, we were keen to explore whether alterations in 14-3-3 expression were affecting 5-HT production. Production of 5-HTP was not significantly affected by age or R18 treatment in both the CB (Figure S4ai, $p = 0.7339$ and $p = 0.0574$ respectively) and VP complexes (Figure S4aii, $p = 0.9464$ and $p = 0.9462$ respectively), although there was a clear trend for R18 to reduce 5-HTP synthesis in the CB complex. There was a significant interaction (age x R18) for CB 5-HT production ($F_{1,10} = 6.206$, $p = 0.0319$). Post hoc analysis showed no effect of R18 in the young CB complex ($p = 0.9976$, Figure S4bi), however, R18 reduced production of 5-HT in the old CB complex (Figure 4bi, $p = 0.0122$). There was a significant effect of R18 on 5-HT production in the VP complex ($F_{1,9} = 6.295$, $p = 0.0334$). Post hoc analysis showed that R18 reduced 5-HT synthesis in the young VP complex (Figure 4bii, $p = 0.0372$), but had no effect in the old VP complex ($p = 0.6997$). Immunohistochemical analysis focused on the CGCs, which are the main source of 5-HT used to regulate the frequency of feeding movements (Yeoman, Pieneman, et al., 1994). Analysis using the 14-3-3β antibody showed faint labelling in the nucleus of the young CGCs (Figure 4ci; inset). In the old CGCs, the weak 14-3-3β labelling was still present in the large nucleus but intense cytoplasmic labelling could now be observed (Figure 4cii; inset). An additional group of cells was consistently labelled on posterior ventral surface of the cerebral ganglia which are most probably the egg-laying caudo-dorsal cells (circles Figure 4ci,ii). Similar experiments using the 14-3-3ε antibody showed very weak labelling in the young CCGs (Figure S4di,ii) which increased in

intensity in the old CGCs (Figure S4diii,iv). This increase in the intensity of labelling in the CGCs is unlikely to be due to differences in the processing of the young and old samples as there was strong labelling of the neuropil in the young cerebral ganglia which was markedly attenuated in the old (circles Figure S4di–iv). To explore whether these changes affected 5-HT content of the CGCs, we performed single-cell HPLC on isolated young and old CGCs. 5-HT expression was significantly increased in the old CGCs ($p = 0.035$; Figure S4e).

4 | DISCUSSION

This study has confirmed that DA is an important regulator of feeding behaviour in the pond snail, *Lymnaea*. DA levels in the CNS of *Lymnaea* decreased with age and levels in the CB complex correlate with sucrose evoked feeding rates consistent with this area of the CNS containing the main feeding circuitry. We have identified changes in two main classes of proteins in this ageing model of motor impairment. These are stress-related proteins and cytoskeletal proteins. A more detailed analysis of one of the stress-related proteins, 14-3-3, characterised a family consisting of at least 3 isoforms. The expression of one of these isoforms, 14-3-3Lym3, was reduced with age in the CB complex but not in the VP complex. Reductions in the CB complex also correlated with decreased feeding rate. We demonstrated in ex vivo CNS tissue that inhibiting 14-3-3 can reduce the synthesis of DA in the CB complex but not in the VP complex. Together these data suggest that region-specific changes in 14-3-3 expression can contribute to the loss of CNS DA and the reduction in motor function associated with increased age.

4.1 | DA can regulate the frequency of feeding in Lymnaea

DA has previously been shown to activate and regulate the frequency of feeding in a range of organisms including *Limax* (Wieland & Gelperin, 1983), *Alpysia* (Serrano et al., 2007; Teyke et al., 1993), *Helisoma* (Quinlan et al., 1997), *Lymnaea* (Kyriakides & McCrohan, 1989), rats (Pitts & Horvitz, 2000; Rowland, 1978) and cats (Jaspers et al., 1984). These findings were confirmed in the current study through the use of two methods that reduced dopaminergic signalling, namely, the competitive D1/D2 antagonist flupenthixol and the dopaminergic neurotoxin 6-OH DA. The observed correlation between CB DA levels and feeding rate across the lifecourse and the reduction in feeding rate seen following two treatments that reduce CNS DA signaling strongly suggests that DA is a major regulator of feeding rate in *Lymnaea*. A similar study in rats demonstrated a significant correlation between the age-related reduction in rat licking behaviour and striatal DA (Stanford et al., 2003). Given that the CV1 cluster of neurons on the ventral surface of the cerebral ganglia are TH positive, it is possible that the effects of DA on feeding rates are due to an impairment in the function of these neurons which have previously been shown to initiate and regulate the frequency of feeding (McCrohan, 1984), through their excitatory inputs to the N1M pattern generating neurons. However, we and others have previously shown that 5-HT is a major regulator of feeding frequency in molluscs and we have previously shown that 5-HT signalling changes with age (Arundell et al., 2006; Patel et al., 2006). It is therefore interesting to note that the CGC expresses excitatory D1A DA receptors (Hernádi et al., 2012) and DA can enhance the excitability of the CGCs (Kemenes et al., 2011), providing a possible link between the two transmitter systems and the regulation of feeding frequency. Therefore, we cannot exclude that decreases in CB DA levels may also decrease the excitability of CGCs and contribute to the decreased frequency of feeding (Yeoman et al., 1996; Yeoman, Pieneman, et al., 1994). DA has also recently been shown to be involved in regulating the snails perception of food value through the activity of a pair of interneurons known as program reversal neurons (prn; Crossley et al., 2018). These prn neurons were capable of biasing the animal towards either ingestion or egestion movements in response to the same input and their effects could block the selective D2 receptor antagonist sulpiride. Interestingly, sulpiride did not affect the frequency of the ingestion or egestion movements. Taken together with our data using the mixed DA receptor antagonist flupenthixol, this strongly suggests that feeding frequency is regulated through the actions of DA on D1 receptors either through the actions of DA on the CGCs or via their direct effects on the N1M pattern generating interneurons.

4.2 | What are the possible causes of the loss of DA in the aged CNS?

In order to understand the causes of the decrease in DA levels in the CNS, we utilised a proteomics approach to identify key candidate molecules. Pooling the CNSs into groups of 20's has allowed us to detect the major proteins that consistently change with age. The proteins identified were abundant proteins, the majority of which could be classed as stress-related proteins. This is consistent with observations in *Lymnaea* and higher organisms which indicated a major role for oxidative stress in the CNS of aged animals (Hermann et al., 2014; Perluigi et al., 2014; Poon et al., 2006; Watson et al., 2012, 2014). In *Lymnaea*, ageing is associated with

a reduction in both spontanoeus and evoked neuronal firing rates (Patel et al., 2006). Previous work by the Wildering laboratory has demonstrated that an age-related increase in oxidative stress drives lipid peroxidation which in turn activates phospholipase A2 releasing inflammatory mediators which reduce neuronal firing rates (Watson et al., 2012, 2014). Our observation that the expression of two reductases were decreased with age and that ageing increased GST (Maurya & Rizvi, 2010) are consistent with oxidative stress being associated with CNS ageing in *Lymnaea*.

Of the list of proteins identified, 14-3-3 has previously been shown to regulate DA synthesis (Ichimura et al., 1987). 14-3-3 proteins are a protein family whose structure has been conserved throughout evolution (Aitken et al., 1995). Three *Lymnaea* isoforms were identified (Lym1-3). Using the 14-3-3β antibody, we observed two clear bands on a Western blot. The bands corresponded well to the predicted molecular weights for the 14-3-3 proteins from their amino acid sequences. These data suggested that the top band represented 14-3-3Lym2, while the bottom band consisted of a combination of 14-3-3Lym1 and Lym3. However, labelling for the evolutionarily conserved 14-3-3ε isoform (14-3-3Lym1) detected a single band in all age groups with an approximate molecular weight of 32 kDa. The shift of 14-3-3ε relative to its predicted molecular weight is seen in all species and may indicate a post-translational modification (Aitken, 2011). Therefore, the 32 kDa band consists of a mixture of 14-3-3Lym1 and 2 while the lower band represents expression of 14-3-3Lym3.

Analysis of the effects of age on 14-3-3 expression showed that 14-3-3Lym 3 was reduced with age and this reduction was limited to the CB complex a region of the CNS that regulates feeding frequency. There was no overall age-related change in the expression of the 32 kD band in either the CB or VP complexes. Given that this band comprises of two 14-3-3 isoforms (Lym 1 and Lym 2), it is possible that the lack of change in the overal density of one isoform is negated by an equal and opposite change in the other. However the observation that there is no age-related change in the expression of 14-3-3Lym 1, as determined by probing the Western blot with the 14-3-3ε antibody, infers a similar lack of a significant change in the 14-3-3Lym 2 isoform. Comparison of the feeding rates of individual animals with levels of 14-3-3Lym3 in the CB complex showed a clear positive correlation, with no such correlation in the VP complex. These data strongly suggest that 14-3-3Lym3 expression in the CB complex influenced feeding rate in *Lymnaea*. As mentioned previously, 14-3-3 proteins have a range of functions. Included in these is their ability to increase the activity of TH the rate-limiting enzyme in the production of DA. Consistent with this was the observation that the 14-3-3 antagonist R18 was able to inhibit L-DOPA and DA formation in the young ex vivo CB complex. However, R18 did not affect L-DOPA/DA synthesis in the old CB complex. These data are strongly suggestive that 14-3-3 significantly augments TH activity in the young but not the old CB complex. Of potentially greater interest was the observation that the 14-3-3 antagonist was without effect in the VP complex of both young and old animals despite age significantly reducing levels of DA in this region of the CNS. Previous work on rat TH has shown that phosphorylation of TH at a range of serine residues can increase its activity. In vitro studies have shown that phosphorylation of both serines at positions 19 and 40 can increase TH activity by 1.5- to 2-fold (Dunkley et al., 2004; Fitzpatrick, 1999) and 14-3-3 is proposed to stabilise this phosphorylation state to maintain the increase in TH activity. Our young CB complex produces about 3 times more L-DOPA and approximately twice as much DA compared to the old CB complex or both the young and old VP complexes, consistent with TH being phosphorylated and stabilised by 14-3-3. The lack of effect of R18 in the old CB complex or in both the young and old VP complexes could be due to the relatively low levels of 14-3-3 or to impaired phosphorylation of TH which would impair the binding of 14-3-3. Impaired phosphorylation is unlikely based on our observation that TpH activity is increased in the old CB complex. TpH activity has been shown to be increased following PKA-dependent phosphorylation and stabilised by binding with 14-3-3 (Winge et al., 2008). Therefore, it is high unlikely that an age-related impairment of phosphorylation is reducing TH activity, given that the same process is still functional in the aged CB complex, albeit in a different neuronal phenotype. Therefore, we strongly believe that the reduced TH activity is due to the inability of 14-3-3 to stabilise the phosphorylated state of the enzyme due in part to the reduced expression of the 14-3-3Lym 3 isoform in these complexes.

Although we have shown that 14-3-3 can regulate both L-DOPA and DA synthesis, we were unable to definitively demonstrate that the effects of R18 on L-DOPA production were through its ability to inhibit the 14-3-3Lym3 isoform as no selective inhibitors of this isoform are available. In addition, attempts to utlise commercially available 14-3-3 α/β antibodies and look for co-localisation with TH-positive neurons also proved unsucessful due to the lack of specificity of the antibody.

In addition to the decrease in 14-3-3 expression and the consequential reduction in TH activity, it is also possible that neurodegeneration could contribute to the the decrease in CB complex DA. We have shown that DA turnover is increased with age and it has been suggested that increased turnover might be an early compensatory mechanism for dopaminergic neurodegeneration in both age (Greenwood et al., 1991) and models of Parkinson's disease (Barrio et al., 1990; Sossi et al., 2002). Although we cannot exclude that the age-related decrease in DA is in part caused by neurodegeneration

of more distant synaptic terminals, this is clearly not the case for the cell soma and axons close to the cell soma, as we have shown that these are maintained with age in a key cluster of CV1 neurons in the cerebral ganglia and the RPeD1 neuron in the right pedal ganglion.

14-3-3 has also been shown to increase the activity of TpH the rate-limiting enzyme in the production of 5-HT. Given the previously well-described role of 5-HT in regulating feeding frequency (Yeoman, Kemenes, et al., 1994; Yeoman, Pieneman, et al., 1994), it was possible that the observed decreases in 14-3-3Lym3 could decrease CB complex 5-HT and this was the main determinant of the age-related decrease in feeding frequency. A number of pieces of evidence argue against this possibility. The first is that 5-HT expression in the CB complex does not change significantly between young and old animals (Patel et al., 2006). Second, and consistent with this was the observation that immunohistochemistry with the 14-3-3β antibody showed a clear up-regulation of cytoplasmic expression 14-3-3 in the old CGCs. This increase could act to stabilise the phosphorylated form of TpH in the cytoplasm and maintain the synthesis of 5-HT in the aged neurons. Using a 14-3-3ε antibody confirmed this up-regulation in the cytoplasm suggesting that in this neuron 14-3-3ε up-regulation may help to main the activity of TpH in old CGCs. Given that the CGCs are the major source of 5-HT in the CB complex, the observed increase in 14-3-3 expression in old CGCs is consistent with the observation that the 14-3-3 antagonist R18 was only capable of inhibiting 5-HT synthesis in old CB complex extracts and the observation that the 5-HT content of the CGCs was increased in old age.

5 | CONCLUSIONS

In summary, the results from this study demonstrate an overall age-related reduction in 14-3-3Lym3 expression in the CB complex of the pond snail, *Lymnaea*. We propose that this decrease reduces DA synthesis, by impairing the activity of the rate-limiting enzyme TH. Although we are unable to state definitively that it is the 14-3-3Lym3 isoform that regulates DA synthesis, the clear correlation of 14-3-3Lym3 expression with both feeding rate and CB complex DA levels and our data showing that the 14-3-3 antagonist R18 can reduce DA synthesis in the young ex vivo CB complex, makes this a strong possibility. We also show that 5-HT expression is maintained in old CGCs in part through an up-regulation of 14-3-3ε. This suggests that the effects of 14-3e-3Lym3 down-regulation in the aged CB complex are not acting through its effects on the synthesis of 5-HT in the CGCs. 14-3-3 levels have been shown to change with age in the CNS of a range of species, however, we believe this is the first time that an age-related decrease in 14-3-3 expression has been correlated with a motor deficit through an impairment in DA synthesis.

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CONFLICTS OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

LDM, AM, BAP, MA, LH and LDB carried out the experiments; LDM, AM, BAP, MA, KJB, LH, AO, LDB, DoH and MSY carried out the data analysis. MSY wrote the manuscript which was critically reviewed and amended by BAP and LDB. Funding for the project was obtained by MSY, DoH and KJB.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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