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Decreases in the serotonin content of a pair of identified neurones can explain the effects of p-chlorophenylalanine on the feeding behaviour of the pond snail, *Lymnaea stagnalis*.

An optimised LCEC technique has been used to measure serotonin (5-hydroxytryptamine, 5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) concentrations in the central nervous system (CNS) of the pond snail, Lymnaea stagnalis*. Detection limits for serotonin and 5-HIAA were 11.9 ng/ml and 119 ng/ml, respectively, allowing detection of these two analytes in a single neurone. pCPA (150 mg/kg) caused a significant decrease in the concentrations of both serotonin and 5-HIAA in the CNS, a result that was confirmed by standard immunohistochemical techniques. These decreases were associated with specific changes in the feeding behaviour of the animal. The role of serotonin in regulating feeding in* Lymnaea *is discussed.*

Introduction

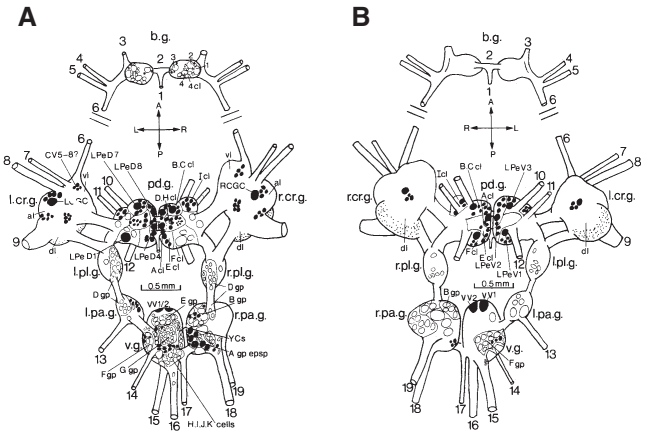
The feeding system of the gastropod molluscs has been widely used to study the neural mechanisms of behaviour. One major interest has been control of the central pattern generator (CPG) responsible for generation of the rhythmic movements involved in the consummatory phase of gastropod feeding.

One class of neurone involved in controlling the activity of the CPG is the modulatory neurones, so called because they could not initiate rhythmic activity in the CPG but could regulate its output. Classic examples of this type of neurone are the paired serotonergic metacerebral giant cells (MCCs) of *Aplysia*, which control the frequency and intensity of the feeding movements but could not drive the basic motor pattern (1).

An equivalent pair of serotonergic cells is present in the pond snail, *Lymnaea*. They are known as the cerebral giant cells and have previously been shown to have a gating function, allowing the animal to respond to food and also to regulate the frequency of feeding movements (2, 3).

In this study serotonin concentrations were depleted in the CNS using *p*-chlorophenylalanine (*p*CPA) and its effects on the feeding behaviour of the animal examined. *p*CPA has been widely used in neurophysiological investigations to diminish serotonin concentrations. It is a competitive inhibitor of the enzyme tryptophan hydroxylase and prevents synthesis of serotonin by stopping production of 5-hydroxytryptophan from tryptophan. Alterations in serotonin concentration were determined using microbore LC with electrochemical detection and standard antibody labelling techniques.

F1. CNS maps of *Lymnaea stagnalis*. A & B show a map of the serotonergic neurones present on the dorsal (A) and ventral (B) surfaces of the CNS. Ganglia: buccal (b.g); cerebral (cr.g.); pedal (pd.g.); pleural (pl.g.); parietal (pa.g.); and visceral (v.g.). L, R and 1, r indicates left and right; A, P indicates anterior and posterior. (19, 36).



Materials and Methods

Animals and Sample Preparation

Three-month-old *Lymnaea stagnalis* were used throughout this study. All animals were kept in copper-free water under constant conditions of 12-hour light/dark cycles with lettuce available *ad libitum*. The methodology for preparation of the tissue samples was adapted from previous methods (4-7).

The CNS was removed from *Lymnaea stagnalis* and pinned out in a silicone elastomer (Sylgard) - lined dish filled with ice-cold HEPES buffer (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) to facilitate dissection of the CNS segments (**F1**). The buccal ganglia and associated lateral and ventral buccal nerves and cerebrobuccal connective tissue were analysed. These samples were homogenised in 200 µl of ice-cold 0.1 M perchloric acid using a hand-held homogeniser and centrifuged at 20000 g at 4 °C. All samples were run between 15 and 20 minutes of preparation, and were stored in ice prior to analysis.

Standards and Accuracy

Standard solutions were prepared from a 0.1 µg/mL stock solution of each analyte, diluted with freshly prepared ice-cold 0.1 M perchloric acid (BDH), and stored in ice. 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 (8, 9).

For each spike and recovery experiment, two snails were used. Recovery measurements were carried out using the whole CNS and various regions of the CNS. The bilaterally symmetrical CNS was divided sagittaly along its midline. The left part of one snail's CNS was matched with the right side of another snail's CNS and vice versa. One half of the CNS was placed in 800 µl of ice-cold 0.1 M perchloric acid, and the second set was placed into 800 µl of ice-cold perchloric acid spiked with a final concentration of 0.04 µg/mL of serotonin and dopamine (DA). For individual CNS regions, samples were placed in 200 µl of 0.1 M perchloric acid and spiked with the same concentration of serotonin and dopamine (0.04 µg/mL). Spiked solutions were also homogenised without tissue and also centrifuged to quantify total sample losses.

Determination of Serotonin and 5-HIAA by LCEC

The determination of serotonin in individual neurons was performed by on-line LCEC using an Agilent HP1050 pump, autosampler and column heater kept constantly at 25 ± 0.15 °C. 5 µL of the sample was injected for all runs. The system incorporated a LUNA® ODS 3 µm 150 X 1.0 mm I.D. analytical column with a 4.0 X 2.0 mm I.D. 5 µm guard column (Phenomenex®, Macclesfield, UK). An Epsilon™ 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, BASi) served as the working electrode and was used with an 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. Sensitivity of the detector was maintained at 50 nA full-scale deflection. Control and data collection/processing were handled through BASi ChromGraph™ software.

The mobile phase composition was similar to previous methods (10, 11). A stock buffer was prepared as follows: 25 mM sodium dihydrogenorthophosphate, 27 µM disodium ethylenediaminetetraacetate (EDTA), 50 mM sodium citrate, 10 mM of diethylamine, 10 mM sodium chloride and 2 mM of decane-sulfonic acid sodium salt were dissolved in 1 L of deionized, distilled water and buffered to pH 3.2 using concentrated phosphoric acid. To prepare the mobile phase, sodium citrate buffer (pH 3.2) was mixed with UV-grade acetonitrile (CHROMSOLV® for HLPC, Riedel de Haën) in a ratio of 82.5: 17.5 v/v, filtered through a 0.20 µm membrane filter, and degassed under vacuum after mixing.

p*CPA Administration and Experimental Procedure

Control animals were kept in conditions identical to those administered with *p*CPA. Each snail weighed approximately 2 g. Animals were injected a total of three times at 12-hour intervals with 0.15 mg/g of tissue, which approximates to a concentration of 150 µM p-CPA per snail. Control animals were injected at the same time with HEPES-buffered saline. Samples were analysed by LCEC or fixed for immunohistochemistry four hours after the last injection of *p*CPA.

Immunohistochemistry

In order to visualise the effects of *p*CPA on the serotonin content of the CGCs, serotonin concentrations were estimated in the CGCs using an anti-serotonin antibody and a fluorescent secondary antibody. Briefly, CNSs were removed from young, middle-aged and old snails and pinned in sylgard-lined dishes. CNSs were then incubated in 0.5% protease solution for 30 minutes at room temperature, after which they were fixed overnight at 4 °C in 1% paraformaldehyde/1% acetic acid solution. The CNSs were then washed hourly for eight hours in supermix containing Triton X-100 (composition 50 mM Tris, 150 mM NaCl, 30.9 mM Triton X-100, adjusted to pH 7.6 with HCl). CNSs were then incubated overnight at 4 °C with the rabbit anti-serotonin antibody (Sigma, UK) diluted 1 in 250 with supermix containing Triton X-100. Preparations were washed hourly, for six hours at 4 °C in supermix without Triton X-100, after which they were incubated overnight at 4 °C with a fluorescein-labelled secondary antibody (Sigma goat anti-rabbit), diluted 1 in 50 with supermix. Prior to viewing under the microscope, preparations were washed every hour for four hours with supermix. CNSs were then mounted on cavity slides in 1% ethylenediamine/75% glycerol solution and inspected using UV light under an inverted microscope (Axiovert, Zeiss). A variety of controls were performed to determine the specificity of binding. These included pre-absorbing the antibody with serotonin prior to incubation with the CNS, and omission of primary antibody to check whether the secondary showed any non-specific binding.

Behavioral Experiments

The effects of age on short-term feeding were examined using a method described previously (12). Briefly, the animals were removed from their home tank and maintained in smaller tanks in copper-free water for 7 days with free access to lettuce until 12 hours before the experiment began, when they were starved. Animals were tested by placing them in a petri dish filled with 90ml of copper-free water. The time taken for them to emerge from their shells (both tentacles visible) was recorded, after which 5 ml of water was pipetted around the lips of the animal and feeding movements recorded over the next 2 min. At the end of the 2-min period, 5ml of sucrose (0.01 M final dish concentration) was added to the dish and feeding movements recorded for a further 2 min. Various feeding parameters were recorded using a software package (12). These were latency to first bite, bite duration, interbite interval and total number of sucrose-evoked bites in a 2-min period.

Data Analysis

Concentrations of all analytes are expressed as the mean ± SEM per cell in each ganglion. The neurophysiology of the pond snail is well known, and distribution of serotonergic neurones has been described previously (4). The differences in the analyte concentrations between saline- and *p*CPA-treated animals were analysed using a one-way ANOVA. Variations among groups were compared using the post-hoc Tukey test. In behavioral experiments, differences between the numbers of non-responding and responding animals were determined using a Chi-square test. All other feeding parameters were statistically analysed using a student's t-test.

For all statistical analyses, a p value of 0.05 or less was considered statistically significant.

Materials

5-hydroxyindole-3-acetic acid (5-HIAA) and *p*-chlorophenylalanine (*p*CPA)were obtained from Sigma-Aldrich and used as received. Serotonin was obtained from ICN Biochemical Inc. All other chemicals used were obtained from Sigma and used as received. All standards were prepared in class A volumetric glassware.

Results

Serotonin and 5-HIAA Concentrations

The retention times of 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HIAA, noradrenaline (NA), adrenaline (AD), DA and serotonin are 2.6, 2.9, 3.3, 3.8, 5.2 and 8.7 min, respectively. Excellent resolution (R > 3) between serotonin, DA and AD is observed, with adequate resolution (R = 1.5) between DOPAC, 5-HIAA, NA AD. **F2** shows a typical chromatograph obtained from the buccal ganglia.

We have used spike and recovery as a means of accounting for sample preparation error. When using small tissue sections such as the buccal ganglia from the CNS, the relative recovery was reduced by half. **T1** shows the percent loss in sample preparation. Recovery is based on sample weight, and the majority of loss occurs during tissue homogenisation. Losses of approximately 50% were observed for serotonin in the buccal ganglia. The loss seems to be correlated to the number of serotonergic cells in each of the ganglia. Based on our antibody labeling data the paired CGCs provide sole serotonergic input to the buccal ganglia, whilst in the cerebral ganglia there are 43 cells, thus variability in the concentration of serotonin within an individual neuron can significantly alter the response from the spike and recovery data.

These calibration curves would allow quantification of serotonin and 5-HIAA in both the cerebral and buccal ganglia, the latter of which contains the terminal processes from just two neurones.

The absolute limits of detection are 2.36 ng/mL for DOPAC, 1.19 ng/mL for 5-HIAA, 1.47 ng/mL for AD, 0.47 ng/mL for NA, 4.20 ng/mL for DA and ng/mL for serotonin at the detector sensitivity of 50 nA full-scale deflection. The LOD of the standards are generally very good for measurement of tissue samples, but results obtained for the responses from DOPAC and serotonin were fit for purpose, but not exceptional. All the standard calibration curves have high correlation coefficients.

Concentrations of Serotonin and 5-HIAA Present Individual Neurons

Serotonin and 5-HIAA concentrations were measured in the two ganglia (buccal and cerebral) shown to regulate feeding behaviour of the pond snail. The number of serotonergic neurons has been well identified in all CNS regions of the pond snail, and results for serotonin and 5-HIAA concentrations are presented per cell.

The buccal ganglion which only contains the axons and terminal processes from the two serotonergic CGCs contained a basal concentration of 9.53 ± 2.55 pmol of serotonin per cell, whilst 754 ± 188 fmol of serotonin were found per cell in the cerebral ganglia. The metabolite of serotonin, 5-HIAA, was also measured. The concentrations of 5-HIAA were found to be 1.12 ± 0.13 pmol per cell for the buccal ganglia, and 94.50 ± 11.25 fmol per cell for the cerebral ganglia. These results indicate the sensitivity of the Epsilon electrochemical detector.

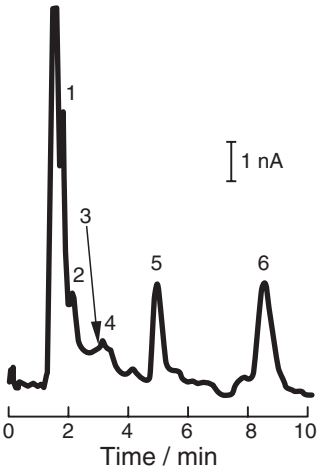
The metabolite concentrations are indicative of the rate of serotonin turnover and provide information about the serotonergic neurone functions. A better estimation of turnover can be obtained by examining the 5-HIAA:serotonin ratio. 5-HIAA:serotonin ratios in the buccal and cerebral ganglia were 0.12 and 0.10, respectively, and were not significantly different from one another.

Effect of p-CPA Administration on Serotonin and 5-HIAA Concentrations

Administration of *p*CPA significantly decreased the serotonin concentration in the buccal ($p < 0.001$) and cerebral ($p < 0.001$; ANOVA) ganglia. The greatest difference to basal concentrations occurred after one dose in both the cerebral ($p < 0.001$; students *t*-test) and buccal ganglia ($p < 0.05$; students *t*-test, **F3**), with serotonin concentrations decreasing by 81.0% in the cerebral ganglia, and by 64.5% in the buccal ganglia. Application of the second and third doses of *p*CPA caused further non-significant reductions in serotonin concentrations in the two ganglia, with concentrations decreasing by 86.7% in the cerebral ganglia and 76.6% in the buccal ganglia when compared to controls.

5-HIAA concentrations were similarly altered by *p*CPA. *p*CPA caused a significant decrease in 5-HIAA concentrations in both the buccal ($p < 0.001$) and cerebral ganglia ($p < 0.001$, ANOVA). The most significant decrease in metabolite concentrations occurred after the first administration of *p*-CPA, where decreases of 91.26% were observed in the

F2. Typical chromatogram obtained from the buccal ganglia sample prepared in 200 µl of 0.1 M perchloric acid and centrifuged for 5 minutes. Solutes: 1, DOPAC; 2, 5-HIAA; 3, noradrenaline; 4, adrenaline; 5, dopamine; and 6, serotonin.



T1. Mean recovery rates of serotonin from various CNS regions. Error bars show ± one standard deviation of the mean ($n = 6$).

CNS region	Mean Recovery Rate (%)
Whole CNS	102.40 ± 2.76*
Buccal ganglia and associated lateral and ventral buccal nerves and cerebrobuccal connective	53.01 ± 2.00
Cerebral ganglia	98.74 ± 2.63

**Recovery results are greater than 100 %, but are within the 2 % of instrumental error, and therefore are assumed to be 100 % recovery.*

cerebral ganglia ($p < 0.001$; student's *t*-test), and 81.49 % in the buccal ganglia ($p < 0.001$; student's *t*-test, **F4**). Further non-significant reductions were seen following the second and third applications of *p*CPA with values decreasing by 99.29 % and 95.76 % for the cerebral and buccal ganglia, respectively.

Immunohistochemistry

The most intense fluorescence can be seen in the axons and terminal processes (**F5**). Application of *p*CPA caused a dramatic reduction in the fluorescence of all parts of the CGCs, consistent with a reduction in serotonin concentrations in the CNS (**F4**).

Behavioral Data

Application of *p*CPA caused a marked decrease in the number of responding animals compared to the control group ($p < 0.05$; Chi-square [**F6A**]). In the animals that did respond to sucrose, *p*CPA increased the bite duration from 0.46 + 0.02 sec to 0.62 + 0.06 sec(**F6B**). Values for duration of the interbite interval also increased from 3.25 + 0.13 sec in saline-injected animals to 5.0 + 0.49 sec in the *p*CPA-treated group ($p < 0.05$; [**F6C**]). The changes in both of these parameters lead to an overall decrease in frequency of sucrose-evoked bites, with values decreasing from 14.1+ 0.69 bites/min to 9.3 + 1.14 bites/min in *p*CPA-treated animals ($p < 0.01$; [**F6D**]).

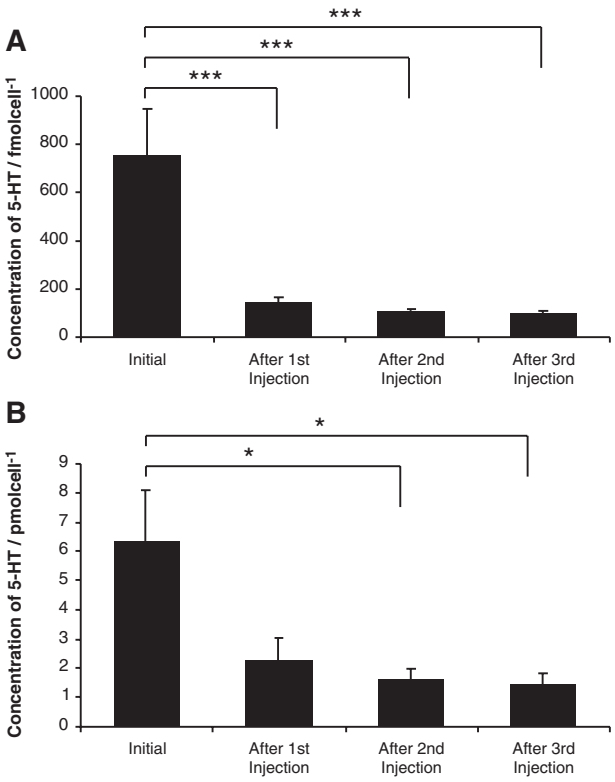
Discussion

An LCEC system was set up to separate and analyze the concentrations of serotonin, 5-HIAA and a variety of other monoamines and their metabolites in CNS tissue from the pond snail, *Lymnaea stagnalis*. The system was optimized allowing measurement of serotonin and 5-HIAA concentrations in the axon and terminals of a single neurone, the CGC. Treatment with *p*CPA caused a significant decrease in the concentrations of both serotonin and 5-HIAA in all regions of the CNS. Previous work in mammals has shown that decreases in serotonin and 5-HIAA were seen following either repeated injections spaced 12 hours apart or single injections using much higher doses than used in the present study (13, 14). In this study significant decreases were seen in both serotonin and 5-HIAA concentrations following a single injection, with no further significant reduction seen following the second and third injections. This difference may reflect the lack of a blood/brain barrier in the snail, allowing better access of *p*CPA to the serotonergic neurons in the CNS, or maybe due to decreases in the rate of metabolism of the *p*CPA in the snail.

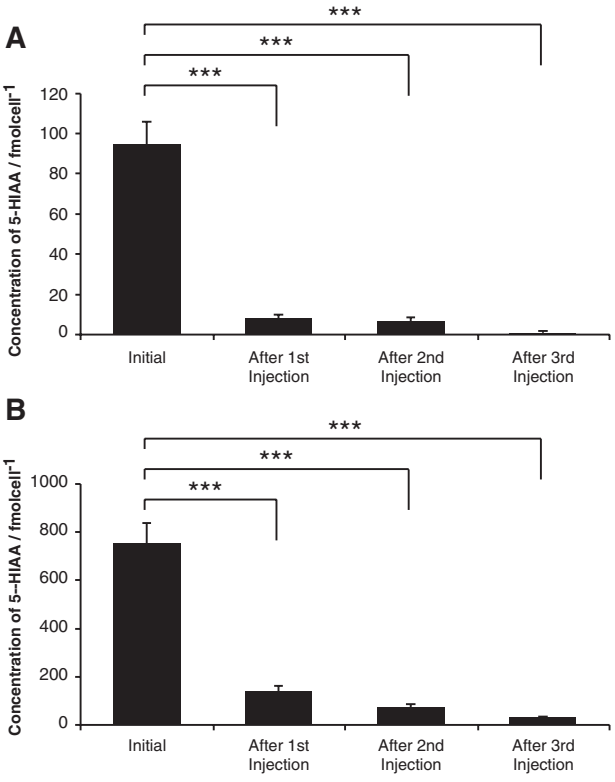
The ability of *p*CPA to decrease serotonin concentrations in the snail's CNS was further supported by immunohistochemical studies. Antibody-labelling of serotonin-containing neurons in the *Lymnaea* CNS of saline-injected animals showed a characteristic distribution of labeled cell bodies and processes consistent with previous studies in this animal (4). Application of *p*CPA caused a complete abolition of labeling, indicating that serotonin concentrations were below the detection limits of the antibody. Although it is impossible to give a precise serotonin concentration at which the antibody looses its sensitivity, antibody detection is completely lost when serotonin concentrations fall below 4 pmoles in the buccal ganglia.

The observed decrease in serotonin and 5-HIAA concentrations was associated with a reduction in the number

F3. Concentrations of serotonin obtained before and after injection with *p*CPA. A – cerebral ganglia and B – buccal ganglia ($n = 4$, $*p < 0.05$ and $***p < 0.001$).



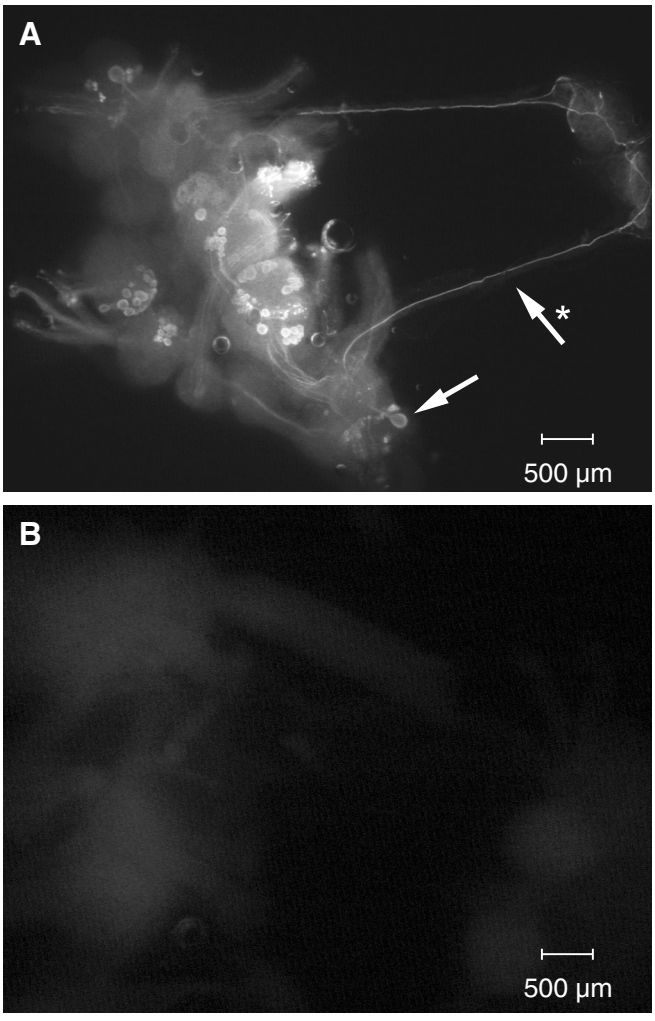
F4. Concentrations of 5-HIAA obtained before and after injection with *p*CPA. A – cerebral ganglia and B – buccal ganglia ($n = 4$, $***p < 0.001$).



of animals that responded to 0.01 M sucrose. In addition, of those animals that did respond to sucrose, *p*CPA caused an increase in both the bite duration and interbite interval and a consequential decrease in the frequency of sucrose-evoked bites. Previous work in *Lymnaea* has documented that the serotonergic CGCs are incapable of initiating a feeding rhythm but have an important modulatory role allowing the feeding CPG to respond to a food stimulus (gating role) and can also regulate the frequency of fictive-feeding (frequency controlling role [2, 3]). Thus, deficits in the function of the CGCs caused by *p*CPA depleting the cells of serotonin could account for the increase in the number of non-responding animals, and for the decrease in sucrose-evoked bites seen in the *p*CPA-treated animals. Additional support for the *p*CPA-induced decreases in serotonin being responsible for the observed changes in feeding behaviour comes from work showing short-term feeding responses in *Lymnaea* that had been pretreated with the serotonergic neurotoxin, 5,6, dihydroxytryptamine (5,6-DHT) (5). 5,6-DHT was shown to decrease serotonin concentrations in the *Lymnaea* CNS and also caused a marked increase in the numbers of non-responding animals, an increase in the interbite interval, and a decrease in the number of sucrose-evoked bites consistent with the data presented in this paper. Thus, two treatments that reduce serotonin concentrations by different mechanisms had similar behavioral effects. Work in a closely related mollusc, *Aplysia*, demonstrated that destruction of the serotonergic metacerebral cells (homologues of the CGCs) caused similar decreases in frequency of biting movements and a decrease in the interbite interval, further substantiating the role of serotonin in regulating feeding behaviour (15).

In summary, LCEC analysis has demonstrated that *p*CPA is capable of decreasing serotonin and 5-HIAA concentrations in the CNS of the pond snail, *Lymnaea stagnalis*, following injection of a single dose equivalent to 150 mg/kg. These decreases were confirmed using standard whole-mount immunohistochemical techniques; however, the absence of labeling in the *p*CPA-treated animals inferred that the LCEC technique was more sensitive than the antibody for detection of serotonin. Based on previous data, decreases in serotonin concentrations in the CNS were responsible for the observed changes in feeding behavior.

F5. *p*CPA reduces anti-serotonin labeling in the CNS of the pond snail, *Lymnaea*. Typical photomicrographs of the CNS of the pond snail, *Lymnaea stagnalis* labelled with an anti-serotonin antibody and visualized using an FITC-conjugated secondary antibody. A) Photomicrograph of the CNS from a saline-injected animal. Clear labelling can be seen in a large number of neurons and processes throughout the CNS. Specifically, labelling can be seen in the CGC (arrows), with the axons of the CGCs clearly visible in the cerebrobuccal connective (*) projecting to the buccal ganglia (bg). Scale bar 500 µm. B) Photomicrograph of the CNS of a *p*CPA-injected animal. Only background fluorescence is visible. Scale bar 500 µm.



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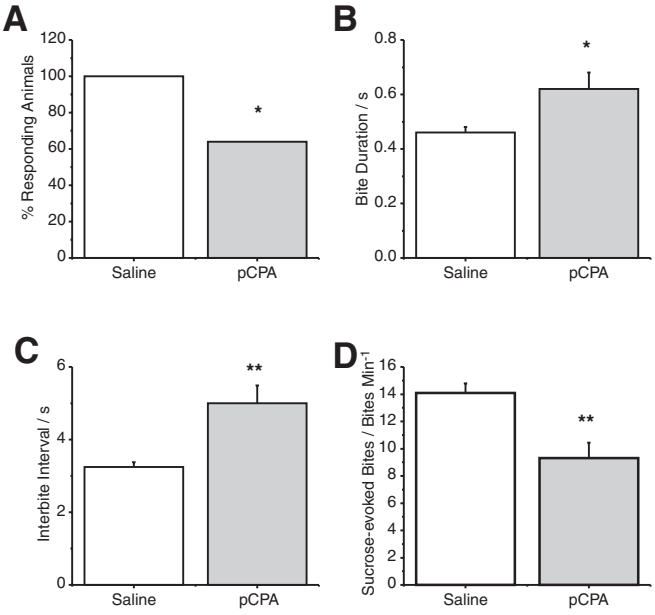
F6. *p*CPA alters the feeding behaviour of the pond snail, *Lymnaea*. A-D Bar graphs comparing feeding parameters from both saline and *p*CPA-injected animals. A) *p*CPA treatment significantly decreases the % responding animals. Of the animals that did respond *p*CPA increased bite duration (B) and interbite interval (C) and reduced frequency of sucrose-evoked bites (D). (*$p < 0.05$; **$p < 0.01$, n = 12 for all groups).



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