Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Behavioural and Biochemical Changes in the Feeding System of Lymnaea Induced by the Dopamine and Serotonin Neurotoxins 6-hydroxydopamine and 5,6-dihydroxytryptamine

G. Kemenes, L. Hiripi and P. R. Benjamin

Phil. Trans. R. Soc. Lond. B 1990 **329**, 243-255
doi: 10.1098/rstb.1990.0168

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 1990 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Behavioural and biochemical changes in the feeding system of *Lymnaea* induced by the dopamine and serotonin neurotoxins 6-hydroxydopamine and 5,6-dihydroxytryptamine

G. KEMENES[1,2], L. HIRIPI[2] AND P. R. BENJAMIN[1]†

[1] *Sussex Invertebrate Neuroscience Group, School of Biology, University of Sussex, Falmer, Brighton BN1 9QG, U.K.*
[2] *Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany H-8237, Hungary*

[Plates 1 and 2]

CONTENTS

	PAGE
1. Introduction	244
2. Materials and methods	244
(*a*) Injection of the snails with 6-hydroxydopamine and 5,6-dihydroxtryptamine	245
(*b*) Behavioural feeding tests	245
(*c*) Measurement of serotonin, dopamine and false transmitter levels in neural tissue	245
(*d*) Do 6-hydroxydopamine and 5,6-dihydroxytryptamine cause release of dopamine and serotonin from snail ganglia?	245
(*e*) Do the neurotoxins inhibit uptake of dopamine and serotonin into snail ganglia?	246
3. Results	246
(*a*) Changes in feeding responses to sugar following drug injection	246
(*b*) Drug-induced changes in dopamine and serotonin fluorescence	247
(*c*) Drug-induced changes in levels of dopamine and serotonin measured by HPLC	249
(*d*) Mechanisms of actions of the drugs	250
4. Discussion	252
References	254

SUMMARY

The neurotoxins 5,6-dihydroxytryptamine (5,6-DHT) and 6-hydroxydopamine (6-OH–DA) were used to examine the role of monoamines in the feeding system of the snail *Lymnaea stagnalis*. Biting responses to sucrose were monitored up to 25 days after injection with drugs. Cerebral and buccal ganglia and cerebro-buccal connectives from the same groups of snails were examined for changes in serotonin and dopamine levels by high performance liquid chromatography and the glyoxylic acid histo-fluorescence technique.

Twelve to eighteen days after injection with 5,6-DHT only 57% of the snails responded to sucrose with biting movements, compared with 98% of controls. Those that did respond had a longer latency to the first bite and the bites were of shorter duration and occurred at a lower rate when compared with controls. This was accompanied by a 39% drop in 5-HT levels in the cerebro-buccal commissure and nerves and a loss of fluorescence in the axons of the paired cerebral giant cells, the main serotonergic neurons involved in feeding. Earlier behavioural effects of injecting 5,6-DHT at 20 min and 3–4 h after injection could not be explained by specific changes in 5-HT levels. Recovery of both behavioural response and serotonin levels occurred between 22–25 days after injection.

Injection of 6-OH-DA also inhibited feeding responses but the effects were quicker (1–3 days) and more dramatic, with only 40% of snails showing any biting response to food compared with a 98% response in controls. Reduction in dopamine (DA) levels of 40%, together with a loss of DA fluorescence in nerve fibres accompanied the reduction of behavioural responsiveness. An early (3–4 h) effect of 6-OH-DA injection could not be correlated with a specific reduction in DA levels. Behavioural responses and DA levels returned to normal by 4–7 days after injection.

Both neurotoxins inhibited uptake of their target monoamines and this appeared to be the main mechanism for depleting 5-HT and DA. Early effects of neurotoxin injection probably directly inhibited monoamine uptake, whereas long-term inhibition was a secondary effect because of degeneration of nerve fibres. Neither neurotoxin caused release of monoamines.

† To whom correspondence should be addressed.

Phil. Trans. R. Soc. Lond. B (1990) **329**, 243–255
Printed in Great Britain

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Philosophical Transactions: Biological Sciences.* www.jstor.org

Downloaded from rstb.royalsocietypublishing.org

Results from both neurotoxins suggest that monoamines play an important role in the initiation and maintainance of the feeding response, the consummatory phase of feeding. Neither neurotoxin prevented the occurrence of the initial appetitive phase of feeding when snails orientate towards food in the experimental chamber.

1. INTRODUCTION

The monoamines dopamine (DA) and serotonin (5-HT) are important in the neural circuits controlling feeding movements of gastropod molluscs (see Pentreath *et al.* 1982; Walker 1986). Both substances are present in the buccal and cerebral ganglia of the most commonly used species and can be localized to the somata or fibres of neurons of the feeding circuitry within these ganglia (Sakharov & Zs-Nagy 1968; Weinreich *et al.* 1973; Granzow & Rowell 1981; Trimble *et al.* 1984; Straub & Kuhlman 1984; Audesirk 1985; Wieland *et al.* 1987; Kemenes *et al.* 1989).

The detailed role of DA is not known but in at least two species (*Helisoma* and *Limax*) its application to the ganglia of the CNS elicited patterns of electrical ativity which probably underlie feeding in the intact animals (Wieland & Gelperin 1983; Trimble & Barker 1984). The interpretation of this data is made difficult by the lack of knowledge of the precise neuronal target of the DA and the fact that neurons containing this transmitter are widespread in the feeding centres (Wieland & Gelperin 1983; Trimble *et al.* 1984; Audesirk 1985).

Serotonin is more restricted in its distribution, with few or no 5-HT-containing neurons occurring in the buccal ganglia where the central pattern-generating circuitry is located (Ono & McCaman 1984; Audesirk 1985; Gadotti *et al.* 1986; Kemenes *et al.* 1989; Hernådi *et al.* 1989). The main serotonergic neurons involved in feeding are a pair of giant neurons which are located in the cerebral ganglia but have synaptic and modulatory actions on neurons of the buccal ganglia or muscles of the buccal mass (reviews Pentreath *et al.* (1982); Benjamin (1983)). The function of these cerebral giant cells (CGCs) (also called metacerebral giant cells in some species) has been extensively investigated in several different species (Granzow & Rowell 1981; Pentreath *et al.* 1982). They are usually considered to be modulatory neurons with a facilitatory role, necessary for full activation of the feeding system. In *Aplysia* it is proposed that these neurons are part of the neural system underlying feeding 'arousal' (Weiss *et al.* 1982). Studies on the pharmacological role of 5-HT in the feeding circuitry are only just beginning but it is already becoming apparent that its actions are complex and difficult to interpret in terms of a simple model. For instance in the snail, *Lymnaea*, 5-HT applied to central ganglia at low concentrations facilitate the production of feeding motor patterns, but at high concentrations will reduce the burst frequency or even inhibit motoneuron activity (Tuersley & McCrohan 1988). Direct effects of serotonin on muscles of the buccal mass are also known in the related mollusc *Aplysia* (Weiss *et al.* 1978). Muscle contractions due to motoneuron actions were facilitated by serotonin directly activating second messenger pathways (Mandelbaum *et al.* 1978).

The main problem with the pharmacological studies so far carried out was that it was very difficult to relate the results to the normal feeding behaviour of the mollusc being studied. Almost all the experiments had to be carried out in isolated or semi-isolated preparations to allow intracellular recording (Weiss *et al.* 1982). Therefore, we decided to use a different approach to compare the roles of DA and 5-HT, one often used in vertebrates. This was to reduce the levels of monoamines in intact animals and then to follow changes in feeding behaviour.

A good method of depleting monoamines is to inject live animals with the neurotoxins 6-hydroxydopamine (6-OH-DA) or 5,6-dihydroxytryptamine (5,6-DHT) to deplete DA or 5-HT, respectively (Berry *et al.* 1974; Baumgarten *et al.* 1975; Sathanantan 1976; Elekes *et al.* 1977; Hiripi *et al.* 1977). We chose the pond snail, *Lymnaea stagnalis*, for study because both the feeding behaviour and the neuronal circuitry underlying feeding were known in considerable detail (Benjamin 1983; Benjamin & Elliott 1989). An understanding of the role of serotonin and dopamine neurons in this species was facilitated by the recent production of maps of serotonin and dopamine-containing neurons (Kemenes *et al.* 1989; figure 3). It was important to measure the actual changes in levels of 5-HT and DA resulting from drug injection, as well as behaviour, and so a parallel biochemical analysis was done with experiments aimed at determining whether 6-OH-DA and 5,6-DHT affected uptake and release of DA and 5-HT.

The results were complex, due to early non-specific effects of the drugs, but it was clear that at periods of time when 5-HT levels were reduced in the fibres of the CGCs, fewer experimental snails than controls responded to food and those that did took longer to respond and had bites of shorter duration and lower frequency compared with controls. This was significant because suppressing CGC electrical activity in electrophysiological experiments produced similar reductions in frequency and duration of bursts in feeding motoneurons (Benjamin & Elliott 1989). Reduction of DA levels led to a dramatic reduction in the number of animals responding to food, again suggesting an important role for monoamines in the activation of the feeding system.

2. MATERIALS AND METHODS

Pond snails, *Lymnaea stagnalis*, were obtained from animal suppliers in the U.K. or collected locally on the Tihany Peninsula in Hungary. No differences in the gross anatomy, behaviour or physiology of specimens of these two geographically distinct populations could be detected. The snails were maintained under standard laboratory conditions (12:12 L:D cycle, 20 ± 2 °C) in aerated standard snail water (Thomas *et*

Downloaded from rstb.royalsocietypublishing.org

al. 1975) and normally fed on a lettuce diet except for three days before the experiments when they were completely starved.

(*a*) *Injection of the snails with 6-hydroxydopamine and 5,6-dihydroxytryptamine*

In separate experiments two groups of 50 snails of 1.5–3.0 g body mass were injected with 25–50 µl solutions of either 5,6-dihydroxytryptamine creatinine sulphate (5,6-DHT) (SIGMA) or 6-hydroxydopamine HCl (6-OH-DA) (Sigma or CALBIOCHEM). The neurotoxins were dissolved in HEPES-buffered saline (Benjamin & Winlow 1981) containing 0.5 mg ml^{-1} ascorbic acid as an antioxidant. The concentration of neurotoxins in the injection solution was 12 mg ml^{-1} and this was equivalent to a 200 mg kg^{-1} dose of the drugs after injection into the snail's body. The syringe needle was inserted into the ventral surface of the anterior part of the foot so that after penetration of the skin the drugs were injected into the haemolymph in the vicinity of the CNS. A group of 50 control snails for each of the two experimental groups was injected with the vehicle (saline + ascorbic acid) alone. To compare the possible immediate effects of injection of the false transmitters with those of the real ones, two groups of 20 snails were separately injected either with DA or 5-HT. The transmitters were dissolved and injected the same way as the neurotoxins to give the same final dose.

(*b*) *Behavioural feeding tests*

Feeding tests were done on control and experimental groups from 25 min up to 28 days after the injection with drug or the vehicle control. Feeding movements (opening of mouth, rasping and closing of mouth) of an individual snail were elicited by pipetting sucrose (0.001 mol l^{-1} final concentration) into an observational chamber and permanently recorded using a penrecorder (Kemenes *et al.* 1986). Snails that did not start feeding within 2 min after the sucrose stimulus were classed as 'non-responding'. A minimum of 7 and a maximum of 25 snails were tested in each experiment and the mean latency to first bite, mean duration of the first 20 individual bites and the mean of the first 20 interbite intervals were computed. One-tailed paired and unpaired *t*-tests (Minitab Statistics Package) were performed on the data to establish significance levels for differences between control and experimental groups.

(*c*) *Measurement of serotonin, dopamine and false transmitter levels in neural tissue*

(i) *Histofluorescence*

We used the histochemical glyoxylic acid procedure (Axelsson *et al.* 1973; Barber 1982) modified for whole mounts in *Lymnaea* by Audesirk (1985) to locate DA and 5-HT in *Lymnaea* neural tissue. The technique stains up yellow (5-HT) and green (DA) fluorescing fibres and cell bodies in the cerebral and buccal ganglia where neurons of the feeding circuit are located. The green fluorescence is not unique to DA and could also indicate noradrenaline. However, we chose certain fibres which we know from separate experiments do not contain biochemically detectable amounts of noradrenaline but stain positively with an antibody to dopamine (Elekes *et al.* in preparation) so we were confident that we were analysing dopamine rather than noradrenaline. Figure 3*b* (within the box) shows the part of the CNS where the main glyoxylic acid analysis was carried out. It contained the axons of the serotonergic CGCs (Kemenes *et al.* 1989) and fibres of DA cells from unidentified neurons of the buccal ganglia. Ganglia from the behaviourally tested animals (control and experimental) were sampled from 1 to 28 days after injection and on each day a minimum of five snails were subjected to the glyoxylic acid assay. Only the presence or absence of fluorescence was noted and any change from one day to another was considered significant only if it occurred in at least 75% of animals. The loss of the fluorescence does not necessarily show complete depletion of the monoamines, rather it shows that the concentration decreased below the sensitivity of the glyoxylic acid technique.

(ii) *High Performance Liquid Chromatography*

More quantitative analysis of DA and 5-HT levels together with neurotoxins was carried out by reverse phase (HPLC) high performance liquid chromatography following the procedures of Saller & Salama (1984). The buccal ganglia, cerebro-buccal connectives together with the latero- and ventro-buccal nerves and cerebral ganglia were analysed as three separate pieces (anatomical arrangement of these structures shown in figure 3).

Tissue samples were homogenized in 40 volumes (mass by volume) of mobile phase and centrifuged at 30000 *g* for 20 min at 4 °C. Portions of the clear supernatant were subjected to HPLC on a reverse phase C18 Nucleosil column (15 cm × 4.6 mm, 5 µm Chrompack) with a mobile phase of 0.1 M sodium phosphate, 1 mM EDTA, 1 mM sodium octane-sulphonic acid, 10% acetonitrile, adjusted to pH 4.5 with citric acid, at a flow rate of 1 ml min^{-1}. The elution of neurotoxins and transmitters was monitored by electrochemical detection at 0.7 V. Quantitative determinations were made by comparisons with appropriate standards. The transmitter concentrations were expressed in picomoles per tissue and then the results from the experimental tissues were expressed as a percentage of the same day's vehicle controls. Each result is the mean of five parallel measurements. Statistical analysis of the differences between experimental snails and same day's vehicle controls was performed by two-sample unpaired two-tailed *t*-tests.

(*d*) *Do 6-hydroxydopamine and 5,6-dihydroxytryptamine cause release of dopamine and serotonin from snail ganglia?*

In these experiments we wished to find out whether the presence of high levels of the neurotoxins, likely to occur following injection into the snail, caused release of monoamines from the *Lymnaea* nervous tissue. This

Downloaded from rstb.royalsocietypublishing.org

might contribute to any behavioural effect of drug injection.

The whole CNS (including the buccal ganglia and nerves) was quickly dissected from 24 untreated snails. The 24 brains were separated into four experimental and two control groups each consisting of four brains. The groups were separately incubated in 2 ml 10^{-7} mol l^{-1} ^{3}H-DA or ^{3}H-5-HT. The ^{3}H-amines were dissolved in HEPES saline containing 0.5 mg ml^{-1} ascorbic acid. After incubation at 25 °C for 1 h the ganglia were rinsed twice in 20 ml saline and placed in a 1 ml chamber. Superfusion was done with saline at 1 ml min^{-1} flow rate. The first fractions were collected after 10 min of superfusion. Twenty fractions were collected and analysed in each group. In the experimental groups, during the collection of the 6th to 10th fraction, the superfusing medium was changed to another fluid of the same composition except that either the K^{+} concentration was raised to 100 mmol l^{-1} or it contained 6-OH-DA or 5,6-DHT in 10^{-5} mol l^{-1} concentration. The radioactivity of the collected fractions in the control as well as experimental groups was measured by liquid scintillography.

(*e*) *Do the neurotoxins inhibit uptake of dopamine and serotonin into snail ganglia?*

As well as causing release, the injected drugs could also be inhibiting the uptake of the monoamines. This was investigated in two types of experiments. In the first type the uptake inhibiting direct effects of the presence of different concentrations of the neurotoxins was examined. In the second one we wished to find out whether the neurotoxins caused persistent uptake impairment in the neural tissue, following more prolonged exposure to the drugs. This would be the result of damage to nerve terminals and axons.

(i) *Concentration-dependent effects*

This examined the direct short-term effects of bathing whole brains in different concentrations of 6-OH-DA or 5,6-DHT, while monitoring DA or 5-HT uptake, respectively.

For controls, 12 whole brains were incubated at 25 °C for 10 min in 2 ml/brain HEPES saline containing ascorbic acid (0.5 mg ml^{-1}) and 10^{-8} mol l^{-1} ^{3}H-DA or ^{3}H-5-HT. In addition to the vehicle and the tritiated monoamines, the incubation mixture of 24 experimental whole brains contained 6-OH-DA or 5,6-DHT at concentrations from 10^{-6} to 10^{-3} mol l^{-1}. At the end of the incubation the brains were recovered with forceps and rinsed twice in 20 ml per brain HEPES saline. After the washing the brains were dissolved in 0.5 ml per brain tissue solubilizer (Protosol, NEN) then 10 ml per brain toluene-based scintillation fluid was added and the radioactivity was measured. The non-specific uptake was measured at 0 °C in Na^{+}-free solution. The specific uptake was defined as the uptake at 25 °C in normal solution minus the uptake at 0 °C in Na^{+}-free solution. The 50% inhibitory concentrations (IC_{50}) were determined graphically from log-probit plots.

(ii) *Persistent changes induced by in vitro and in vivo exposure to the neurotoxins*

Uptake measurements were done on isolated whole brains exposed *in vitro* to the effect of neurotoxins for a period of time estimated to be equivalent to 3–4 h *in vivo* exposure. In these experiments the *in vitro* test developed by Björklund *et al.* (1975) was used. Ten freshly dissected brains were exposed for 1 h to 10^{-5} mol l^{-1} concentration of either 6-OH-DA ($n = 5$) or 5,6-DHT ($n = 5$) at 25 °C. Ten control brains were incubated in 2 ml of brain HEPES saline containing ascorbic acid (0.5 mg ml^{-1}). After the exposure to drugs or vehicle the brains were rinsed in 20 ml physiological solution then the uptake of ^{3}H-5-HT or ^{3}H-DA was measured in both controls and experimentals as described before.

In vitro uptake measurements were also done on whole brains dissected from snails injected with vehicle ($n = 5$) or neurotoxins *in vivo* three days (6-OH-DA injection, $n = 5$) and 14 days (5,6-DHT injection, $n = 3$) before the biochemical assay.

3. RESULTS

(*a*) *Changes in feeding responses to sugar following drug injection*

(i) *Snails injected with 6-hydroxydopamine*

6-OH-DA had an immediate severe paralysing effect on the snails that lasted for about 1 h. In this period, the only behavioural response that could be induced was the opening of the pneumostome (entrance to lung) when it was artificially exposed to the surface of the water. None of the snails showed any response to sucrose, which normally induced a 98% response in preinjected snails (figure 1). Injection of DA produced the same paralysing effect so it appeared that 6-OH-DA was mimicking the effect of the natural transmitter.

Three to four hours after injection the 6-OH-DA injected snails had recovered from general paralysis and showed normal locomotory and breathing behaviour and withdrew from tactile and shadow stimuli. However, only 27% of the snails responded to sucrose compared with 98% in vehicle injected controls (figure 1). The latency to bite was also slightly greater than in controls ($p < 0.125$). No difference was found in the other parameters measured.

Between 1–3 days after injection the experimental group continued to show reduced tendency to respond to food (figure 1). In addition, those snails that did respond (40%) had an increased interbite interval ($p < 0.01$). Given that there was no difference in the duration of individual bites (figure 1) this meant that the snails were feeding at a lower rate.

After 4–7 days a complete recovery of response to sugar had occurred and experimental snails returned to pre-injection response levels and resembled same-day controls (figure 1).

(ii) *Snails injected with 5,6-dihydroxytryptamine*

5,6-DHT induced an immediate change in general behaviour of the snails which lasted for about 20 min. Rapid uncoordinated bending movements were the

Downloaded from rstb.royalsocietypublishing.org

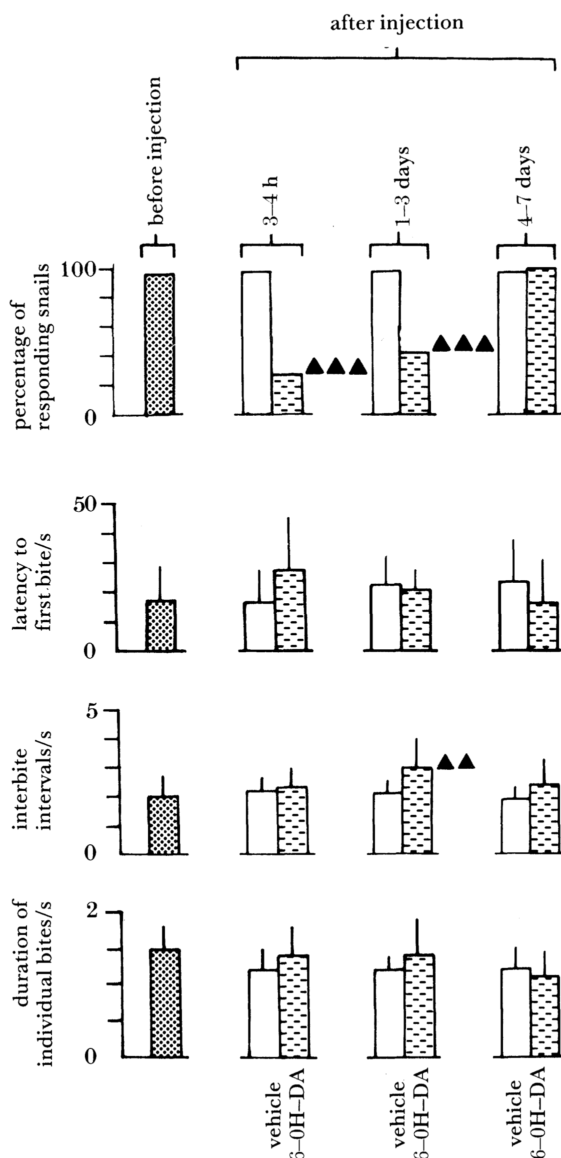


Figure 1. Changes in four different parameters of the feeding response to sucrose in *Lymnaea* after injection with 6-hydroxydopamine (6-OH-DA) (dashed bars) or vehicle control (empty bars). Pre-injection values shown by dotted bars. Solid triangles indicate statistically significant differences between experimental and same-time vehicle injected control values (one-tailed t-tests, $n = 7$–25) (▲▲, $p < 0.01$; ▲▲▲, 0.001).

most obvious feature of this abnormal behaviour and the animal was incapable of carrying out normal locomotory movements or responding to food. Injection of 5-HT induced the same behaviour so it was likely that 5,6-DHT had an immediate pharmacological effect similar to that of 5-HT. In the period afterwards (20–30 min) the snails' response to food appeared to be facilitated. Like pre-injected snails, almost all responded to sugar (figure 2) but they responded more rapidly (reduced latency) compared with either control or pre-injected groups. At the same time there was a slight tendency for the duration of bites to be reduced compared with vehicle injected controls. However, the response to food in the experimental group was peculiar in one specific aspect. The snails only responded with a limited number of bites (7–18) and then they stopped despite the fact that the food was continuously present. Normally, snails carried out feeding movements for as long as they were left in the experimental dish or for the normal 20 responses, which were routinely monitored. The interpretation of the data from this 20–30 min period was made difficult by the results from the control group which was injected with the ascorbic acid vehicle alone. The vehicle or the injection-induced trauma inhibited responses to food. Thus significantly fewer snails responded and those that did responded more slowly compared with pre-injection controls. This effect of the vehicle or the injection itself was not seen at any of the later stages of the experiment and from 3–4 h until the end of the observation period (25 days) the controls showed normal behaviour (figure 2).

Experimental snails at 3–4 h and again when they were tested at 12–18 days showed a reduction in responsiveness to sucrose (figure 2). The percentage of snails responding to food was reduced significantly (36 % in the 3–4 h group and 57 % in the 12–18 days group compared with vehicle injected controls (98 %)). Those that did respond were slower to respond (latency to first bite increased), had shorter bites and greater interbite intervals ($p < 0.01$–0.001) (figure 2). That these changes occurred at 12–18 days was particularly significant because this was shown to be accompanied by reductions in serotonin levels (see below). In between these periods of reduced responsiveness, the experimental animals were similar to controls (2–9 days, see figure 2) and after 22 days the behaviour of the snails again returned to normal except for a slight but statistically significant ($p < 0.05$) reduction in the duration of the bites which persisted from the 12–18 day period.

An interesting general observation was that at periods when the biting responses to food were inhibited or reduced by 5,6-DHT, the increased general exploratory activity associated with the introduction of sucrose into the experimental dish still persisted. The same phenomenon was observed in the snails injected with 6-OH-DA. This implies that only the consumatory phase of feeding was affected by toxins and not the initial appetitive phase when the snails were searching for food (Kupfermann 1974; Benjamin 1983).

(*b*) *Drug-induced changes in dopamine and serotonin fluorescence*

Serotonin (yellow) and dopamine (green) fluorescent fibres and cell bodies were examined for more than 30 days after injection with either 6-OH-DA or 5,6-DHT and compared with vehicle-injected controls. Throughout the period of the experiment no diminution of fluorescense was seen in the cell bodies of DA or 5-HT cells (figure 3) with either drug treatment (figure 4, plate 1). These examples of cell bodies from 1 and 15 day snails injected with 6-OH-DA (figure 4*a*, *b*) and 5,6-DHT (figure 4*c*, *d*), respectively, contrast strongly with the absence of fluorescence seen in fibres on the same days (figure 5*c*, *e*, plate 2). Pre-injection

Downloaded from rstb.royalsocietypublishing.org

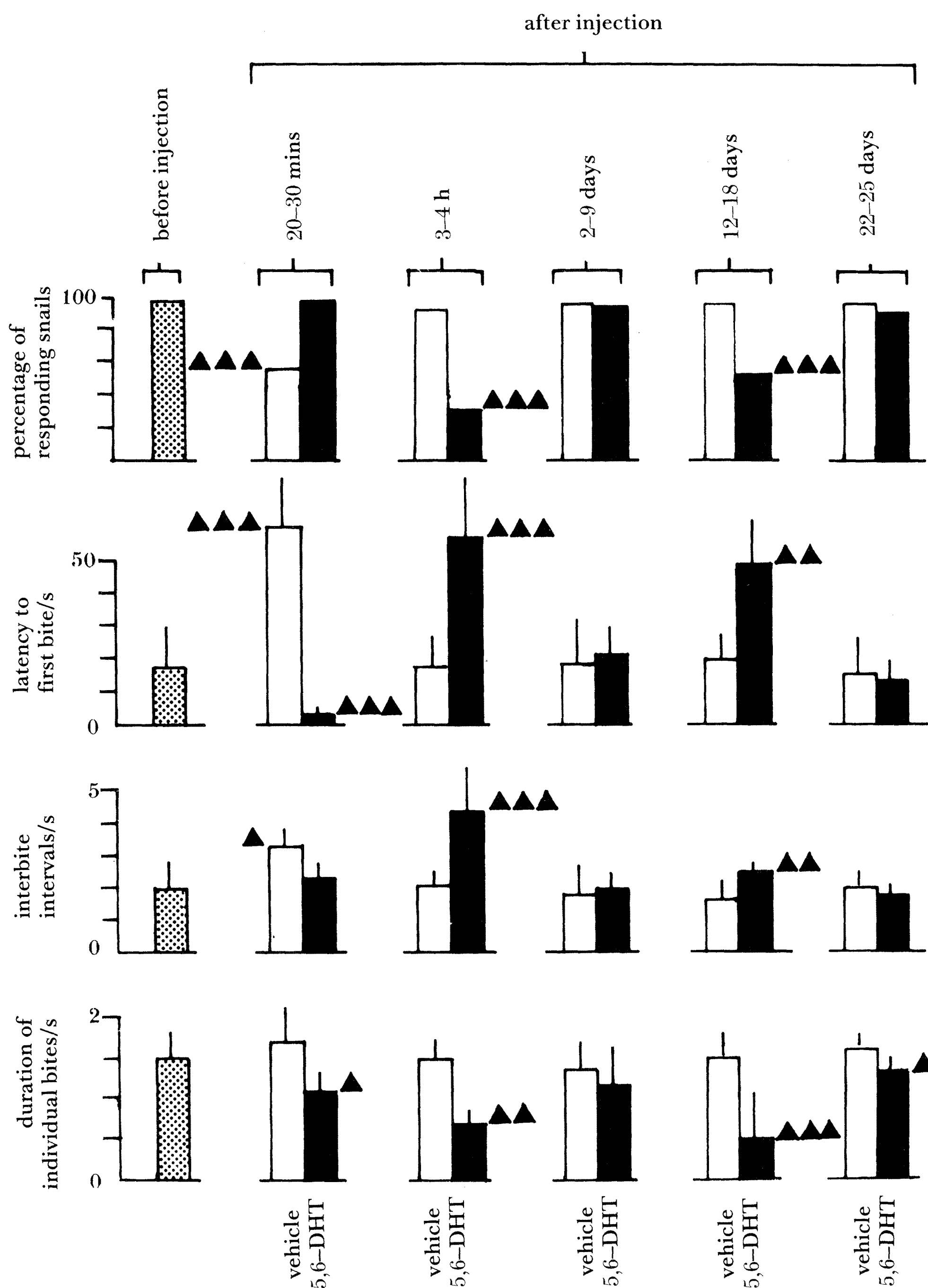


Figure 2. Changes in four different parameters of the feeding response to sucrose in *Lymnaea* following injection with 5,6-dihydroxytryptamine (5,6-DHT) (solid bars) or vehicle control (empty bars). Pre-injection values shown by dotted bars. Solid triangles to the right of the bars indicate statistically significant differences between experimental and same-time vehicle injected control values (two-sample, unpaired t-tests, $n = 7$–25). Solid triangles to the left of the bars indicate statistically significant differences between vehicle control and pre-injection control values (one-sample, paired t-tests, $n = 20$) (▲, $p < 0.05$; ▲▲, $p < 0.01$; ▲▲▲, 0.001).

controls (figure 5*a*, plate 2) or vehicle injected controls (figure 5*b*, *d*, plate 2) showed normal fluorescence. This implied that the monoamines had been lost from or their levels were greatly reduced in the axons and/or the axons themselves had disappeared. Our detailed analysis of fibres concentrated on the part of the cerebrobuccal connective near the latero- and ventro-buccal nerves (within the square shown in figure 3*b*). This contained the axons of the CGCs, the main serotonin cells in the feeding system, and a bundle of DA fluorescing axons which probably originated from the extensive population of cells in the buccal ganglia (figure 3*a*). The disappearance (and subsequent reappearance) of the two types of monoamine fluorescence followed a different timecourse. After 6-OH-DA injection the DA fluorescence was clear until the first day after injection but by the end of the first day no DA (green) fluorescing fibres could be seen (figure 5*c*, plate 2) until they reappeared at day four (figure 5*f*, plate 2). Serotonin (yellow) fibres persisted throughout

Downloaded from rstb.royalsocietypublishing.org

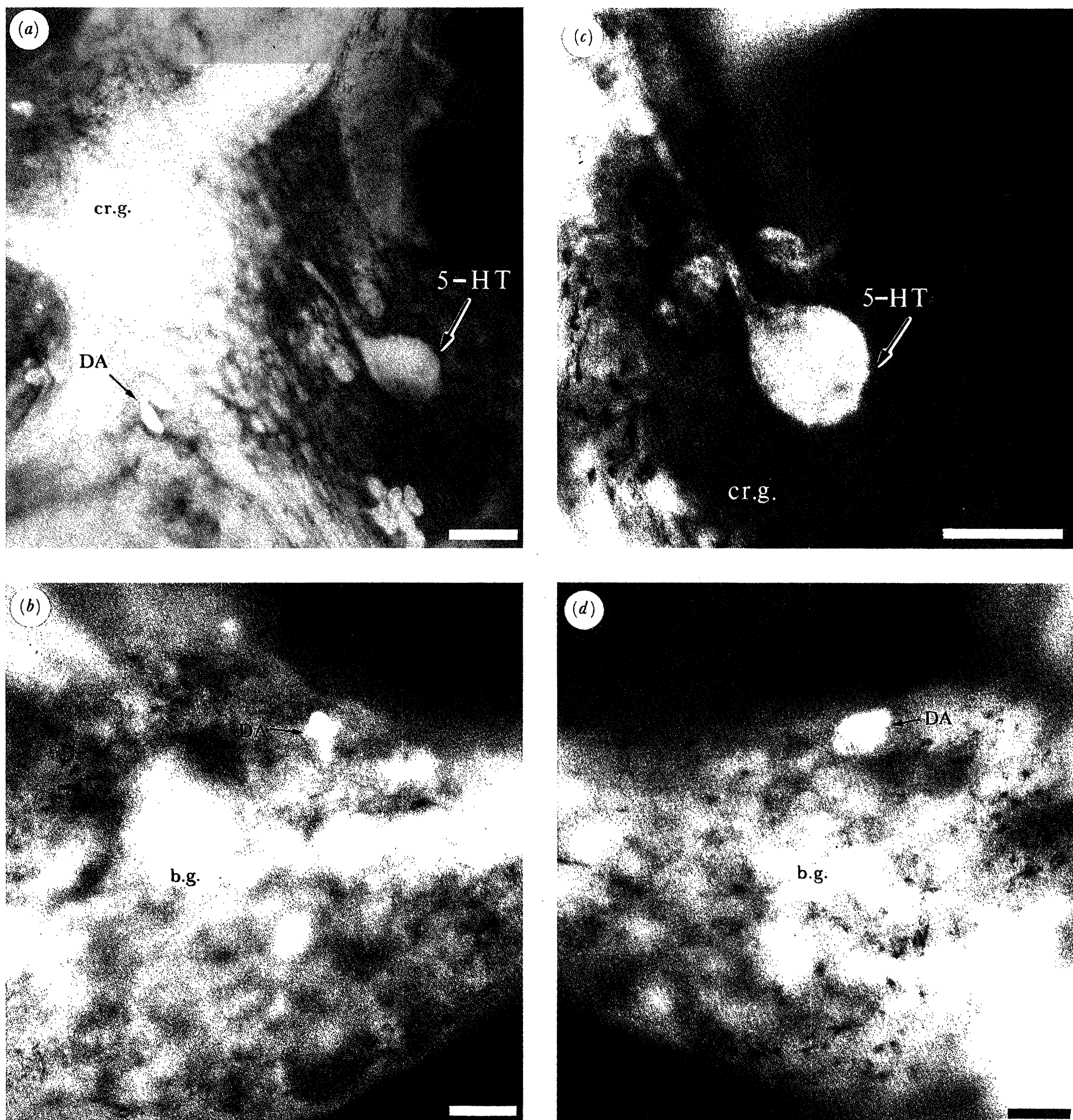


Figure 4. Serotonin (5-HT), yellow, and dopamine (DA), green, fluorescent cell bodies in the cerebral (cr.g.) and buccal ganglia (b.g.) of *Lymnaea* revealed by the glyoxylic acid method. The largest cell in the cerebral ganglion (arrowed 5-HT) is the cerebral giant cell, one of a pair of large serotonin-containing neurons involved in the feeding circuit. None of the dopamine cells are identifiable. Injection of snails with 6-hydroxytryptamine (6-OH-DA + 1 day) (figure 4*a*,*b*) or with 5, 6-dihydroxytryptamine (5, 6-DHT + 15 days) (figure 4*c*,*d*) had no effect on the fluorescence of the cell bodies. On the same days, clear reductions in axonal fluorescence occurred (figure 5*c*,*e*, plate 2). (Calibration bars: (*a*,*c*), 100μm; (*b*,*d*), 30μm.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

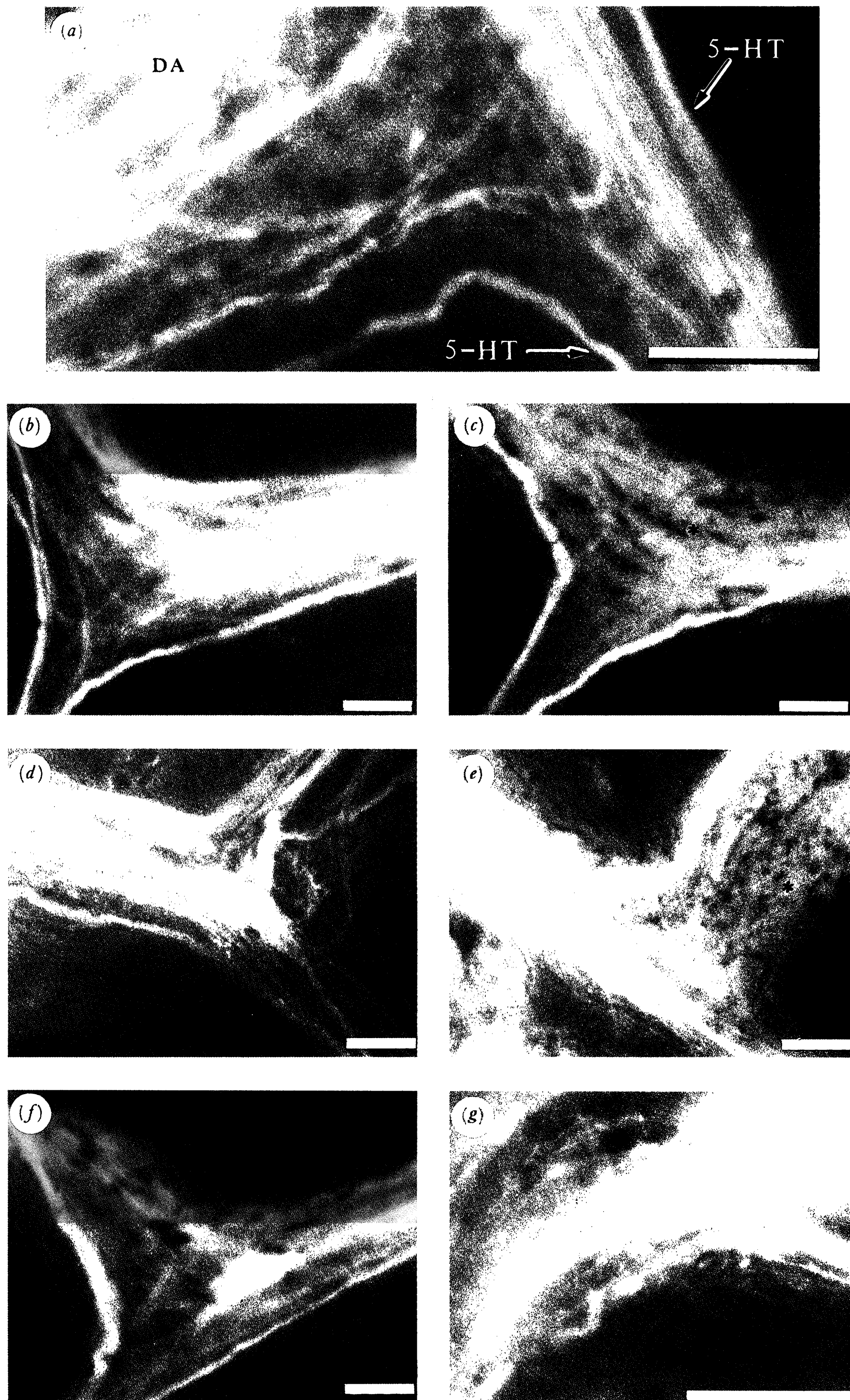


Figure 5*a* – *g*. For description see opposite.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

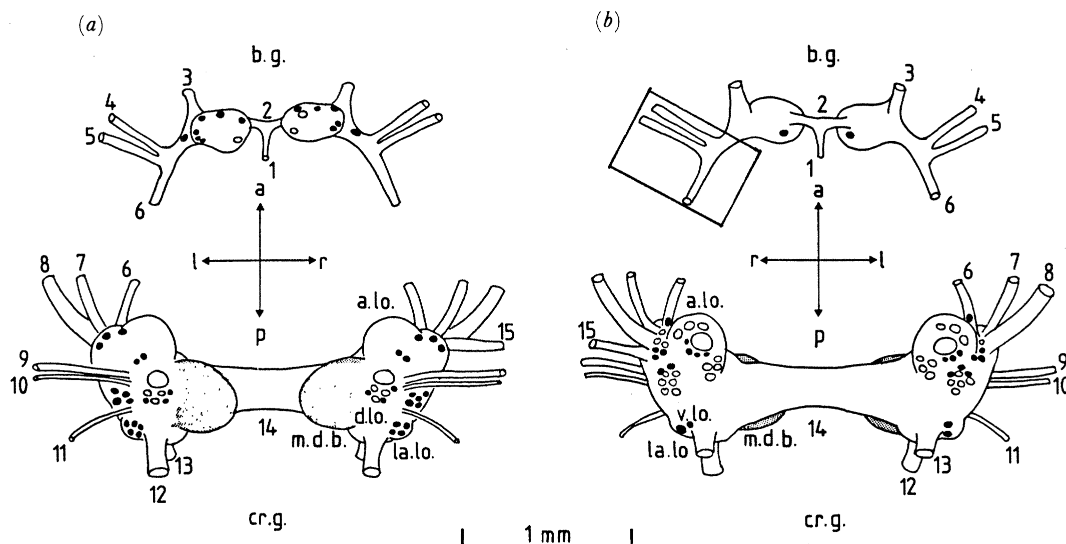


Figure 3. Serotonin (empty circles) and dopamine (solid circles) containing neurons in the buccal ganglia (b.g.) and cerebral ganglia (cr.g.) of *Lymnaea* shown in dorsal (*a*) and ventral view (*b*). The serotonin content was shown by immunocytochemistry, glyoxylic acid technique and 5,6-DHT-induced pigmentation (Kemenes *et al.* 1989). The dopamine-containing cells were mapped by glyoxylic acid technique (Audesirk 1985, and present work) and immunocytochemistry (Elekes *et al.* in preparation). Changes in glyoxylic acid induced fluorescence following 6-OH-DA, 5,6-DHT or vehicle injection were monitored on the neuronal somata in the ganglia (figure 4) and in fibres of the nerve structures shown in the rectangle (figure 5). (a.lo., d.lo., la.lo., anterior, dorsal and lateral lobes, respectively; m.d.b., medio-dorsal body; a, anterior; p, posterior; l, left; r, right.) Nerves, commissures and connectives: post-buccal nerve (1), buccal commissure (2), dorso-buccal nerve (3), latero-buccal nerve (4), ventro-buccal nerve (5), cerebro-buccal connective (6), superior lip nerve (7), median lip nerve (8), tentacle nerve (9), optic nerve (10), nuchal nerve (11), cerebro-pleural connective (12), cerebro-pedal connective (13), cerebral commissure (14), penis nerve (15).

the 0–4 days after 6-OH-DA injection (figure 5*a*, *c*, *f*, plate 2). With 5,6-DHT injection the CGC axonal fluorescence could still be seen up until 12–14 days after injection. From 12–18 days after injection serotonin could not be detected in the region of the cerebro-buccal connective where the CGC axons normally occurred (example from 15-day control and experimental shown in figure 5*d*, *e*), although it was still present in controls. By the 22nd to the 25th day following injection, serotonin-containing fibres had reappeared (figure 5*g*, plate 2). 5,6-DHT injection had no effect on DA-containing fibres which were seen throughout the 25 days.

(*c*) *Drug-induced changes in levels of dopamine and serotonin measured by* HPLC

(i) *Snails injected with 6-hydroxydopamine*

Histofluorescence data showed a loss of DA fluorescence in the fibres from 1 to 3 days after injection. This was significant because behavioural tests showed that feeding responses to food were inhibited on the same days. Biochemical measurements of DA levels in the cerebro-buccal commissure and nerves also showed a significant reduction in DA (40 %) compared with controls ($p < 0.05$) on the same days (figure 6), giving further evidence that a reduction in DA levels might be responsible for the behavioural change. No significant changes in levels of 5-HT were seen in the cerebro-buccal connectives on the same days. Neither did the buccal ganglia or cerebral ganglia show any significant differences in monoamine levels compared with controls.

Behavioural tests showed that feeding behaviour was inhibited in the first 3–4 h after injection but DA fibres could still be detected by histofluorescence indicating that there was still some transmitter present. There was a small drop in biochemically measured DA levels in the same tissue (figure 6) but it was not statistically significant. However, interpretation was made more

DESCRIPTION OF PLATE 2

Figure 5. Effects of 6-hydroxydopamine (6-OH-DA), 5,6-dihydroxytryptamine (5,6-DHT) and vehicle injection on dopamine (DA), green, and serotonin (5-HT), yellow, containing nerve fibres in the cerebro-buccal connective and nerves of *Lymnaea* revealed by glyoxylic acid staining. Area within bracket of figure 3*b* was photographed. The serotonin-containing fibres originate from the identified cell type called the cerebral giant cell. The dopamine-containing fibres mainly originate from unidentified cell bodies in the buccal ganglia. (*a*) Pre-injection control shows both types of fibre. (*b*) Vehicle control one day after injection compared with one day experimental injected with 6-OH-DA (*c*). (*c*) This shows a loss of green dopamine fluorescence (*), but a retention of yellow serotonin fluorescence. (*d*) 15-day vehicle control compared with 15-day experimental (*e*) injected with 5,6-DHT. (*e*) Yellow serotonin-containing fibres are lost (*), but the green dopamine-containing fibres are retained. By four days with 6-OH-DA (*f*) and 22 days with 5,6-DHT (*g*) both types of monoamine-containing fibres can be seen again. (Calibration bars: 50 μm.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

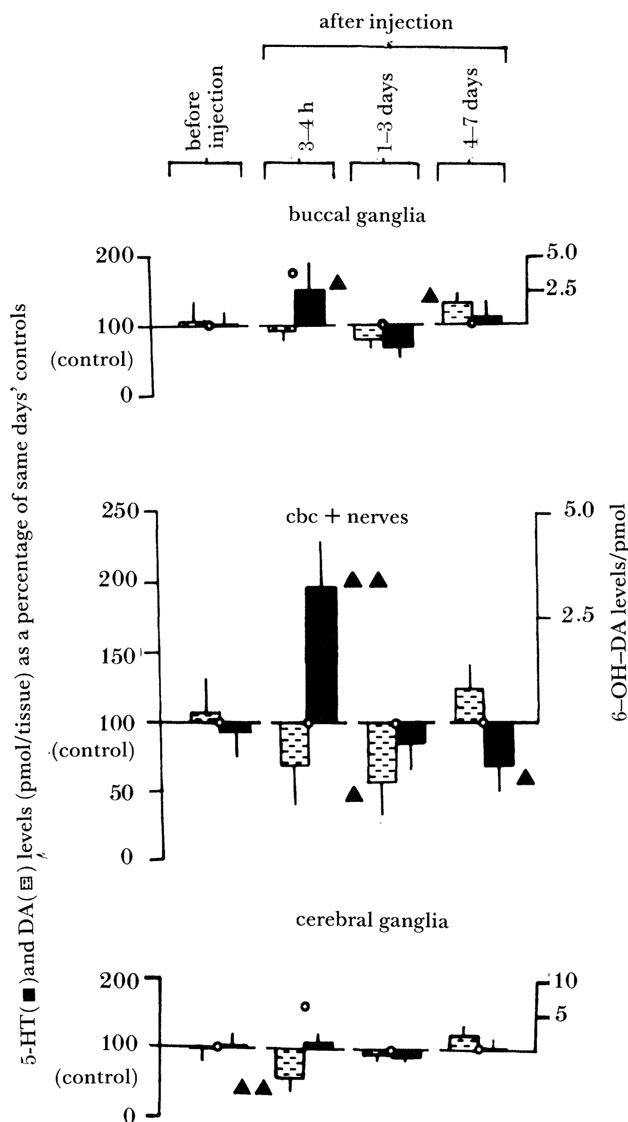


Figure 6. Changes in levels of dopamine (DA, dashed bars), serotonin (5-HT, solid bars) and 6-hydroxydopamine (6-OH-DA, open dots) following injection of snails with 6-OH-DA measured by HPLC. Values are expressed as a percentage of same-time vehicle-injected controls (±s.d.) for dopamine and serotonin (left-side axis) but as picomoles per tissue piece for 6-OH-DA (right-side axis). Solid triangles to the left of the DA bars and to the right of the 5-HT bars show statistically significant differences between experimental and same-time vehicle control values (two-tailed t-test, $n = 5$) (▲, $p < 0.05$; ▲▲, $p < 0.01$). The most important result is the drop in DA concentration in the cerebro-buccal connective and nerves (cbc+nerves) at 1–3 days after injection. This coincides with a loss of dopamine fluorescence in the same connectives (figure 5*c*) and an inhibition of feeding responses (figure 1).

difficult by an increase in 5-HT occurring at the same time. Analysis of the cerebral and buccal ganglia also revealed complex results with increases in 5-HT occurring in the buccal ganglia and decreases in DA in the cerebral ganglia. A major factor in these 3–4 h animals was the high levels of the neurotoxin itself in the buccal and cerebral ganglia (figure 6, open circles). This was only present in measurable quantities at this time and may have been the main factor causing the inhibition of feeding. 6-OH-DA has well known neurotoxic effects in other organisms (Richards 1971; Berry *et al.* 1974; Elekes *et al.* 1977; Hiripi *et al.* 1977).

DA levels in 4–7-day-old animals were similar to normal apart from an increase in the buccal ganglia (figure 6). This corresponded with the recovery of normal behavioural responses to food and the presence of DA histofluorescent fibres in the cerebro-buccal connectives and nerves.

(ii) *Snails injected with 5,6-dihydroxytryptamine*

Again, there was a significant period after injection when a change in behaviour could be correlated with changes in levels of a monoamine. Between 12–18 days, the snails responded less strongly to sugar and this corresponded with a loss of 5-HT histofluorescent fibres. Biochemical measurements of the 5-HT levels in cerebro-buccal connectives and nerves, containing the same fibres, showed a similar drop of 5-HT (figure 7, 40% compared with vehicle injected controls). A complicating factor was that DA levels increased considerably at the same time. Thus even high levels of DA could not counteract the behavioural effects of reduced 5-HT levels. It was unlikely that high levels of DA would actually inhibit feeding responses as increasing levels of DA does the opposite in physiological experiments (see for example, Wieland & Gelperin (1983)). No significant changes in levels of DA and 5-HT in the cerebral and buccal ganglia were seen in the 12–18 day period.

Levels of monoamines before (2–9 days) and after (22–25 days) this critical period were not significantly different from those of vehicle injected controls (figure 7) and the snails behaved normally. This showed that recovery from neurotoxin injection had occurred.

Behavioural tests at 3–4 h after injection showed that, like in the later 12–18 day period, feeding responses were reduced in frequency, duration and latency. However, like the early effects with the other neurotoxin, the results were confounded by non-specific changes in drug levels. Biochemical measurements showed that there was a high level of the neurotoxin itself in all three neural tissues examined (figure 7). This reduced to zero after 3–4 h. Also both 5-HT and DA levels went up together. Thus, interpretation of the data in relation to specific monoamine concentration changes was impossible.

(*d*) *Mechanisms of actions of the drugs*

(i) *Do 5,6-dihydroxytryptamine and 6-hydroxydopamine cause the release of monoamines?*

As both neurotoxins caused immediate behavioural effects following injection, we tested the hypothesis that this was caused by the release of monoamines present in neural tissue. Tritiated DA or 5-HT was loaded into the ganglia, followed by steady perfusion of saline to allow the sampling of released radioactivity. At a specific point in time, 6-OH-DA or 5,6-DHT or high K^+ saline were added to the perfusing medium. Figure 8 shows that neither toxin could cause the release of their target monoamines. However, depolarizing nerve

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

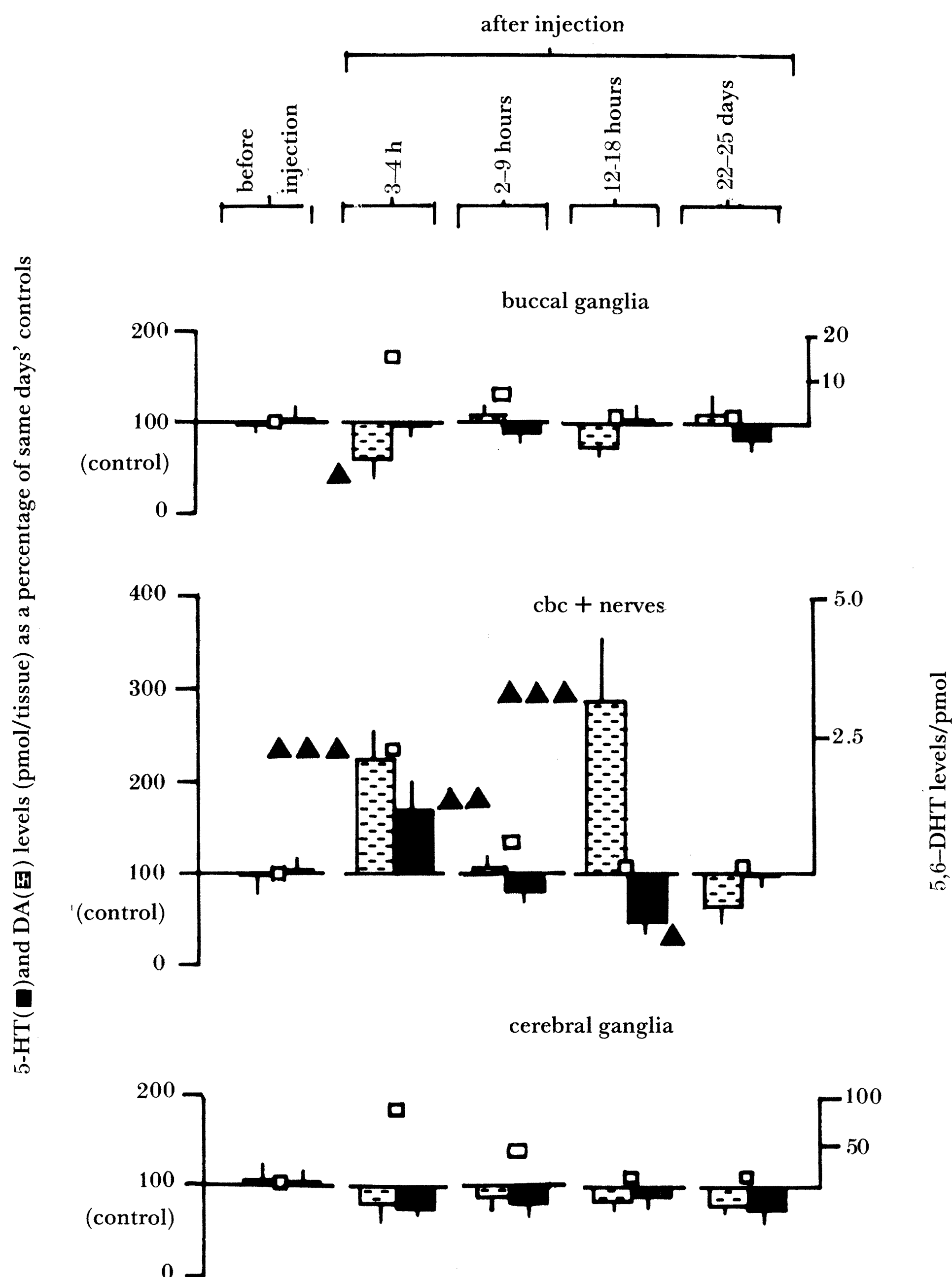


Figure 7. Changes in levels of serotonin (5-HT, solid bars), dopamine (DA, dashed bars), and 5,6-dihydroxytryptamine (5,6-DHT), open squares) following injection of snails with 5,6-DHT measured by HPLC. Values are expressed as a percentage of same-time vehicle-injected controls ($\pm$s.d.) for dopamine and serotonin (left-side axis) but as picomoles per tissue piece for 5,6-DHT (right-side axis). Solid triangles to the left of the DA bars and to the right of the 5-HT bars indicate statistically significant differences between experimental and same-time vehicle control values (two-tailed t-test, $n = 5$) (▲, $p < 0.005$; ▲▲, 0.01; ▲▲▲, 0.01). The most important result is the drop in 5-HT concentration in the cerebro-buccal connective and nerves (cbc+nerves) at 12–18 days after injection. This coincides with a loss of serotonin fluorescencing fibres in the same connectives (figure 5*d*) and an inhibition of feeding responses (figure 2). The large increase in dopamine levels occurring in the cerebro-buccal connectives and nerves on the same days cannot explain the reduction in behavioural responsiveness (see text).

fibres using the high K^+ saline caused release of the monoamines (figure 8) showing that the normal release mechanisms were still intact.

(ii) *Effects on uptake of monoamines*

Initial experiments examined the short-term effects on monoamine uptake of directly bathing the whole CNS in different concentrations of neurotoxins. The 10-min *in vitro* incubation period used would be equivalent to the initial loading of haemolymph with neurotoxins produced by the *in vivo* injection of intact snails. Both toxins inhibited uptake but the IC_{50} value of 5,6-DHT for 5-HT (indicating the concentration of drug necessary to cause a 50% reduction of uptake) was high (10^{-4} mol l^{-1}) suggesting that uptake would not be significantly affected in the first minutes after *in vivo* injection. This was also the case for 6-OH-DA effects on DA uptake which gave a value of 2×10^{-4} mol l^{-1}.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

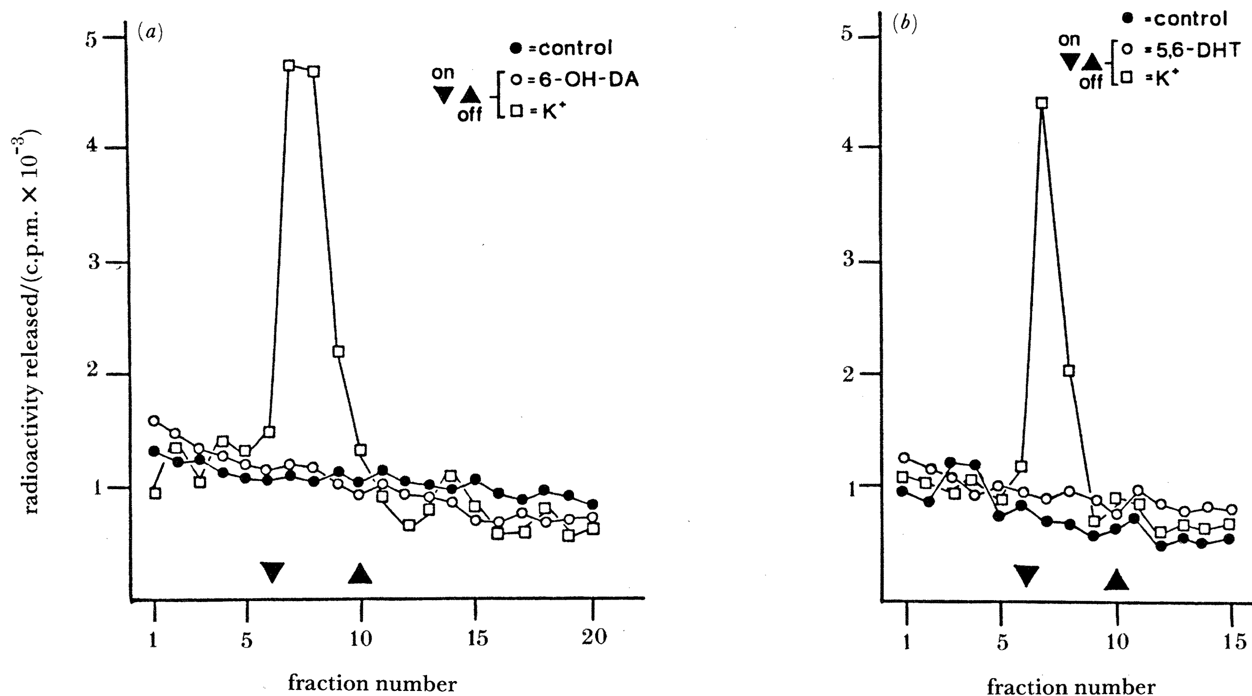


Figure 8. Release of ^{3}H-labelled monoamines from isolated *Lymnaea* ganglia which were perfused with normal saline (solid dots), toxin (open dots) or high potassium saline (open squares). (*a*) 6-hydroxydopamine (6-OH-DA) failed to induce the release of dopamine. (*b*) 5,6-dihydroxytryptamine (5,6-DHT) failed to induce the release of serotonin. In both (*a*) and (*b*) normal release of monoamines occurred following perfusion with high potassium saline.

More significant effects were obtained after 1 h incubation. Preliminary uptake measurement experiments with brains dissected from snails injected 3–4 h before the tests allowed us to calculate that this would be equivalent to 3–4 h after *in vivo* injection when high levels of neurotoxins were shown to be present in the neural tissue (figures 6 and 7). This difference in the time necessary for the toxins to exert the same effect in *in vitro* perfused ganglia and in brains of *in vitro* injected animals was probably because of the different speed by which the directly perfused and haemolymph injected drug solutions reached the central nervous tissue. The result of *in vitro* incubation for 1 h with 10^{-5} mol l^{-1} 6-OH-DA was a 23.0% (±5.19 s.d.) reduction of DA uptake whereas 10^{-5} mol l^{-1} 5,6-DHT reduced uptake of 5-HT by 30.2% (±8.2 s.d.). These reductions in monoamine uptake were both significant compared to vehicle perfused controls ($p < 0.05$). If it is assumed that the same phenomena are occurring *in vivo*, we can conclude that the presence of neurotoxins in the feeding ganglia in the first few hours after toxin injection was causing significant reductions in the uptake of monoamines.

A further type of experiment was done on isolated ganglia from snails which had been previously injected with neurotoxins or vehicle control solutions, either 3 days (6-OH-DA) or 14 days (5,6-DHT) before the *in vitro* assay. These days were chosen because it was known that the concentrations of neurotoxins in the ganglia were below measurable levels (figures 6 and 7), and no fluorescent nerve fibres could be seen in nerves or connectives leaving the feeding ganglia (figure 5). Despite the absence of neurotoxins, the uptake of the monoamines were still reduced compared with controls. In the 6-OH-DA group uptake was reduced by 23.7% (±4.2 s.d.) and in the 5,6-DHT group by 24.1% (±7.4 s.d.). This significant ($p < 0.05$ for both drugs) level of inhibition of uptake cannot be due directly to the presence of the neurotoxins in the ganglia but probably resulted from a toxin-induced degeneration of nerve terminals or axons of the monoamine-containing cells. Degenerative changes have been shown to occur in other molluscan nerve tissue because of the injection of 5,6-DHT or 6-OH-DA (Berry *et al.* 1974; Elekes *et al.* 1977).

We conclude that uptake of monoamines was inhibited by neurotoxin injection both in the short- and long-term with the short-term effects due to the presence of neurotoxins in the ganglia tissue and those in the long-term because of degeneration of the monoamine containing fibres.

4. DISCUSSION

The objective of this paper was to gain information concerning the role of monoamines in the feeding system of the snail, *Lymnaea stagnalis*. The approach was to inject neurotoxins into snails and measure concurrent changes in behaviour and levels of monoamines in central ganglia and nerves. These data could then be compared with our knowledge of the feeding circuitry obtained from physiological experiments (Benjamin 1983; Benjamin & Elliott 1989). The results were complex due to non-specific effects of the neurotoxins on more than one monoamine, the effects of the carrier/antioxidant or the injection-induced trauma at the early stages of the experiment and the likely toxic effects of the injected substances themselves when at

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

certain stages of the experiments they were present in the neural tissue at high concentrations.

Non-specific behavioural effects of ascorbic acid present in 1 mg ml^{-1} concentration in the vehicle and mimicking those of the administration of 6-OH-DA were earlier reported in rats (Waddington & Crow 1979). However, both the biochemical and behavioural quantities measured after the initial 20–30 min in our experiments in vehicle-treated control snails showed that these were 'normal' controls. It could not be excluded that the 0.5 mg ml^{-1} ascorbic acid concentration used in snails had a similar effect in the short-time to that found in rats but it is clear that it did not produce long-term changes.

Despite the problems with the non-specific effects there were periods in the experiments when clear correlations could be made between specific behavioural deficits, loss of fluorescence in axons and decreases in the levels of DA or 5-HT in neural structures containing the feeding circuitry. These correlations were observed only in the neurotoxin-injected groups.

The data from the 6-OH-DA injection experiment were the simplest to interpret. From 1 to 3 days after the injection 60% of the snails failed to respond to sucrose, a stimulus that produced a 98% response in normal animals. This could be correlated with a drop in DA levels in the feeding system. DA fluorescence disappeared from fibres in the cerebro-buccal connectives and latero- and ventro-buccal nerves and biochemical measurements showed a 40% drop in DA levels in the same structures. The neurotoxin did not significantly influence the biochemically measured levels of DA or DA fluorescence in the ganglia where the cell bodies of the neurons occurred. This suggested that the neurotoxins were mainly targeting the fibres of neurons or the levels of DA were too high in the cell bodies to show significant depletion. The response to sucrose recovered by 4–7 days after injection with 6-OH-DA.

The only other period in the experiments with 6-OH-DA when feeding was affected was at 3–4 h after injection. However, inconsistent changes in the levels of both DA and 5-HT occurred in several neural structures and significantly there were high levels of the neurotoxin in neural tissue. This made it unlikely that the behavioural inhibition could be due simply to changes in DA levels.

The only other interesting result occurred immediately after the initial injection of 6-OH-DA. Complete paralysis of the snails was seen and lasted for about an hour. Only respiratory movements involving opening of the pneumostome could be induced. This behavioural response was mimicked by injection of DA and so it appears as if the neurotoxin was having an immediate pharmacological effect similar to DA. DA in high concentration must play some role in the inhibition of locomotory behaviour, but which specific system was involved was unknown.

DA appears to be necessary for a basic feeding response to food to occur, and pharmacological experiments support this hypothesis. In the closely related pond snail, *Helisoma*, application of DA to the isolated ganglia activated bursts of activity in feeding motoneurons (Trimble & Barker 1984) or increased the frequency of motor bursts in the slug, *Limax* (Wieland & Gelperin 1983). A preliminary report in *Lymnaea* suggests that similar activation of feeding patterns could occur (Kyriakides & McCrohan 1989) on application of DA to isolated ganglia. The records of Kyriakides & McCrohan (1989) showed that both the motoneurons and the rhythm-generating interneuronal network could be the target for DA.

The results from 5,6-DHT injected animals suggested that 5-HT, like DA, was necessary for full activation of the feeding system. However, the effects of reduction in 5-HT levels in fibres of the feeding system were more subtle than those with DA and easier to interpret in terms of neuronal mechanisms. From 12–18 days after injection, when behavioural changes could be correlated with falls in 5-HT levels and loss of 5-HT fluorescing fibres, the majority of snails (57%) responded to sucrose. However, they had a longer latency to the first bite, lower frequency and shorter bite duration compared with controls. The target for the 5,6-DHT was much easier to determine than for DA. The only serotonergic neurons known to be involved in feeding are the CGCs (McCrohan & Benjamin 1980) and it was the loss of 5-HT from the fibres of these specific neurons that was monitored in the histofluorescence experiments. This loss of 5-HT was accompanied by reduction of efficacy of the synaptic connections from the CGCs to the feeding motoneurons (Kemenes *et al.* 1988), and so a breakdown in the neural transmission from the CGCs to neurons of the rest of the feeding network can account for the reduction of behavioural responsiveness. Similar impairment of the CGC-buccal neurons synaptic pathway was observed after 5,7-DHT treatment in *Helisoma* (Gadotti *et al.* 1986) and after 5,6-DHT treatment in *Helix* (Vehovszky *et al.* 1988).

Changes in the response to food after 5,6-DHT injection also occurred in the early stages of the experiment. However, the interpretation of these results was confounded by the non-specific effects of carrier/antioxidant or injection-induced trauma at 20–30 min and the presence of high levels of the neurotoxin in neural tissue at 3–4 h (cf. 6-OH-DA above). Inhibition of neuronal activity by 5,7-DHT (a serotonin neurotoxin closely related to 5,6-DHT) is known in the vertebrate nervous system (Segal 1986) and similar inhibitory effects on snail neurons probably accounted for the inhibition of feeding response seen in the snails at 3–4 h.

An immediate response to 5,6-DHT injection, which was mimicked by 5-HT injection, consisted of exaggerated bending movements of the body. Muscular movements of the body were associated with locomotory turning movements (Haydon & Winlow 1986) and serotonergic mechanisms have been shown to play a role in locomotion (Syed *et al.* 1988) and so conceivably 5,6-DHT could be activating circuits associated with movement control. This was supported by work in *Aplysia* where injection of 5,6-DHT induced pedal locomotory movements (Jahan-Parwar *et al.* 1987).

Downloaded from rstb.royalsocietypublishing.org

Injection with 6-OH-DA resulted in the depletion of DA much sooner than the depletion of 5-HT occurred after 5,6-DHT treatment. This is consistent with earlier suggestions that, unlike 5-HT-containing neurons, those containing DA have a higher proportion of their transmitter content in their axons and nerve terminals than in their cell bodies (Swann *et al.* 1982). Since both 6-OH-DA and 5,6-DHT are taken up by nerve terminals first and transported retrogradely along the fibres (Elekes *et al.* 1977) it follows that axonal DA would be depleted first. Our measurements of the levels of neurotoxins in neural tissue showed that the DA neurotoxin itself is used up more rapidly by this process and the recovery also appeared sooner.

Depletion of the monoamines in fibres of the *Lymnaea* feeding circuitry probably came about by the impairment of uptake processes. This was a short latency effect of bathing the *Lymnaea* ganglia with neurotoxins, but it also persisted for up to 3 (6-OH-DA) and 14 days (5,6-DHT) following injection of the whole animal with the drugs. The persistent effects occurred in the absence of neurotoxins in central ganglia and nerves and so we presumed that it must be a secondary effect due to degeneration of nerve terminals and fibres.

No direct evidence was obtained for this phenomenon in *Lymnaea* but in two other molluscan species (*Anodonta cygnea*: Elekes *et al.* 1977; *Helix pomatia*: S.-Rózsa *et al.* 1986; Hernádi *et al.* 1988) ultrastructural evidence for neurotoxin-induced degeneration was obtained. The timecourse of the degeneration and its subsequent recovery (Elekes *et al.* 1977) correlated well with the sequence of biochemical and behavioural changes induced by the neurotoxins in *Lymnaea*. Re-uptake is part of the normal mechanism for maintaining levels of monoamines in pre-synaptic terminals and its inhibition could account for the gradual reduction in monoamines in *Lymnaea* neural tissues. The neurotoxins did not cause the release of monoamines, an alternative method for depleting fibres.

This work was supported by the SERC and a SOROS/MTA (HUNGARY) short-term fellowship. We thank Mark Yeoman for his expert comments on this manuscript. The skilful assistance of Ms K. Komáromi, Ms Zs. Zámbó and Mr B. Balázs is much appreciated.

REFERENCES

Audesirk, G. 1985 Amine-containing neurons in the brain of *Lymnaea stagnalis*: distribution and effects of precursors. *Comp. Biochem. Physiol.* A **81**, 359–365.

Axelsson, S., Björklund, A., Falck, B., Lindwall, O. & Svensson, L. A. 1973 Glyoxylic acid condensation: a new fluorescence method for the histochemical demonstration of biogenic monoamines. *Acta Physiol. Scand.* **89**, 57–62.

Barber, A. 1982 Monoamine-containing varicosities in the neural sheath of a gastropod mollusc demonstrated by glyoxylic acid histofluorescence. *Cell Tiss. Res.* **226**, 267–273.

Baumgarten, H. G., Björklund, A., Nobin, A., Rosengren, E. & Schlossberger, H. G. 1975 Neurotoxicity of hydroxylated tryptamines: structure-activity relationship. *Acta Physiol. Scand.* **429**, 1–27.

Benjamin, P. R. 1983 Gastropod feeding: behavioural and neural analysis of a complex multicomponent system. In *Neural origin of rhythmic movements* (ed. A. Roberts & B. Roberts), pp. 159–193. Cambridge University Press.

Benjamin, P. R. & Elliott, C. J. H. 1989 Snail feeding oscillator: the central pattern generator and its control by modulatory interneurones. In *Cellular and neuronal oscillators* (ed. J. Jacklet), pp. 173–214. New York: M. Dekker.

Benjamin, P. R. & Winlow, W. 1981 The distribution of three wide-acting inputs to identified neurones in the isolated brain of *Lymnaea stagnalis*. *Comp. Biochem. Physiol.* A **70**, 293–307.

Berry, M. S., Pentreath, V. W., Turner, J. D. & Cottrell, G. A. 1974 Effects of 6-hydroxydopamine on an identified dopamine-containing neuron in the central nervous system of *Planorbis corneus*. *Brain Res.* **76**, 309–324.

Björklund, A., Horn, A. S., Baumgarten, H. G. & Schlossberger, H. G. 1975 Neurotoxicity of hydroxylated tryptamines: structure-activity relationships. 2. *In vitro* studies on monoamine uptake inhibition and uptake impairment. *Acta Physiol. scand.* (suppl.) **429**, 29–60.

Elekes, K., Hiripi, L. & Nemcsök, J. 1977 Ultrastructural effects of 6-hydroxydopamine and 5,6-dihydroxytryptamine on the central nervous system of fresh-water mussel, *Anodonta cygnea* L. *Acta biol. Acad. Sci. hung.* **28**, 259–272.

Gadotti, D., Bauce, L. G., Lukowiak, K. & Bulloch, A. G. M. 1986 Transient depletion of serotonin in the nervous system of *Helisoma*. *J. Neurobiol.* **17**, 431–447.

Granzow, B. & Rowell, C. H. F. 1981 Further observations on the serotonergic cerebral neurons of *Helisoma* (Mollusca, Gastropoda): the case for homology with the metacerebral giant cells. *J. exp. Biol.* **90**, 283–305.

Haydon, P. G. & Winlow, W. 1986 Shell movements associated with locomotion in *Lymnaea* are driven by a central pattern generator. *Comp. Biochem. Physiol.* A **83**, 23–25.

Hernádi, L., Vehovszky, A. & S.-Rózsa, K. 1989 5,6-dihydroxytryptamine induced ultrastructural changes as a specific marker of the serotonergic system in the CNS of *Helix pomatia*. In *Neurobiology of invertebrates: transmitters, modulators and receptors* (ed. J. Salånki & K. S.-Rózsa), pp. 173–183. Budapest: Akadémiai Kiadô.

Hernádi, L., Elekes, K. & S.-Rózsa, K. 1989 Distribution of serotonin-containing neurons in the central nervous system of the snail *Helix pomatia*. Comparison of immunocytochemical and 5,6-dihydroxytryptamine labelling. *Cell Tiss. Res.* **257**, 313–323.

Hiripi, L., Nemcsók, J., Elekes, K. & Salánki, J. 1977 Monoamine level and periodic activity in 6-OHDA treated mussel *Anodonta cygnea* L. *Acta biol. Acad. Sci. hung.* **28**, 175–183.

Jahan-Parwar, B., S.-Rózsa, K., Salánki, J., Evans, M. L. & Carpenter, D. O. 1987 *In vivo* labelling of serotonin-containing neurons by 5,7-dihydroxytryptamine in *Aplysia*. *Brain Res.* **426**, 173–178.

Kemenes, G., Benjamin, P. R. & Hiripi, L. 1988 5,6-dihydroxytryptamine-induced changes in the serotonergic modulation of feeding in *Lymnaea*. In *Neurobiology of invertebrates: transmitters, modulators and receptors* (ed. J. Salánki & K. S.-Rózsa), pp. 415–431. Budapest: Akadémiai Kiadó.

Kemenes, G., Elekes, K., Hiripi, L. & Benjamin, P. R. 1989 A comparison of four techniques for mapping the distribution of serotonin and serotonin-containing neurons in fixed and living ganglia of the snail, *Lymnaea*. *J. Neurocytol.* **18**, 193–208.

Kemenes, G., Elliott, C. J. H. & Benjamin, P. R. 1986 Chemical and tactile inputs to the *Lymnaea* feeding system: effects on behaviour and neural circuitry. *J. exp. Biol.* **122**, 113–137.

Kupfermann, I. 1974 Dissociation of the appetitive and

Downloaded from rstb.royalsocietypublishing.org

consummatory phases of feeding in *Aplysia*: a lesion study. *Behav. Biol.* **10**, 89–97.

Kyriakides, M. & McCrohan, C. R. 1989 Effect of putative neuromodulators on rhythmic buccal motor output in *Lymnaea stagnalis*. *J. Neurobiol.* **20**, 635–650.

Mandelbaum, E. D., Koester, J., Schonberg, M. & Weiss, K. R. 1979 Cyclic AMP mediation of the excitatory effect of serotonin in the heart of *Aplysia*. *Brain Res.* **177**, 388–394.

McCrohan, C. R. & Benjamin, P. R. 1980 Synaptic relationships of the cerebral giant cells with motoneurones in the feeding systems of *Lymnaea stagnalis* *J. exp. Biol.* **85**, 169–186.

Ono, J. K. & McCaman, R. E. 1984 Immunocytochemical localization and direct assays of serotonin-containing neurons in *Aplysia*. *Neuroscience* **11**, 549–560.

Pentreath, V. W., Berry, M. S. & Osborne, N. N. 1982 The serotonergic cerebral cells in gastropods. In *Biology of serotonergic transmission* (ed. N. N. Osborne), pp. 457–513. New York: Wiley.

Richards, J. G. 1971 Ultrastructural effects of 6-hydroxydopamine on catecholamine containing neurons in the rat brain. In *6-hydroxydopamine and chatecholamine neurons* (ed. T. Malmfors & H. Thoenen), pp. 151–161. Amsterdam: North-Holland and Publ. Co.

Sakharov, D. A. & Zs-Nagy, I. 1968 Localization of biogenic monoamines in the cerebral ganglia of *Lymnaea stagnalis*. *Acta biol. Acad. Sci. hung.* **19**, 145–157.

Saller, C. R. & Salama, A. J. 1984 Rapid automated analysis of biogenic amines and their metabolites using reversed-phase high performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **309**, 287–298.

Sathananthan, A. H. 1976 Degeneration of monoamine nerves in anterior byssus retractor muscle of *Mytilus* induced by 5,6-dihydroxytryptamine. *Cell Tiss. Res.* **172**, 425–429.

Segal, M. 1986 5,7-Dihydroxytryptamine modifies excitability of rat dorsal raphe serotonergic neurons *in vitro*. *Neurosci. Lett.* **71**, 306–310.

Straub, H. & Kuhlmann, D. 1984 Identification and quantitative measurements of biogenic monoamines in the central nervous tissue of some gastropods. *Comp. Biochem. Physiol.* C **78**, 319–323.

Swann, J. W., Sinback, C. N., Kebabian, P. R. & Carpenter, D. O. 1982 Motoneurons which may utilize dopamine as their neurotransmitter. *Cell. Molec. Neurobiol.* **2**, 309–324.

Syed, N. I., Harrison, D. & Winlow, W. 1988 Locomotion in *Lymnaea* – role of serotonergic motoneurones controlling the pedal cilia. In *Neurobiology of invertebrates: transmitters, modulators and receptors* (ed. J. Salánki & K. S.-Rózsa), pp. 415–431. Budapest: Akadémiai Kiadó.

Thomas, J. D., Lough, A. A. & Lodge, R. W. 1975 The chemical ecology of *Biomphalaria glabrata* Say the snail host of *Schistosoma mansoni* Sambon: the search for factors in media conditioned by snails which inhibit their growth and reproduction. *J. appl. Ecol.* **12**, 421–434.

Trimble, D. L. & Barker, D. L. 1984 Activation by dopamine of patterned motor output from the buccal ganglia of *Helisoma trivolvis*. *J. Neurobiol.* **15**, 37–48.

Trimble, D. L., Barker, D. L. & Bullard, B. J. 1984 Dopamine in a molluscan nervous system: synthesis and fluorescence histochemistry. *J. Neurobiol.* **15**, 27–36.

Tuersley, M. D. & McCrohan, C. R. 1988 Serotonergic modulation of patterned motor output in *Lymnaea stagnalis*. *J. exp. Biol.* **135**, 473–486.

Vehovszky, Á., Kemenes, G., Hiripi, L., Hernádi, L. & S.-Rózsa, K. 1988 Reversible effect of 5,6-DHT treatment on *Helix*: a combined behavioural, electrophysiological and biochemical study. In *Neurobiology of invertebrates: transmitters, modulators and receptors* (ed. J. Salánki & K. S.-Rózsa), pp. 403–414. Budapest: Akadémiai Kiadó.

Waddington, J. L. & Crow, T. J. 1979 Drug-induced rotational behaviour following unilateral intracerebral injection of saline-ascorbate solution: neurotoxicity of ascorbic acid and monoamine-independent circling. *Brain Res.* **161**, 371–376.

Walker, R. J. 1986 Transmitters and Modulators. In *The mollusca, 9, neurobiology and behaviour* (ed. A. O. D. Willows), pp. 279–484. New York: Academic Press.

Weinreich, D., McCaman, M. W., McCaman, R. E. & Vaughn, J. E. 1973 Chemical enzymatic and ultrastructural characterization of 5-hydroxytryptamine containing neurons from ganglia of *Aplysia californica* and *Tritonia diomedia*. *J. Neurochem.* **20**, 969–976.

Weiss, K. R., Cohen, J. L. & Kupfermann, I. 1978 Modulatory control of buccal musculature by a serotonergic neuron metacerebral cell in *Aplysia*. *J. Neurophysiol.* **41**, 181–203.

Weiss, K. R., Koch, U. T., Koester, J., Rosen, S. C. & Kupfermann, I. 1982 The role of arousal in modulating feeding behavior of *Aplysia*: neural and behavioral studies. In *The neural basis of feeding and reward* (ed. B. G. Hoebel & D. Novin), pp. 25–57. Brunswick: Haer Institute.

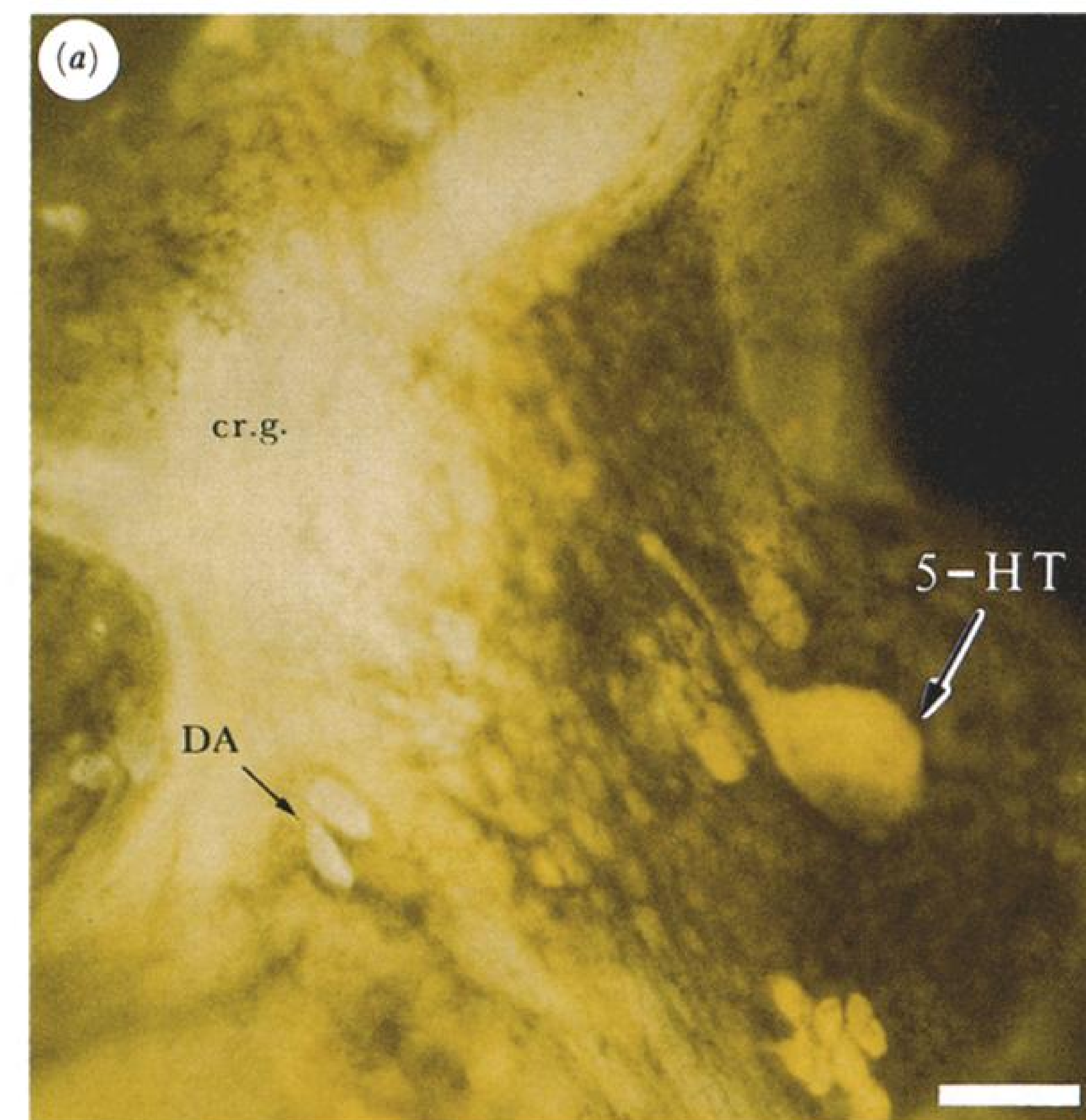
Wieland, S. J. & Gelperin, A. 1983 Dopamine elicits feeding motor program in *Limax maximus*. *J. Neurosci.* **3**, 1735–1745.

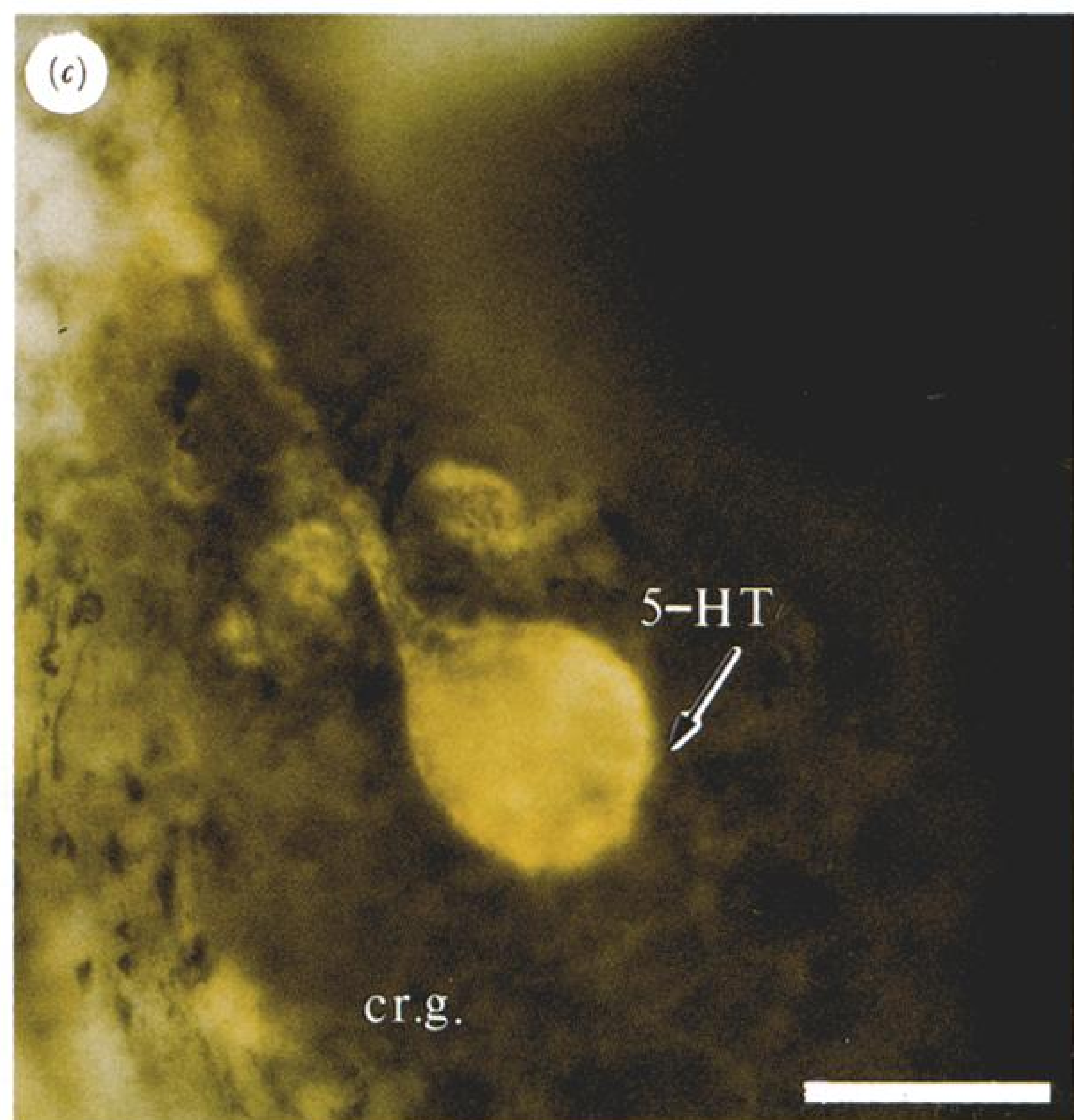
Wieland, S. J., Jahn, E. & Gelperin, A. 1987 Localization and synthesis of monoamines in regions of *Limax* CNS controlling feeding behavior. *Comp. Biochem. Physiol.* C **86**, 125–130.

(*Received 8 March 1990; revised 6 June 1990; accepted 2 July 1990*)

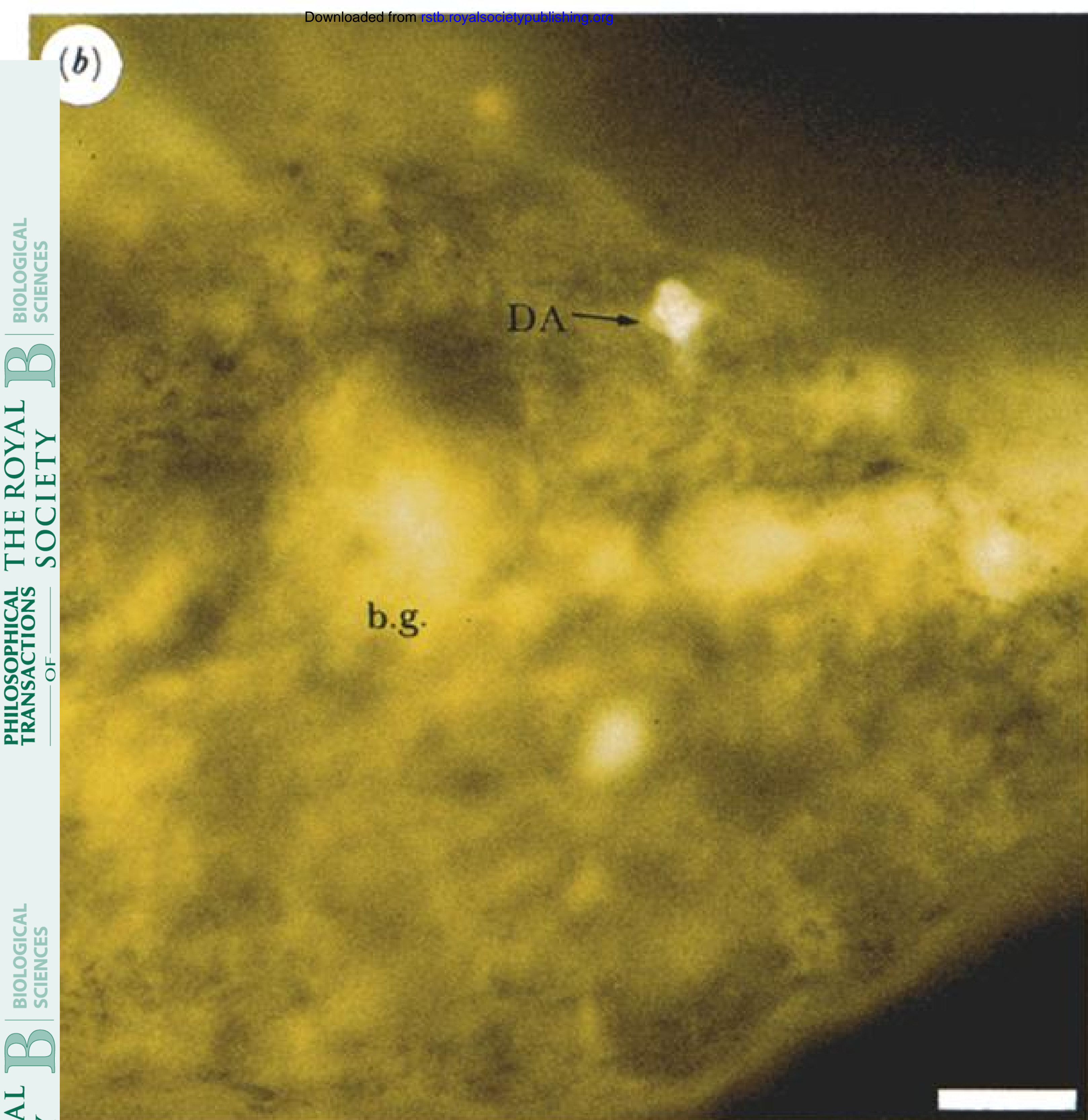
Plates 1 and 2 were printed by George Over Ltd. Rugby, U.K.

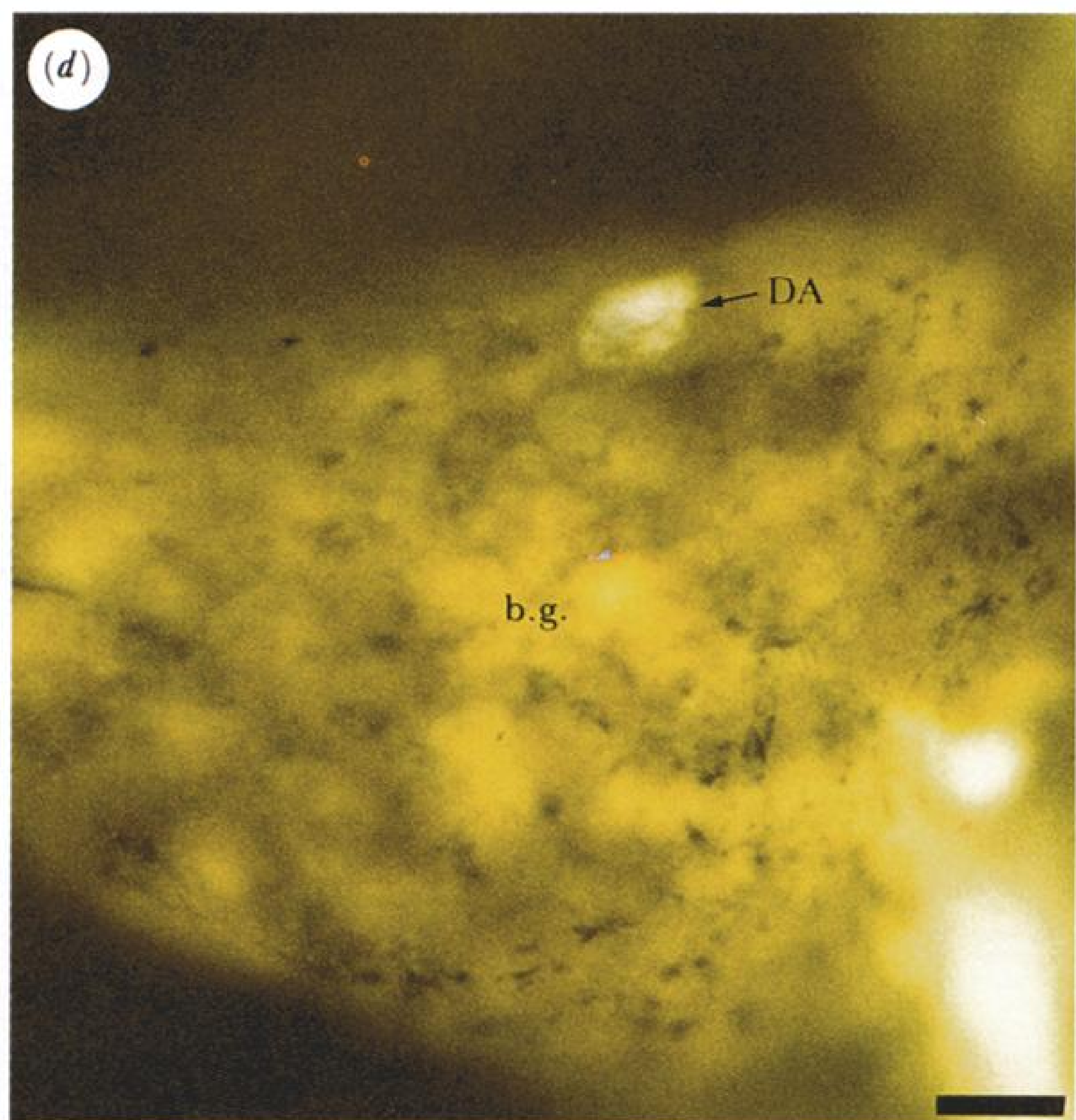
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES





Downloaded from rstb.royalsocietypublishing.org

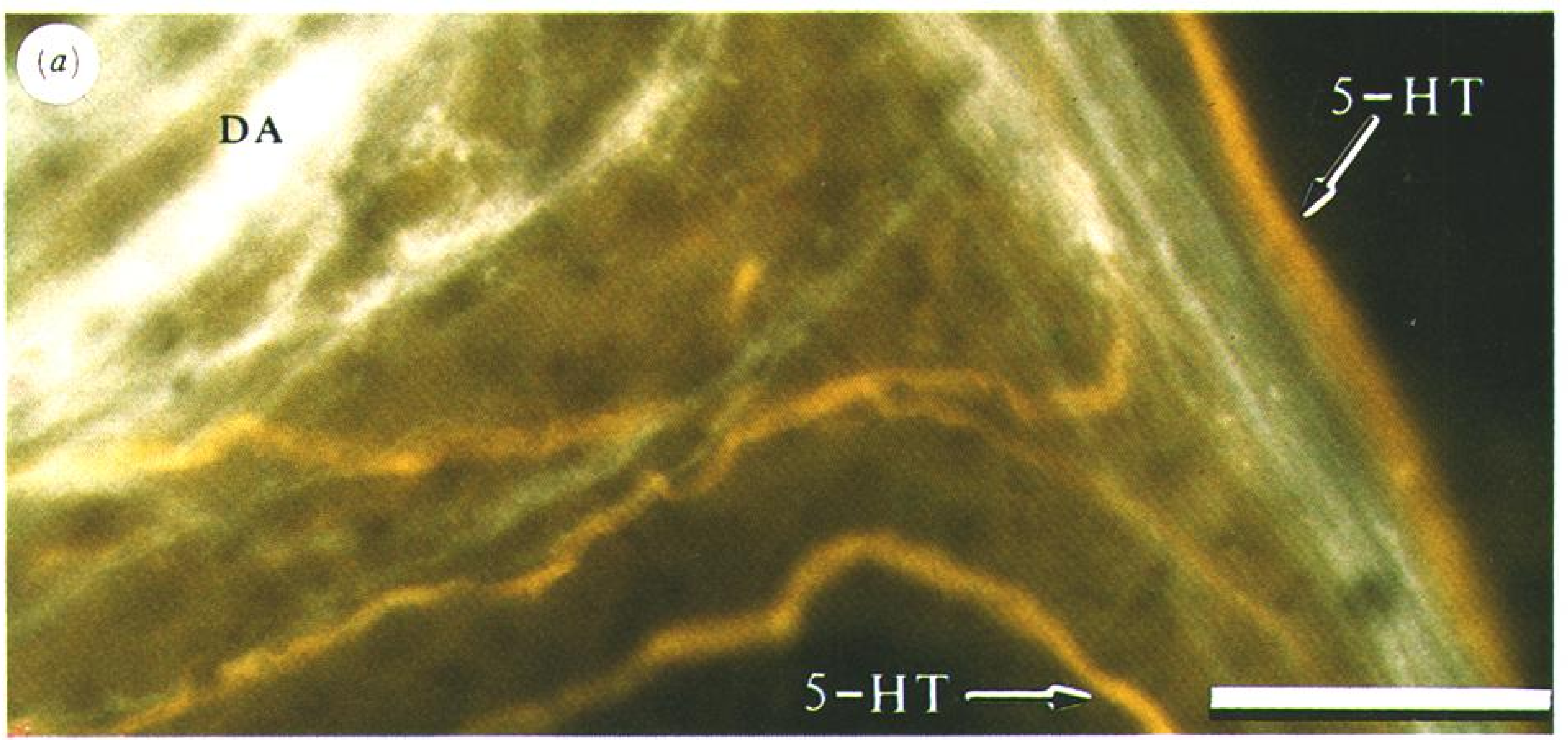


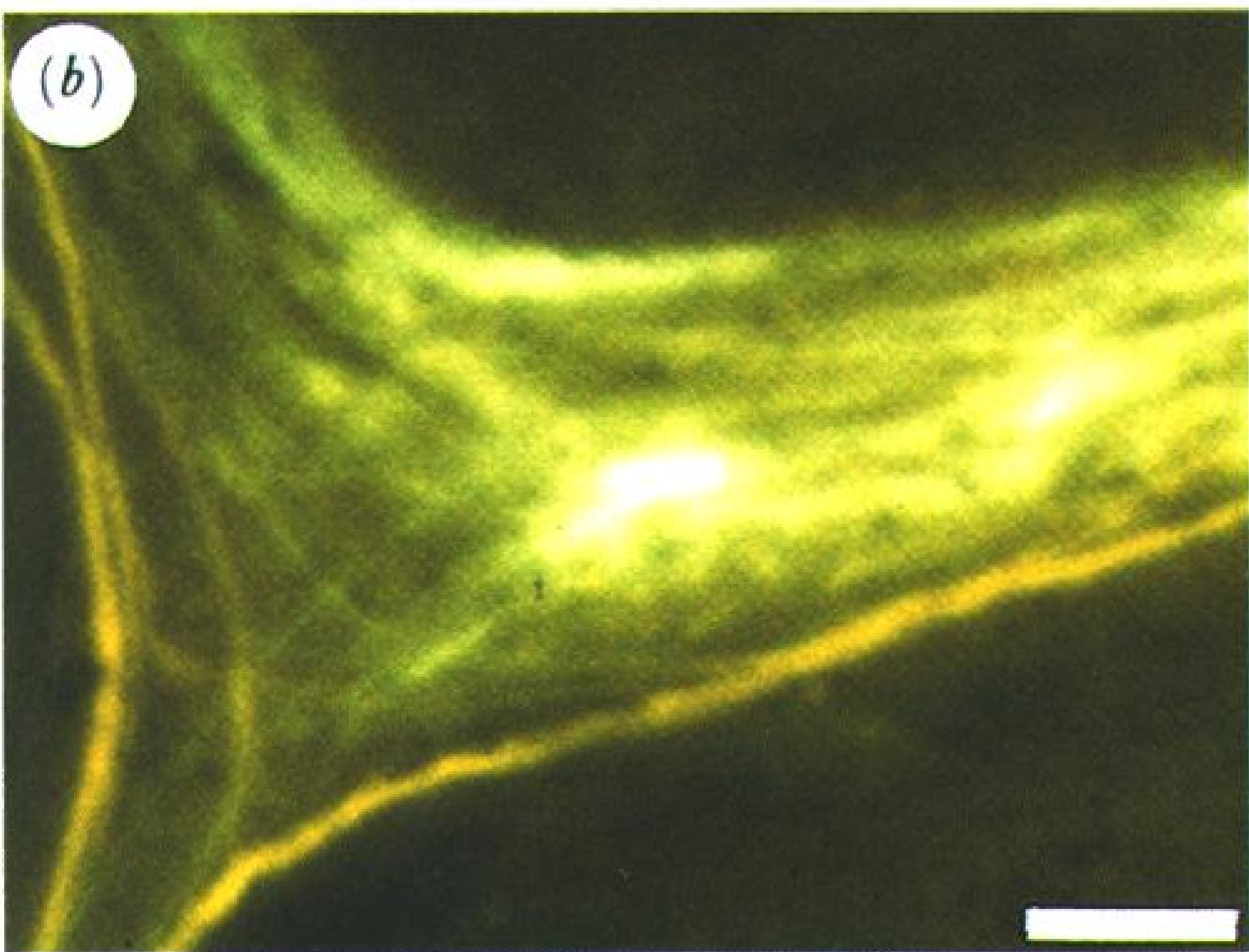


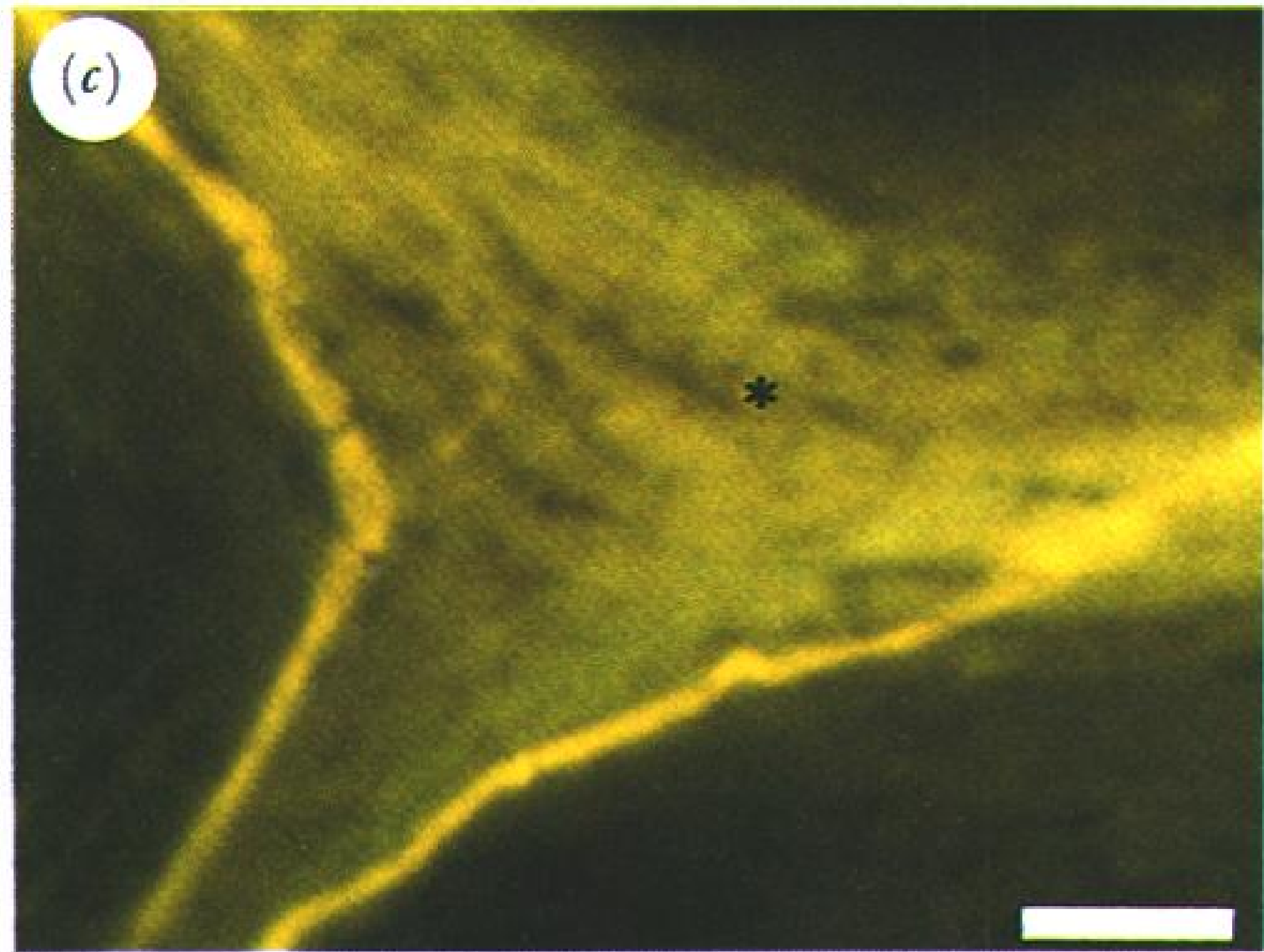
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

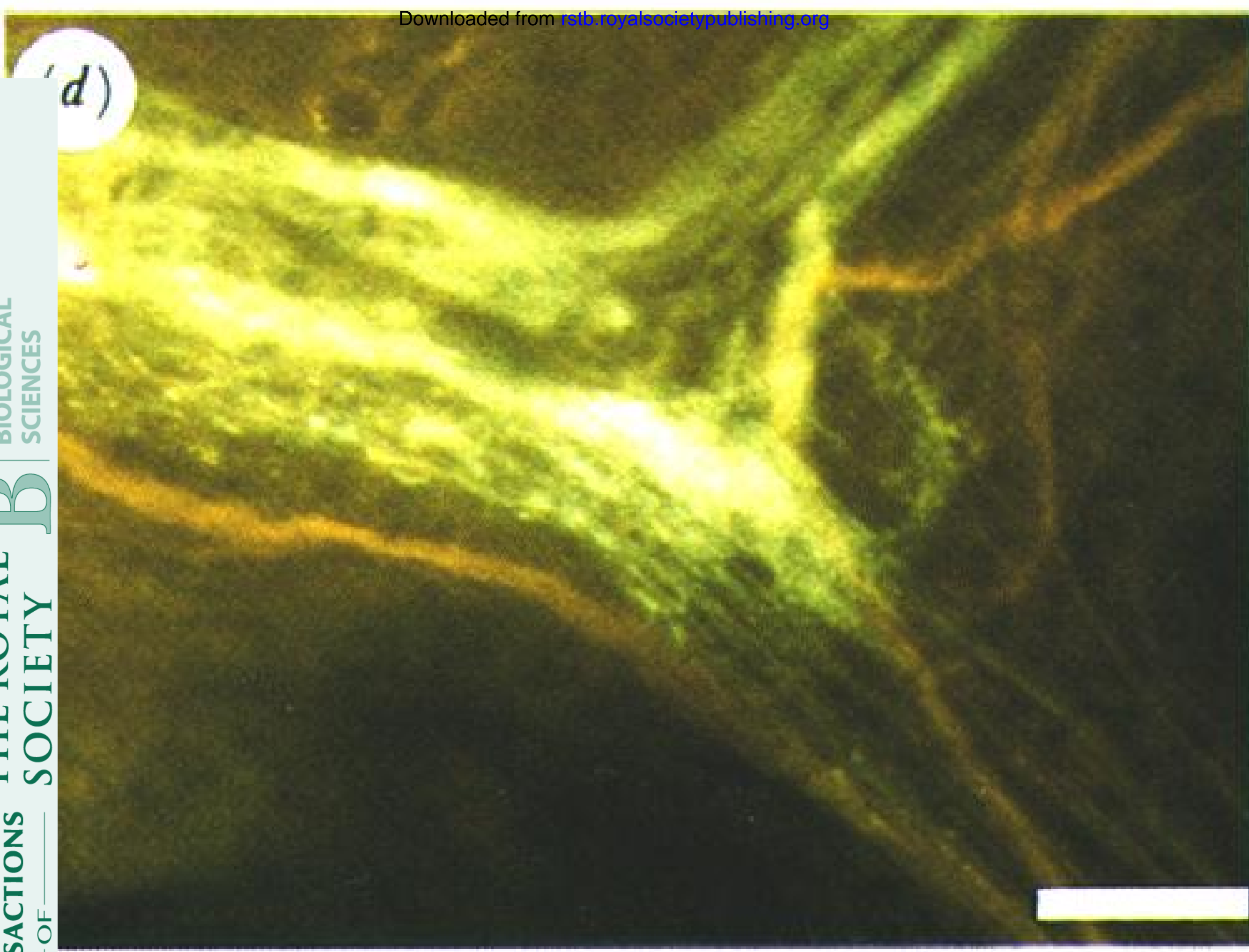
igure 4. Serotonin (5-HT), yellow, and dopamine (DA), green, fluorescent cell bodies in the cerebral (cr.g.) and uccal ganglia (b.g.) of *Lymnaea* revealed by the glyoxylic acid method. The largest cell in the cerebral ganglion ırrowed 5-HT) is the cerebral giant cell, one of a pair of large serotonin-containing neurons involved in the feeding rcuit. None of the dopamine cells are identifiable. Injection of snails with 6-hydroxytryptamine (6-OH-DA + day) (figure 4*a*,*b*) or with 5, 6-dihydroxytryptamine (5, 6-DHT + 15 days) (figure 4*c*,*d*) had no effect on the uorescence of the cell bodies. On the same days, clear reductions in axonal fluorescence occurred (figure 5*c*,*e*, late 2). (Calibration bars: (*a*,*c*), 100μm; (*b*,*d*), 30μm.)

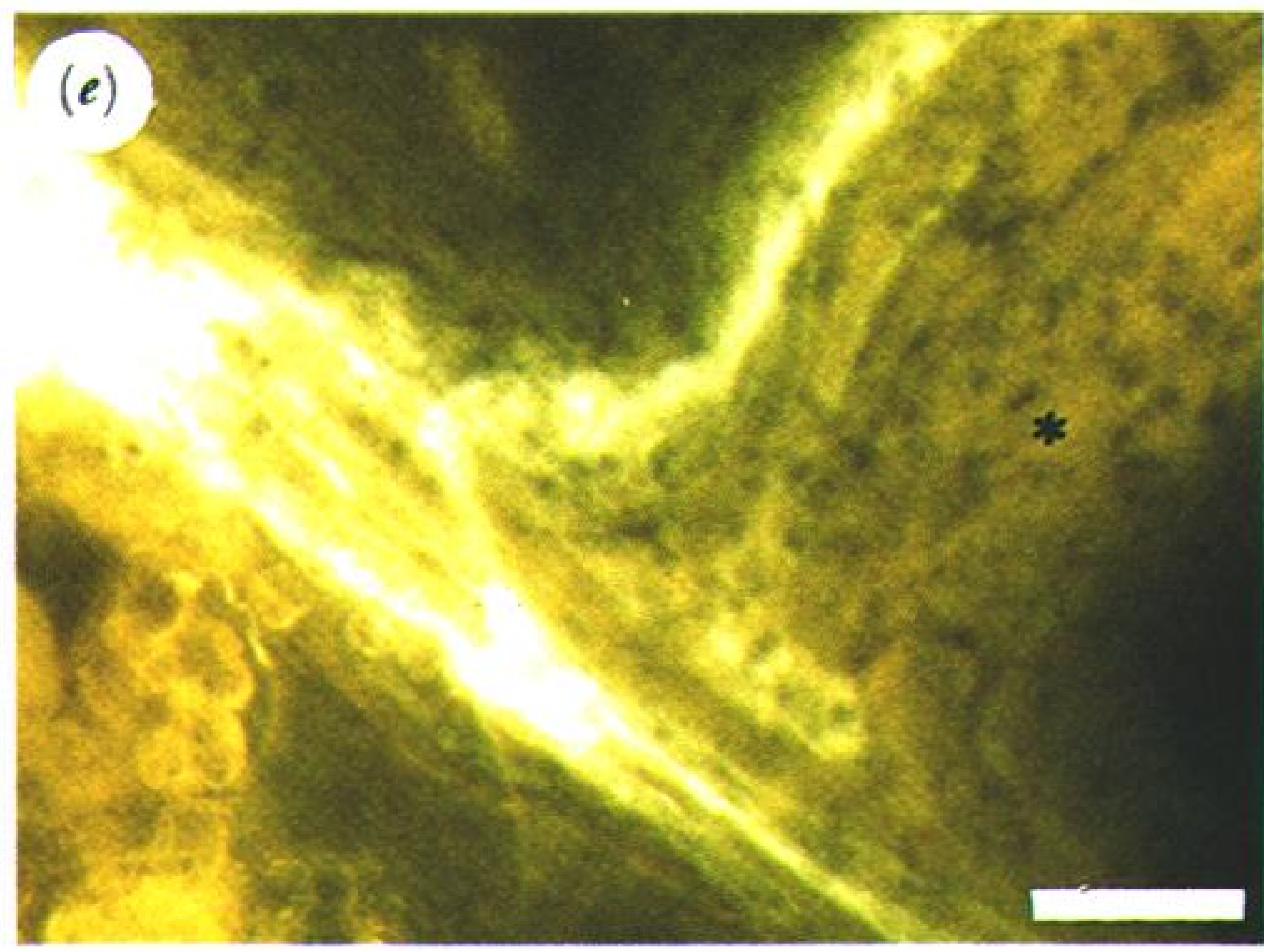


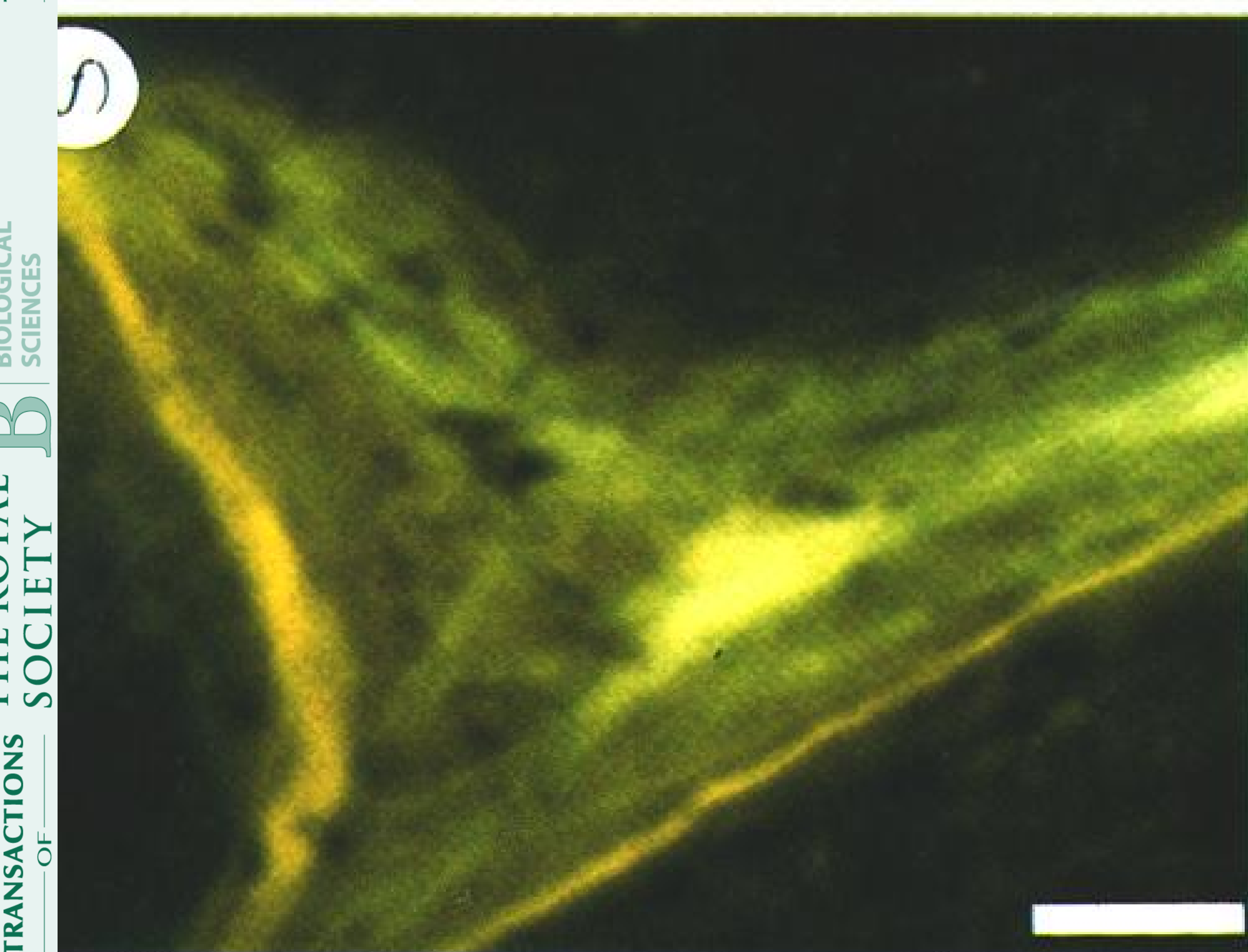


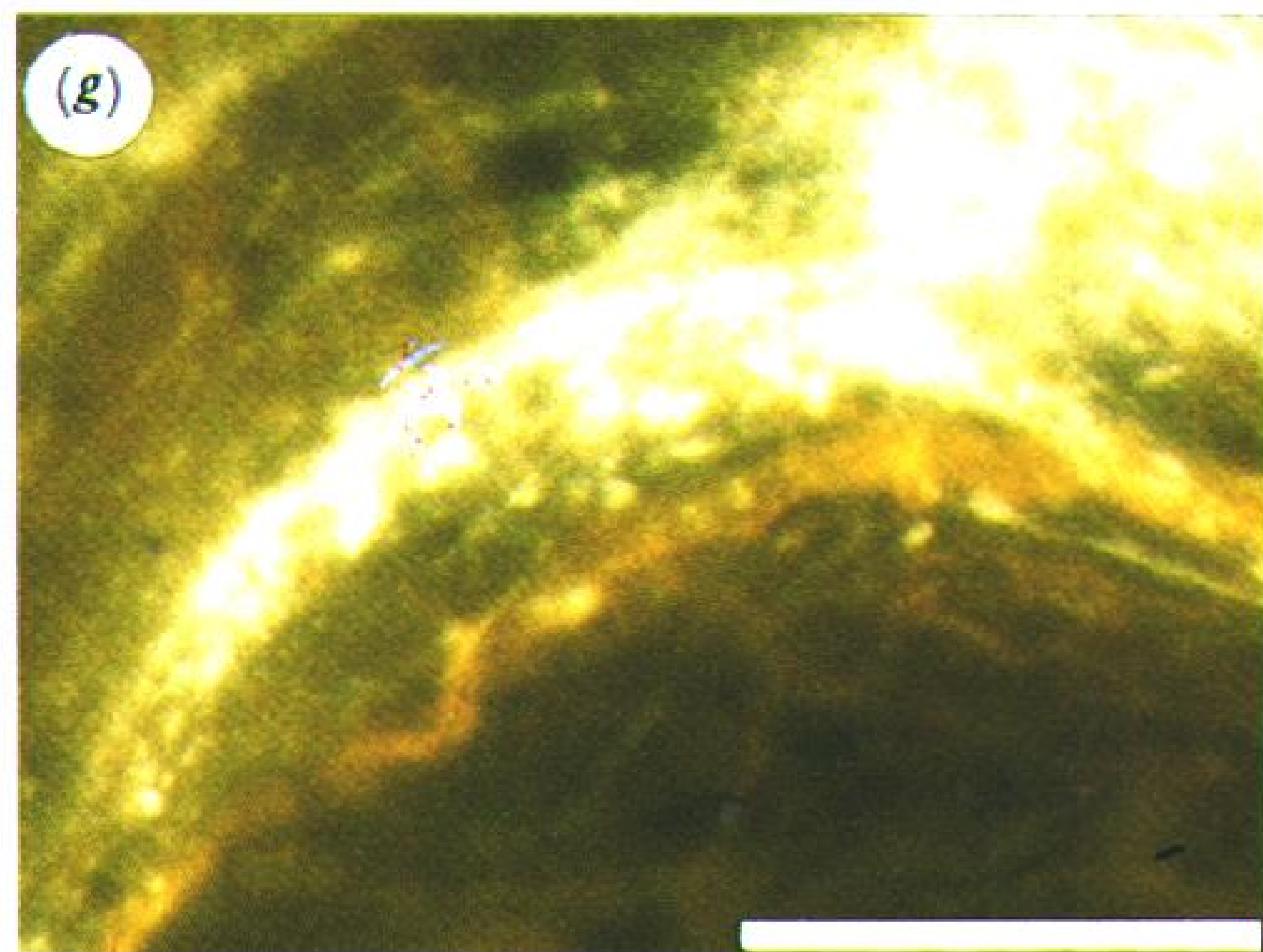


Downloaded from rstb.royalsocietypublishing.org









PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Figure 5*a* – *g*. For description see opposite.