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# Immunocytochemical localization of neurosecretory amines and peptides in the free-living nematode, *Goodeyus ulmi*

L. LEACH[1], D. L. TRUDGILL[2] and P. B. GAHAN[1*]

[1]*Department of Biology, King's College London, Kensington Campus, Campden Hill Road, London W8 7AH, UK*
[2]*Scottish Crop Research Institute, Invergowrie, Dundee, Scotland*

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## Summary

Mammalian antibodies to the neuroamines, serotonin and γ-amino-butyric acid (GABA) and to the neuropeptides, adrenocorticotrophic hormone (ACTH) and FMRF-amide evoked a response to *Goodeyus ulmi*, a free-living nematode. Serotonin-like immunoreactivity was found in cell bodies in the nerve ring and in the ventral nerve cord in all developmental stages. Neurons in the vulva, implicated in egg-laying, were immunoreactive to anti-serotonin in *G. ulmi* females, while in males serotonergic nerve fibres was found in the spicular region. Immunoreactivity to ACTH was also seen to differ depending on the developmental stage of *G. ulmi*, being present only in the ventral cord from the late L3 stage. Anti-GABA immunoreactivity was localized in two cell bodies near the amphids in all life stages and FMRF-amide immunoreactivity was seen in the nerve ring in all developmental stages. No reactivity was found with antibodies to vasointestinal peptide and somatostatin-14.

## Introduction

Although a variety of biochemical, histochemical and electrophysiological experiments have been used to determine the presence and localization of neurotransmitters in nematodes, few of these studies have succeeded. The presence of acetylcholine has been indicated by electrophysiological experiments conducted on the larger animal parasitic nematodes, e.g. *Ascaris* (Debell, 1965). Paraformaldehyde-induced fluorescence has been used to show that serotonin and octopamine are present in the soil nematode *Caenorhabditis elegans* (Horvitz *et al.*, 1982) while dopamine has been implicated as a neurotransmitter in *C. elegans* (Sulston *et al.*, 1975). However, there appears to be no literature concerning either the presence or the localization of peptidergic neurotransmitters in nematodes. As a first step in the study of the control mechanisms of growth, development and moulting in *Goodeyus ulmi*, immunocytochemistry has been employed to study the localization and variety of such molecules in the free-living nematode *G. ulmi*, an ectophoretic associate of the bark beetles, *Scolytus scolytus* and *S. multistriatus*, which transmit dutch elm disease.

In this study the presence of the neuroamines serotonin and γ-amino-butyric acid, the conventional neurotransmitters, were investigated together with peptides like somatostatin, vaso-intestinal peptide, adrenocorticotrophic hormone and phenylalanylmethionylarginylphenylalanimide, which have recently been implicated as neurotransmitters in both vertebrate and invertebrate systems. Localization of immunoreactivity of amines and especially peptides with known hormonal functions would facilitate comparative studies to understand control mechanisms involved in the post-embryonic development of *Goodeyus ulmi*.

## Materials and methods

### CULTURE

*Goodeyus ulmi* were obtained from bark beetle frass found in diseased elm (Dutch elm disease) from near Perth, Scotland and cultured on nutrient agar plates inoculated with *Escherichia coli* (B9) (Leach *et al.*, 1986).

### IMMUNOCYTOCHEMISTRY

*Tissue preparation*

Whole nematodes were fixed in freshly prepared 4%

*To whom correspondence should be addressed.

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paraformaldehyde solution in 0.1 M phosphate buffer for 24 h at 4° C, squashed and frozen (Gossett *et al.*, 1980). In order to increase cuticle permeability, the squashes were hydrated with a graded alcohol series before washing in 0.01 M phosphate buffered saline (PBS) at pH 7.2. To further facilitate entry of each antibody and reduce non-specific binding in the tissue, 0.6% Triton ×100 and 1 mg/ml bovine serum albumin (BSA) were added to each antibody dilution and intermediate PBS washes (Polak & Van Noorden, 1985).

*Immunocytochemical reactions*

Mammalian antisera to serotonin (5-HT), γ-aminobutyric acid (GABA), adrenocorticotrophic hormone (ACTH), somatostatin-14 (SS14), vasointestinal peptide (VIP) and FMRF-amide (Phe-Met-Arg-Phe-$NH_2$) were used. The antibodies were obtained from two different sources, commercial (Immuno-nuclear Inc.) and from Professor J. Polak, Royal Postgraduate Medical School, London.

The indirect immunofluorescence technique (Coons *et al.*, 1955) was used for anti-neuroamines and the peroxidase–anti-peroxidase method (Sternberger & Joseph, 1979) was used to localize neuropeptides. In the indirect reaction the background reaction was blocked by the addition of goat serum (1:30 dilution), the serum of the donor animal of the second layer, to the squashes and incubation for 30 min at 37° C. The goat serum was drained off and the primary antibody (rabbit) at a dilution of 1:1000 was placed on the squashes and incubated for 24 h at 20° C followed by 2 h at 37° C. The primary antibody was removed and the specimens were washed in Triton-PBS three times each for 30 min, at 37° C. The squashes were again incubated for 30 min at 37° C in goat serum and finally rhodamine isothiocyanate goat anti-rabbit was placed on the squashes and incubated for 24 h at 4° C, followed by 30 min at 37° C. The specimens were rinsed thoroughly in Triton–PBS and mounted in 80% glycerol with 0.2% propyl gallate added as a free radical scavenger (pH 8.0).

In the peroxidase–anti-peroxidase method the squashes were first immersed in excess 0.3% hydrogen peroxide for 30 min at 37° C to block endogenous peroxidase. They were then incubated in goat serum, as for the indirect method, and then in primary rabbit antibody (1:1000 dilution). After rinsing in Triton–PBS, the squashes were incubated in a second layer, the peroxidase-conjugated goat anti-rabbit globulin (1:200 dilution) for 60 min at 37° C. The specimens were again rinsed (3 × 30 min) and a third layer, the peroxidase–anti-peroxidase (1:500 dilution) was added, the squashes being incubated for 60 min at 37° C. All specimens were rinsed thoroughly and the peroxidase label was visualised using a reaction medium containing 25 mg of 3,3′ diaminobenzidine tetrahydrochloride in 100 ml PBS to which 50 µl of 100 vol (30%) $H_2O_2$ was added. The optimal reaction time was found to be 15 min at 37° C. The squashes were rinsed in PBS and mounted in Farrants' medium.

*Controls*

In order to block the antigenic sites, the primary antibody layer was substituted with either non-immune serum from the species donating the second-layer antibody, e. g. normal goat serum at a 1:1000 dilution or 1 mg/ml of serum bovine albumin in PBS. Control tissues from the rat brain were used to check the viability of the antibodies. Serum specificity of the antisera used was tested by preabsorbing the antisera with corresponding artificial antigens for 1 h at 37° C prior to usage as the primary antibody layer.

*Microscopy*

The peroxidase–anti-peroxidase preparations were viewed with a Zeiss II photomicroscope and the fluorescent preparations with a Leitz Dialux 20 EB microscope using a filter block M2 which incorporates a BP 546/14 nm excitation filter, RKP 580 beam splitting mirror and LP 850 nm suppression filter.

Photomicrographs of fluorescent images were taken on Kodak Ektachrome ASA 200 film, using a Wild MPS45 Photo automat system. Photomicrographs of the peroxidase–anti-peroxidase reaction were taken on Ilford Contrast FF film.

## Results and discussion

Immunoreactivity to anti-5HT, anti-GABA, anti-ACTH and anti-FMRF amide was found. Their localization in different developmental stages of *G. ulmi* is shown in Table 1 and Fig. 1–3. No immunoreactivity was found with either anti-SS14 or anti-VIP.

Different regions of the nervous system, such as the cell bodies and nervous fibres in the circumpharyngeal nerve ring or the longitudinal cords, show immunoreactivity to different types of antisera, whether anti-peptidergic or anti-aminergic. However, limited immunoreactivity was seen to the classical inhibitor of motor neurons, GABA, only two neurons in the head showing GABA-like reactivity. This may reflect either the absence of GABA from other neurones, or a limited cross-reactivity between the mammalian antibody to GABA and the nematode GABA molecule, or technical difficulties, e.g. low levels of binding, washing out of the antibodies during the tissue preparation, and permeability barriers to these molecules. The latter may well be important in nematodes with their relatively impermeable cuticle. Recent studies on the use of collagenase and elastase as a pretreatment step during tissue preparation (McIntyre, personal communication) indicate that enzyme treatment enhances the number of anti-GABA immunoreactive neurons and fibres found in *Caenorhabditis elegans*. No reactivity was found with anti-VIP or anti-SS14 in *G. ulmi*. Intraneuronal peptides are not often present in as high concentrations as, for example, hormones in endocrine cells. Thus the negative results may be due to technique insensitivity or an absence of these peptides in *G. ulmi*.

Serotonin (5-HT)-like immunoreactivity was located in the ventral nerve cord, the major longitudinal

fibre bundle of motor neurons in *G. ulmi* (Fig. 1a) as well as in the two major ganglionic regions of the nerve ring, which comprises motor and sensory neurons (Table 1, Fig. 1d) in all developmental stages. Serotonergic immunoreactivity has also been located in four group C motor neurons in the vulva (Table 1, Fig. 1c), which in *G. ulmi* resemble by virtue of their position and lineage the hermaphrodite specific neurons of *C. elegans*, a soil nematode morphologically very similar to *G. ulmi* (Sulston *et al.*, 1983). This is a first demonstration of serotonin-like immunoreactivity in the vulval neurons of nematodes. Additional serotonergic-like immunoreactive nerve fibres were localized in male *G. ulmi* near the spicule in the tail and were similar in localization to the male specific neurons identified by Nomarski interference microscopy. A similar male/female difference in serotonergic-like immunoreactivity has been implicated in *C. elegans* (McIntyre & Horvitz, 1985).

In *G. ulmi*, exogenously applied serotonin stimulated vulval activity three-fold (Leach *et al.*, in preparation). Similar activity has been observed in wild-type hermaphrodites of *C. elegans*; whilst *C. elegans* mutants deficient in hermaphrodite specific neurons do not lay eggs, egg-laying has been induced by application of serotonin, implicating the hermaphrodite specific neurons as an endogenous source of serotonin in wild types (Trent *et al.*, 1983). The additional serotonergic neurons seen in the caudal region of male *G. ulmi* resemble those seen in *C. elegans*; these neurons have been implicated in copulatory behaviour in the latter nematode (White *et al.*, 1975). The immunolocalization of serotonin-like reactivity in the vulval and tail neurons of adult *G. ulmi* as well as in the ventral nerve cord of all developmental stages indicates that serotonin may be present in *G. ulmi* and may be involved in the egg-laying and copulatory behaviour of *G. ulmi* as well as functioning as a general motor neurotransmitter.

Immunoreactivity to ACTH, a hormone involved in gonadal development in mammals (Bell *et al.*, 1980) was localized in the ventral nerve cord in *G. ulmi* from the late L3 juvenile stage to the adult stage, corresponding to the period of maximum gonadal development which culminates during the L4 moult. No ACTH-like immunoreactivity was seen in the early juvenile stage, i.e. L1, L2 and early L3. The temporal specificity of ACTH localization in the ventral cord, which is adjacent to the gonadal regions, indicates a possible involvement of ACTH in gonadal development in *G. ulmi*. ACTH and 5HT immunoreactivity were seen at similar sites in the ventral nerve cord, in the tail region and in the part of the cord just posterior to the retrovesicular ganglion.

FMRF-amide-like immunoreactivity was seen in some of the nerve fibres in the nerve ring in all developmental stages of *G. ulmi* (Fig. 3). The tetrapeptide FMRF-amide, first characterized as a molluscan cardioexcitatory compound, has been found

**Table 1.** Localization of immunoreactivity to anti-5HT, anti-GABA, anti-ACTH and anti-FMRF amide in the various life stages of *G. ulmi*.

| *Life stages* | *Anti-5HT* | *Anti-GABA* | *Anti-ACTH* | *Anti-FMRF amide* |
|---|---|---|---|---|
| First larval stage (L1) | Ventral nerve cord and six cell bodies along the cord adjacent to the gonadal regions; four cell bodies in the ventral and lateral ganglia of the nerve ring | Two cell bodies in the amphidial region of the head, situated opposite each other in the dorsal and in the ventral plane | No reaction | Longitudinal nerve fibres leading both into and posterior to the nerve ring, and in fibres within the nerve ring |
| Second larval stage (L2) | " " | " " | No reaction | " " |
| Third larval stage (L3) | " " | " " | Ventral nerve cord | " " |
| Fourth larval stage (L4) | " " | " " | " " | " " |
| Female | Same as larvae and four cell bodies and associated commissures in the vulval region | " " | " " | " " |
| Male | Same as larvae and additional nerve fibres near the spicule | " " | " " | " " |

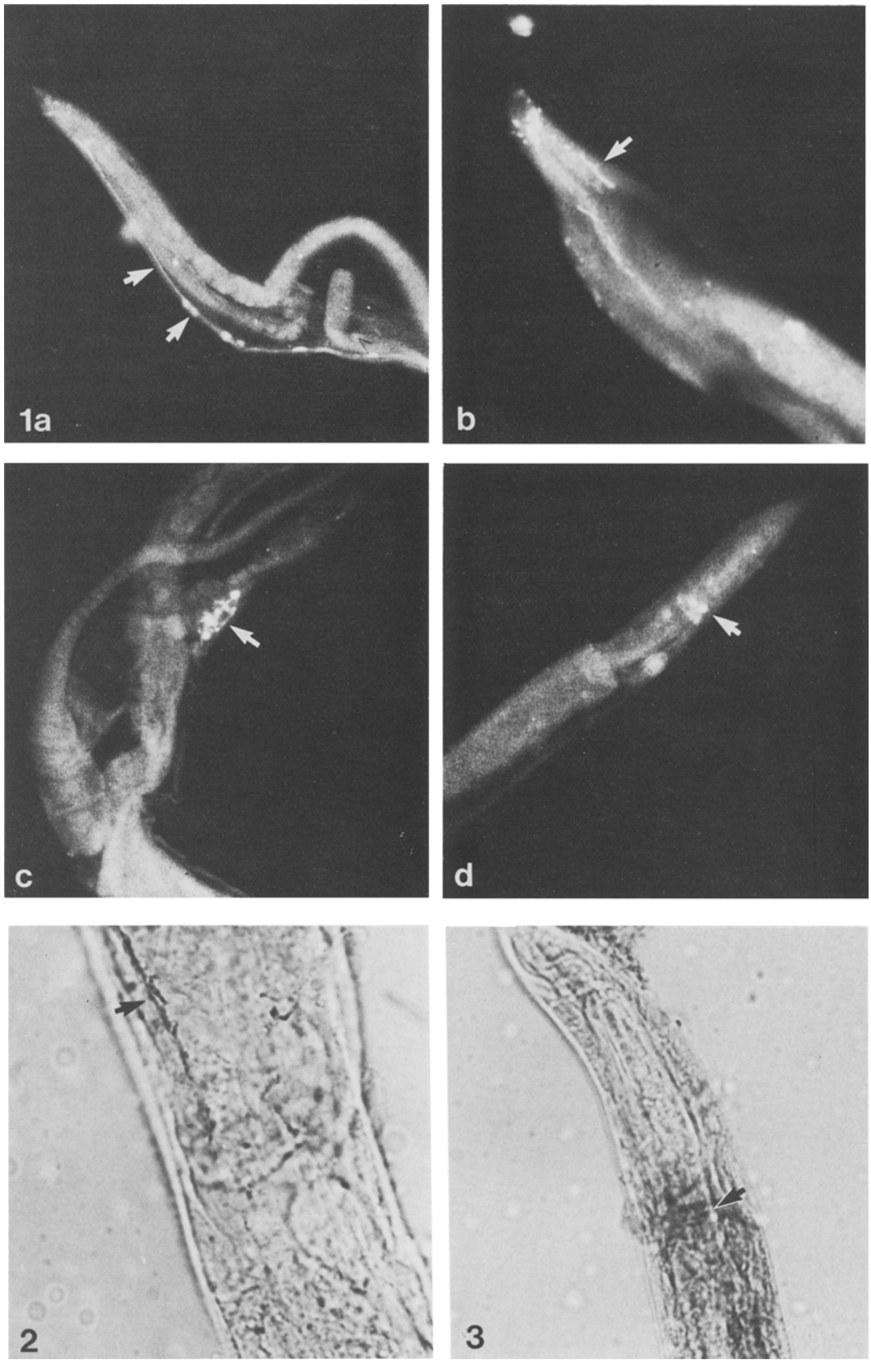


**Fig. 1.** Localization of 5HT-like immunoreactivity in *G. ulmi* squash preparations by the indirect fluorescence method. All × 250. Arrows show areas of immunoreactivity. (a) 5HT-like immunoreactivity in the ventral nerve cord and associated cell bodies. (b) 5HT-like immunoreactivity in spicular regions of the male. (c) 5HT-like immunoreactivity in the vulval region of the female. (d) Cell bodies in the ventral and lateral ganglia of *G. ulmi* showing 5HT-like immunoreactivity.

since in a variety of animals where it has numerous physiological effects. Price & Greenberg (1977a, b) suggested that FMRF-amide might act as a long duration mimic of the classical molluscan cardioexcitatory transmitter 5HT. In mammals, FMRF-amide-like immunoreactivity can be localized both in neurons of the central nervous system and in gut endocrine cells, implying a dual role as both a central neuroregulator and a gut hormone (Dockray *et al.*, 1981; Sorenson *et al.*, 1984). FMRF-amide-like immunoreactivity has been reported for *C. elegans* (Li & Chalfie, 1986) where the immunoreactivity has been visualized in several motor neurons of the ventral cord as well as in the nerve ring. Thus, the immunoreactivity to anti-FMRF-amide found in all of the *G. ulmi* individuals suggests that it may also be present in nematodes, localization in the central nerve ring indicating a neuro-regulatory function.

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**Fig. 2.** ACTH-like immunoreactivity in the ventral nerve cord adjacent to the gonadal regions. (PAP method, × 345.)

**Fig. 3.** FMRF amide-like immunoreactivity in the nerve ring of *G. ulmi.* (PAP method, × 345.)
Arrows show areas of reactivity.