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Monoamines in the nervous system of the tube-worm *Chaetopterus variopedatus* (Polychaeta): Biochemical detection and serotonin immunoreactivity

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Summary. The presence and distribution of biogenic monoamines in the tube-worm *Chaetopterus variopedatus* were investigated by a radioenzymatic method and HPLC with electrochemical detection, and the cellular localization of serotonin by peroxidase-antiperoxidase (PAP) immunohistochemistry with an antibody against serotonin-formaldehyde-protein conjugate. Dopamine, norepinephrine, epinephrine, serotonin (5-HT) and some of their metabolites were detectable, dopamine and norepinephrine being present in substantially larger amounts than 5-HT and epinephrine. With few exceptions, the largest amounts of amines were localized in the most nerve-rich tissues such as tentacles, and those containing cerebral ganglia and the ventral nerve cord. Serotonin-immunoreactive unipolar neurons were widely distributed in the dorso-lateral cerebral ganglia, the neurosecretory pharyngeal ganglion and the segmental ganglia of the anterior (dorsolateral) and posterior (medioventral) nerve cords. Some nerve-fiber tracts stained in the cerebral ganglia, but the neuropiles of segmental ganglia were the most intensely reactive CNS structures. Numerous reactive fibers were also present in connectives, commissures and segmental nerves. All peripheral sensory structures included serotonin-immunoreactive cells and neurites, especially the parapodial cirri and the bristle receptors of the setae. Trunk and parapodial muscles contained reactive varicose fibers and neuronal somata. These results suggest that monoamines are abundant and widespread in these worms and that 5-HT appears to have a key sensory role.

Key words: Monoamines – Serotonin – Polychaetes – Neurochemistry – Immunoreactivity – *Chaetopterus variopedatus* (Annelida)

Biogenic monoamines are currently considered to be major neuroactive substances in invertebrates as well as vertebrates (Klemm 1985). Although the phenolic amine octopamine appears to predominate in the arthropod nervous system (Robertson and Juorio 1976; David and Coulon 1985), dopamine and serotonin (5-HT) are commonly detected in arthropod as well as non-arthropod nervous systems (Klemm 1985). Similarly, octopamine (Robertson 1975; Webb and Orchard 1980; Belanger and Orchard 1986) as well as monoamines (Welsh and Moorhead 1960; von Euler 1961; Osborne et al. 1972; McAdoo and Coggeshall 1975) are present in nervous tissues or identified neurons of annelid worms, whose nervous system organization grossly resembles that of arthropods (Bullock and Horridge 1965). Little is known of the functional significance of these amines in annelid nervous systems (Gardner and Walker 1982), except that octopamine released by Leydig neurons in leech seem to play a neurohormonal role (Belanger and Orchard 1988), and 5-HT in Retzius cells has been implicated in the control of mucous release and inhibitory modulation of somatic muscles (Gardner and Walker 1982, for review).

Even less is known regarding monoamines in polychaetes. Catecholamines (Manaranche and L'Hermite 1973) and 5-HT (Welsh and Moorhead 1960; Manaranche and L'Hermite 1973) were estimated in two species of the errant polychaete *Glycera*, by use of fluorescence derivatization techniques, but these results have not been confirmed by more sensitive techniques such as radioenzymatic assays or HPLC. The large size of the chaetopterid tube-worms and the availability of these assay techniques prompted us to undertake a comprehensive analysis of monoamine contents in various tissues of a single sedentary species, *Chaetopterus variopedatus*.

To corroborate such an analysis, it is important to visualize the cellular distribution of monoamines in the same polychaete species. Catecholamines and 5-HT have been tentatively identified and visualized with the Falck-Hillarp fluorescence technique in various errant polychaetes (Clark 1966; Warembourg and Dhainaut-Courtois 1969; Manaranche and L'Hermite 1973; White and Marsden 1978). The visualization of 5-HT-containing neurons was largely confirmed and expanded recently by immunohistochemistry in another errant polychaete, the scale-worm *Harmothoe imbricata* (Miron et al. 1987; Miron and Anctil 1988). These studies have revealed that serotonergic neurons and fibers are widely distributed in the brain, ventral nerve cord and peripheral nervous system of this species, and that 5-HT is present in interneurons, motoneurons and a few sensory neurons. The contrasting lifestyles of errant versus sedentary polychaetes is reflected in major differences in brain development and nervous system organization (Bullock and Horridge 1965; Martin and Anctil 1984). We were interested to know whether such differences extend to the distribution of 5-HT immunoreactivity. The present immunohistochemical investigation was undertaken to compare the distribution of serotonergic neuronal elements of a seden-

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tary polychaete, *Chaetopterus*, with that of errant polychaetes, in order to gain further insight into the functional significance of 5-HT in polychaetes. We report in this study the presence of hithertho unsuspected serotonergic neurosecretory and sensory structures.

Materials and methods

Specimens of *Chaetopterus variopedatus* were obtained from Pacific Bio-Marine Laboratories and placed in an aquarium filled with cooled (14–16° C) filtered, aerated and recirculated artificial sea water (ASW, Wards). Before either biochemical or immunohistochemical processing, all worms, still residing inside their tubes, were anesthetized by immersion in isotonic $MgCl_2$ (0.37 M). After 30–60 min, the relaxed worms were removed gently from the tubes and the body parts of interest were dissected out and processed as described below.

Biochemical assays

Catecholamines were assayed in tissues of 30 sexually mature worms by a radioenzymatic method as described by De Waele et al. (1987). Tissues from 6 different body regions (see Table 1) were homogenized in 0.1 N perchloric acid, and extracts centrifuged for 20 min at 6000 rpm and 4° C. The supernatant was stored at −20° C until assayed. The assay involved a transmethylation from a donor, [^{3}H]S-adenosylmethionine (New England Nuclear, 55–85 Ci/mmol) to catecholamines in the presence of the enzyme catechol-O-methyltransferase (COMT, Sigma). This reaction was carried out in a buffer containing 0.2 M Tris base, 30 mM $MgCl_2$, 2.4 mM EGTA and 0.2% O-benzylhydroxylamine at pH 8.2 and 37° C. The tritiated, methylated products were then separated by applying the samples on thin-layer chromatography (TLC) plates (Rediplate GF, Fisher) with a multispotter (AIS Instrumentation). The migrated TLC spots, revealed by their fluorescence, were recovered and their radioactivity measured with an LKB 1217 liquid scintillation counter. The linearity of the enzymatic reaction, recovery of catecholamines and specificity of the assay were determined as reported by De Waele et al. (1987).

Catecholamines, 5-HT and one each of their metabolites were also simultaneously determined by HPLC with electrochemical detection (HPLC-ED). Tissues from 5 animals were extracted in 0.1 N perchloric acid and extracts were centrifuged at 52000 g and 4° C for 25 min. The supernatant was filtered with preparative disc-membrane filters (Bio-Rad, 0.45 µm), and 50-µl samples injected in the HPLC column. The HPLC equipment consisted of a BIO-RAD system including the Model 1350 soft-start pump, the AS-48 automatic sampler and a Bio-Sil ODS-5S reversed phase column (250 × 4 mm) maintained at 29° C in a column heater. A 30 × 4.6 mm ODS-5S reversed phase guard column and an SSI precolumn filter (porosity: 0.5 µm) were placed in-line between the sampler and the analytical column. Column effluents flowing at 1.2 ml/min passed through the carbon paste electrode of an EG & G-Princeton Model 400 electrochemical detector operated at +0.72 V. The signals were split between a Pharmacia recorder and an analog-to-digital computer interface for further analysis by use of the BIO-RAD Model 600 chromatography workstation. The buffer used as mobile phase was composed of 0.75 mM sodium phosphate, 1 mM 1-octane sulphonic acid, 50 µM EDTA, 12% methanol and 3% acetonitrile at pH 3.25. The freshly made mobile phase was vacuum filtered with nylon membrane filters (porosity: 0.22 µm) before use. All reagents and water were of HPLC grade. Amine standards were obtained from Sigma.

Immunohistochemical procedure

The procedure for 5-HT immunostaining was identical to that reported by Miron and Anctil (1988). Tissues were fixed for 18 h at 4° C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), and embedded in O.C.T. compound (TissueTek, Miles Laboratories) by freezing with

Table 1. Concentration of endogenous monoamines and some of their metabolites in tissues of *Chaetopterus variopedatus*

Amines	Tissues sampled					
	Tentacles	Cerebr. gangl.	Notopods	Intestine	Fans	Nerve cord
DA	380.9±37.8 (776.9±187.1)	290.9±63.8 (770.9±201.0)	64.2±17.8 (434.5±144.8)	201.1±33.3 (107.8±31.6)	261.3±39.9 (152.1±25.9)	229.7±27.6 (159.6±58.0)
DOPAC	traces	traces	traces	27.6± 5.2	14.9± 5.9	48.8± 4.9
NOR	346.7±27.9 (176.4±77.3)	204.4±34.6 (322.5±83.6)	71.6±14.4 (212.2±45.9)	144.5±24.4 inhibition	159.3±19.7 (205.9±63.8)	227.1±16.7 (100.2±23.4)
NMN	93.8±28.4	95.1±29.2	ND	ND	ND	ND
EPI	ND (32.8± 8.4)	ND (14.3± 5.8)	ND (5.2± 3.0)	ND inhibition	ND (9.5± 0.3)	ND (21.8±15.3)
MN	83.4±15.0	ND	47.8±18.1	ND	ND	78.4±21.1
5-HT	83.2±13.8	99.4±24.7	11.6± 3.1	19.8± 7.1	53.5±13.8	94.8±16.7
5-HIAA	ND	ND	ND	13.5± 6.5	13.8± 6.0	20.3±13.8

Values are means±SD in pg/mg tissue (wet weight). Values in parentheses are from radioenzymatic assays (n=30 animals), all others from HPLC-ED (n=5 animals). ND=not detectable; traces=barely at detection level; inhibition=not detectable because of inhibition of radioenzymatic reaction by extract

The abbreviated amines are dopamine (*DA*), 3,4-dihydroxyphenylacetic acid (*DOPAC*), norepinephrine (*NOR*), normetanephrine (*NMN*), epinephrine (*EPI*), metanephrine (*MN*), 5-hydroxytryptamine (*5-HT*), and 5-hydroxyindole-3-acetic acid (*5-HIAA*)

isopentane cooled in liquid nitrogen. Sections of 10–15 μm, cut with a Hacker-Bright cryotome, were rinsed in phosphate-buffered saline (PBS) and immersed in 0.3% H_2O_2 in PBS to eliminate endogenous peroxidase reactions. The rabbit 5-HT antibody (Incstar) was diluted 1:500 or 1:800 in PBS containing 0.1% Triton-X-100 and 33% nonantigenic swine serum. Sections were incubated in this primary antibody solution for 42 h at 4° C in a moist atmosphere. The sections were then incubated for 1 h in a 1:50 dilution of an antiswine antibody raised against rabbit immunoglobulin (Dimension). The reaction was finalized by immersing the sections for 1 h in the peroxidase-antiperoxidase complex (PAP, Dimension) diluted 1:100. The reaction was revealed with 0.05% diaminobenzidine and 0.015% H_2O_2 in PBS for 7 min. Semi-serial sections of different body regions were mounted in Aquamount (BDH) and examined with a Zeiss Axiomat microscope.

For controls, the primary or secondary antibodies, or the PAP complex were omitted from the protocol. The specificity of the antibody was tested as reported by Miron et al. (1987).

Results

Monoamine contents

All three biogenic catecholamines and 5-HT were detected in tissues of *C. variopedatus* (Table 1, Fig. 1). Dopamine appeared to be the quantitatively predominant catecholamine in *Chaetopterus* tissues assayed by the radioenzymatic method. However, the amounts of norepinephrine detected by HPLC-ED were roughly equivalent to those of dopamine (Table 1). Epinephrine levels were consistently marginal in radioenzymatic assays, and undetectable by HPLC-ED due to the masking effect of a solvent peak overlapping with the adrenaline peak at low epinephrine concentrations in our chromatographic conditions (Fig. 1). In addition, metabolites of dopamine (DOPAC), norepinephrine (normetanephrine), epinephrine (metanephrine) and 5-HT (5-HIAA) were simultaneously detected by HPLC-ED in some tissues (Table 1, Fig. 1). Except for epinephrine, the metabolites were present in amounts smaller than those of their corresponding biogenic amines.

Six tissue regions were dissected out for both radioenzymatic and HPLC analysis: the tentacles, the cerebral ganglia and surrounding tissue, the aliform notopods, the anterior intestine, the ventral nerve cord flanked by two strips of longitudinal muscle, and the modified notopods known as fans (including segmental ganglia). The tentacles, cerebral ganglia and ventral nerve cord were the most nerve-rich tissues sampled, and they contained the largest amounts of catecholamines and 5-HT (Table 1). Dopamine, norepinephrine and 5-HT levels measured by HPLC-ED were lowest in the aliform notopods and intestine in which the nerve cord was absent. Tissues such as the fans in which muscle masses dominate, contained lower levels of norepinephrine and 5-HT than nerve-rich tissues. In the heavily pigmented anterior intestine, norepinephrine and epinephrine were undetected radioenzymatically due to the presence of factors in the samples that inhibited the COMT-mediated reactions, as shown by the inhibitory effect of intestinal samples on recovery of internal standards (see also De Waele et al. 1987).

Localization of 5-HT immunoreactivity in the central nervous system

The major neuroanatomical features and patterns of 5-HT immunostaining of *C. variopedatus* are illustrated in Fig. 2, and the terminology used in this account follows those of Bullock and Horridge (1965) and Martin and Anctil (1984).

Specific 5-HT immunoreactive (5-HT-IR) elements were widely distributed in the nervous system of *C. variopedatus*. They were present in the cerebral, pharyngeal and segmental ganglia as well as in their connectives and commissures. Segmental nerves and the subepidermal nerve plexus also contained 5-HT-IR neurites. Numerous 5-HT-IR neurites were also localized in sensory structures associated with parapodial cirri and setae. In addition, 5-HT-IR fibers were distributed along and inside trunk and pharyngeal muscles. Control sections were usually free of staining except for some sections in which the neuropiles, peripharyngeal and subepidermal nerve plexuses as well as epidermal cells exhibited non-specific staining. In the plexuses, this staining was coarsely granular in texture (Fig. 5C) and appeared to be associated with granule-containing glio-interstitial

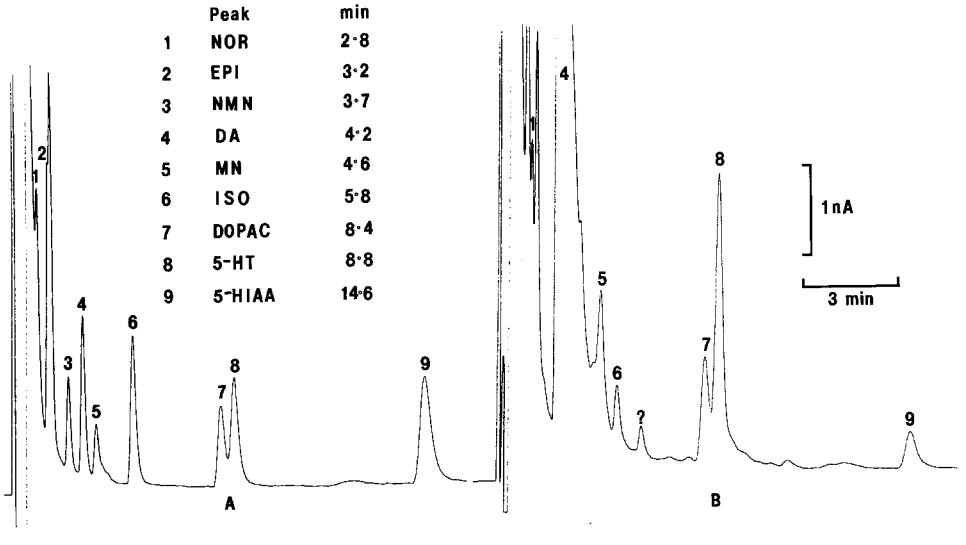


Fig. 1 A, B. Representative electrochromatograms of 1 ng each of amine standards (**A**) and of an extract of the ventral nerve cord (**B**), prepared as described in Materials and Methods. Time of elution peak (*min*) for each substance is given in **A**. Abbreviations are identified in Table 1, except for the internal standard isoproterenol (*ISO*). Note in **A** that elution peak for epinephrine standard (2) overlaps with a late solvent peak; an unidentified peak is shown in **B** (?)

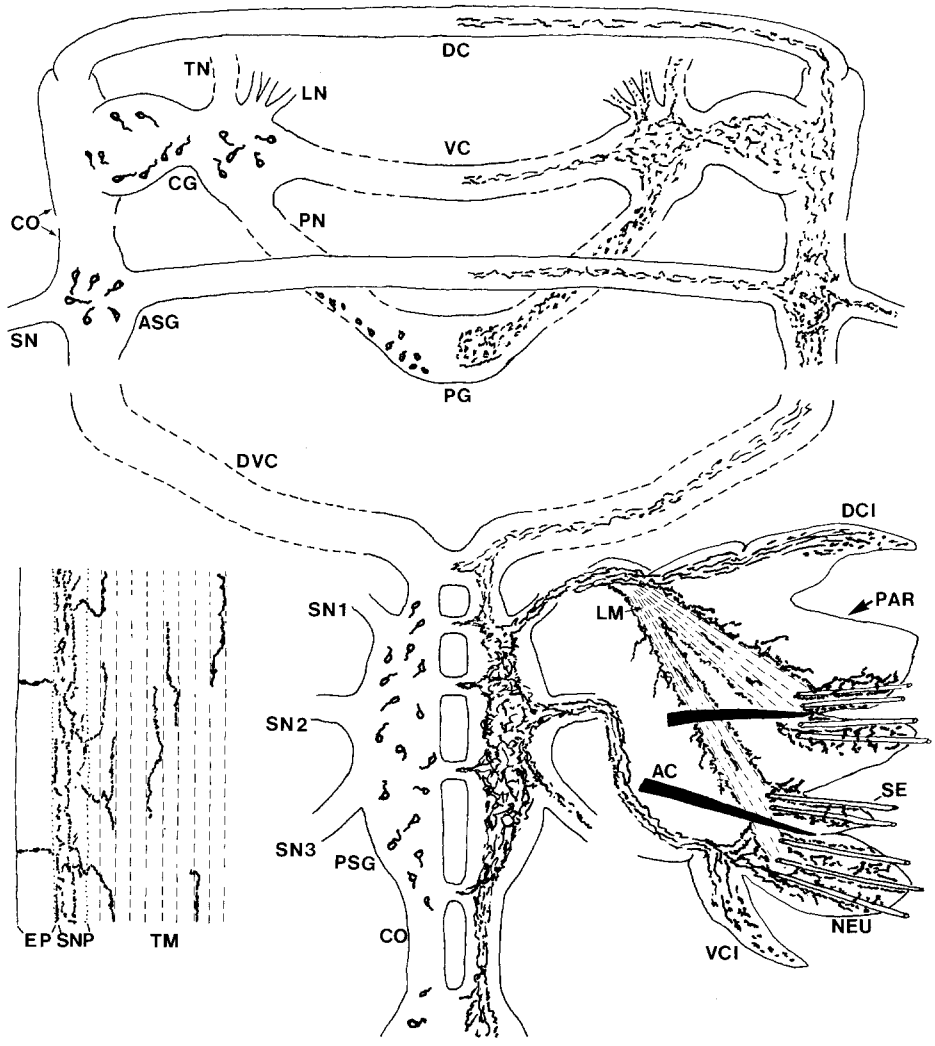
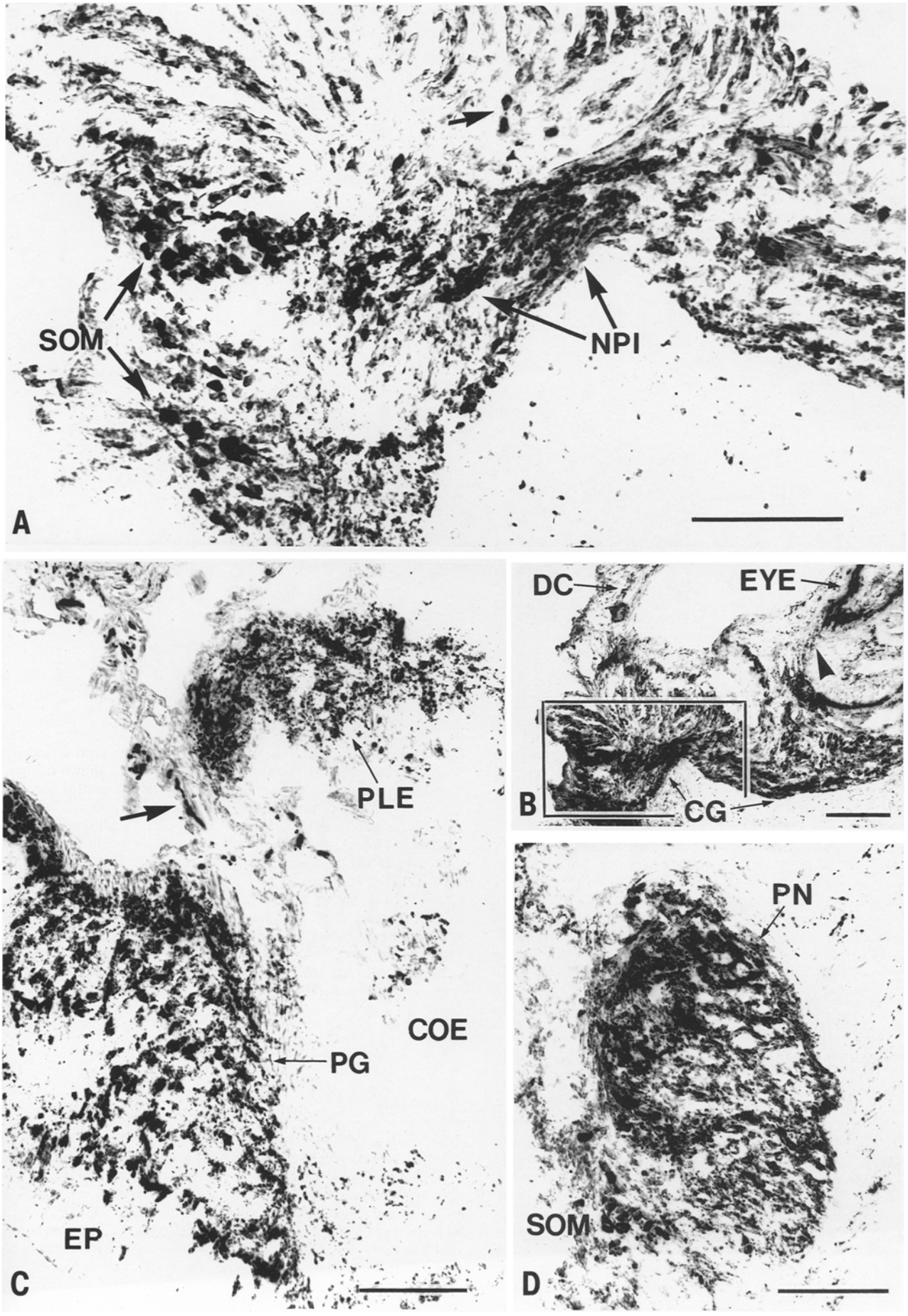


Fig. 2. Schematic representation of main components of nervous system of *Chaetopterus variopedatus*, with synopsis of distribution of 5-HT-immunoreactive elements. In the central nervous system, note cerebral ganglia (*CG*) interconnected by dorsal (*DC*) and ventral commissure (*VC*), and connected with pharnygeal ganglion (*PG*) by pharyngeal nerve. Root of tentacular nerve (*TN*), three labial nerves (*LN*). ASG second anterior segmental ganglion with its single commissure and single segmental nerve (*SN*), located in 4th body segment containing pharyngeal ganglion. Lateral connectives (*CO*) interconnect segmental ganglia and connect first anterior segmental ganglion to cerebral ganglion. Dorso-ventral connectives (*DVC*) descend from dorso-laterally situated 9th anterior segmental ganglia to first pair of posterior segmental ganglia (*PSG*) in 12th body segment with their 3 segmental nerves (*SN1, 2, 3*). Trajectories between dorsal and ventral locations represented by dashed lines. Serotonin-immunoreactive cell bodies shown in right half, and neuropiles and fiber tracts in left half of central nervous system. At left of posterior segmental ganglion, note one component of peripheral nervous system, the parietal wall consisting of epidermis (*EP*) with 5-HT-IR sensory cells, subepidermal nerve plexus (*SNP*) with a few 5-HT-IR fibers, and underlying trunk muscles (*TM*) also containing 5-HT-IR neurites. At right, parapod (*PAR, arrow* pointing to notopod) with skeletal elements, aciculi (*AC*) and setae (*SE*), and levator muscles (*LM*) in both dorsal notopod and ventral neuropod (*NEU*). Drawing adapted from Fig. 2 in Dorsett (1964). Drawings, not to scale, also show immunoreactive neurons and neurites associated with these elements as well as with dorsal (*DCI*) and ventral (*VCI*) sensory cirri

Fig. 3A–D. Transverse sections through cerebral ganglion (**A, B**), pharyngeal ganglion (**C**) and nerve (**D**) showing distribution of 5-HT-IR elements. **A** Enlargement of boxed portion in **B**, showing major foci of intense staining in neuropiles (*NPI*) and groups of small somata (*SOM*) in lateral portion of cerebral ganglion. Note also scattered somata (*arrow*). ×230. **B** Overview of cerebral ganglion (*CG*) shown in **A**, with dorsal commissure (*DC*) invading upper lip of mouth, and pigmented eyecup (*EYE*) whose optic tract includes some 5-HT-IR fibers (*arrowhead*). ×50. **C** Pharyngeal ganglion (*PG*) overlying epidermis (*EP*) and associated nerve plexus (*PLE*) located underneath pharynx, both structures bordering coelomic cavity (*COE*) and containing numerous 5-HT-IR neurites and small somata. Note reactive neurites (*arrow*) in connective tissue strand between the two structures. ×170. **D** Ventral half of trajectory of pharyngeal nerve (*PN*). Heavily stained fiber tracts and neuropile; small somata (*SOM*) located at ventro-lateral margin. ×170. *Bars*: 100 (**A, C, D**) and 200 μm (**B**)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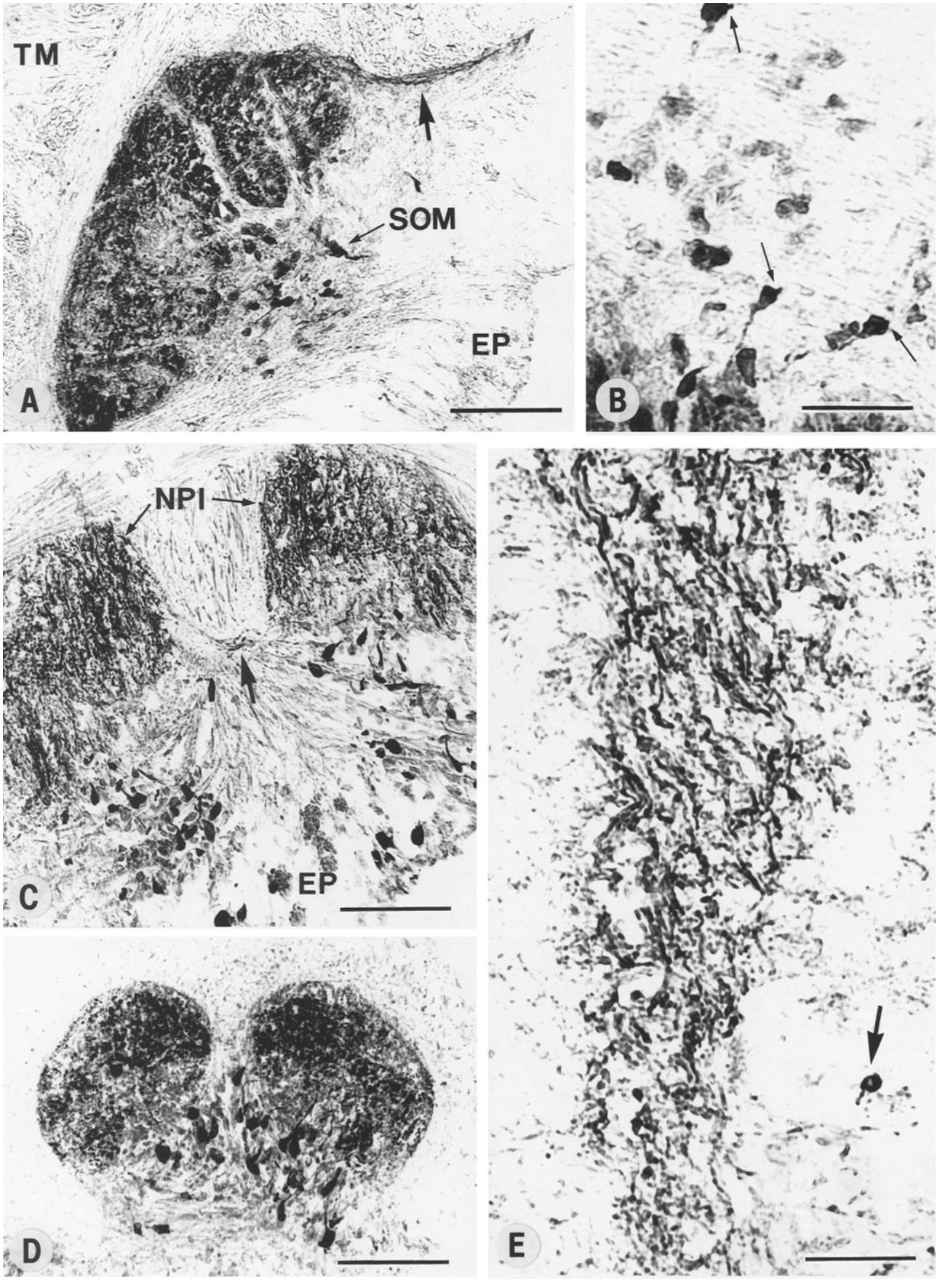
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cells of the nervous tissue (see Anctil 1979). In the epidermis, the non-specific staining was present in the gland cells described by Anctil (1979).

5-HT-IR elements were unevenly distributed within the elongated cerebral ganglion (Fig. 3B). They comprised numerous, unipolar neurons with small somata (5–7 μm diameter) and associated neurites, mostly concentrated in patches located posterior to the eyecup and to the roots of the labial nerves, and in the lateral lobe of the ganglion (Fig. 3A, B). Reactive nerve fibers were usually scattered throughout the neuropile, but a few bundles of intensely stained fibers were also present (Fig. 3A). A few 5-HT-IR neurites extended into the tractus opticus and eyecup (Fig. 3B) and into the roots of the tentacular and labial nerves (Fig. 2) where they merged into the local subepidermal plexuses. Both the dorsal (Fig. 3B) and ventral commissures emerging from these ganglia contained several reactive fibers. The pharyngeal nerves also included numerous reactive fibers, but in addition a few small somata stained at the margin of the nerve (Fig. 3D). The bilateral pharyngeal ganglion and median unpaired pharyngeal plexus were intensely reactive to the 5-HT antibody (Fig. 3C). The neuropile of the ganglion contained scattered, heavily stained neurites, with a few small reactive somata situated ventro-laterally to the neuropile. In the pharyngeal plexus, overlying a large coelomic cavity, a few small (3–4 μm diameter) somata and numerous short beaded neurites stained intensely (Fig. 3C). A few stained neurites lied in the connective tissue connecting the pharyngeal ganglion to its plexus (Fig. 3C).

The 5-HT immunoreactivity of anterior segmental ganglia was especially intense and widespread in the neuropile as well as in the long dorsal commissure interconnecting the bilateral neuropiles (Fig. 4A). Several 5-HT-IR unipolar neurons were localized in the ganglionic cell mass lying dorsally to the neuropile (Fig. 4A). From some of these somata, whose diameter reached up to 10–12 μm, emerged proximally a small apical knob and distally a thin axonal process usually oriented toward the neuropile (Fig. 4B). As in commissures, many 5-HT-IR fine varicose fibers were seen in the segmental nerve coursing underneath the epidermis. In addition, several small, beaded neurites emerging from the ventral surface of the ganglionic neuropile and innervating adjacent muscle sheets, were 5-HT-IR.

The pair of posterior ventromedian nerve cords also included considerable 5-HT immunoreactivity. Numerous varicose fibers (1–2.5 μm diameter) stained intensely in the neuropile of their ganglia (Fig. 4C–E), and a few stained also in the small commissures connecting the pair of neuropiles (Fig. 4C). Several intensely stained ganglionic somata were scattered ventrally to each neuropile, reaching inside the ventral epidermis (Fig. 4C). In regions of the ganglia where the bilateral neuropiles were more closely apposed (Fig. 4E), the reactive somata were straddled over the median space separating the two neuropiles. These unipolar neurons were similar in shape and size to those of the anterior ganglia. All three segmental nerves emerging from these ganglia contained numerous 5-HT-IR fibers (see Fig. 2 and below); a staining pattern similar to that of the long anterior commissures shown in Fig. 4A.

Localization of 5-HT immunoreactivity in the peripheral nervous system

Thin, elongated bipolar cells of the epidermis, as well as a few of the thin varicose neurites comprising the subepidermal nerve plexus, reacted with the 5-HT antibody (Fig. 5A). In the trunk, the ventral plexus contained more stained neurites than in the dorsal plexus. Where the plexus appeared more densely stained, it was attributable to neurites of segmental nerves or commissures running through it (Fig. 5B). Some of the neurites from these segmental nerves and commissures appeared to make incursions into the underlying musculature (Fig. 5B). Numerous 5-HT-IR fibers ran deep into longitudinal trunk muscles (Fig. 5E). These fine, variably varicose fibers run parallel to the long axis of the muscle fibers proper. In addition, some of the stained neurites in muscle sheets were part of intramuscular unipolar or pseudounipolar neurons endowed with small (3–4 μm diameter) somata capped with an apical knob (Fig. 5B, E).

Similarly, the pharyngeal epithelium contained thin 5-HT-IR bipolar cells endowed with a small ovoid soma. The outer process of these cells ended with a tiny apical knob at the epithelial surface lining the pharynx lumen (Fig. 5C). The peripharyngeal nerve plexus, like its subepidermal counterpart, contained a few thin reactive neurites (Fig. 5D). Unlike the subepidermal plexus, however, very few of its constituent reactive neurites made incursions into the underlying musculature. The intestine, in contrast, was devoid of 5-HT immunostaining.

Several parapodial structures contained 5-HT-IR elements (Figs. 2, 6, 7). The skeletal system of these locomotory structures, the basal aciculi and especially the distal setae (Fig. 2), were innervated by numerous reactive fibers (Fig. 6B). The network of setal neurites appeared to project into that of the subepidermal nerve plexus (Fig. 6A). The great density of small 5-HT-IR fibers in these regions formed a meshwork wrapping the setae (Fig. 6B), and some of these fibers in fact invaded the inner core of the setae (Fig. 6C). Many of these fibers were parallel to each other and maintained a definite orientation with respect to the long axis of the setae (Fig. 6B). The sensory cirri were the most richly innervated parapodial structures. Their apex and the adjacent parapodial region contained numerous epidermal immunostained cells, 4–10 μm in diameter (Fig. 6D), some of which clearly showed an axonal process. From these cellular masses individual reactive fibers become clustered into small bundles of intensely stained fibers

Fig. 4A–E. Transverse (**A–D**) and horizontal (**E**) sections through nerve cord, showing 5-HT-IR elements of ganglia, connectives and commissures. **A** Dorsolaterally situated anterior segmental ganglion with large, heavily stained neuropile and few 5-HT-IR somata (*SOM*) between neuropile and epidermis (*EP*). Note trunk musculature (*TM*) and stained fibers in commissure (*arrow*). ×190. **B** Enlarged view of 5-HT-IR neuronal somata from another anterior segmental ganglion. Note unipolar appearance and apical knobs (*arrows*). ×380. **C** Center of pair of bilateral, posteror segmental ganglia, comprised of two neuropiles (*NPI*) with numerous 5-HT-IR fibers, and of two associated masses of somata, some of them heavily stained and located in epidermis (*EP*). Few stained fibers in commissure (*arrow*). ×190. **D** Posterior end of pair of posterior ganglia with two heavily stained neuropiles and masses of somata more closely apposed. ×190. **E** Slightly oblique, horizontal section through neuropile of posterior connective with irregularly varicose, intertwined 5-HT-IR fibers. Note single stained elements (*arrow*) lateral to neuropile. ×380. *Bars*: 100 (**A, C, D**) and 50 μm (**B, E**)

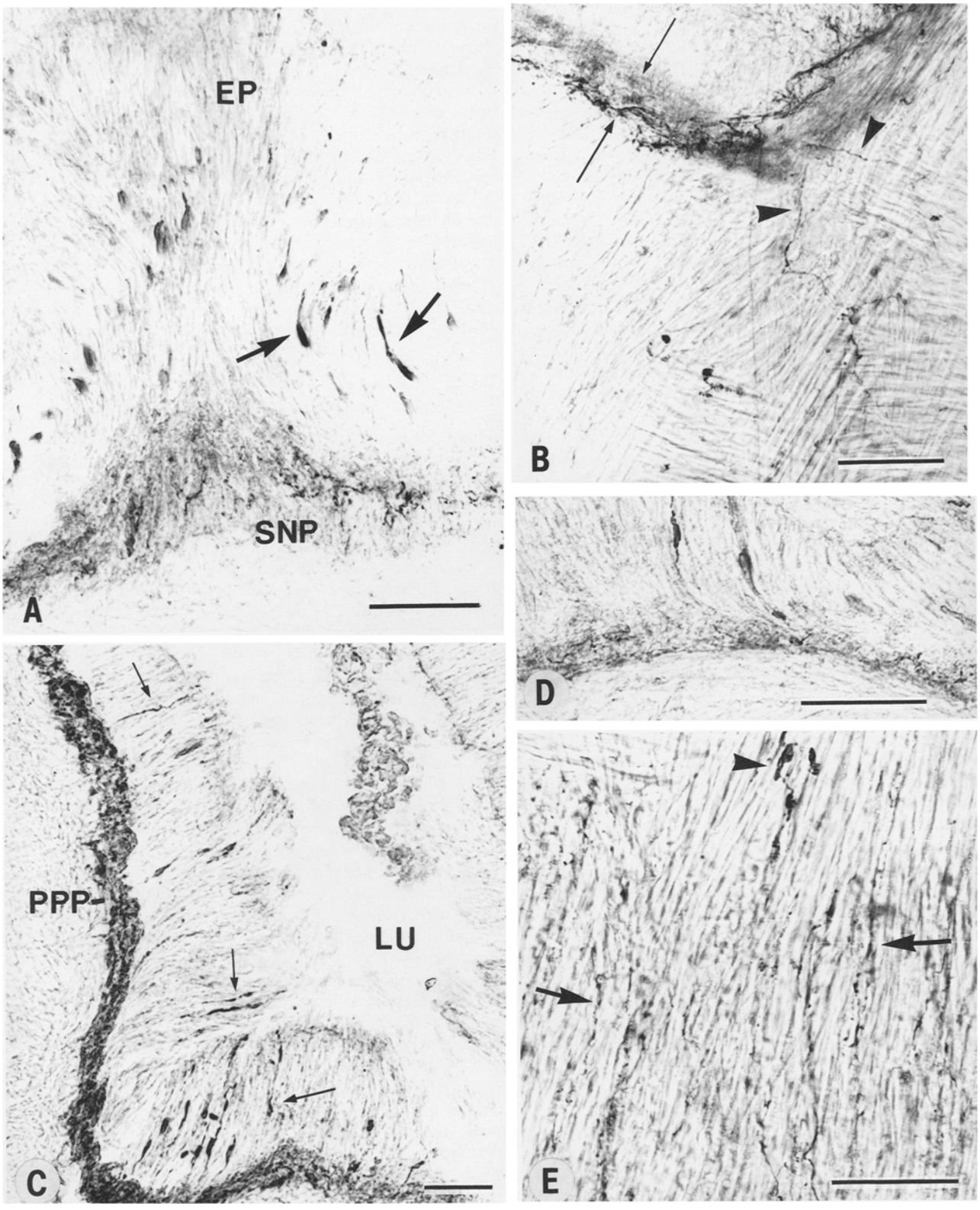

Fig. 5A–E. Transverse sections through trunk of anterior body segments, showing 5-HT-IR elements in body wall and musculature (**A, B, E**) and in pharynx (**C, D**). **A** Dorsal body wall flanking median ciliary groove, showing thin, elongated 5-HT-IR cells (*arrows*) in epidermis (*EP*) and few stained varicose fibers in subepidermal nerve plexus (*SNP*). ×360. **B** Commissure (between *arrows*) coursing underneath dorsal epidermis with meshwork of 5-HT-IR fibers, from which emerge stained varicose fibers (*arrowheads*) invading trunk musculature. Note also reactive neurones with small somata deeper in muscle. ×340. **C** Pharyngeal wall containing fine, elongated reactive cells (*arrows*) whose apical knob reaches lumen (*LU*). Peripharyngeal plexus (*PPP*) heavily stained, but mostly unspecific. ×230. **D** Peripharyngeal plexus with all visualized neurites specifically 5-HT-IR. Note also two reactive pharyngeal cells. ×400. **E** Numerous varicose 5-HT-IR fibers (*arrows*) scattered in longitudinal trunk muscles, and occasional reactive neurons with small soma (*arrowhead*). ×400. *Bars*: 50 μm

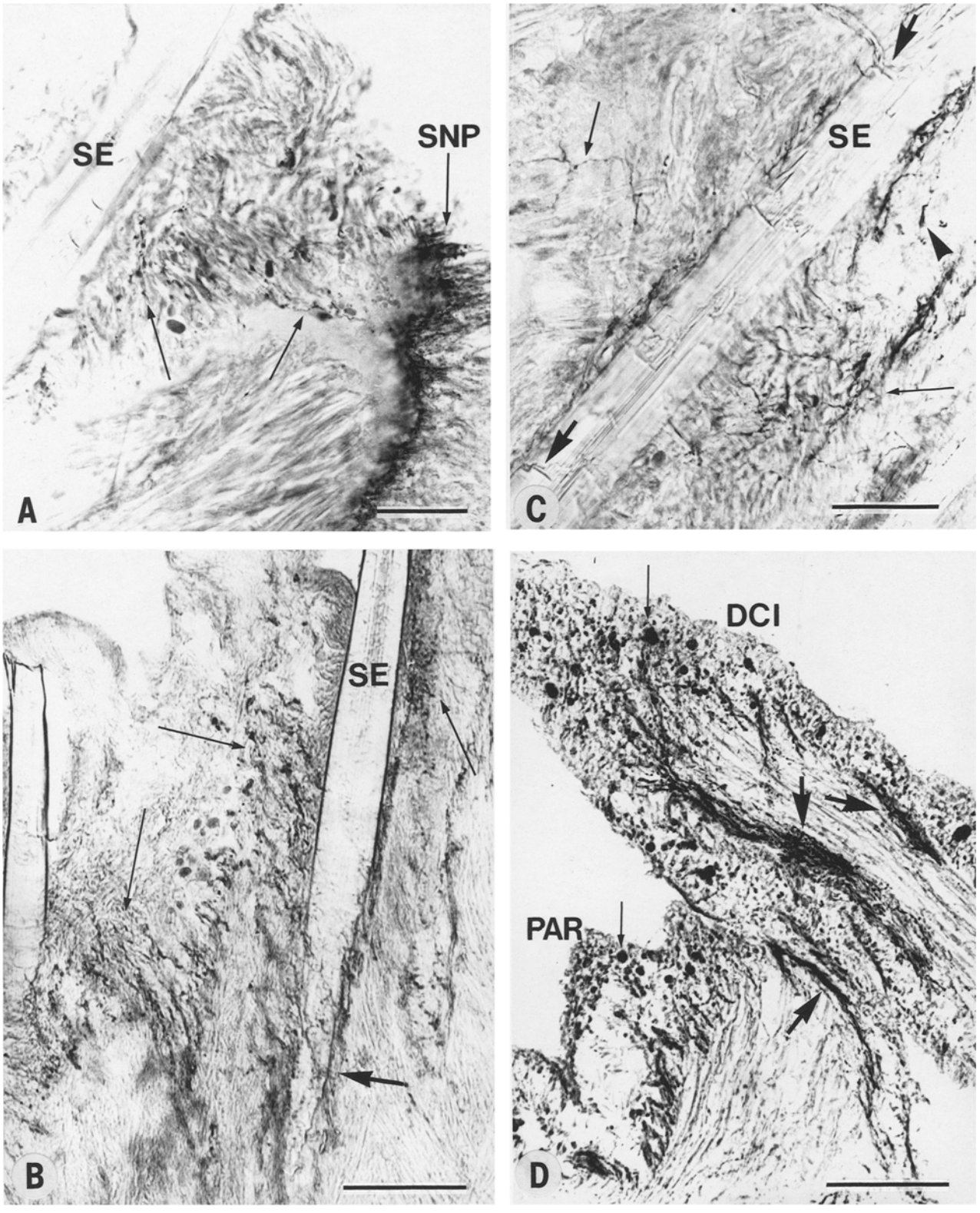

Fig. 6 A–D. Sagittal sections through parapods showing distribution of 5-HT-IR elements in their sensory structures. **A** Reactive neurites (*arrows*) forming meshwork over connective tissue spanning across from setae (*SE*) to reactive neurites in subepidermal nerve plexus (*SNP*) of neuropod. ×350. **B** Numerous small reactive neurons and neurites (*small arrows*) closely associated with two setae (*SE*) of notopod. Setae enwrapped by reactive neurites (*large arrow*). ×235. **C** Reactive neurites (*small arrows*) associated with setae (*SE*), some invading core of setae (*large arrows*). Note small soma of sensory neuron (*arrowhead*) whose varicose neurite reaches setal surface. ×400. **D** Dorsal cirrus (*DCI*) and adjacent region of its parapod (*PAR*) containing numerous reactive epidermal cells with small somata (*small arrows*), and reactive fibers condensed into heavily stained tracts (*large arrows*). ×235. *Bars*: 50 (**A**, **C**) and 100 μm (**B**, **D**)

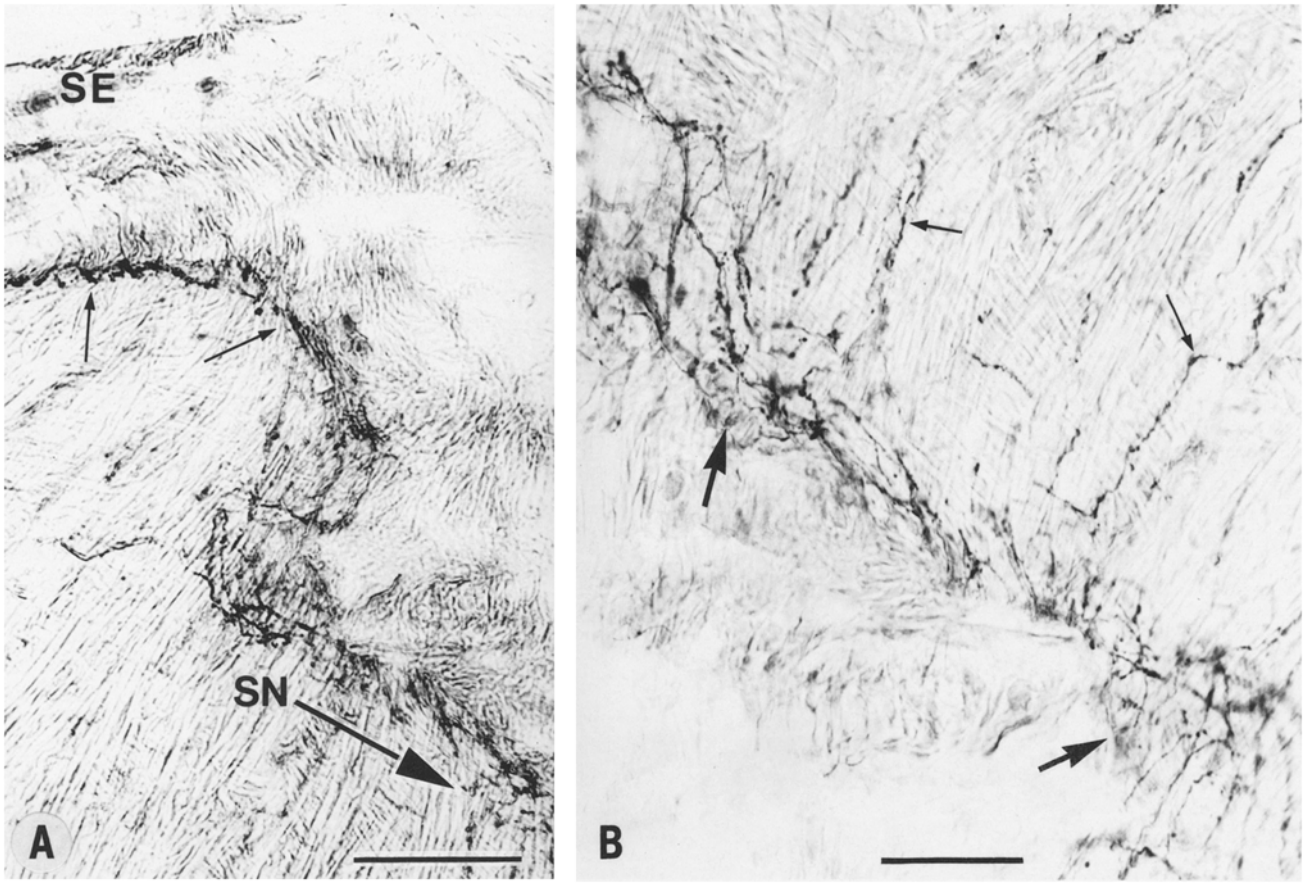

Fig. 7A, B. Transverse sections through posterior body segments at base of parapods. **A** Mesh-like tract of 5-HT-IR fibers (*small arrows*) coursing centripetally and sinuously from setae (*SE*) toward segmental nerve (*SN*) of corresponding segmental ganglion. ×235. **B** Closer view of similar reactive meshwork (*large arrows*) with merging varicose fibers embedded in parapodial muscle (*small arrows*). ×400. *Bars*: 100 (**A**) and 50 μm (**B**)

coursing centripetally for long distances (Fig. 6D). Finally, meshworks of immunostained varicose fibers coursed away from setae and were sprawled along the edge between parapodial muscles and connective tissue (Fig. 7A). Some of the 5-HT-IR fibers lying in the muscle mass of parapods appeared to merge into this meshwork (Fig. 7B). The latter, as well as the cirral fiber bundles, eventually merged into the segmental nerves 1 (notopod fibers) and 2 (neuropod fibers) of the corresponding segmental ganglion (Fig. 2). The 5-HT-IR fibers in segmental nerve 3 (Fig. 2) were found to be associated with fibers in the subepidermal nerve plexus and underlying muscle of the ventro-medial lobe of the neuropod (not shown) previously described by Joyeux-Laffuie (1890).

Discussion

The biochemical analyses provided clear evidence for the presence of biogenic catecholamines as well as 5-HT in tissues of *C. variopedatus*. In the case of catecholamines, the confirmatory evidence was obtained with two different and sensitive detection techniques and thus strenghtens our confidence in the positive identification of dopamine and norepinephrine in the tissues. In the case of 5-HT, the positive immunoreactivity (see below) supports the evidence obtained by HPLC-ED. The high levels of dopamine and norepinephrine, and the small amounts of epinephrine, in the cerebral or segmental ganglia are in agreement with similar data in earthworms (Gardner and Walker 1982, for review). Our and other measurements in non-polychaete invertebrates (Klemm 1985) contradict the results of the fluorimetric determinations of Manaranche and L'Hermite (1973) which indicated that epinephrine was more abundant than norepinephrine and dopamine in the cerebral and segmental ganglia of the polychaete *Glycera*.

The presence of relatively large amounts of dopamine and norepinephrine in the cerebral ganglia supports their possible involvement as neuroactive substances in the brain of *C. variopedatus*, and their very high levels in the tentacles, especially in the HPLC-ED analysis, are suggestive of a sensory role. Clark (1966) and White and Marsden (1978) have described with the Falck-Hillarp method green-fluorescent, presumably catecholaminergic cells in both the brain and cephalic sensory structures of other polychaetes. Clark (1966) noted that these neurons were substantially more abundant in the cerebral than segmental ganglia of *Nephthys*, yet we found no clear biochemical evidence in the tube-worm to corroborate this observation. In the case of the HPLC measurements of the ventral nerve cord, where more care was exercised than in radioenzymatic assays to remove extraneous tissues, catecholamine levels were comparable to those of cerebral ganglia. On the other hand, the substantial amounts of dopamine and norepinephrine

in the anterior intestine, despite the absence of nerve cord in the samples, raise the possibility of a physiological role for these amines in gut motility. In support of this contention, norepinephrine and epinephrine were found to induce contractures and increase the frequency of peristaltic waves of anterior intestine preparations of *C. variopedatus* (Anctil et al. 1984).

The existence of inactivation mechanisms for all three biogenic catecholamines is suggested by the HPLC detection in *C. variopedatus* of known vertebrate metabolites for these substances (see Vaughan 1988). The detection of significant amounts of normetanephrine and metanephrine suggests the involvement of some COMT activity in the inactivation of norepinephrine and epinephrine, respectively. Similarly, the presence of DOPAC and 5-HIAA implies the involvement of a monoamine oxidase in dopamine and 5-HT metabolism. This is the first evidence of such metabolites in an annelid, suggestive of an ancient evolutionary history for these metabolic pathways in invertebrates. It is interesting that these methylation and oxidation pathways appear to be largely inactive in arthropods (Dewhurst et al. 1972; Vaughan and Neuhoff 1976; Kennedy 1978).

5-HT was also detected in all sampled tissues of *C. variopedatus*. This was confirmed by the widespread distribution of 5-HT-IR neurons, fiber tracts and neuropile elements in all regions of the nervous system of this worm. Except for stomatogastric and sensory structures, these staining patterns correspond roughly to those identified in the errant polychaete *Harmothoe* (Miron and Anctil 1988).

As in *Harmothoe* ganglia, the ubiquity of reactive unipolar somata and of reactive neuropile elements in *C. variopedatus* is suggestive of an important role for 5-HT in the CNS of this worm. 5-HT has been shown to increase electrical activity recorded with suction electrodes on deafferented segmental ganglia of *C. variopedatus*, and this reponse was blocked by methysergide (Martin 1983). While serotonergic interneurons and motoneurons were distinguishable in *Harmothoe* ganglia, this was not achieved in the tube-worm. The size of these neurons was strikingly smaller than that of *Harmothoe* ganglionic neurons (Miron and Anctil 1988): 5–7 μm compared with 10–20 μm in cerebral ganglia, 10–12 μm with 10–50 μm in segmental ganglia. Therefore, uniquely identifiable, serially homologous motoneurons could not be traced. Although it is reasonable to assume that some of the reactive ganglionic cells are interneurons, the visualization of numerous reactive neurites in muscles suggests that at least part of this neuronal population consists of motoneurons.

The significance of the distribution of stained elements within the cerebral ganglia is not clear. The brain of *C. variopedatus* is much less complex than, and is not easily homologized with, that of errant polychaetes (see Martin and Anctil 1984). The immunoreactivity found in the optic tract, tentacular and labial nerves, however, suggests that the brain of the tube-worm receives serotonergic inputs from cephalic sense organs or sends serotonergic modulatory efferent projections to those sensory structures. The neuropile of the cerebral ganglia has fewer stained elements than the compact, heavily stained neuropile of segmental ganglia. In the latter, it is likely that a large fraction of the immunostaining is contributed by afferents, given the relatively small number of reactive ganglionic somata. The source of these afferents is to be found in the numerous reactive sensory neurons localized in parapods. The extent of this immunoreactivity was unsuspected because of the commonly held view, based on Falck-Hillarp histofluorescence visualizations, that serotonergic neurons are primarily motor and catecholaminergic ones sensory in annelids (Clark 1966; Myhrberg 1967; Gardner and Walker 1982). Relatively few 5-HT-IR sensory bipolar neurons were noted in *Harmothoe* (Miron et al. 1987; Miron and Anctil 1988). In contrast, the majority of the sensory innervations described in *Harmothoe* by Horridge (1963) and in nereids by Dorsett (1964) include 5-HT-IR elements in *C. variopedatus*. Prominent among these are the bipolar or tripolar neurons whose meshworks of neurites, sprawled around and within groups of setae, are characteristic of bristle (stretch) mechanoreceptors. The cirral cells with their heavily stained axon bundles also correspond to the abundant bipolar sensory cells described in cirri of other polychaetes. There is some experimental evidence that these cells are chemoreceptors (Horridge 1963). Thus our observations provide the first indication of a major serotonergic component to a sensory feedback system, stretch receptors, known to be involved in the coordination of the locomotory cycle of these worms. The possible involvement of 5-HT in avoidance reflex responses also deserves further study in view of our observations of 5-HT-IR epidermal cells resembling touch mechanoreceptors described by Dorsett (1964) and of presumptive 5-HT-IR cirral chemoreceptors. Mildly acid solutions, but not touch, applied to cirri induced escape responses in *Harmothoe* (Horridge 1963), and a similar stimulus applied to *C. variopedatus* inside its tube caused a typical "rejection" response involving parapodial muscles (Sumida and Case 1983).

The presence of numerous 5-HT-IR varicose fibers within muscles, originally branching off from segmental nerves, commissures and the subepidermal nerve plexus, supports a motor role for 5-HT. However, the motor end-plates of polychaetes follow the "en grappe" pattern of arthropods (Dorsett 1964), whereas the serotonergic muscle innervation of *Harmothoe* (Miron and Anctil 1988) and *C. variopedatus* is of the "en passant" type. This finding suggests that 5-HT may act as a modulator, not neurotransmitter, in polychaete somatic muscles. Inhibitory effects of 5-HT on muscle tone have been reported in the leech (Mason et al. 1979) as well as polychaetes (Alvarez et al. 1969). On the other hand, the presence of small reactive neuronal somata, also within somatic muscles of *C. variopedatus*, suggests that 5-HT is involved in the proprioceptive modulation of muscle activities of this worm. This appears to be the first reported evidence of intrinsic neurons in annelid muscles.

The pharyngeal ganglion of *C. variopedatus* appears to be a unique neurosecretory center among annelids (Martin and Anctil 1984). The significance of the intense 5-HT immunostaining of this ganglion and its associated nerve and plexus is unclear. Electron micrographs show that some of the intraganglionic neurosecretory cells (Martin and Anctil 1984) include, in addition to large granules usually associated with neuropeptides, small diameter (60–70 nm) granules typical of monoamine-containing vesicles. The widespread distribution of a meshwork of 5-HT-IR neurites in the pharyngeal plexus suggests that 5-HT is somehow involved in pharyngeal activities related to feeding in this worm. It is interesting in this regard that a plexus of serotonergic neurites was visualized by glyoxylic acid histochemistry in the pharyngeal wall of the leech (Lent and Dickenson 1984).

The 5-HT-IR neurites in the peripharyngeal nerve plexus of *C. variopedatus* seem to be peculiar to these tubeworms since they were unnoticed in *Harmothoe imbricata* (Miron and Anctil 1988). Both species, however, share the presence of 5-HT-IR bipolar cells in the pharyngeal epithelium, but it is not clear why these stained cells were detected in the intestine of *H. imbricata*, but not of *C. variopedatus*.

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