The Journal of Neuroscience, January 1988, 8(1): 242-255

Identification of the Neuropeptide Transmitter Proctolin in *Drosophila* Larvae: Characterization of Muscle Fiber-Specific Neuromuscular Endings

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The cellular localization of the peptide neurotransmitter proctolin was determined for larvae of the fruitfly *Drosophila melanogaster*. Proctolin was recovered from the CNS, hindgut, and segmental bodywall using reverse-phase HPLC, and characterized by bioassay, immunoassay, and enzymatic analysis. A small, stereotyped population of proctolin-immunoreactive neurons was found in the larval CNS. Several of the identified neurons may be efferents. In the periphery, proctolin-immunoreactive neuromuscular endings were identified on both visceral and skeletal muscle fibers. On the hindgut, the neuropeptide is associated with endings on intrinsic circular muscle fibers. We propose that the hindgut muscle fibers are innervated by central neurons homologous to previously described proctolinergic efferents of grasshoppers. The segmental bodywall innervation consists of a pattern of segment-specific junctions on several singly identifiable muscle fibers. While it is generally accepted that *Drosophila* muscle fibers are innervated by glutamatergic motoneurons, our data indicate that a specialized subset of muscle fibers are also innervated by peptidergic efferents.

The fruitfly *Drosophila melanogaster* is an attractive organism for the cellular and molecular analysis of neural development. The study of synaptogenesis in *Drosophila* has been impeded by the small number of described monosynaptic connections. Although adult synapses of the jump circuit have been characterized (Gorcyzka and Hall, 1984; Thomas and Wyman, 1984), and embryonic neurons have been identified by homology to those of grasshopper (Thomas et al., 1984), there are as yet no identified monosynaptic connections in fly larvae or embryos. In this paper we describe the neuromuscular innervation of visceral and bodywall muscle fibers by neurons expressing the pentapeptide transmitter proctolin.

The most accessible synapses in *Drosophila* are the neuromuscular junctions. The larval musculature is simple, consisting of no more than 30 singly identifiable muscle fibers in each hemisegment (Hertweck, 1931; Crossley, 1978). The fibers are also large and can be studied electrophysiologically (Jan and Jan, 1976a, b, 1982; Wu and Haugland, 1985). At present however, little is known about the CNS locations of larval *Drosophila* motoneurons, and with the exception of glutamate (Jan and Jan, 1976a, b), little is known about the transmitters they use.

Insect neurons use a wide variety of neurotransmitters, especially neuropeptides (O'Shea and Schaffer, 1985). A diverse array of neuropeptides is also found in ganglia of other invertebrates (Mahon and Scheller, 1983) and in the CNS of vertebrates (Snyder, 1980; Krieger, 1983). In insect ganglia a subset of the motoneurons specialize to express neuropeptides as transmitters (Bishop and O'Shea, 1982; Adams and O'Shea, 1983; Keshishian and O'Shea, 1985a; Myers and Evans, 1985). Using immunocytochemistry it is possible to map the specific muscle fibers innervated by specialized peptidergic motoneurons. With this method, Witten and O'Shea (1985) surveyed the leg muscles of the cockroach and identified muscle bundles innervated by a specific peptidergic motoneuron. We reasoned that if similar motoneuronal transmitter specializations occur in *Drosophila,* muscle fibers with peptidergic neuromuscular junctions could also be identified by transmitter immunocytochemistry.

At present, the best characterized insect neuropeptide transmitter is proctolin (Arg-Tyr-Leu-Pro-Thr). The peptide was discovered in the cockroach *Periplaneta americana* and proposed as a neurotransmitter of insect visceral motoneurons (Brown, 1967, 1975; Brown and Starratt, 1975; Starratt and Brown, 1975; Holman and Cook, 1979). Proctolin has since been identified in a broad spectrum of arthropods (Brown, 1977; Sullivan, 1977; Bishop et al., 1984; Schwarz et al., 1984; Siwicki et al., 1987). There is also indirect evidence for the presence of proctolin in Diptera. Brown (1977) succeeded in partially purifying a proctolin-like material from houseflies using high-voltage paper electrophoresis, but the molecule has yet to be analyzed in *Drosophila.* Proctolin may also be located in the mammalian CNS, where immunocytochemistry indicates that it is colocalized with 5-HT and norepinephrine in neurons of the hypothalamus and spinal cord (Holets et al., 1984). The peptide is also bioactive on the rat ileum (Penzlin et al., 1981).

O'Shea and Adams (1981) first identified neurons in insects containing proctolin. The distribution of proctolinergic cells in the cockroach and grasshopper nervous systems has been described (Bishop and O'Shea, 1982; Keshishian and O'Shea, 1985a; Witten and O'Shea, 1985; Lange et al., 1986). Proctolin is found in uniquely identified skeletal motoneurons of insects (O'Shea and Bishop, 1982) and in the postural motoneurons of the crayfish (Bishop et al., 1984, 1987). Adams and O'Shea (1983) demonstrated that proctolin can be released together with

Received Mar. 18, 1987; revised June 29, 1987; accepted July 7, 1987.

We thank Drs. John Carlson, Jørgen Johansen, Douglas Kankel, William McGinnis, and Robert Wyman for comments on the text, and thank Michael O'Shea and Cindy Bishop for a generous gift of antiserum. Research is supported by grants from the National Science Foundation and the McKnight Foundation to H.K., a NIMH predoctoral fellowship to M.D.A., and a Canadian NSERC postgraduate scholarship to M.E.H.

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a second neurotransmitter, probably glutamate. Using physiological and cytochemical methods, the peptide also has been shown to be active at skeletal neuromuscular junctions in the grasshopper (May et al., 1979; Evans and Myers, 1986; Worden and O'Shea, 1986).

While immunocytochemical mapping is a powerful and efficient technique for characterizing neuronal phenotypes, a chromatographic assay of the putative peptide is essential to confirm its identify. In addition to the problems of antibody access and cross-reactivity, neuropeptides present in the CNS can share partial sequence homologies. For example, O'Shea et al. (1984) have identified 2 myoactive peptides in insects (MI and MII) that they found had partial sequence homology to a third peptide, AKH. Unless the antibody is specific to unique sequences, immunocytochemistry may fail to distinguish between neurons expressing structurally related peptides.

To confirm the immunocytochemistry described in this study, we assayed the proctolin in *Drosophila* larvae. The peptide was recovered from the CNS, hindgut, and segmental bodywall musculature of larvae using reverse-phase HPLC and was characterized by bioassay, immunoassay, and enzymatic analysis. Using immunocytochemistry, we found the peptide is expressed by a small and highly conserved population of individually identifiable central neurons, several of which may be motoneurons. The major proctolinergic innervation is confined to segment-specific endings on identified abdominal bodywall muscle fibers. Other immunoreactive processes innervate the intrinsic circular muscle fibers of the hindgut. The results show that a stereotyped subset of *Drosophila* muscle fibers are targets of proctolin-expressing efferents. Some of the material in this study has been reported in abstract form (Keshishian, 1985; Halpern et al., 1986).

Materials and Methods

Animals. Larvae of wild type (Oregon-R) *Drosophila melanogaster* were used for the HPLC analyses. Immunocytochemistry is based on descriptions of both Oregon-R and Canton-S strains. No systematic differences were observed between them. Crawling 3rd instar larvae were collected from standard cornmeal-molasses-agar bottles. Grasshoppers (*Schistocerca americana*) were obtained from a breeding colony. Metathoracic legs for the extensor tibiae bioassays were taken from adults who had molted during the preceding few days.

Dissections. Larvae were dissected in osmotically balanced insect saline (140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 4 mM $NaHCO_3$, 1 mM $MgCl_2$, 5 mM N-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 5 mM trehalose, 100 mM sucrose, pH 7.2). For chromatographic extractions the brain hemispheres and ventral ganglion were dissected with the peripheral nerves cut short and the imaginal discs removed. Hindguts were cut at the level of the Malphigian tubules. Bodywalls were dissected with a longitudinal cut down the dorsal or ventral midline with all internal tissues except the bodywall musculature removed.

Immunocytochemistry. The tissues were fixed in cold 4% paraformaldehyde for 2–12 hr. The proctolin-specific antiserum #9 (Bishop et al., 1981) was used for immunocytochemistry, and labeling was demonstrated using either the peroxidase–antiperoxidase (PAP) reaction or with fluorescein- or tetramethyl rhodamine-labeled goat anti-rabbit IgG (FITC-GAR or TRITC-GAR), as described by Keshishian and O'Shea (1985a). This serum has been used in numerous studies (Bishop and O'Shea, 1982; Keshishian and O'Shea, 1985a; Witten and O'Shea, 1985), and its specificity for proctolin has been demonstrated (Bishop et al., 1981; O'Shea and Bishop, 1982). Colchicine injections did not measurably improve the CNS staining, as they do in other insects (Bishop et al., 1981). Incubation of the antiserum with proctolin (1 mM for 24 hr at 4°C) abolishes all observed immunoreactivity in the CNS, hindgut, and bodywall.

The immunocytochemical descriptions are based upon observations, photomicrographs, and camera lucida drawings of 56 ganglia, 48 hindguts, and 36 bodywall preparations. The larval bodywall dissection damages about one-fifth of the muscle fibers. Consequently, both dorsal and ventral dissections were used to guarantee that all fibers would be assayed. In 20 larvae we undertook a detailed examination of approximately 6000 myofibers for motor endings. The myofibers were scanned with a 63× NA 1.4 planapochromatic objective by 2 observers. We usually could not assay segment A8 because of damage.

Cobalt backfills. The cut ends of ventral ganglion nerves were immersed in small drops of 0.1 M hexammine cobalt chloride at room temperature for 6–12 hr. The ganglia were washed in saline, and the cobalt developed using 1% ammonium sulfide. The ganglia were transferred to Carnoy's fixative, rehydrated, and intensified using the Gallyas silver/tungsten protocol (Davis, 1982). The ganglia were dehydrated in ethanol, cleared with methyl salicylate, and mounted in DPX (Gurr/BDH Ltd., Poole, UK).

HPLC analysis of dissected tissues. Larval tissues dissected in physiological saline were pooled at 4°C in 500 μl of methanol-water-glacial acetic acid (90:9:1) extraction medium. The tissues were ground in either a siliconized glass or polypropylene plastic homogenizer and then further homogenized by probe sonication. The homogenate was spun for 5 min at 15,000 × *g*, and the supernatant was saved. The pellet was washed in an additional 500 μl of extraction medium. The pooled supernatants were dried under vacuum at 60°C. The dried supernatant was dissolved in 1 ml of distilled water and loaded onto a C-18 Sep-Pak cartridge (Waters Associates). Following 5 ml distilled water and 15% methanol washes, a crude peptide-containing fraction was eluted in 4.5 ml of 80% methanol. The eluate was dried under vacuum at 60°C. This method recovers at least 90% of the proctolin present in the homogenate (Keshishian and O'Shea, 1985a).

To control for spurious proctolin contamination, in several extractions the tissue homogenization was preceded by incubating the homogenizing vessel for about 10 min with 1 ml of extraction medium. The control medium was then Sep-Pak extracted and used as a blank control for the HPLC run. Otherwise, a sample of distilled water was injected for a blank control. A single syringe was used for all HPLC column injections.

The crude peptide fractions were chromatographed in reverse phase using a 2-pump HPLC with a 30 cm C-18 Bondapak column. Three kinds of separation were used: (1) a gradient elution of 5–50% acetonitrile against an aqueous buffer of 50 mM ammonium acetate, pH 4.5; (2) a gradient elution of 5–50% acetonitrile in the presence of a constant aqueous 0.1% trifluoroacetic acid, pH 2.3; and (3) an isocratic elution with 12% acetonitrile in 50 mM ammonium acetate, pH 4.5. In each case, the HPLC was run at 1 ml/min, with 30 min gradients. Fractions of 1 ml were collected except for 2 ml before and after the proctolin peak, when 500 μl fractions were collected.

The elution time was determined using synthetic proctolin with a tritium label on the Tyr residue (New England Nuclear). Each tissue analysis consisted of 4 chromatographs: (1) the tritiated proctolin standard, to determine the retention time of the peptide, (2) 25 μl blank control (see above), (3) the Sep-Pak-treated tissue fraction, and (4) a second tritiated proctolin standard, to detect shifts in the peak.

Proctolin assay. The eluates of the control blank and tissue chromatographs were dried at 60°C in vacuum and dissolved into 25 μl of physiological saline. The samples were quantified using the grasshopper extensor tibiae bioassay. This method has a threshold sensitivity of about 5×10^{-17} mol proctolin. The bioassay measures the peak response frequency of the myogenically rhythmic, proctolin-sensitive muscle bundle of the extensor tibia. The acceleration of the rhythm was compared to dose–response curves made using proctolin standards (Keshishian and O'Shea, 1985a).

Peptidase sensitivity. Proctolin is readily cleaved by the exopeptidase leucine aminopeptidase but is unaffected by chymotrypsin (Brown, 1977). Putative *Drosophila* proctolin was recovered by HPLC from 50 3rd instar larvae using a 5–50% acetonitrile gradient against 50 mM ammonium acetate buffer. Aliquots, 10 μl, from the proctolin peak adjusted to a concentration equivalent to 5×10^{-10} M proctolin were incubated for 10 min at 37°C with either 1 unit of leucine aminopeptidase (EC 3.4.11.1), 1 unit of α-chymotrypsin (EC 3.4.21.1), or the respective heat-inactivated enzymes as controls. The reaction mixtures were heated for 5 min at 60°C to inactivate the enzymes followed by a spin for 5 min at 15,000 × *g*. Samples (1 μl) from the supernatants were applied to the leg bioassay. The above experiment was also performed using synthetic proctolin as a control.

Antibody binding of proctolin extracts. The putative *Drosophila* proc-

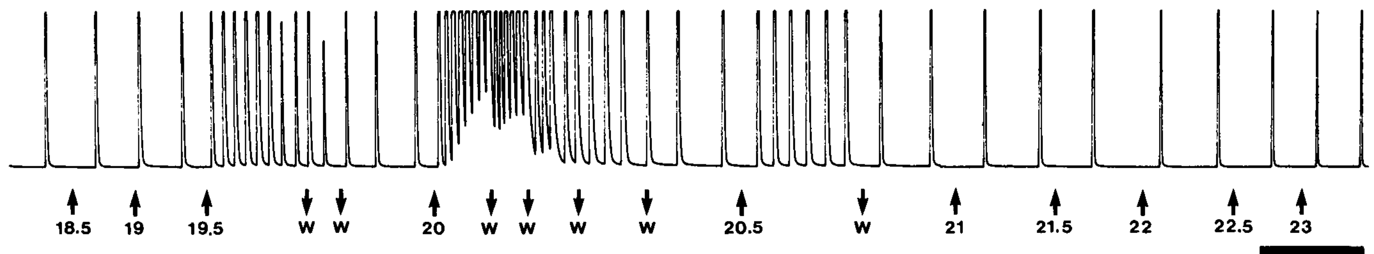

Figure 1. Extensor tibiae bioassay response to a proctolin-like material recovered by HPLC from whole 3rd instar larvae. Proctolin mediates a reversable acceleration of the myogenic rhythm of the test muscle, with an increase in the tibial extension. The larval material was chromatographed with a 5–50% acetonitrile gradient over 30 min. The *Drosophila*-derived bioactivity has a peak elution time of 20 min, which matches authentic proctolin (see Fig. 2). The response to fractions collected from 18.5 to 23 min are shown here. The *upward arrows* indicate the time when the test sample was applied, *W* when the bioassay muscle was washed with saline. The *upward deflections* indicate tibial extensions (not calibrated). Scale bar, 1 min.

tolin was recovered from 50 3rd instar hindguts by gradient HPLC as described above. Aliquots, 10 μl, containing a final concentration equivalent to 5×10^{-10} M proctolin were incubated at 20°C for 10 min in the presence of a 1:500 dilution of either normal rabbit serum or an immune rabbit serum generated against proctolin-BSA (#13; Bishop et al., 1981). The antibody–peptide complex was centrifuged for 5 min at $15,000 \times g$. Samples (1 μl) from the supernatant were bioassayed.

Results

We provide 6 lines of evidence for proctolin's presence in *Drosophila* larvae. (1) With reverse-phase C-18 HPLC, a bioactive substance can be extracted from *Drosophila* having the same elution time as tritiated proctolin, when tested in either an isocratic or a gradient acetonitrile–ammonium acetate solvent system. The elution time of the molecule is shifted in a fashion identical to proctolin when the gradient HPLC is performed in the presence of constant 0.1% trifluoroacetic acid. (2) The bioactive substance recovered from *Drosophila* larvae evokes motor responses that are indistinguishable from those of proctolin when it is applied to the grasshopper extensor tibiae muscle, a sensitive bioassay. (3) The molecule is heat stable and, like proctolin, is rapidly degraded by the leucine aminopeptidase but not by α-chymotrypsin. (4) The bioactivity of the HPLC-purified molecule is abolished by antiserum specific to proctolin. (5) Focal application of the peptide on several visceral muscles in *Drosophila* causes or accelerates contractions. (6) Immunocytochemistry with proctolin-specific antisera reveals neuronal staining in tissues where proctolin-like bioactivity is located.

Chromatographic evidence for proctolin in Drosophila

Authentic proctolin has potent effects on the myogenic rhythm of the grasshopper extensor tibiae muscle (May et al., 1979; Keshishian and O'Shea, 1985a). The effects include an acceleration of the myogenic rhythm, an increase in the muscle's basal tension, and a restoration within 1–2 min of the normal rhythm following a saline wash (Figs. 1, 3, and 4). Other substances affecting the extensor tibiae, such as the peptides AKH, MI, or MII (O'Shea et al., 1984), have effects on the muscle distinct from proctolin.

A substance with extensor tibiae muscle bioactivity typical of proctolin was recovered from homogenates made from whole 3rd instar *Drosophila* larvae, as well as from tissue extracts made from the CNS, hindgut, and bodywall muscle fibers, with a threshold detectability of 0.1 fmol/tissue ($n = 25$ tissues). The bioactive substance was recovered from acid-methanol homogenates and eluted between the 15 and 80% methanol organic solvent washes of C-18 Sep-Pak cartridges. The substance is heat stable, losing negligible biological activity after 12 hr at 60°C.

Each tissue extract with bioactivity was chromatographed using high-resolution, reverse-phase HPLC, with a 30 cm analytical C-18 column (Figs. 1, 2). For each tissue fraction the recovered bioactivity was limited to a single sharp peak having the same elution time as authentic proctolin. As a control for cross-contamination, the chromatograph of the tissue extract was preceded by a control blank (Fig. 3, *A* and *B*). In each case we preceded and followed the 2 runs with chromatographs of tritiated proctolin to calibrate the column elution time for the peptide.

Three chromatographic methods were adopted in order to demonstrate cochromatography of the bioactive peak with authentic peptide under different solvent or running conditions. These were (1) a gradient of 5–50% acetonitrile against an aqueous 50 mM ammonium acetate buffer, pH 4.5; (2) a gradient of 5–50% acetonitrile against a constant 0.1% trifluoroacetic acid (TFA), pH 2.3; and (3) an isocratic separation at 12.5% acetonitrile against an aqueous 50 mM ammonium acetate buffer, pH 4.5. The acetonitrile/TFA chromatograph shifts the peak to a longer elution time, presumably because of the formation of ion pairs with the peptide. Authentic proctolin, chromatographed using the 3 HPLC regimes, had elution times of 20, 23, and 26 min, respectively (Fig. 2). Proctolin standard was recovered in ±0.5 min of the peak for the gradient elutions, and ±1 min for the isocratic elutions.

The single biologically active peak, as assayed by its effect on the extensor tibiae muscle, cochromatographed with authentic proctolin in each of the 3 types of HPLC protocols. A total of 28 tissue and control HPLC runs were performed, with proctolin bioassays performed on each tissue and control eluate (1 ml fractions, shifting to 500 μl fractions ±2 min of the peak) over the first 30 min of the chromatographs. The recovered bioactive substance elicited effects on the extensor tibiae muscles that closely resembled authentic proctolin (compare Fig. 4, *A* and *D*). For each protocol there were no significant discrepancies in the elution times of standards and unknowns. Nor were differences observed in the elution times of the bioactive substance as a function of the tissue source (Fig. 2). Figure 2 illustrates sample HPLC runs, with the proctolin-like bioactivity (PLB) equivalence shown. Proctolin is probably less efficiently recovered from homogenized whole larvae than from dissected tissues; thus, whole larval measures tended to lower levels than the sum of the tissue fractions. We recovered from 3rd instar larvae proctolin activity equivalent to 2.29 ± 1.0 fmol/animal

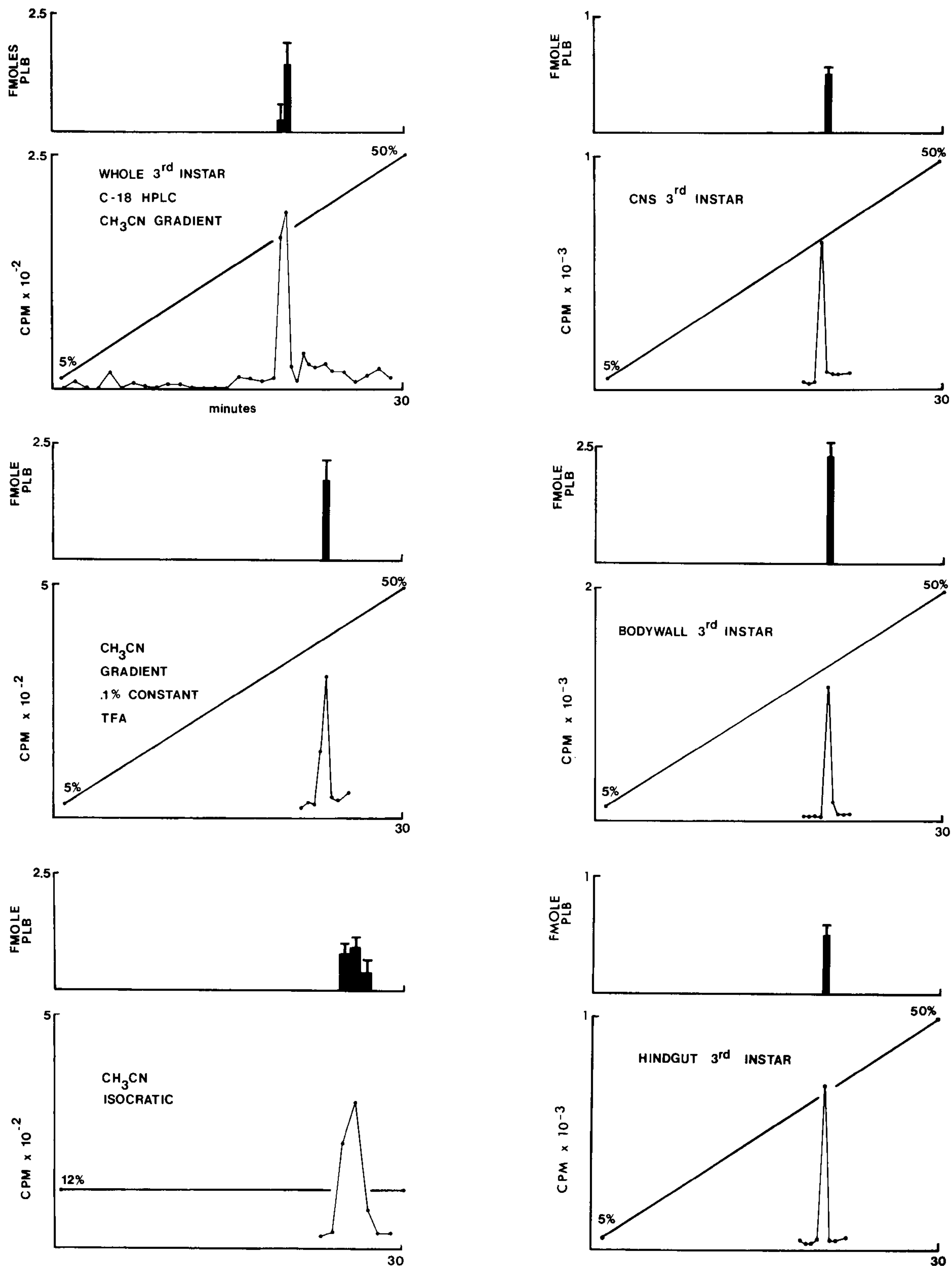

Figure 2. A substance with proctolin-like bioactivity is recovered by C-18 reverse-phase HPLC from *Drosophila.* The peak bioactivity cochromatographs with authentic tritiated proctolin in both gradient and isocratic protocols. *Left,* sample HPLC results from whole 3rd instar larvae (25 larvae pooled for each of the gradient runs, and 15 larvae for the isocratic run). *Right,* HPLC results from larval CNS (10 pooled ganglia), bodywalls (16 pooled tissues), and hindguts (10 pooled tissues). The *top* of each frame shows fractions where proctolin-like bioactivity (*PLB*) was recovered, as tested by the extensor tibiae muscle response (estimated from bioassay dose–response curves, with levels corrected to the PLB present in 1 larva or tissue). The *bottom* of each frame indicates where a tritiated proctolin standard eluted. A blank control chromatograph (see Materials and Methods) preceded the chromatograph of each unknown. Tissue extracts were run using either a 5–50% acetonitrile gradient or isocratically with 12% acetonitrile. The error bars indicate the SD of the bioassay PLB equivalence.

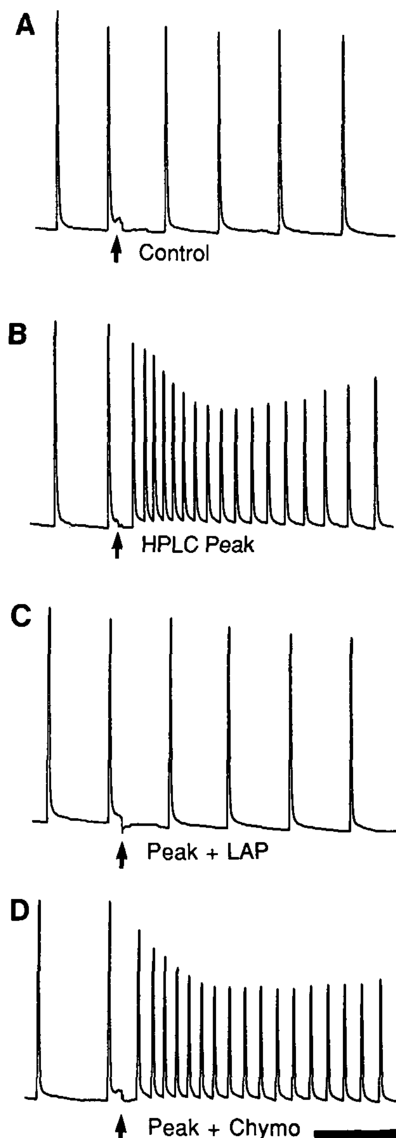

Figure 3. The *Drosophila* proctolin-like substance recovered by HPLC is degraded by leucine aminopeptidase. Pooled 3rd instar whole larvae were chromatographed by reverse-phase gradient HPLC. The peak bioactivity, which cochromatographs with authentic proctolin, was recovered and incubated with either leucine aminopeptidase or chymotrypsin. All biological activity was abolished by the peptidase but not by chymotrypsin. *Arrows* indicate the time of application of samples. *A,* Bioassay response to a control HPLC run at the elution time for proctolin (20 min). *B,* The bioassay response to pooled 3rd instar larvae, at the elution time for proctolin. The sample aliquot is equal to 0.2 larvae, and the response is equivalent to approximately 0.5 fmol proctolin. *C,* Same as in *B,* with the fraction incubated with 0.1 units of leucine aminopeptidase. Bioactivity was abolished. *D,* Same as in *B,* but where the HPLC peak was incubated with chymotrypsin. Here, the response is relatively unchanged. Time scale, 1 min.

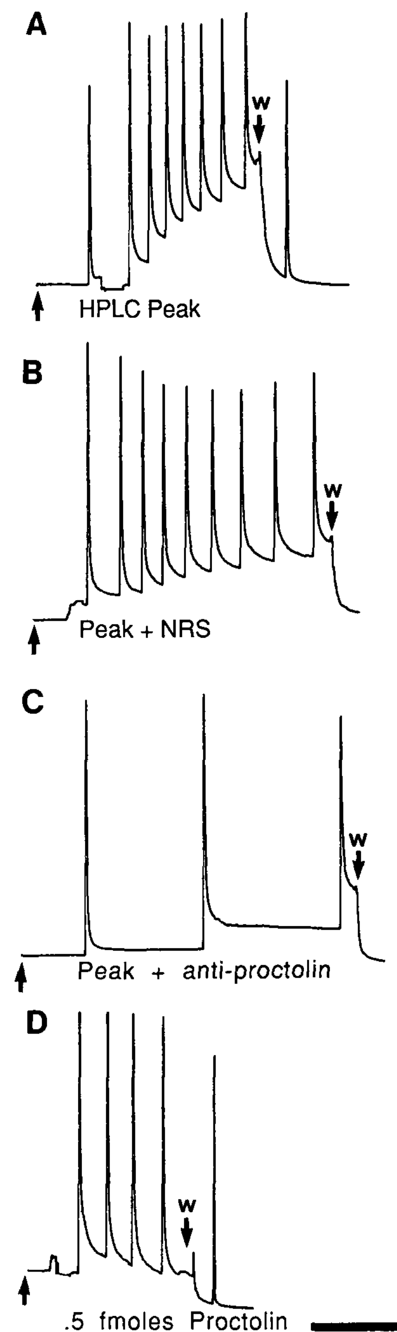

Figure 4. The *Drosophila* proctolin-like bioactivity recovered by reverse-phase HPLC is abolished by antibodies specific to proctolin. *A,* Bioassay response to the HPLC fraction at the proctolin elution time (20 min) from pooled 3rd instar larval hindguts. The sample aliquot is equivalent to 1 hindgut and evokes a response equal to approximately 0.5 fmol proctolin. *Arrowheads* indicate the time the samples were applied, *W* the washout with saline. *B,* Same test as in *A,* but following a 10 min incubation of the aliquot with normal rabbit serum, 1:500 serum dilution. *C,* Same test as in *B,* but following a 10 min incubation in serum from a rabbit immunized with proctolin–BSA conjugates, 1:500 serum dilution. *D,* Response of the extensor tibiae muscle to 0.5 fmol of proctolin standard. Time scale, 1 min.

(mean + SD; $n = 4$ HPLC runs). For tissues with proctolin-like activity, the bodywall averaged 1.65 ± 0.39 fmol ($n = 3$); the CNS 0.82 ± 0.37 fmol ($n = 4$); and the hindgut 0.61 ± 0.21 fmol ($n = 3$). The cochromatography of the bioactive substance with a tritiated proctolin standard in 3 HPLC protocols is strong evidence for the presence of proctolin in *Drosophila.*

We were able to show that the molecule behaved like proctolin using enzyme degradation and antibody affinity tests. Proctolin is not degraded by trypsin or chymotrypsin but is rapidly cleaved by the exopeptidase leucine aminopeptidase (LAP) (Brown, 1977). The degree of degradation of the peptide by LAP can be monitored by bioassaying the residual proctolin following a brief incubation of the peptide with the enzyme. Using gradient HPLC, the proctolin-like bioactive substance from whole larvae was

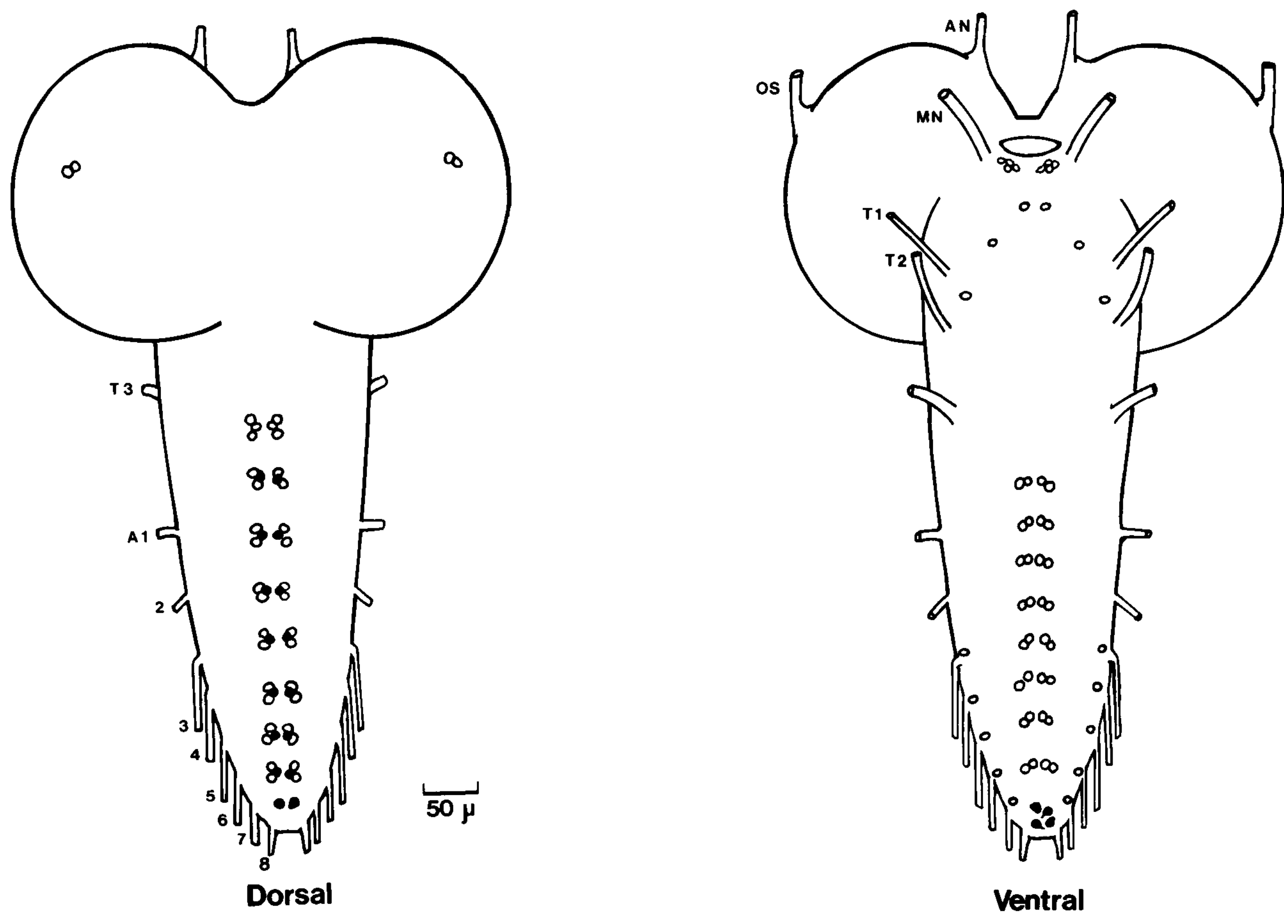

Figure 5. Diagram of the principal proctolin-immunoreactive neurons of the 3rd instar larval CNS (brain hemispheres and ventral ganglion). The cells with consistent strong immunoreactivity are drawn in *black.* The dorsal-midline neurons are drawn as clusters of 3 cells, although in some cases clusters of up to 5 cells were stained (refer to Fig. 6*F*). The ganglionic nerve anatomy is adapted from Hertweck (1931). Abbreviations: *AN,* antennal nerve; *MN,* mandibular/maxillary nerve; *OS,* optic stalk; *T1–3,* thoracic nerves 1–3; *A1–8,* abdominal nerves 1–8.

recovered at the elution time for the peptide (20 min). An aliquot of the eluate with an activity equivalent to approximately 0.5 fmol proctolin/µl evoked a 5-fold increase in the bioassay frequency response [Fig. 3*B,* as compared with the HPLC blank control eluate (Fig. 3*A*)]. Incubation of the aliquot in the presence of 0.1 units of LAP for 10 min abolished all bioactivity, yielding responses indistinguishable from the blank control (Fig. 3*C*). The identical experiment, using 0.1 units of chymotrypsin, failed to abolish the bioactivity (Fig. 3*D*), indicating that most of the *Drosophila* bioactivity was resistant to that enzyme. The sensitivity of the bioactivity to LAP suggests that the bioactive substance is a peptide.

We next determined whether incubation with proctolin-specific antiserum can abolish the bioactivity of the *Drosophila* peptide. The bioactive molecule was purified by HPLC from larval hindguts. An aliquot of the eluate with an activity equivalent to approximately 0.5 fmol proctolin/µl (Fig. 4, *A* and *D*), showed little loss of activity when incubated 10 min in the presence of a 1:500 dilution of normal rabbit serum (NRS; Fig. 4*B*). When the aliquot was incubated with a 1:500 dilution of an antiserum specific for proctolin, the bioactivity disappeared, with the bioassay frequency dropping to baseline levels ($n = 4$ trials; Fig. 4*C*). This indicates that virtually all the *Drosophila* bioactivity that chromatographs with the same elution time as proctolin can be abolished by serum antibodies made to that peptide.

The preceding evidence indicates that proctolin is probably present in small but readily measurable levels in *Drosophila.* The regional distribution of proctolin in *Drosophila* larvae resembles that described for lower insects (grasshopper: Keshishian and O'Shea, 1985a; cockroach: Bishop et al., 1981; Penzlin et al., 1981; Witten and O'Shea, 1985). Over half of the larval proctolin in *Drosophila* is located in the bodywall, with the remainder found in the hindgut and the CNS.

CNS neurons with proctolin-like immunoreactivity

The CNS of *Drosophila* larvae consists of 2 bilaterally symmetrical brain hemispheres and a compound ventral ganglion (Fig. 5). The ventral ganglion fuses during embryonic development from at least 14 segmental ganglia (Campos-Ortega and Hartenstein, 1985), and as a result, the intersegmental cortical boundaries are not clearly defined. In this study the neurons are described by their position relative to the levels of the segmental nerve roots and commissures. Positively scored neurons had consistent and bilaterally symmetrical cell body staining. Larval immunoreactivity was restricted to cell bodies, with occasionally revealed neurites. The somata were uniform in size, ranging from 8 to 10 µm in diameter. The staining pattern was stable through the larval stages (data not presented here). The following description is of the 3rd instar larva (summarized in Fig. 5).

Brain, subesophageal, and thoracic immunoreactivity. The brain hemisphere immunoreactivity was limited to 2 adjacent

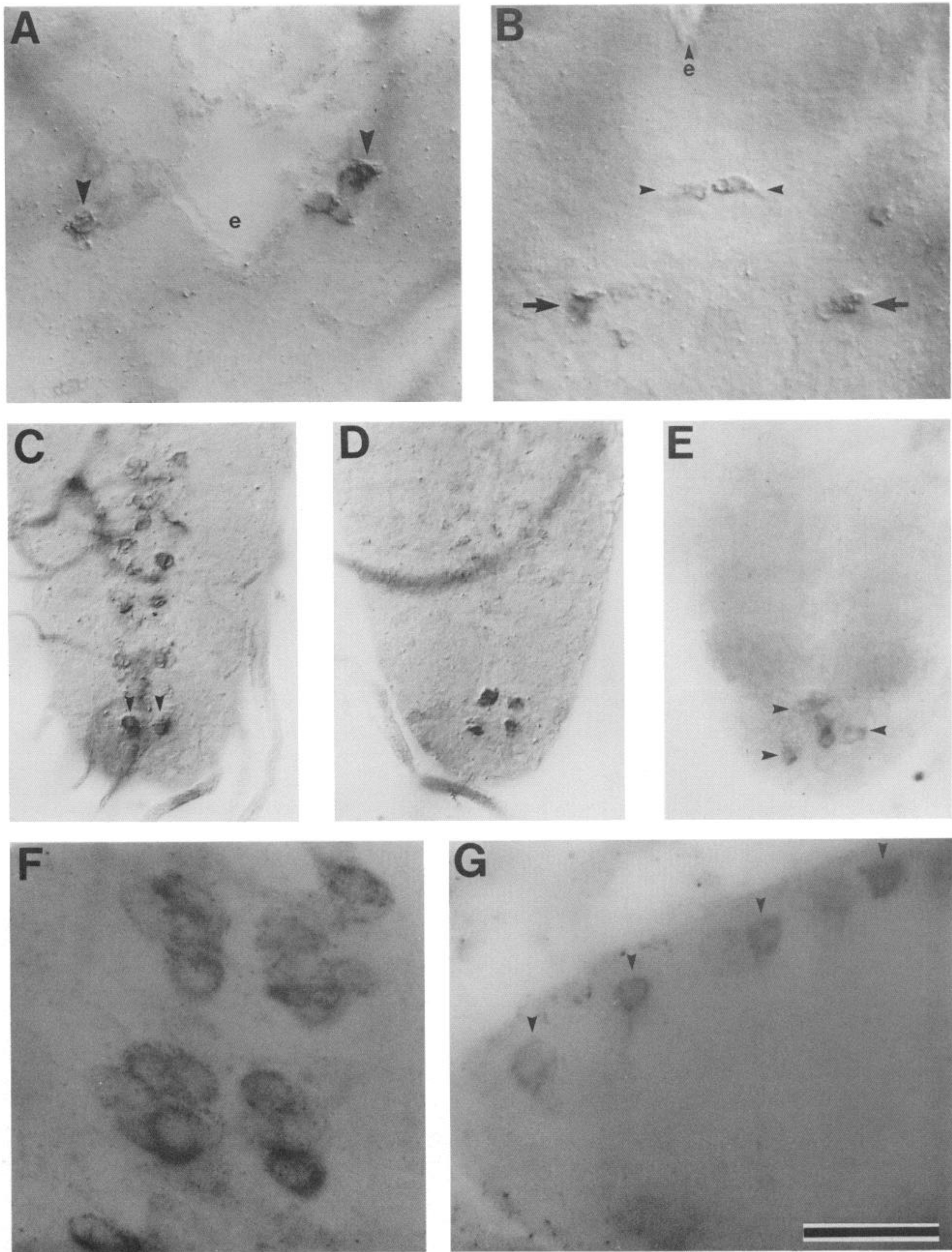

Figure 6. Proctolin-immunoreactive neurons of the 3rd instar larval CNS. *A,* Ventral proctolinergic cell cluster (*arrowheads*) located near the base of each mandibular/maxillary nerve. The neuronal clusters are positioned adjacent to the esophageal canal (*e*). *B,* Two of the 3 cell pairs located in the gnathal and anterior thoracic segments. The *arrowheads* point to a pair of ventral medial cells located anterior to the T1 nerve roots. The *arrows* indicate probable T1 cell pairs. A third cell pair (not visible) lies at the level of the T2 nerve roots (refer to Fig. 5). *C,* Dorsal view of the abdominal ventral ganglion, with its characteristic cell clusters. Usually in each cluster one cell stained strongly. At the posterior end of the pattern

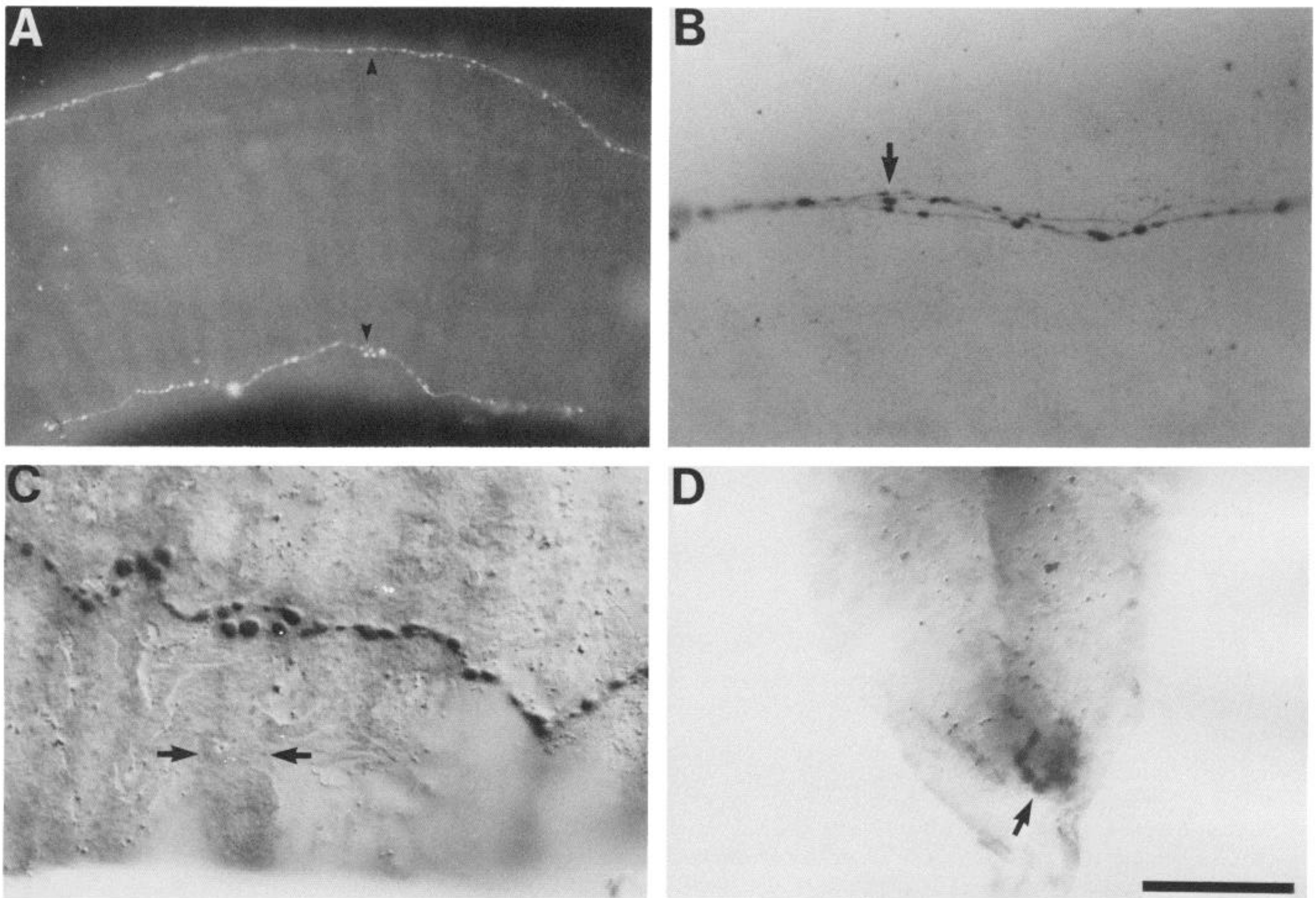

Figure 7. Proctolin-immunoreactive endings of the hindgut. *A*, Pair of varicose immunoreactive fascicles (*arrowheads*) project longitudinally on opposite sides of the hindgut. *B*, Detail demonstrating 3 immunoreactive fibers diverging from the fascicle (*arrow*). Up to 3 stained fibers diverge from each fascicle. *C*, The immunoreactive endings project superficially over the circular muscles of the hindgut (*arrows* indicate edges of a circular muscle fiber). *D*, Small cluster of efferent somata is revealed at the posterior end of the ganglion (*arrow*), when the 8th nerve branches near the hindgut were cobalt backfilled. Scale bars: *A* and *D*, 100 μm; *B* and *C*, 25 μm.

cell bodies located laterally on the dorsal surface (Fig. 5). A cluster of 3–4 ventral somata was observed at the root of the mandibular/maxillary nerve (Fig. 6*A*, arrowheads) adjacent to the esophageal canal (*e*, Fig. 6*A*). Other anterior CNS immunoreactivity consisted of 3 symmetrical pairs of neurons in the subesophageal and thoracic regions (Fig. 5; the anterior 2 pairs are illustrated in Fig. 6*B*). The somata are located at the levels of the labial, prothoracic (T1), and mesothoracic (T2) nerves. The spacing of the cells along the anterior–posterior (A-P) axis suggests that they reside in different and perhaps successive segments.

Abdominal immunoreactivity. Three segmentally repeated clusters of neurons were found in the abdominal segments of the ganglion. As with the subesophageal/thoracic pairs, evidence of the metameric arrangement of these neurons was supported by even spacing along the A-P axis. The first set of *dorsal medial* (DM) neurons consisted of 8–9 serially repeated bilateral clusters of 2–5 cells (Fig. 6, *C* and *F*), extending from the level of the T3 nerve roots to the level of segment A7 (Figs. 5, 6*C*). A single cell body on each side stained prominently (Fig. 6*C*), and in some ganglia only these DM cells were observed in the abdominal segments.

The remaining 2 patterns of metameric immunoreactive neurons were located on the ventral side of the ganglion. A pair of *ventral medial* (VM) neurons was observed in each hemisegment from a level between the T3 and A1 nerve roots to the A7 level (Figs. 5, 6*G*). As with the DM neurons, one of the cells on each side was prominently revealed (sagittal view in Fig. 6*G*). The remaining metameric neurons were the *ventral lateral* (VL) cells. A single moderately immunoreactive neuron was stained in each

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there is typically a single conspicuous pair of cells (*arrowheads*). *D*, The ventral view of the ganglion in *C*, revealing 4 strongly stained neurons located directly opposite the dorsal pair. *E*, In some ganglia the terminal 6 neurons lie on the ventral side. *F*, Detail of the segmental clusters on the dorsal midline. Up to 5 cells could be observed within each cluster, although 3 were typical. *G*, Sagittal view of the ventral midline cells (*arrowheads*). Some of the cells are accompanied by a lighter staining neuron. *A–D*, PAP demonstration; *E*, fluorescein; and *F*, rhodamine immunofluorescence. Scale bars: *A* and *B*, 63 μm; *C–E*, 100 μm; *F*, 25 μm; *G*, 40 μm.

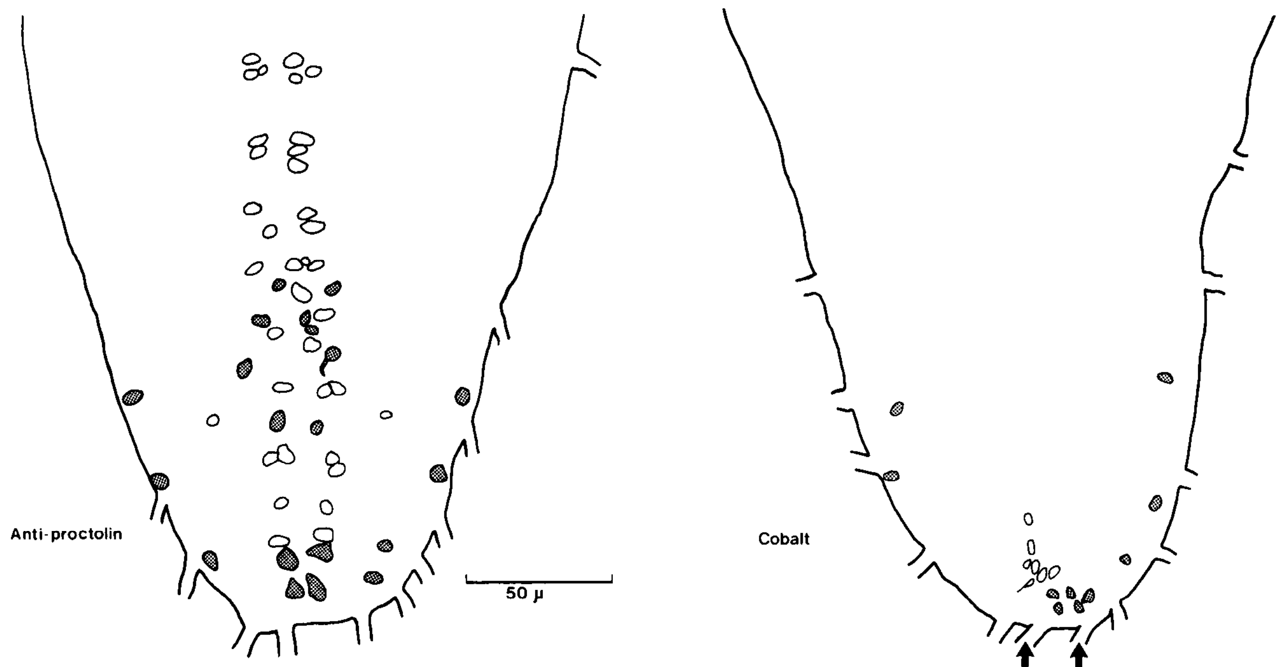


Figure 8. Comparison of camera lucida drawings of neurons with proctolin-like immunoreactivity with the cell bodies revealed by bilaterally backfilling the 8th nerves near the hindgut (*arrows* point to the 8th nerve roots). Ventral cell bodies *shaded.*

hemisegment from the levels of the A3–A7 nerve roots. The VL cells were moderately immunoreactive and were usually located near the base of each nerve root.

At the most posterior (probably A8) segment, we usually stained 4 ventral cells (Fig. 6*D*). The *posterior medial* (PM) neurons were the most strongly immunoreactive cells in the CNS. The 4 neurons lie directly opposite 2 dorsal A8 cells, which were also prominently stained (Figs. 5, 6*C*, arrowheads). Although 4 ventral and 2 dorsal PM cells were typical, in some cases the dorsal pair was missing, and 6 cell bodies were located on the ventral side (Fig. 6*E*). In grasshopper embryos a similar cluster of 6 strongly immunoreactive proctolinergic neurons is located in the region of the 8th abdominal segment of the terminal ganglion (Keshishian and O'Shea, 1985b). The grasshopper neurons have been shown to be proctolinergic efferents to the hindgut.

In conclusion, CNS proctolin immunoreactivity is rare, limited to approximately 50 neurons. Because the staining was usually limited to cell bodies, it was not possible to tell by immunocytochemistry alone which neurons were efferents or interneurons. However, as the HPLC analysis demonstrated that most of the larval proctolin is located in the periphery, we surveyed putative efferent targets.

Hindgut neuromuscular endings

Hindgut innervation. Proctolin was first discovered in the cockroach by Brown (1967) by virtue of its potent contractile effects on visceral tissues, especially the hindgut. *Drosophila* tissues are also sensitive to proctolin. Micromolar bath application of proctolin to isolated larval hindguts results in pronounced longitudinal contractions (Halpern et al., 1986). Other tissues responsive to proctolin include the proventriculus and dorsal organ (aorta), where the peptide induces or accelerates rhythmic contractions. These effects can be reversed by brief washes with physiological saline.

Hindgut proctolin-like immunoreactivity. The larval *Drosophila* hindgut consists of an outer layer of circular muscles and inner longitudinal muscle fibers (Crossley, 1978). The hindgut is innervated by branches of the 2 segmental 8th abdominal nerves of the ventral ganglion (Campos-Ortega and Hartenstein, 1985). Proctolin immunocytochemistry revealed 2 fascicles that projected superficially on opposite sides of the larval hindgut (Fig. 7*A*). The fascicles consisted of immunoreactive processes measuring about 0.5 μm in diameter (Fig. 7*B*). Each fascicle tended to unravel and rejoin over the length of its trajectory, revealing 3 stained processes (Fig. 7*B*). These stained projections were highly varicose (Fig. 7, *B* and *C*), with 2–3 μm blebbings spaced 3–5 μm apart. They projected on the outer circular muscle fibers (Fig. 7*C*, arrows indicate edges of a circular fiber), and the varicose morphology of the projections resembled the shorter contacts made by motor endings on skeletal muscle fibers (see next section). We did not observe any collateral branches or lateral sprouts from the fascicles. Their simple, blebbed trajectories on the circular muscles suggest that they make *en passant* release sites over the circular muscle fibers.

Backfills. We wished to test whether the 6 proctolin-immunoreactive processes on the hindgut could be axons from the 6 strongly immunoreactive PM cell bodies found at the posterior end of the ganglion. A cluster of 6 proctolinergic 8th segment neurons has been shown to innervate the hindgut in the grasshopper embryo (Keshishian and O'Shea, 1985b).

To reveal the CNS location of *Drosophila* hindgut efferents, the two 8th nerves were isolated and backfilled with hexammine cobalt chloride ($n = 3$). A small cluster of dorsal and ventral cell bodies was revealed by this method, lying in the same general location as the PM cells (Fig. 7*D*). The camera lucida drawings of Figure 8 compare the backfilled cell bodies to proctolin immunocytochemistry of the posterior ventral ganglion, illustrating positional overlap that is supportive, but not conclusive, evidence that the PM neurons are hindgut efferents. Also, note

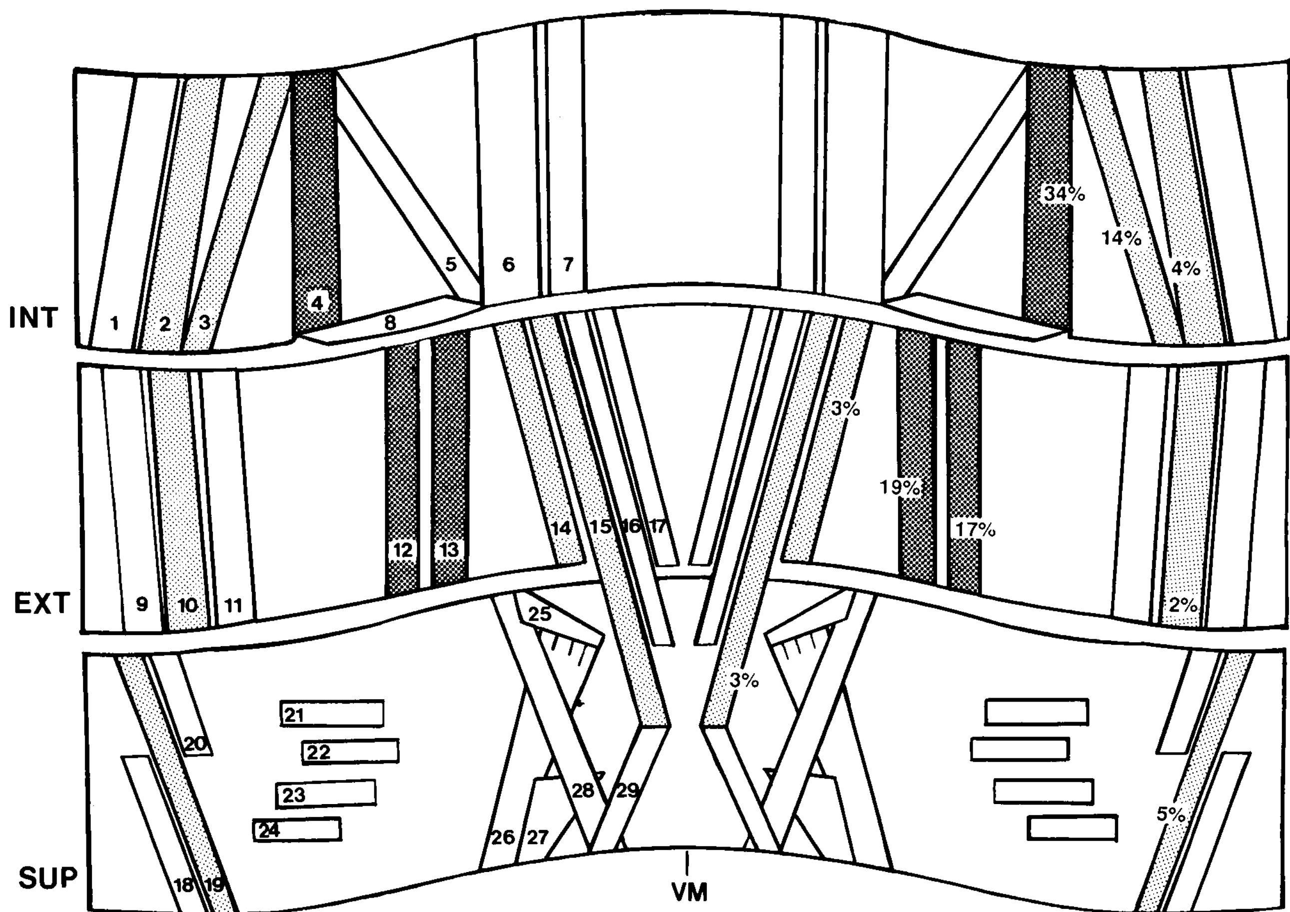


Figure 9. Schematic representation of the muscle fibers and the observed proctolinergic motor endings of the abdominal bodywall. Each segment has 3 layers of muscle fibers. Here, 3 adjacent segments are shown. For clarity, only the internal fibers (#1–8) are drawn in the top segment, the externals (#9–17) in the middle segment, and the superficials (#18–29) in the bottom segment. The fibers with proctolinergic endings are *shaded,* with the major muscle targets *shaded darkly.* The percentages at *right* show the fraction of all immunoreactive nerve endings in segments A3–A7 found on the indicated fiber. The frequency of observed endings on a segment-specific basis is given for each muscle fiber in Table 1.

that cells anterior and lateral to the PM neurons were also backfilled. Direct colabeling of the PM neurons is in progress.

Segmental bodywall neuromuscular endings

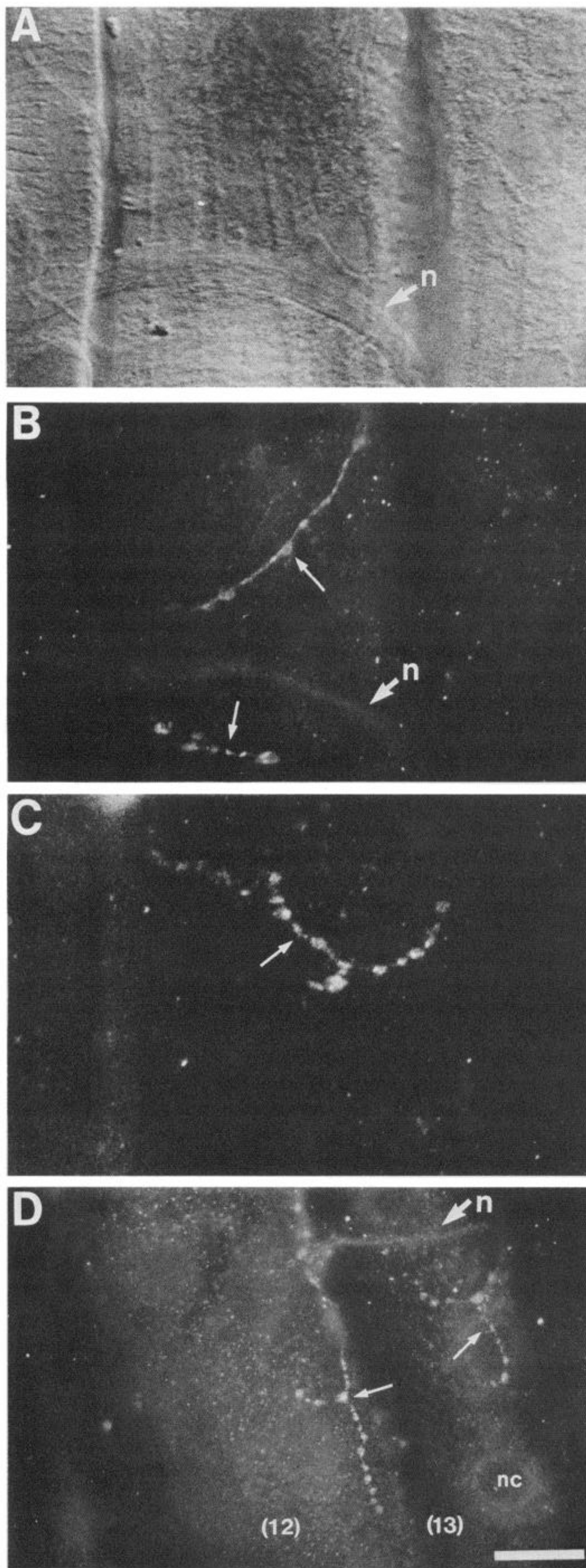
More than half of the larval proctolin is associated with the segmental bodywall musculature. Therefore, we examined the whole-mount immunoreactivity of the bodywall muscles to identify which muscle fibers were innervated by proctolinergic efferents. The segmental musculature of *Drosophila* embryos and larvae is remarkably simple, consisting of 7–30 uniquely identifiable muscle fibers per hemisegment. The muscle fibers vary in size, with the largest measuring 300–500 μm in length and 50–80 μm in width. There are 5 patterns of larval fibers, specific to segments T1, T2, T3, A1–7, and A8. The muscle

Table 1. Abdominal segmental distribution of proctolinergic synapses[a,b]

Segment	Ventral	Muscle fiber(s)				Dorsal	
	14 + 15	12 + 13	4	2 + 3	19	10	Total
A1	–	–	–	–	–	–	–
A2	–	–	1 (3%)	–	–	–	1
A3	–	4 (6%)	21 (58%)	2 (3%)	–	–	27
A4	–	15 (22%)	27 (80%)	12 (18%)	5 (15%)	1 (3%)	60
A5	6 (12%)	29 (56%)	22 (85%)	15 (29%)	7 (27%)	3 (12%)	82
A6	9 (18%)	38 (76%)	20 (80%)	15 (30%)	3 (12%)	1 (4%)	86
A7	5 (25%)	31 (78%)	14 (70%)	13 (33%)	–	–	63
Total	20	117	105	57	15	5	319

[a] Shown is the number of muscle fibers with positively scored endings. There were no observed endings in thoracic segments. Segment A8 was not assayed.

[b] In parenthesis, for each muscle fiber, is the success rate for finding positively stained endings.



fiber pattern in each abdominal segment from A1–A7 is essentially the same, the only difference being found in segment A1, where there is an additional ventral internal oblique fiber (#31) and a missing ventral external oblique muscle fiber (#17) (Hertweck, 1931; Crossley, 1978; Campos-Ortega and Hartenstein, 1985; Hooper, 1986).

The bodywall muscle fibers are arranged in internal, external, and superficial layers, with the majority running longitudinally or obliquely (Fig. 9). Although there are no muscle fiber bundles, several of the muscle fibers are closely aligned. It is possible that closely aligned muscle fibers behave synergistically, and they may share similar kinds of synaptic inputs.

Distribution of proctolin-immunoreactive endings. The immunoreactively revealed contacts were restricted to 9 segment-specific muscle fibers, illustrated in Figure 9 and Table 1. The proctolinergic innervation was discerned with high reliability in 3 segmental muscle fibers: the dorsolateral internal longitudinals (fiber #4) and the 2 pleural external longitudinal muscle fibers (#12 and #13). Muscle fiber #4 represented the most reliable and consistent proctolinergic target in the body wall. With the exception of a single example in segment A2, all the endings on muscle fiber #4 were restricted to segments A3–A7. Of the 141 examined muscle fiber #4s of segments A3–A7, 104 bore proctolinergic motor endings ($n = 20$ larvae; 74% of the fibers bearing immunoreactive endings). The probability of finding endings varied among specific segments. Only 58% of the examined #4s in segment A4 had discernible endings, while the remaining segments averaged over 80%. The pleural internal longitudinals (#12 and 13) had similar percentages over the same range of segments (refer to Table 1). In most cases, the stained endings were found on the bilateral homologs. For example, in 31 of 39 examined bilateral pairs of muscle fiber #4, stained endings were found on both sides ($n = 10$ larvae, segments A3–A7).

In contrast to the high reliability of observed endings on muscle fibers #4, 12, and 13, the remaining 6 proctolin-innervated muscle fibers had endings that were observed with low frequency (fibers #2, 3, 10, 14, 15, and 19). We also noted that the endings were distributed within fewer segments of the abdomen than fibers #4, 12, and 13, although remaining between segments A3 and A7 (see Table 1). We found only 20 endings on 142 examined muscles (14%) for fibers #14 and 15; 15 out of 85 (18%) for fiber #19; and 5 out of 85 (6%) for fiber #10. We did not observe any correlation between the presence of endings on the low-frequency fibers and the absence of endings elsewhere.

Anatomy of segmental motoneuron endings. Each hemisegment receives a large segmental nerve from the ventral ganglion, running laterally across the muscle fibers (Hertweck, 1931; Campos-Ortega and Hartenstein, 1985). Branches emerge from the nerve as it projects from the ventral to dorsal sides of the segment to innervate the muscle fibers (Fig. 10*A*). Proctolinergic motoneuron endings were usually limited to 1 or 2 varicose

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Figure 10. Proctolin-immunoreactive endings on the abdominal bodywall muscle fibers. *A*, Nomarski optics view of muscle fiber #4 in segment A5 with a small nerve fascicle (*n*) projecting across it. *B*, Immunofluorescence view of *A*, demonstrating 2 short proctolin-immunoreactive endings (*arrows*). *C*, Endings (muscle fiber #4 in segment A6) revealing the prominent immunoreactive varicosities. *D*, Endings on fibers #12 and 13 in segment A6. The endings on both muscles could be traced from the nerve fascicle (*n*). The outline of a muscle nucleus is visible (*nc*). Rhodamine immunofluorescence. Scale bars: *A*, *B*, and *D*, 25 μm; *C*, 16 μm.

processes on the fiber (Figs. 10, *B–D*). The immunoreactive processes never wrapped a muscle fiber, but were found on the internally facing surface. Also, the endings rarely extended the full length of a fiber. In a few examples, Y-shaped branches were found on the fibers (Fig. 10*C*), but elaborate branching or clusters of boutons were not seen. On muscle fiber #4, the endings were usually found at the midsection (Fig. 10*A*), while on fibers #12 and 13, endings were usually found closer to the anterior ends (Fig. 10*D*).

The stained endings measured less than 0.5 μm in diameter, and were varicose, with the blebbings spaced each 3–5 μm. The varicosities ranged in diameter between 1 and 3 μm (Fig. 10*B–D*). On fibers #12 and 13 we often observed a single immunoreactive axon projecting across both fibers, with endings on each. It is possible that the 2 muscle fibers are innervated by a common axon (Fig. 10*D*).

Discussion

This study has demonstrated the presence of the neuropeptide proctolin in the larvae of *D. melanogaster,* identifying both central neurons and neuronmuscular endings. The evidence for proctolin in *Drosophila* is strongly supported by our data. The bioactivity detected by the extensor tibiae assay is restricted to a single peak comigrating with authentic tritiated proctolin in both isocratic and gradient high-pressure liquid chromatography. The peak is shifted identically to authentic proctolin when the HPLC solvent system is altered. The heat-stable *Drosophila* molecule can be cleaved by leucine aminopeptidase, indicating that it is likely a peptide. Proctolin-dependent bioactivity is eliminated by incubation of the *Drosophila* substance with antiserum specific to proctolin, suggesting that the molecule closely resembles proctolin. Motor responses to the peptide can be demonstrated in several muscular systems of the larvae, as well as in muscle bundles in grasshopper known to be sensitive to proctolin. The cytochemical localization of proctolin with antisera is consistent with the regional localization of the molecule as demonstrated by HPLC. The staining of motor endings is specific to tissues where proctolin can be recovered. In conclusion, either proctolin itself or a molecule extremely similar to it is distributed in a highly stereotyped fashion in the central and peripheral nervous system of *Drosophila.*

At present, several other neurotransmitter phenotypes have been described in *Drosophila* using immunocytochemical methods and enzyme histochemistry. The descriptions include ACh (Hall et al., 1979; Greenspan et al., 1980; Salvaterra and McCaman, 1985), 5-HT (Valles and White, 1986), and glutamate (Chase and Kankel, 1987). Using mutants deficient in catalytic enzymes, the roles of ACh, 5-HT, and glutamate in normal morphogenesis and function have been tested (Greenspan et al., 1980; Valles and White, 1986; Chase and Kankel, 1987).

Immunocytochemical evidence for neuropeptide expression in the *Drosophila* CNS has been provided by White et al. (1986) for the tetrapeptide FMRFamide and by Jan and Jan (1982) and White and Valles (1985) for substance P. However, the immunoreactive antigens remain to be recovered and assayed from *Drosophila,* and they could be sequence analogs to these peptides (see Myers and Evans, 1985; O'Shea and Schaffer, 1985). There is also little information concerning the synaptic targets of these peptidergic neurons in *Drosophila.*

This study provides a direct demonstration of a peptide neurotransmitter substance in *Drosophila* by HPLC recovery and assay, and it demonstrates that *Drosophila* motoneurons may use transmitters other than glutamate at neuromuscular junctions. The presence of proctolin in motor endings suggests that the peptide functions as a synaptic transmitter or neuromodulator, as has been shown in other arthropods. The role of proctolin at the dipteran neuromuscular junctions is not understood, but this peptide potentiates glutamate-evoked muscle contractions in crustacea (Schwarz et al., 1980; Bishop et al., 1984, 1987) and directly evokes long-lasting muscle contractions in skeletal muscles of lower insects (May et al., 1979; Adams and O'Shea, 1983).

Ganglionic immunoreactivity

Whole-mount proctolin immunocytochemistry is an efficient method for assaying the major immunoreactive neurons of insect ganglia. We observed positive immunoreactivity in about 50 bilaterally symmetrical neurons. The criteria we used for identifying neurons were conservative and depended on several histological methods. The pattern of immunoreactive neurons in *Drosophila* larvae suggests that proctolin expression is segmentally repeated and located principally within the abdominal segments of the CNS. In contrast to this arrangement is the cluster of strongly immuoreactive neurons, usually 6 in number, located at the posterior end of the CNS.

Other neurotransmitter systems in *Drosophila* are also expressed in a highly specialized fashion. The adult distribution of FMRFamide cytochemistry is limited in each hemiganglion to about 20 neurons in 12 clusters, with a similar pattern for the larval CNS (White et al., 1986). The serotonergic neurons of *Drosophila* are also few in number (Valles and White, 1986). In other insects, similar observations have been made for proctolin (Bishop and O'Shea, 1982; Keshishian and O'Shea, 1985a), FMRFamide (Myers and Evans, 1985), GABA (Hoskins et al., 1986), 5-HT (Bishop and O'Shea, 1982; Taghert and Goodman, 1984), and octopamine (Goodman et al., 1979). These studies suggest that the CNS of invertebrates may contain a wide variety of neurotransmitter substances, each expressed by a small, specialized subset of neurons.

Hindgut innervation

Six axonlike processes that project in 2 symmetrical fascicles are proctolin immunoreactive. To determine the total number of axons projecting over the hindgut, and whether the stained processes are single axons, electron microscopic immunocytochemistry will be needed. The simple varicose anatomy of the stained hindgut processes suggests that they make repeated *en passant* release sites along the circular muscles of the hindgut. Similar varicosities have been described for larval *Drosophila* muscle fiber endings using vital labeling techniques (Hardie, 1975; Yoshikami and Okun, 1984).

The 6 strongly stained PM cell bodies of the posterior end of the ganglion are good candidates for hindgut efferents. In addition to their numerical correspondence to the hindgut processes, the cell bodies are copositional to efferents revealed by backfilling the hindgut nerves. A similar pattern of 6 terminal ganglion efferents has been described for the grasshopper embryo by Keshishian and O'Shea (1985a). The 6 grasshopper AM neurons express proctolin and are located within the 8th abdominal segment of the grasshopper CNS. They project to the hindgut, as demonstrated by Lucifer yellow–proctolin immunocytochemistry colabeling experiments. It is interesting to note that if the hindgut innervation in *Drosophila* and grasshopper

prove to be homologous, then there has been conservation of a synaptic system between 2 species separated by over 300 million years of evolution (Thomas et al., 1984).

Bodywall innervation

Proctolinergic motor endings were confined to segments A3–A7 (based on the analysis of 6000 muscle fibers in segments T1–A7). Within each hemisegment only a subset of the muscle fibers is innervated by proctolinergic processes. There are 9 specific innervated muscle fibers that can be grouped into the 6 sets listed in Table 1. The innervated muscle fibers do not appear to fall into clearly defined functional classes, nor is there a preference with respect to the dorsoventral axial location of the muscle fibers. However, all the innervated muscle fibers are either obliques or longitudinals, extending the entire length of the segment. None of the short or transverse fibers that represent about a third of the entire musculature are innervated by proctolinergic endings. The presence within a segment of fiber-specific proctolinergic endings strongly suggests that the fibers are singled out by the nervous system for as yet undetermined motor specializations.

Structurally, in larval *Drosophila* all the abdominal segments other than the most posterior 8th have identical muscle fiber patterns (with the exception of a minor difference in A1). Thus, the muscle fibers of segments A2 and A3 are essentially indistinguishable anatomically. Despite this apparent similarity, A2 and A3 are dramatically different in the frequency of observed proctolin-immunoreactive endings. An important conclusion from the bodywall staining is that, between segments, structurally homologous bodywall muscle fibers do not necessarily have identical synaptic inputs, as defined by transmitter phenotype. Rather there are segment-specific synaptic specializations on muscle fibers that are otherwise indistinguishable. An issue remaining to be resolved is whether the differences in neurotransmitter staining seen between homologous muscle fibers are due to differences in transmitter phenotype of segmentally reiterated motoneurons or to different or novel motoneuronal projections.

Both motoneurons and muscles are highly stereotyped in insects. In this study we show that there is comparable precision in the expression of specific neurotransmitters at the motor endings. It is probable that the expression of several different neuromuscular transmitters is a strategy to obtain specializations in muscle fiber behavior within the limits of a structurally conserved arrangement of motoneurons and muscle fibers.

In conclusion, we have demonstrated the presence of the neuropeptide transmitter proctolin in *Drosophila* larvae and have determined its cellular localization in the CNS and at neuromuscular junctions. The data indicate that a subset of larval visceral and bodywall muscle fibers is innervated by neurons expressing a peptide transmitter. The pattern of endings is restricted in a segment-specific fashion to several singly identifiable muscle fibers. The results show that by examining specialized neurotransmitters it is possible to characterize efficiently a fiber-specific pattern of synapses on the *Drosophila* musculature.

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