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FMRF-amide-like Substances in the Leech

I. Immunocytochemical Localization[1]

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Abstract

FMRF-amide-like immunoreactivity (FLI) was localized to approximately 50 neurons in each segmental ganglion of the medicinal leech using immunocytochemical techniques. Although most of these neurons were iterated in each segmental ganglion, some were more restricted in their segmental distribution. The head and tail ganglia likewise contained numerous FMRF-amide-like immunoreactive cells. In addition to cell bodies, many nerve processes and varicosities were also immunoreactive throughout the ganglion. All labeling of FLI was blocked by preabsorption of the anti-FMRF-amide antiserum with synthetic FMRF-amide.

Using a combination of Lucifer Yellow cellular injection and indirect immunofluorescence techniques, we identified several of the neurons possessing FLI. Identified neurons included excitatory motor neurons (HE, RPE, LPE, AE, and L), the HA modulatory neuron, interneuron cell 204, and cells of unknown function (AP). The processes of HE motor neurons and HA modulatory neurons which innervate the heart tubes were also immunoreactive. These results indicate a role for FMRF-amide-like substances as neurochemical signals in the leech.

The tetrapeptide FMRF-amide (Phe-Met-Arg-Phe-NH_2), first characterized as a molluscan cardioexcitatory substance, has since been found in a variety of animals where it has numerous physiological effects. Early investigations (Kerkut and Laverack, 1960; Jaeger, 1966; Frontali et al., 1967) showed that heart and brain extracts of snails contained peptides which increased the force of contraction and frequency of beating in the normally myogenic hearts of the clam *Mercenaria mercenaria*. FMRF-amide was later isolated from *Macrocallista nimbosa* and sequenced by Price and Greenberg (1977a, b), who suggested that FMRF-amide might act as a long-duration mimic of the classical molluscan cardioexcitatory transmitter, serotonin.

Studies examining a large number of species have shown that FMRF-amide is predominantly but not exclusively a cardioexcitatory substance (Painter and Greenberg, 1982). Nanomolar concentrations of FMRF-amide elicit excitatory effects in the isolated heart of *M. mercenaria* (Price and Greenberg, 1980). Equally low FMRF-amide concentrations cause contractions in the isolated radula protractor muscle of *Busycon contrarium* (Greenberg and Price, 1979), the isolated tentacle muscle of *Helix aspersa* (Cottrell et al., 1983), and the anterior byssus retractor muscle of *Mytilus edulis* (Painter, 1982). Acetylcholine (ACh) is the classical excitatory neuromuscular transmitter in all three of these non-cardiac muscle preparations.

Immunocytochemical techniques have been the primary tools used for studying the distribution of FMRF-amide-like substances in molluscs (Boer et al., 1980; Painter, 1982; Cottrell et al., 1983) and other non-molluscan species (Boer et al., 1980; Weber et al., 1981; Ali-Rachedi et al., 1982; Grimmelikhuijzen et al., 1982; O'Donohue et al., 1984; Watson et al., 1984). These studies indicate multiple roles of FMRF-amide activity in both invertebrate and vertebrate systems. In mammals, FMRF-amide-like immunoreactivity can be localized both to central neurons and to gut endocrine cells, suggesting that FMRF-amide-like peptides may have dual roles as both central neuroregulators and gut hormones (Dockray et al., 1981; O'Donohue et al., 1984; Sorenson et al., 1984).

The organization and accessibility of the leech CNS makes it particularly amenable to studies of neuropeptide function at a cellular level. The approximately 10^4 neurons in the CNS (Macagno, 1980) are reproducibly identifiable from animal to animal and are large enough to penetrate with intracellular microelectrodes. Many neurons have been characterized morphologically and electrically and have known physiological roles (see Muller et al., 1981).

In the present study, FMRF-amide-like substances are localized to a specific subset of leech central neurons with immunocytochemical techniques. Using double-labeling techniques to positively identify some of these cells, neurons important in the control of behaviors such as heartbeat (Calabrese and Peterson, 1983), swimming (Stent et al., 1978; Weeks and Kristan, 1978), penile eversion (Zipser, 1979), and annulus erection (Stuart, 1970) are confirmed as showing FMRF-amide-like immunoreactivity (FLI). Evidence for a physiological role for FMRF-amide-like substances as chemical transmitters within the leech heartbeat system is presented in the second paper of this series (Kuhlman et al., 1985).

Materials and Methods

Animals. Adult specimens of the leech *Hirudo medicinalis* were purchased from European suppliers (Ricarimpex and Blutegelimport und Versand). Leeches were kept at 15°C in artificial pond water for up to 6 months before use.

Dissections. Isolated nerve cords and isolated heart tubes were used for immunocytochemical procedures. Chains of ganglia and their associated roots and connectives were removed from the animal as described by Muller et al. (1981) and pinned in clear Sylgard-coated dishes. The glial sheaths encapsulating the ganglia were mechanically removed from one surface of the ganglia using fine scissors and forceps either prior to or following fixation. Because the antisera often failed to penetrate the entire thickness of the ganglia, ventrally desheathed preparations were used to detect FLI on the

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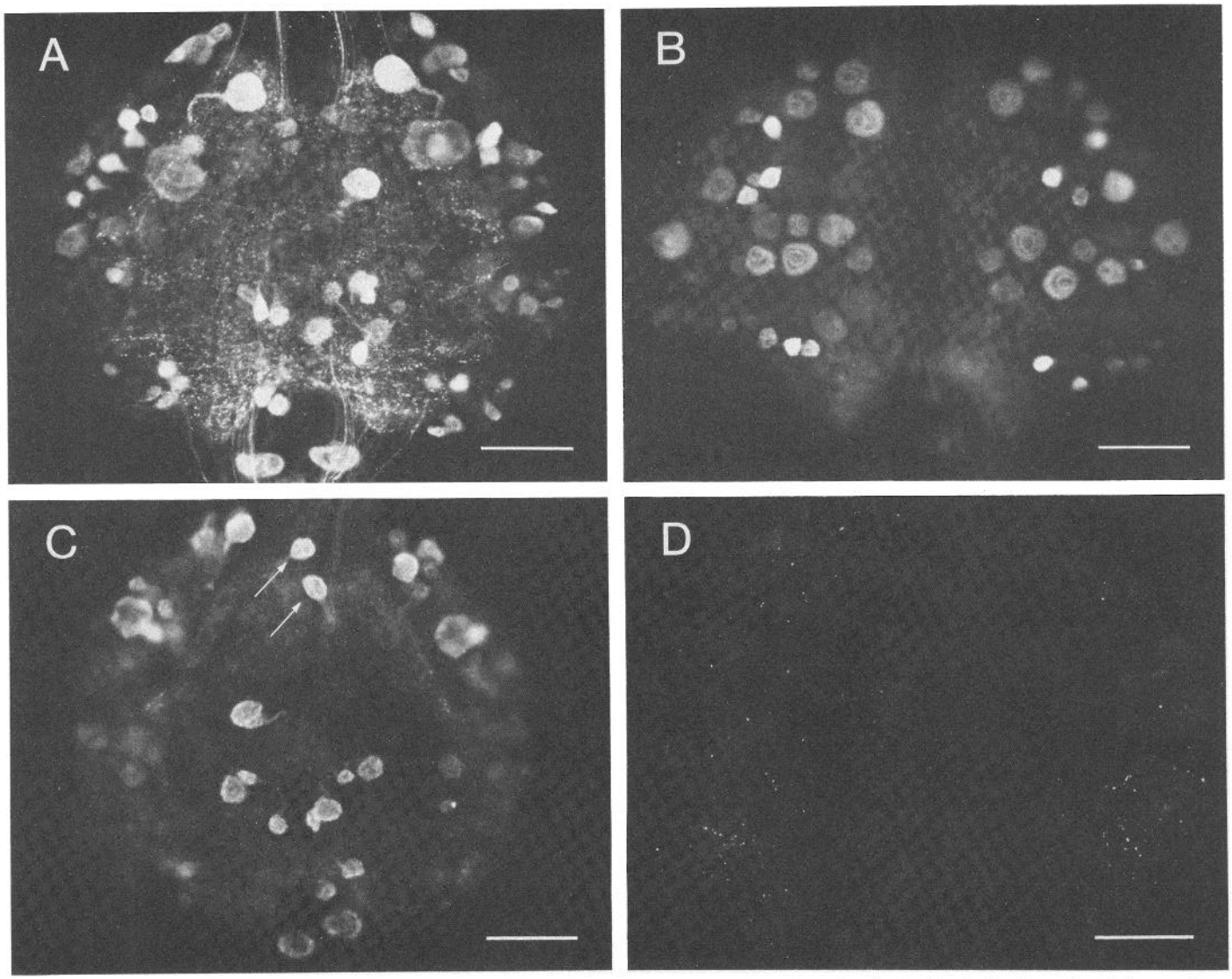

Figure 1. The distribution of FMRF-amide-like immunoreactive cells in a typical midbody ganglion, visualized with rhodamine-conjugated secondary antibody. *A*, Labeling of FLI in G8. The ganglion was desheathed ventrally, and the ventral cell bodies are in focus. *B*, Staining of dorsal cell bodies in a dorsally desheathed G7 preparation. *C*, Staining of ventrally desheathed G7 preparation after preabsorption of the primary antibody with methionine-enkephalin. The typical FLI distribution pattern is not affected. *Arrows* indicate a ventral pair of neurons which stain only in G7 and G14. *D*, Staining of segmental G15 after preabsorption of the primary antibody with synthetic FMRF-amide. No specific staining is seen. The low level of background staining is due to nonspecific binding of the antisera and is found in all preparations. In this and all subsequent figures ganglia are oriented with anterior upward. *Calibration bars* = 0.1 mm.

ventral surfaces of the ganglia, whereas dorsally desheathed preparations were used to detect FLI in dorsally lying cells and neuropil.

Three to five segments of the lateral heart tubes were dissected out of the animal and similarly pinned. Excess connective tissue on the heart tubes was then carefully removed using fine scissors and forceps.

To label processes on the heart tubes by intracellular injections of central neurons, the body wall preparation described by Maranto and Calabrese (1984b) was used with several segments of body wall and their associated CNS connections dissected for each preparation.

Immunocytochemistry. Immunocytochemical procedures were performed on whole mounts of nerve cord and heart tube preparations as described by Li and Calabrese (1985). A polyclonal antibody raised in rabbits against synthetic FMRF-amide conjugated to succinylated bovine thyroglobulin (O'Donohue et al., 1984) was used in most of the isolated nerve cord preparations and in all of the heart tube preparations. A second antibody to FMRF-amide was obtained commercially (Cambridge Research Biochemicals) and used on several occasions with isolated nerve cord preparations. Binding of the primary antibody was visualized through either the indirect immunofluorescence technique (Coons, 1958) or a peroxidase-conjugated secondary antibody followed by reaction with 3,3′-diaminobenzidine (DAB) (Sternberger, 1979).

Absorption controls for all immunocytochemical experiments were performed by incubating tissues with antiserum that had been preincubated for ½ to 3 hr with synthetic FMRF-amide (Peninsula Laboratories). Antisera at working dilutions were combined with FMRF-amide to give a final FMRF-amide concentration of 100 μg/ml of antiserum. Methionine-enkephalin and leucine-enkephalin were similarly tested for their ability to cross-react with the antisera, and were incubated with the antiserum at concentrations of 200 μg/ml and 150 μg/ml, respectively.

Intracellular injections. Intracellular injection of the dye Lucifer Yellow (Stewart, 1978) or of horseradish peroxidase (HRP) (Muller and McMahon, 1976) was used in conjunction with indirect immunofluorescence to positively identify immunoreactive cells and processes. Cells chosen for dye injection were first penetrated with standard glass microelectrodes (Haer) backfilled with 4 M potassium acetate. Following physiological identification, iontophoresis of Lucifer Yellow or pressure injection of HRP was performed as described by Li and Calabrese (1985). Lucifer Yellow-injected preparations were immediately processed for indirect immunofluorescence, whereas HRP-injected preparations were cultured for 5 to 6 days before reaction with DAB (Li and Calabrese, 1985). Following immunocytochemical staining, heart tubes were viewed successively under brightfield optics to visualize HRP-

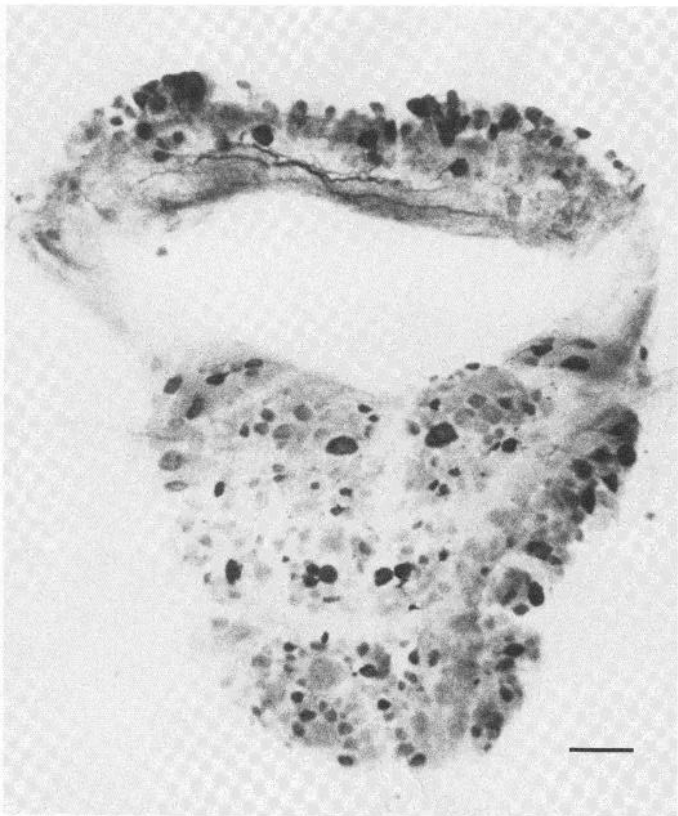

Figure 2. The distribution of FMRF-amide-like immunoreactive cells and processes in the head brain, visualized with peroxidase-conjugated secondary antibody. Labeling of the left side of the brain is weak, most likely due to incomplete removal of the glial sheath. *Calibration bar* = 0.1 mm.

filled processes and under rhodamine fluorescence optics to visualize immunoreactive processes.

Results

Specificity of immunocytochemical staining

About 50 cells in a typical midbody ganglion of the leech show FLI (Fig. 1, *A* and *B*). The pattern of cells showing FLI was similar for both primary antisera tested, although the antibody provided by O'Donohue et al. (1984) generally produced more intense and more consistent labeling. Visualization of the primary antibody by either indirect immunofluorescence or the DAB reaction product (peroxidase-conjugated secondary antibody) showed comparable distributions of immunoreactive cells.

Absorption controls provided further evidence for the specificity of the immunocytochemical labeling. Preabsorption of the primary antisera with synthetic FMRF-amide as described under "Materials and Methods" completely eliminated all labeling of immunoreactive cells and processes (Fig. 1*D*), indicating a high affinity of the antisera for the FMRF-amide sequence. Preabsorption of the antisera with other peptides (methionine-enkephalin and leucine-enkephalin) had no effect on the intensity or pattern of labeling (Fig. 1*C*).

Distribution of immunoreactive neurons

Immunoreactive cell bodies were divided approximately equally between the ventral and dorsal surfaces of a typical midbody ganglion (Fig. 1, *A* and *B*). Although all of the dorsal cells and most of the ventral cells showing FLI occurred as bilateral pairs, either one or two unpaired immunoreactive cells were observed in the anterior medial packet on the ventral surface of midbody ganglia. A large unpaired neuron (>30 μm in diameter) with a thick primary neurite was labeled in all 21 segmental ganglia. An additional unpaired ventral cell was found only in the more posterior segmental ganglia beginning with ganglion 9 (G9). This additional labeled neuron could be distinguished from the first by its smaller size.

There were other more localized deviations from the typical distribution pattern. The ganglia which innervate the genitalia (G5 and G6) contained immunoreactive cells not found in any other ganglia, including a bilateral pair of large cells in the ventral anterior lateral packets. Ganglion 6 contained the most immunoreactive cells of any segmental ganglion, including a pair of large, conspicuous neurons which labeled intensely in the most rostral portion of this ganglion only. These differences in distribution patterns of FLI are summarized schematically in Figure 5. The segmental ganglia nearest the two ends of the animal, namely G1 and G2 and G19 to G21, contained fewer immunoreactive cells than did the midbody ganglia. An interesting deviation from the typical FLI distribution pattern occurred in G7 and G14, where an additional pair of immunoreactive neurons was found on the ventral surface in the rostral medial region of these ganglia only (Fig. 1*C*). Additional labeled cells were also observed in the ventral anterior medial packets of G3 (bilateral pair) and G4 (unpaired cell), although with less consistency.

Like the segmental ganglia, the head and tail brains of the leech contained FMRF-amide-like substances. FLI was localized to numerous neurons in both the supraesophageal and subesophageal ganglia of the headbrain (Fig. 2). Although immunoreactive cells have been observed in the tailbrain, difficulties in effectively desheathing the tailbrain prevented an accurate determination of its distribution pattern of FLI.

Immunoreactive processes and varicosities in nerves and neuropil

Not only cell bodies but also many processes and varicosities show FLI in the leech CNS (Fig. 1*A*). Immunoreactive varicosities were found throughout the neuropil but were most concentrated in the caudal portion of the ganglion. Immunoreactive processes were visible both in the roots and in the connectives. Intensely labeled processes were also seen running transversely in the supraesophageal portion of the headbrain (Fig. 2).

Identification of immunoreactive cells

By combining indirect immunofluorescence labeling with Lucifer Yellow dye injection, some of the neurons showing FLI were positively identified. Included in this list of identified cells are neurons which occur in all segmental ganglia as well as neurons found in a particular subset of the ganglia.

Neurons innervating the hearts. The lateral heart tubes of the leech are innervated by heart excitor (HE) motor neurons located in G3 to G18 (Thompson and Stent, 1976; Maranto and Calabrese, 1984a) and by heart accessory (HA) modulatory neurons located in G5 and G6 (Calabrese and Maranto, 1984; Maranto and Calabrese, 1984a). Both the HE and HA cells show FLI (Fig. 3). FLI labeling of the HA cells accounts for the presence of the bilateral pairs of large immunoreactive cells seen only in the ventral lateral packets of G5 and G6.

Swim-initiating interneurons. The unpaired immunoreactive neuron found in segmental ganglia posterior to G9 was identified as the swim-initiating interneuron cell 204 (Weeks and Kristan, 1978) by double labeling (Fig. 4, *C* and *D*). Although the additional unpaired neuron showing FLI in G9 was not double-labeled, its size and position within the ganglion indicate that it is a homologous swim-initiating interneuron, cell 205 (Weeks, 1982).

Other identified neurons. As previously stated, G6 innervates the genitalia and contains more FMRF-amide-like immunoreactive cells than do other segmental ganglia. Both the rostral penile evertor (RPE) and the lateral penile evertor (LPE) motor neurons (Zipser, 1979) of ganglion 6 show FLI (Fig. 4, *A* and *B*; data for LPE not shown). A bilateral pair of cells in G5 which are morphologically and electrically similar to the LPE neurons of G6 (Zipser, 1982) was not double-labeled with the FMRF-amide antibody.

The annulus erector (AE) and large longitudinal (L) motor neurons

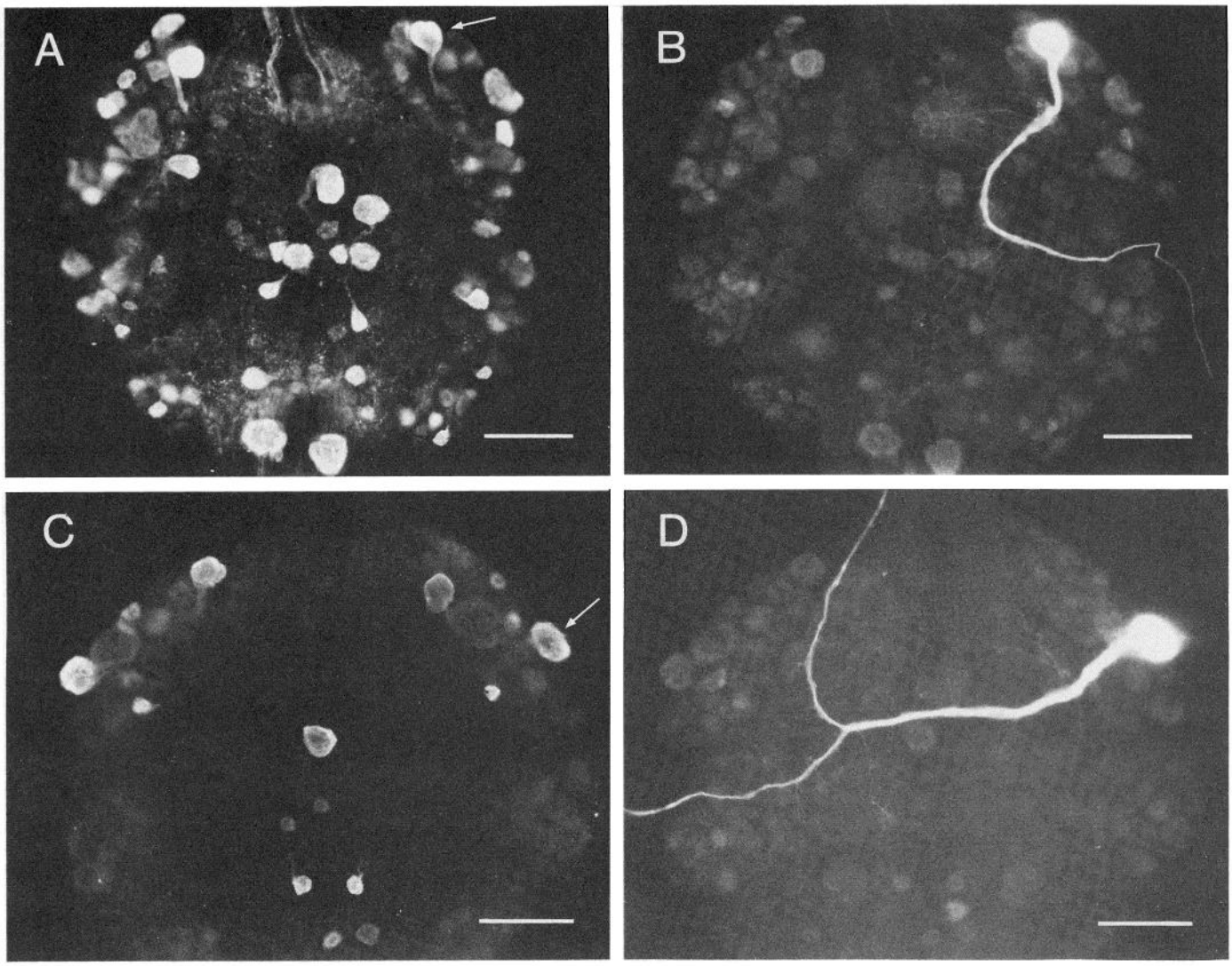

Figure 3. Identification of neurons showing FLI by double labeling: The heart motor neuron and the heart modulatory neuron. *A*, FLI labeling of a ventrally desheathed G10 viewed with rhodamine optics. The *arrow* indicates the cell injected with Lucifer Yellow as seen in *B*. *B*, Lucifer Yellow injection of an HE cell as viewed with fluorescein optics. This same cell shows intense FLI when viewed under rhodamine optics as seen in *A*. *C*, FLI labeling of a ventrally desheathed G5. The *arrow* indicates the cell injected with Lucifer Yellow as seen in *D*. *D*, Lucifer Yellow injection of an HA cell as viewed with fluorescein optics. This same cell shows intense FLI when viewed under rhodamine optics as in *C*. *Calibration bars* = 0.1 mm.

(Stuart, 1970) and the anterior pagoda (AP) neurons (Sunderland, 1980; Fuchs et al., 1981) showed FLI in all segmental ganglia.

Summary of central staining

A schematic summary of the cells showing FLI is presented in Figure 5. Both the typical segmental distribution pattern and ganglia with atypical staining patterns (G5, G6, and G7) are shown. Also indicated on this diagram are cells which have been positively identified by double labeling.

Immunoreactive processes on the heart

Labeling of isolated heart tubes by the indirect immunofluorescence technique revealed immunoreactive processes on the muscle cells of the hearts (Fig. 6, *A* and *B*). Often two or more distinct processes were seen running longitudinally along the heart tubes; these processes occasionally sent off branches which in turn branched into many finer processes (Fig. 6*A*). In some cases, the processes followed the contours of the underlying cardiac muscle cells closely, causing a labeled process to appear scallop-shaped against the dark background (Fig. 6*B*; representative muscle cells are visible in Fig. 6*D*).

HE and HA cells (in different preparations) were injected with HRP to visualize their processes on the heart tubes (Fig. 6, *D* and *F*). Such processes were found in all of the HE preparations ($n = 3$) and in half of the HA preparations ($n = 4$). These processes run longitudinally along the hearts, branching occasionally, and closely resemble those processes seen by Maranto and Calabrese (1984a) using similar techniques. Heart tubes showing HRP-labeled processes were labeled with FMRF-amide antibody by the indirect immunofluorescence technique. Although the HRP label (DAB reaction product) slightly obscured the fluorescent signal, overlap of immunoreactive processes with processes originating in the HE and HA cells was clearly demonstrated (Fig. 6, *C* and *D* and *E* and *F*). Thus, at least some of the immunoreactive processes on the heart are peripheral branches of the HE and HA cells.

Discussion

Based on immunocytochemical studies, FMRF-amide-like substances have been localized to a subset of central neurons in the leech. The distribution of cell bodies showing FLI was similar from ganglion to ganglion, although deviations from a general distribution pattern were found in a number of ganglia. Distribution patterns have

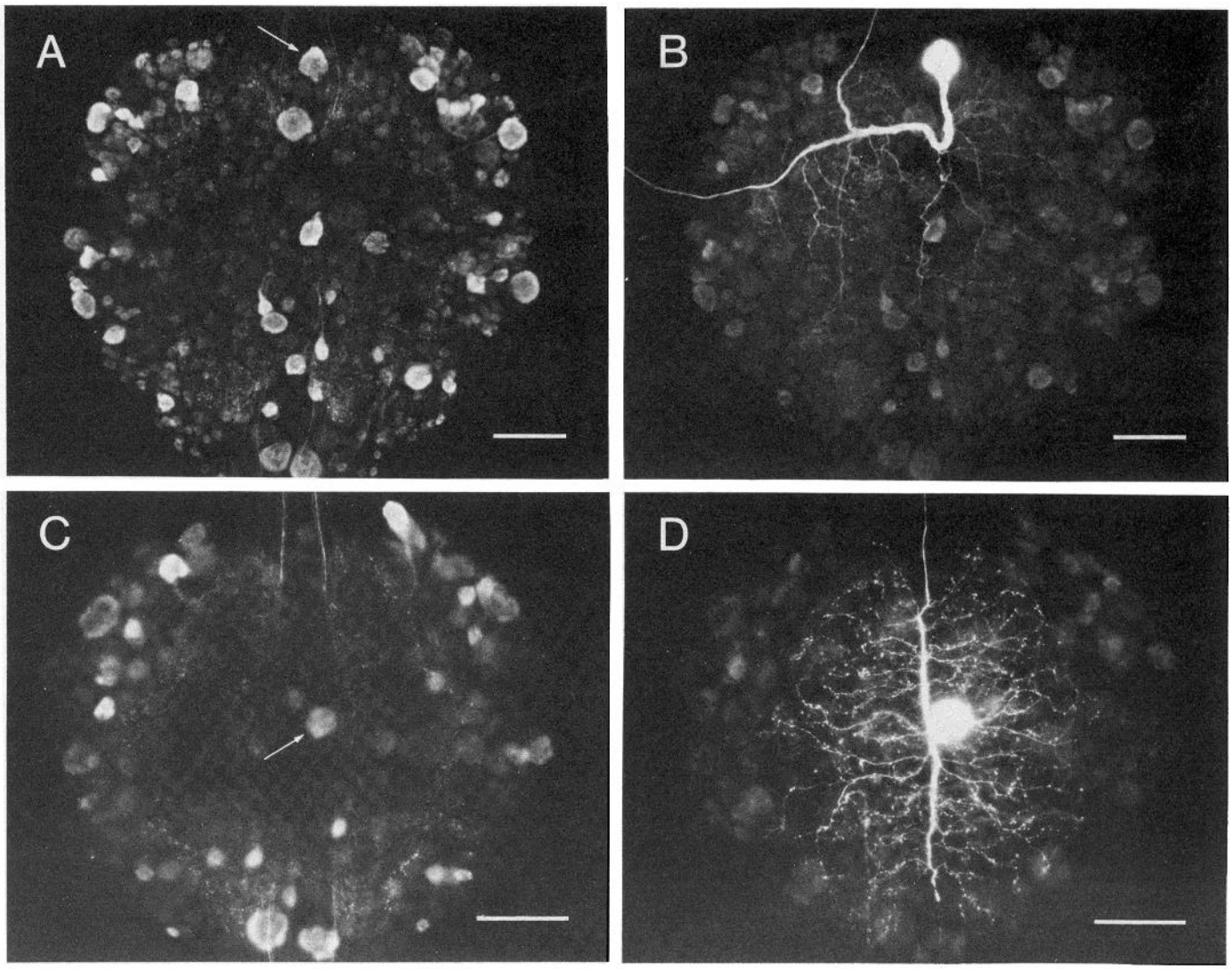

Figure 4. Identification of neurons showing FLI by double labeling: The RPE motor neuron and the swim-initiating interneuron cell 204. *A,* FLI labeling of a ventrally desheathed G6. The *arrow* indicates the cell injected with Lucifer Yellow as seen in *B. B,* Lucifer Yellow injection of an RPE motor neuron as viewed with fluorescein optics. This same cell shows intense FLI when viewed under rhodamine optics as in *A. C,* FLI labeling of a ventrally desheathed G13. The *arrow* indicates the cell injected with Lucifer Yellow as seen in *D. D,* Lucifer Yellow injection of cell 204 as viewed with fluorescein optics. This same cell shows intense FLI when viewed under rhodamine optics as in *C.* In both *C* and *D,* the cell body is photographed slightly out of focus in order to display the full arborization of the Lucifer Yellow-filled cell 204. *Calibration bars* = 0.1 mm.

also been determined for other antigens found in the leech CNS (Zipser, 1980; Zipser and McKay, 1981; Zipser, 1982; Johansen et al., 1984; Li and Calabrese, 1985). Localized deviations from a general distribution pattern were found in these studies as well and in some cases aided in the discovery of physiologically interesting neurons (Zipser, 1982). Localization of FMRF-amide-like substances in the leech CNS has revealed uniquely occurring neurons in G5 and G6 and in G7 and G14.

The specificity of immunocytochemical labeling was demonstrated by absorption controls and use of various primary antisera and immunocytochemical techniques. Nevertheless, the existence of true FMRF-amide in the leech CNS has not yet been demonstrated. Previous work with polyclonal FMRF-amide antisera has shown that the similarity between the recognized antigen and true FMRF-amide need not be greater than an -Arg-Phe-NH_2 carboxyl terminal (Weber et al., 1981). As this C-terminal requirement is sufficient not only for FLI but also for FMRF-amide-like bioactivity (Price and Greenberg, 1980), only biochemical analysis can reveal the exact identity of the active peptide. In both *Helix aspersa* (Price, 1982) and *Limulus polyphemus* (Watson et al., 1984), biochemical analysis of CNS extracts has revealed that FLI in these preparations is not due to true FMRF-amide but rather to closely related peptides. We have begun work on characterizing the FMRF-amide-like peptide of leech using high performance liquid chromatography and radioimmunoassay.

By using intracellular marking in conjunction with immunocytochemistry, some of the neurons showing FLI were positively identified. Among these identified neurons are many excitatory motor neurons (HE, RPE, LPE, AE, and L neurons). In addition, most of the as yet unidentified dorsal neurons showing FLI occupy the characteristic positions within the ganglia of excitatory motor neurons to the body wall musculature (Figs. 1*B* and 5). Pharmacological evidence suggests that the excitatory motor neurons of the leech use ACh as their primary transmitter (Kuffler, 1978). Biochemical studies have provided further evidence for this claim. Using the enzymes choline acetyltransferase and acetylcholinesterase as markers, a complete map of putative cholinergic neurons in the leech CNS has been constructed (Sargent, 1977; Wallace, 1981a,b; Wallace and Gillon, 1982). These neurons include: the HE, AE, and circular ventrolateral motor neurons, the excitatory motor neurons of

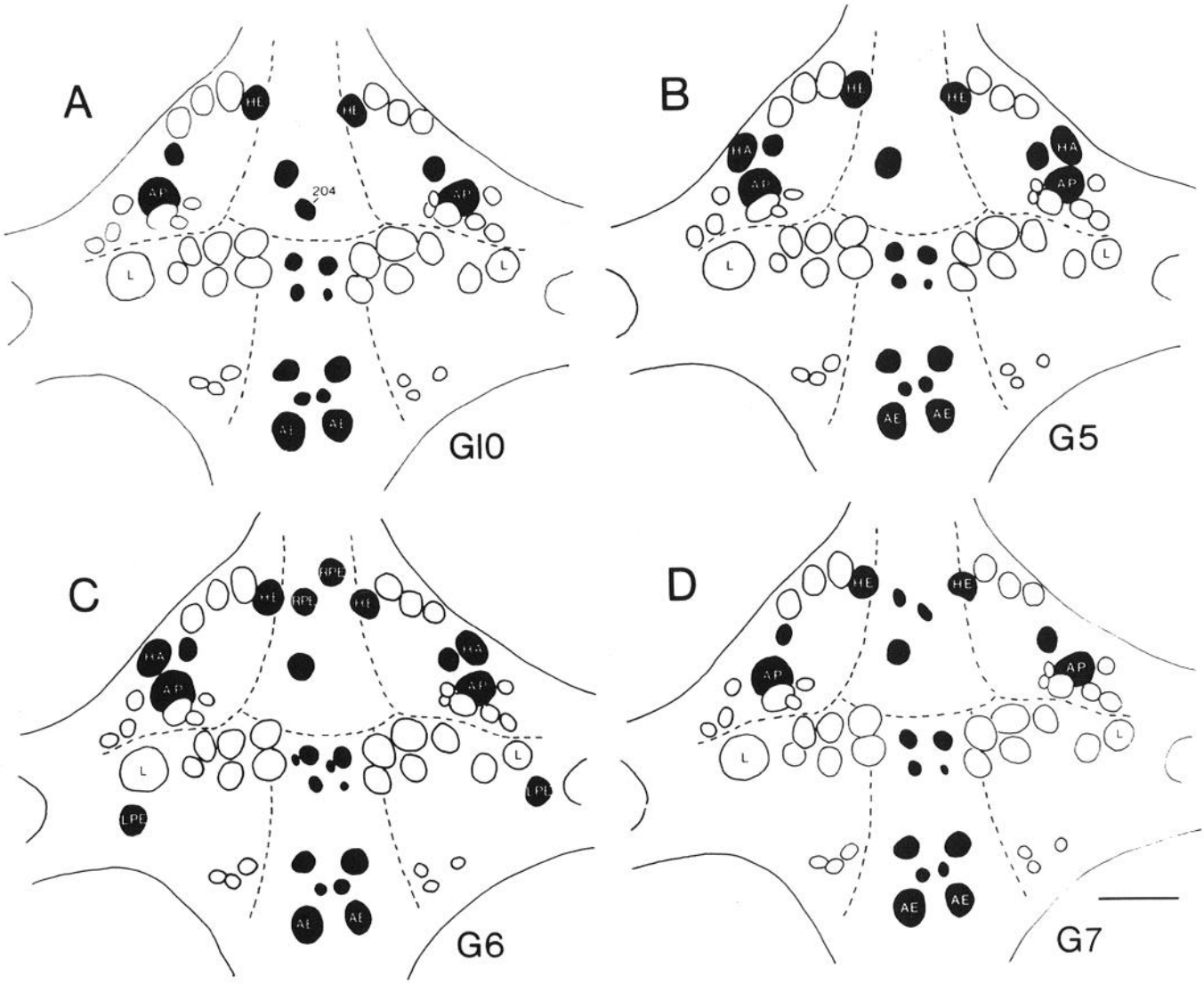

Figure 5. Maps of neurons showing FLI in typical and atypical ganglia. In these maps, ventral cell bodies are indicated by *solid circles*, and dorsal cell bodies are indicated by *open circles*. Cells positively identified by double labeling are indicated by name or number. The relative positions and sizes of the cells are adapted from a ganglionic map by Muller et al. (1981). *A*, FLI in a typical midbody ganglion. Both unpaired ventral medial immunoreactive cell bodies are included. *B*, FLI in G5. *C*, FLI in G6. *D*, FLI in G7, which includes a ventral cell pair not found in the typical segmental ganglion. *Calibration bars* = 0.1 mm.

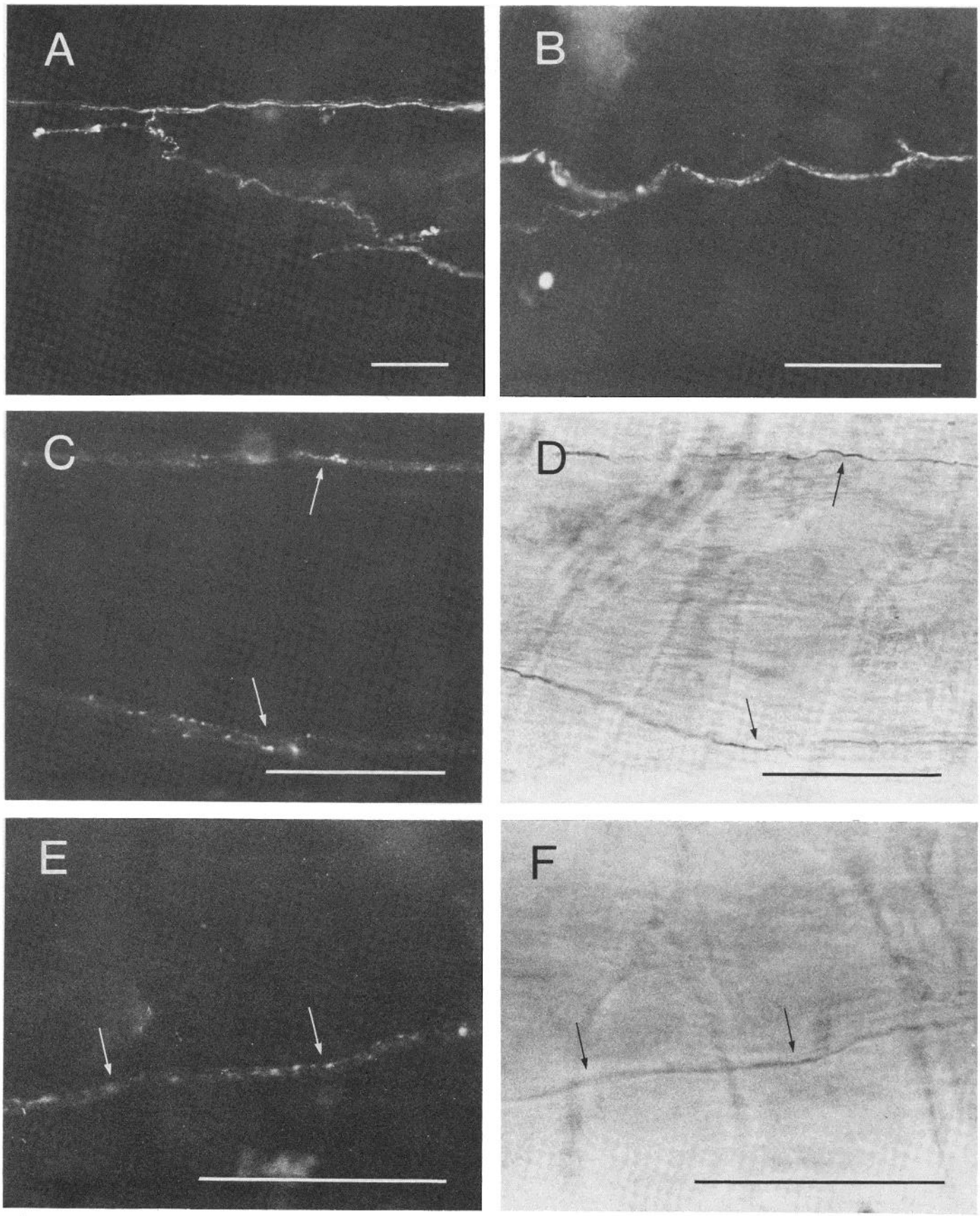

Figure 6. Processes on the heart tubes showing FLI: Identification by double labeling. *A,* Representative segment of a heart tube showing FLI. The labeled process consists of a single main fiber which sends off many smaller branches. *B,* A section of a labeled process as viewed under higher magnification. The scallop-shaped appearance of the fiber is due to the closeness with which the process follows the contour of the underlying muscle cells. Muscle cells as viewed under brightfield optics with similar magnification can be seen in *D. C* and *D,* Double labeling (FLI/rhodamine-conjugated secondary antibody and HRP/DAB) of a heart motor neuron's processes on the heart tube. The processes of the HE cell, which had been injected with HRP, show FLI when viewed under rhodamine optics (*C*) and a DAB reaction product when viewed under brightfield (*D*). The two labeled processes arose from a single labeled process. The fluorescent label is partly obscured by the DAB reaction product. *E* and *F,* Double labeling (FLI/rhodamine-conjugated secondary antibody and HRP/DAB) of an HA cell's process on the heart tube. The process of the HA cell, which had been injected with HRP, shows FLI when viewed under rhodamine optics (*E*) and a DAB reaction product when viewed under brightfield (*F*). *Calibration bars* = 0.05 mm.

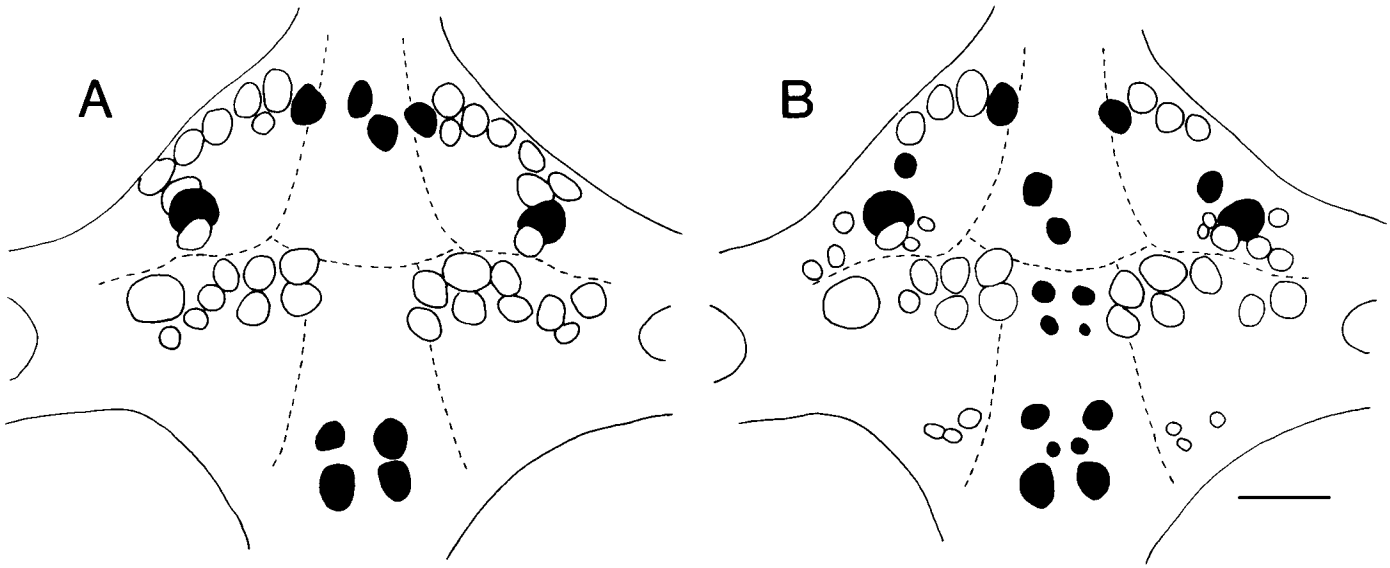

Figure 7. Overlap of the distributions of putative cholinergic neurons and neurons showing FLI. *A,* Schematic map of the putative cholinergic neurons in a typical midbody ganglion. Putative cholinergic neurons are characterized as containing either the synthetic enzyme choline acetyltransferase or the catabolic enzyme acetylcholinesterase. Ventral cell bodies are indicated as *solid circles,* whereas dorsal cell bodies are indicated as *open circles.* The relative positions and sizes of the cells are adapted from a ganglionic map by Muller et al. (1981). *B,* Schematic map of the neurons showing FLI in a typical midbody ganglion (G10). *Calibration bar* = 0.1 mm.

the dorsal surface (cells 3, 4, 5, 7, 8, 11, 12, 17, 106, 107, 108, 109, 110, 111, 112, 117, and L), and two ventral cell pairs of unknown function (AP neurons and cells 251) (Fig. 7*A*). Although many of the FMRF-amide-like immunoreactive neurons have yet to be positively identified, there appears to be a substantial overlap between the putative cholinergic neurons and the neurons showing FLI (Fig. 7, *A* and *B*). If co-release of ACh and an FMRF-amide-like peptide is a general phenomenon of leech motor neurons, then there exists a role for FMRF-amide in the periphery as a general modulator or regulator of muscle tension. Evidence for an FMRF-amide-like peptide as a regulator of the tension produced by leech cardiac muscle is presented in the following paper (Kuhlman et al., 1985).

Figure 7, *A* and *B*, clearly indicates that not all neurons showing FLI are putative cholinergic neurons. Two such neurons (cell 204 and HA) have been positively identified. Cell 204 is a swim-initiating interneuron which has no peripheral targets but synapses only on central neurons of the swim circuit (Weeks and Kristan, 1978). A role for a FMRF-amide-like substance as a central neurotransmitter is therefore indicated. The HA modulatory neurons synapse on the hearts, and activity in the HA neurons modulates the output of the hearts (Calabrese and Maranto, 1984). This suggests the possibility of peripheral modulation of muscle tension by release of an FMRF-amide-like peptide as a primary transmitter. Hence, FMRF-amide may serve as a primary transmitter at some synapses (such as those between HA neurons and heart muscle) and as a co-transmitter with ACh or other molecules at other synapses (such as those between HE neurons and heart muscle). The following paper (Kuhlman et al., 1985) addresses the various roles of FMRF-amide in chemical transmission within the leech heartbeat system.

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