# Evidence for Homologous Peptidergic Neurons in the Buccal Ganglia of Diverse Nudibranch Mollusks

**Winsor H. Watson III[1,*] and A. O. Dennis Willows[2]**

[1]Department of Zoology, University of New Hampshire, Durham, New Hampshire 03824; and
[2]University of Washington, Friday Harbor Laboratories, Friday Harbor, Washington 98250-9218

## SUMMARY

**The buccal ganglia of seven nudibranches (*Aeolidia papillosa, Armina californica, Dirona albolineata, D. picta, Hermissenda crassicornis, Melibe leonina,* and *Tritonia diomedea*) were examined to explore possible homologies between large cells that reacted with antibodies directed against small cardioactive peptide B ($SCP_B$). The buccal ganglion of each species possessed a pair of large, dorsal–lateral, whitish neurons that contained an $SCP_B$-like peptide. We refer to these neurons as the *SLB* (*$SCP_B$-immunoreactive Large Buccal*) cells. In all species examined, the SLB cells project out the gastroesophageal nerves and appear to innervate the esophagus.**

**In each species, an apparent rhythmic feeding motor program (FMP) was observed by intracellular recording from both SLB neurons and other neurons in isolated preparations of the buccal ganglia. SLB cells often fire at a high frequency, and usually burst in a specific phase relation to the FMP activity. Stimulation of SLB cells enhances expression of the feeding motor program, either by potentiating existing activity or eliciting the FMP in quiescent preparations. Finally, perfusion of isolated buccal ganglia with $SCP_B$ excites the SLB cells and activates FMPs. Thus, both the immunohistochemical and electrophysiological data suggest that the SLB cells within three suborders of the opisthobranchia (*Dendronotacea, Arminacea, and Aeolidacea*) are homologous. A comparison of our data with previously published studies indicates that SLB cell homologs may exist in other gastropods as well.**

***Keywords:*** **$SCP_B$, immunohistochemistry, nudibranchs, peptides, buccal ganglia, feeding motor program, central pattern generators, homologous.**

## INTRODUCTION

A primary feature of gastropod mollusks that has promoted their use in neurophysiology is the individual identifiability of their central nerve cells, based upon both morphological and physiological criteria. Studies on diverse species have led to identification of many specific neurons and determination of roles they play in behavioral responses. As such information becomes available from a sufficient number of different preparations, it is possible to look across species for evidence of conservation and for evolution of neural functions at the cellular level. For example, one distinctive pair of neurons on or near the anterior face of the cerebral ganglia can be identified in two subclasses based upon morphological, physiological, and pharmacological criteria (Senseman and Gelperin, 1974; Weiss and Kupfermann, 1976) suggesting that these serotonin-containing metacerebral giant cells are homologous (Weiss and Kupfermann, 1976; Granzow and Kater, 1977). Homologies have also been reported amongst neurons controlling gill movements in opisthobranch mollusks by Dickinson (1979, 1980).

The buccal ganglia of several gastropod mollusks contain a pair of large (100–300 $\mu$m in diameter), small cardioactive peptide B- ($SCP_B$) containing neurons. The opisthobranch *Aplysia californica* has two large, paired neurons in the buccal

Received January 21, 1992; accepted January 22, 1992
Journal of Neurobiology, Vol. 23, No. 2, pp. 173–186 (1992)

CCC 0022-3034/92/020173-14$04.00
* To whom correspondence should be addressed.

ganglion (B1 and B2) that innervate the gut and contain and synthesize SCPs ($SCP_A$ and $SCP_B$) in large amounts (Lloyd et al., 1985; Lloyd, Schacher, Kupfermann, and Weiss, 1986; Lloyd, Frankfurt, Stevens, Kupfermann, and Weiss, 1987). Both B1 and B2 are active during the swallowing cycle, but not during biting or food rejection, and appear to regulate gut motility (Lloyd, Kupferman, and Weiss, 1988a). The buccal ganglion of another opisthobranch, *Tritonia diomedea,* also has two distinctive pairs of neurons (B11 and B12) that contain SCP-like peptides (Lloyd, Masinovsky, and Willows, 1988b). Both B11 and B12 elicit cyclic motor output of the swallowing pattern generator and drive contractions of the gut (Lloyd and Willows, 1988; Willows, Lloyd, and Masinovsky, 1988). Identified SCP-containing neurons are also present in the buccal ganglion of *Tritonia festiva,* and they may be homologs to B11 and B12 (Masinovsky, Kempf, Calloway, and Willows, 1988). Two other nudibranches, *Hermissenda* and *Dentronotus,* also have very prominent $SCP_B$-immunoreactive neurons that project to the esophagus (Masinovsky et al., 1988). Pulmonate gastropods have also been shown to have large identifiable SCP-containing neurons in their buccal ganglia. *Limax maximus,* a terrestrial slug, contains a number of buccal ganglion cells with $SCP_B$ antigenicity. The largest of these are the lateral B1 cells. (Identification of neurons by particular numbers, e.g., B1 in *Aplysia, Limax, Helisoma,* and *Lymnaea,* does not imply that evidence for homology exists.) In *Limax,* the B1 cells modulate several aspects of feeding behavior (Prior and Watson, 1988). In *Helisoma trivolvis,* a freshwater pulmonate, seven to eight pairs of dorsal and four to five pairs of ventral $SCP_B$-containing neurons have been identified. The largest, located dorsolaterally, have been designated B1 (Murphy, Lukowiak, and Stell, 1985). The buccal ganglion of a close relative to *H. trivolvis, Lymnaea stagnalis,* contains two large and three medium-sized pairs of $SCP_B$-immunoreactive cells. The large lateral B1 cells and fibers appear to project to the anterior gut and salivary glands (Masinovsky et al., 1988). Thus, the available evidence indicates that many gastropod buccal ganglia have large identifiable SCP-containing neurons with similar biochemical, anatomical, and physiological properties. This has led to the hypothesis that they are homologous (Watson and Willows, 1986; Willows and Watson, 1986; Lloyd et al., 1988a,b; Masinovsky et al., 1988).

In order to explore this homology hypothesis further, we examined the buccal ganglia of *Aeolidia papillosa, Armina californica, Dirona albolineata, D. picta, Hermissenda crassicornis, Melibe leonina,* and *Tritonia diomedea.* We report here that the buccal ganglia of all seven species, representing three major nudibranch suborders (*Aeolidacea, Arminacea,* and *Dendronotacea*), have buccal ganglion neurons that are reidentifiable and probably homologous, based upon morphological characteristics, electrophysiological properties, and SCP-like immunoreactivity.

## METHODS

### Animals

*Tritonia* were obtained by trawling in Bellingham Bay, WA at depths of about 25 m. All other species were collected in near-shore locations near the Friday Harbor Laboratories, in the San Juan Islands of Washington, using SCUBA. Animals were maintained in flow-through seawater aquaria at approximately 10°C.

### Immunohistochemistry

Animals received an injection of 0.3 *M* $MgCl_2$ to anesthetize them 20 min prior to dissection. Buccal ganglia and associated gut tissue (esophagus, stomach, salivary glands, etc.) were removed, pinned out in a Sylgard-lined glass petri dish filled with cold seawater, and then fixed for 12 h in a solution of 4% paraformaldehyde in 0.1 *M* phosphate-buffered saline (PBS) (0.9% NaCl, pH 7.4). Fixations, and all the following procedures, were carried out on a shaker table at 8°C. After fixation, tissues were washed for 1 h in PBS, dehydrated through a graded series of alcohols, cleared in toluene or xylene, and then rehydrated (Costa, Buffa, Furness, and Soccia, 1980; Beltz and Kravitz, 1983; Prior and Watson, 1988). Following dehydration/rehydration, tissues were washed for 1 h in PBS containing 0.3% Triton X-100 and 0.1% sodium azide (PTA), and incubated for 12 h in PTA containing 6% nonimmune goat serum.

Spin media from hybridoma cell cultures producing IgG monoclonal antibodies against $SCP_B$ (Masinovsky et al., 1988) was diluted 1:20 in PTA containing 6% nonimmune goat serum, and tissues were incubated in this solution for 36–48 h. They were then washed for 24 h in PTA (four to six changes) and incubated for 24–36 h in goat anti-mouse secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and diluted 1:100 in PTA containing 6% nonimmune goat serum. Finally, tissues were washed in PTA for 12 h and PBS for 1 h.

Preparations were mounted on slides using a medium that consisted of one part 50 m*M* Tris buffer (pH 9.5), and nine parts glycerol. Whole mounts were viewed on a

Nikon Optiphot microscope using epifluorescent illumination. A B2 excitation-barrier filter and reflector combination cube was used for FITC observations (460–490 nm interference excitation filter, 505 dichroic mirror, 511–545 barrier), and a G-cube (520–550 nm interference excitation filter, 575 nm dichroic mirror, 580 nm barrier) was used for visualizing antibodies conjugated to RITC (see below). Preparations were photographed with Tri-X film (ASA 400).

Staining with the monoclonal $SCP_B$ antibodies was partially inhibited by preincubation of the antibodies with $10^{-5}$ *M* $SCP_B$ for 24 h, and completely eliminated when we used $5 \times 10^{-5}$ *M* $SCP_B$. Preincubation with $10^{-5}$ *M* FMRFamide had no effect on subsequent immunohistochemical staining with $SCP_B$ antibodies. Further details about the $SCP_B$ monoclonal antibodies used in this study are available elsewhere (Masinovsky et al., 1988). Secondary antibodies and normal goat serum were obtained from Cappel Worthington Biochemicals (Malvern, PA).

### Lucifer Yellow Injections

The tips of microelectrodes (1 mm, thick walled) were filled with 4% Lucifer Yellow dissolved in 1% LiCl. Then, the electrode was backfilled with 3 *M* LiCl, and the tip was beveled to a resistance of 20–30 MΩ using a squirt bottle filled with a suspension of micropolish (0.05 μ gamma alumina, Buehler, Lake Bluff, IL). Dye was introduced into the cell by either pressure injection (Picospritzer, General Valve Co.), or application of hyperpolarizing current pulses (10 nA, 50% duty cycle). Buccal ganglia with injected cells were incubated in seawater overnight at 4°C and then fixed and processed as described above for immunohistochemistry, except with rhodamine-conjugated secondary antibodies. Lucifer and rhodamine were visualized and photographed in the same cells by switching filter cubes (G-cube for rhodamine and B2 cube for Lucifer Yellow).

### Electrophysiology

Buccal ganglia were removed from animals as stated above and pinned out in 1-ml Sylgard- or wax-lined recording chambers. Seawater at 12°C perfused the preparations at a rate of approximately 5 ml/min.

Neurons were impaled through the epineurium with 10–30 MΩ microelectrodes filled with 3 *M* KCl. Continuous intracellular records were obtained using Gould–Brush chart recorders. Neurons were stimulated intracellularly through the recording electrodes using a bridge–current circuit in the amplifiers.

$SCP_B$ stock solutions were made up in acidified distilled water each week. For pharmacological experiments, the stock solutions were diluted at least 1:1000 in seawater. $SCP_B$ was prepared by diluting a stock solution in 5–10 ml of filtered seawater and then perfusing the appropriate dilution through the recording chamber.

## RESULTS

### Immunohistochemistry

In every nudibranch we examined, there was a pair of large neurons in the buccal ganglion that contained $SCP_B$-like immunoreactive material (Figs. 1, 2). In live ganglia these neurons usually had a white or pale-white pigmentation when viewed with epiillumination. We call these neurons *$SCP_B$-immunoreactive Large Buccal* (SLB) cells. In some species they are also referred to by numbers. For instance, the B11 cells in *Tritonia diomedea* and the B1 cells in *Limax* are also SLB neurons, according to our terminology. In four species (*Tritonia, Melibe, Dirona albolineata,* and *Aeolidia*), we confirmed that the large, white cells were the same cells that contained an $SCP_B$-immunoreactive peptide by injecting them with Lucifer Yellow and then processing the buccal for SCP-like material using rhodamine-conjugated secondary antibodies.

In addition to the SLB cells, a number of other neurons in the buccal ganglion of each species reacted with the $SCP_B$ antibodies. There were three basic patterns of staining. The buccal ganglia of *Dirona picta, Dirona albolineata, Hermissenda, Tritonia,* and *Armina* each contained at least eight pairs of immunoreactive neurons in addition to the SLB cells (Fig. 1). Typically, there were two or three pairs of very intensely fluorescent cells, and the rest of the immunoreactive neurons were only weakly stained. In contrast, only three pairs of SCP-immunoreactive neurons were observed in the buccal ganglion of *Aeolidia;* one pair of SLB cells, and four much smaller cells that have not been identified (Fig. 1). Finally, in *Melibe,* which lacks an extensive buccal mass musculature and has a very small buccal ganglion, the paired SLB cells were the only buccal neurons that contained an $SCP_B$-immunoreactive peptide (Figs. 1, 2).

SLB cells had processes within the buccal ganglia (Fig. 1) and the peripheral nerves (Figs. 2, 3). Projections in the buccal ganglion produced a dense network within the ipsilateral hemiganglion, and sometimes passed to the neuropile of the contralateral side [note the contralateral projection of the SLB cell in *Melibe,* in Fig. 3(D)]. Large axons from the SLB cells project in the ipsilateral gastro-

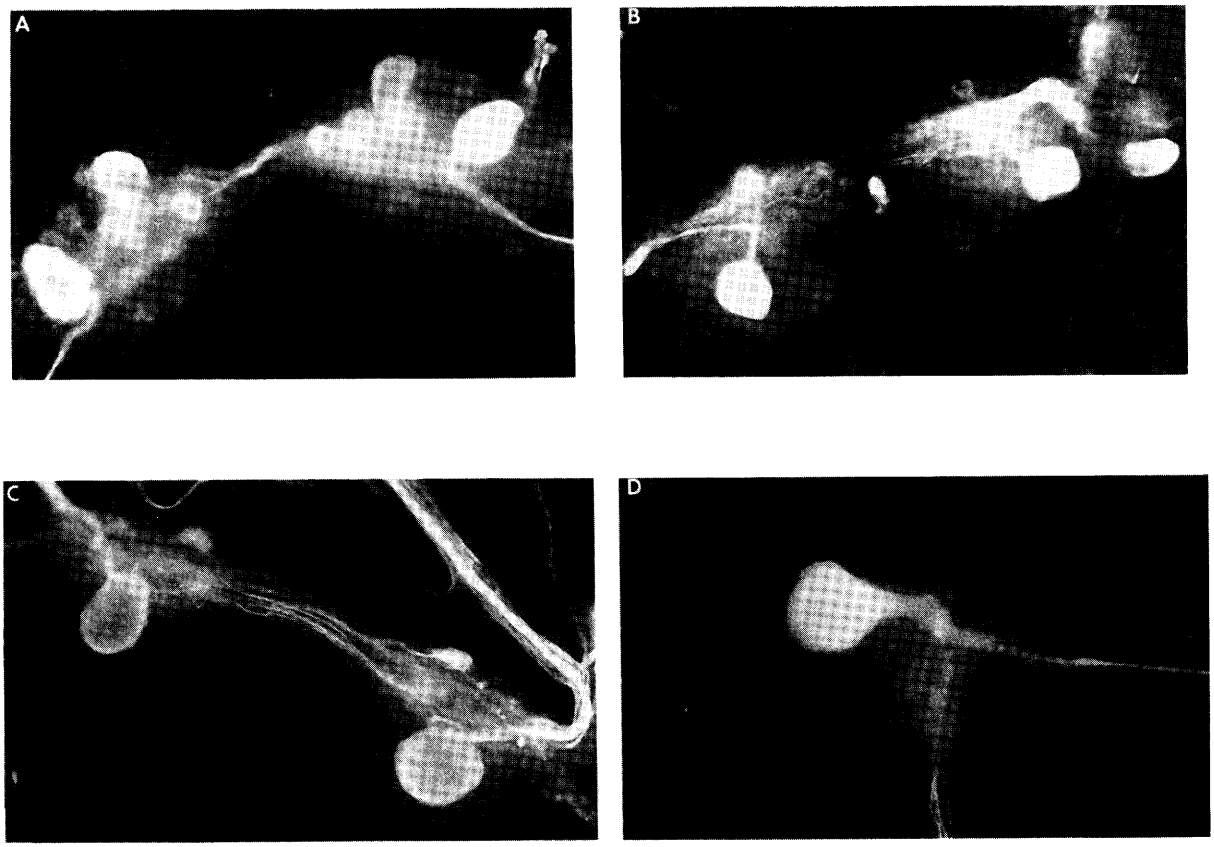


**Figure 1** $SCP_B$-immunoreactive neurons in the buccal ganglia of *Dirona picta* (A), *Hermissenda crassicornis* (B), *Aeolidia papillosa* (C), and *Melibe leonina* (D). All fixed whole mounts were treated with monoclonal antibodies directed against $SCP_B$. The SLB cells are the largest of the immunoreactive neurons in each ganglion. The large axons leaving the buccal ganglia in each figure arise from the SLB neuron somata. Other neurons containing an $SCP_B$-like peptide are also apparent in the ganglion of each species except *Melibe*. There are two additional pairs of small cells in *Aeolidia*, but one pair is not in focus, and six to eight smaller neurons in *Dirona* and *Hermissenda*. The pattern of staining in *Dirona picta*, shown here, is nearly identical to the pattern observed in *Dirona albolineata*, pictured in Figure 3. The large immunoreactive neuron just outside the right buccal ganglion of *Hermissenda* is located in one of the gastroesophageal ganglia. Anterior is up for all plates, but the large SLB cell axon in C was twisted during fixation so it appears to project anteriorly, although it actually projects posteriorly to the esophagus, as shown in Figures 2 and 3. Scale bar = 100 $\mu$m for A–C and 50 $\mu$m for D.

esophageal nerves to the esophagus and other regions of the gut (Figs. 2, 3). Some species (*Armina, Tritonia, Dirona, Aeolidia,* and *Hermissenda*) had a single large $SCP_B$-immunoreactive neuron in each gastroesophageal ganglion (GE-1) (Masinovsky et al., 1988) [Fig. 1(B) and 3(A)]. In these animals, the axons from the SLB cells and the $SCP_B$-positive neuron in the gastroesophageal ganglion project to the esophagus in parallel. Possible synaptic interactions between the gastroesophageal and SLB cells have only been examined in *Dirona,* and we found no observable morphological contacts between the cells, no electrical coupling, and no evidence of either excitatory or inhibitory synaptic interactions.

The peripheral SLB cell axons in all species gave rise to numerous branches that produced a dense network of processes and varicosities on the surface of the gut musculature (Fig. 4). In some species, such as *Armina* [Figs. 4(B)], a number of immunoreactive cell bodies were visible on the esophagus and they gave rise to a nerve plexus.

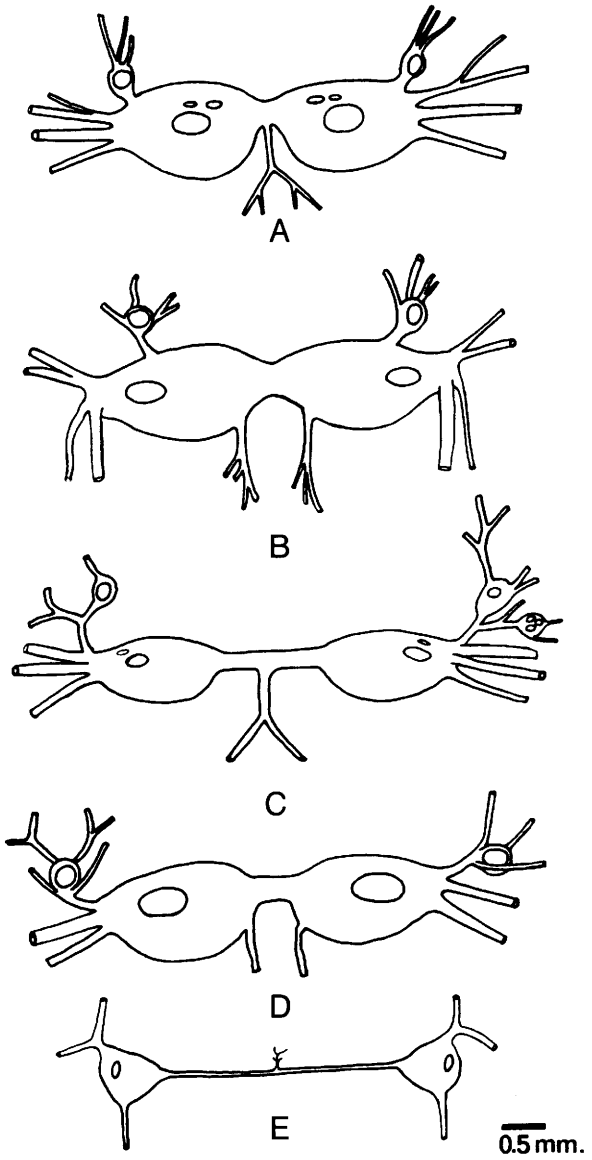


**Figure 2** Diagrammatic representation of the buccal ganglia of (A) *Tritonia,* (B) *Aeolidia,* (C) *Armina,* (D) *Dirona,* and (E) *Melibe* indicating the relative sizes and positions of the primary nerve trunks, and the major $SCP_B$ immunoreactive neurons. Scale bar = 1.0 mm for (A), 0.5 mm for (B–E).

Thus, while many of the varicosities on the esophagus represented SLB cell terminals, it is likely that some came from cells in the intrinsic nerve plexus.

## Electrophysiological Properties

***Relationship of SLB Cell Activity to the Feeding Motor Program.*** In all five genera, an apparent feeding motor program (FMP; identified by comparison with the FMP in *Tritonia diomedea,* Willows, 1980) could be recorded from any of several relatively large neurons in the buccal ganglion (examples from *Armina, Tritonia,* and *Melibe* shown in Fig. 5). These presumptive motor neurons were usually located dorsally, near the postero-medial margins of each ganglion. The burst pattern recorded across all species included spontaneous FMP bouts 2–10 min in duration which were composed of recurrent impulse bursts with 5- to 30-s interburst intervals, and each individual burst was often preceded by a hyperpolarizing wave.

When intracellular electrical activity in SLB cells was monitored during these spontaneous FMPs, a common pattern was observed (*Aeolidia, Dirona,* and *Tritonia* shown in Fig. 6). SLB cells which were silent, or firing irregularly, fired at a higher frequency during bouts of FMP. In some instances, SLB cells would also burst, and these bursts had a particular phase relationship with feeding motor neurons. Increases in the firing frequency of SLB cells often preceded increases in the intensity of motor neuron bursting (spikes per burst and/or burst frequency).

Changes in the rate of firing in both SLB cells occurred together, but spiking was not synchronous. In most species this was probably due to common synaptic input because we found no evidence for electrical coupling of left and right SLB cells, except in *Melibe.*

***Response of the FMP to Stimulation of SLB Neurons.*** In all the species, except for *Melibe,* intracellular stimulation of SLB neurons with sufficient current to cause firing at 5–10 Hz for more than 10 s usually elicited activity in previously inactive FMP neurons, or increased the rate and intensity of a spontaneously active preparation (Fig. 7). However, hyperpolarization of the SLB neurons, during spontaneously occurring FMP output, did not prevent expression of the FMP. Accordingly, SLB activity is sufficient, but not necessary for elicitation of FMP output.

***$SCP_B$ Exposure Excites SLB Neurons and Elicits FMP.*** Earlier work with the SLB neurons of *Tritonia diomedea* indicated that they contain and use $SCP_B$ (Lloyd et al., 1988b; Lloyd and Willows, 1988; Willows et al., 1988). Therefore, we perfused the buccal ganglion of each species with $SCP_B$ and examined the effect on the FMP and SLB cell activity. In each species, concentrations of $3 \times 10^{-5}$ to 3

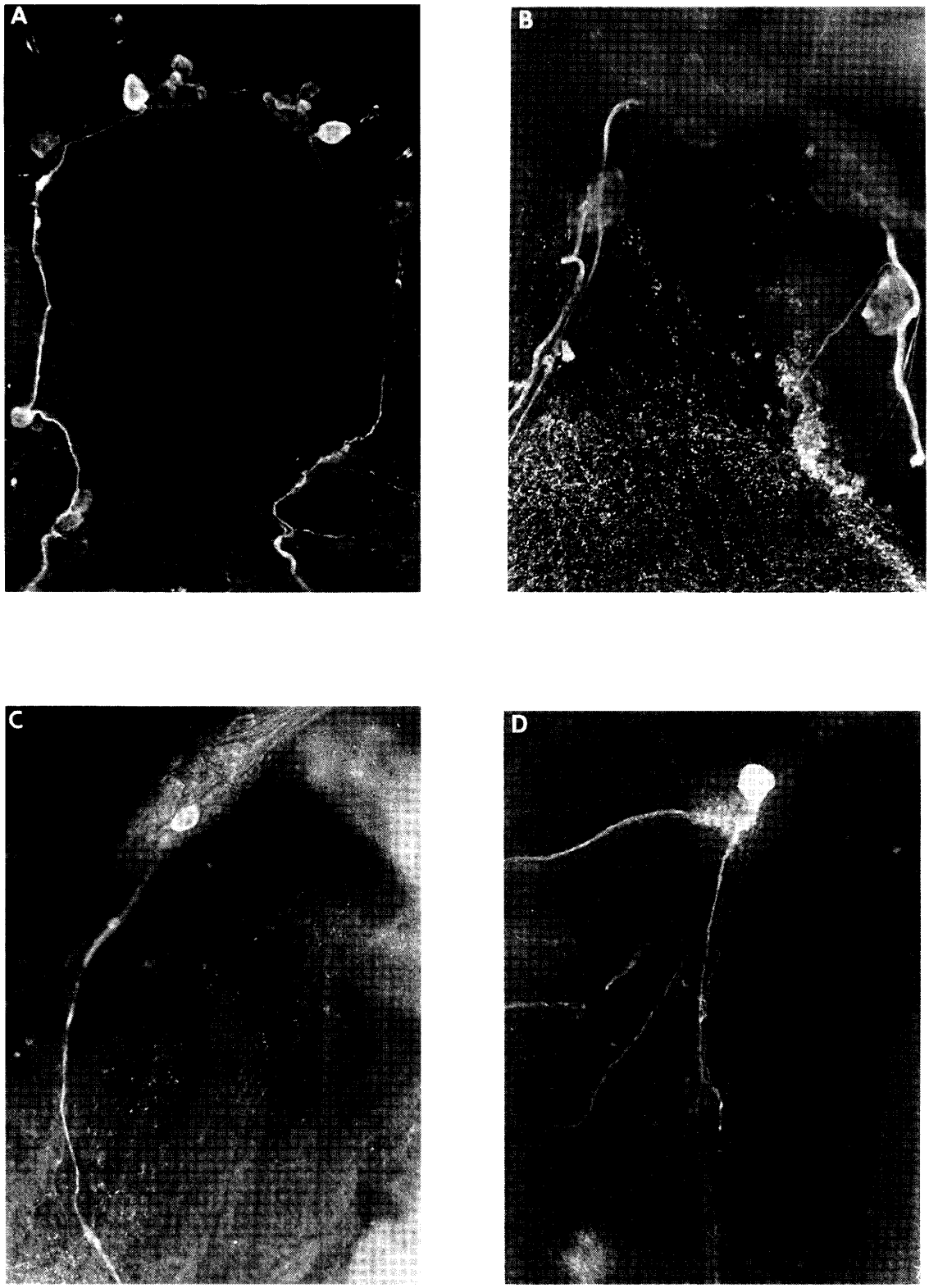


**Figure 3** Projections of SLB cells in *Dirona albolineata* (A), *Aeolidia* (B), *Armina* (C), and *Melibe* (D). These preparations were processed similarly to the ones shown in Figure 1, except the buccal ganglia remained attached to the esophagus during processing so the axonal projec-