Characterization of the Aplysia californica Cerebral Ganglion F Cluster

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INTRODUCTION

The cerebral ganglia of Aplysia californica play an essential integrative role for receiving, transmitting, and processing information originating from the pedal, pleural, buccal ganglia, and peripheral sensory sites such as rhinophores, anterior tentacles, mouth, and eyes. To date, a number of cerebral ganglion neurons, neuronal groups, and pathways have been identified and their functional properties described (Clatworthy and Walters 1994; Kuenzi and Carew 1994; Rosen et al. 1983; Strack and Jacklet 1993; Teyke et al. 1989; Xin et al. 1995; Xin et al. 1996).

Although considerable progress has been made in determining the functional properties of several neurons and neuronal groups, most cerebral neurons and neuronal clusters are poorly characterized. Perhaps the least-characterized clusters on the cerebral ganglia dorsal side are the medially located F clusters. According to Jahan-Parwar (Jahan-Parwar and Fredman 1976), the F cluster contains $\geq 50$ small ($<50 \mu m$), whitish neurons that have mostly silent or irregular spontaneous discharges, and 40–60% of studied F-cluster neurons respond to electrical stimulation of cerebral nerves. The F clusters may be involved in egg-laying behavior; Ferguson (Ferguson et al. 1989) found that local electrical stimulation of F clusters induced a short afterdischarge in bag cell clusters. After the cerebral ganglion was backfilled from the right pleuroabdominal connective, a group of four to six stained cells was visible in the F-cluster region. Except for demonstration of angiotensinogen-, urotensin I- and urotensin II-like immunoreactivities of F-cluster neurons (Gonzalez et al. 1992, 1995), little is known about the biochemical properties of F-cluster cells.

We have performed comparative study of morphological, electrophysiological, and chemical characteristics of the F-cluster neurons. We find that the cluster contains at least three distinct neuronal subpopulations and that these neurons are organized into a unique layered structure within the cluster. Using mass spectrometry, we show that all three populations of neurons have distinct and unique mass of peptides, and these peptides are transported out of the ganglion to specific nerves. The morphologically distinct neurons in the top layer of the F cluster (CFT neurons) appear to be electrically coupled neuronal groups that are similar to the light green cells of Lymnaea stagnalis.

METHODS

Animal and cell preparation

Forty-six cerebral ganglia were used to study the morphological and electrophysiological properties of the cerebral F-cluster neurons. A. californica (100–200 g) were obtained from Aplysia Research Facility (University of Miami, Miami, FL) and kept in an aquarium containing continuously circulating, aerated and filtered artificial sea water (ASW) at 14–15°C until used. Animals were anesthetized by injection of isotonic MgCl$_2$ (30–50% of body wt) into the body cavity. The head ganglia (cerebral, pedal, and pleural) were dissected and placed in ASW containing (in mM) 460 NaCl, 10 KCl, 10 CaCl$_2$, 22 MgCl$_2$, 6 MgSO$_4$, and 10 HEPES, pH 7.7 or in ASW-antibiotic solution: ASW containing 100 units/ml penicillin G, 100 µg/ml streptomycin, and 100 µg/ml gentamicin, pH 7.7. In some experiments Ca$^{2+}$-free ASW was used, which contained (in mM) 460 NaCl, 10 KCl, 0.5 CaCl$_2$, 32 MgCl$_2$, 6 MgSO$_4$, and 10 HEPES, pH 7.7.

Removing the nervous system sheath without protease treatment or in hyperosmotic ASW resulted in damage of the top layer neurons in the F cluster. Therefore the sheath was digested enzymatically by incubating the ganglia in ASW-antibiotic solution containing 1% of protease (Type IX: Bacterial; Sigma, St. Louis, MO) at 36°C for 1–3 h depending on animal size. Next, the ganglia were washed in fresh ASW, and the dorsal side of the cerebral ganglia was desheathed.
Using 0.38-μm-diam tungsten wire (WPI, Sarasota, FL), the ganglia were pinned dorsal side up to a silicone elastomer (Sylgard, Dow Corning, Midland, MI) layer in a recording chamber containing 3–4 ml of ASW-antibiotic media. The isolated ganglia preparation was incubated in ASW-antibiotic solution at 14°C for ≥1 h before each experiment.

**Morphology**

To visualize the vascular system within the cerebral ganglia, Indigo (Sigma) dispersed in ASW was injected by syringe in right pedal or parapodial artery. Cross-sections of the cerebral ganglia were cut in cryostat at 40–70 μm thickness after dissection, fixation in 4% paraformaldehyde, and freezing.

To study neuronal morphology, the cells were filled with 2% Lucifer yellow (Sigma) or 15% lysine-fixable biotin dextran amine (NeuroTrace BDA-10,000 Neuronal Tracer Kit, Molecular Probes, Eugene, OR). The dyes were injected iontophoretically into neurons using glass pipettes with resistance 1–2 MΩ for 1–2 h. The neurons retained physiological properties (action potentials and electrical coupling) during and after injection. After 24–48 h incubation at 14°C in ASW-antibiotic solution, the ganglia were fixed with 4% paraformaldehyde in 0.1 M PBS pH 7.5 for 2 h at 4°C. The avidin-horseradish peroxidase solution (1.0 μg/ml avidin-horseradish peroxidase in PBS containing 0.3% Triton-X100) was applied for 3 h after washing away excess fixative with PBS. Rinsing the ganglia with PBS was followed by addition of hydrogen peroxide to a final concentration of ∼0.5% for ∼30 s and replacing it with 0.05% diaminobenzidine and ∼0.5% H₂O₂ solution for 3–5 min. Wash, dehydration in ascending concentrations of alcohols and clearing in methyl salicylate finalized preparation of the tissue for microscopic study. The cells were visualized with fluorescence and transmission microscopy using an Olympus IMT-2 inverted microscope (Lake Success, NY).

**Electrophysiology**

Small-diameter polyethylene suction electrodes, an A-M Systems (Carlsborg, WA) differential AC amplifier (Model 1700) and an isolated pulse stimulator (Model 2100) were used for extracellular recording and stimulation of the anterior tentacular nerve (AT) (AT, Jahan-Parwar and Fredman 1976; C2, Hoffmann 1939) nerve. Intracellular recording and stimulation were made using glass microelectrodes pulled from 1 mm borosilicate glass capillaries (WPI, Sarasota, FL) and filled with 506.2 mM KCl and 5 mM HEPES solution (pH 7.6, 8–15 MΩ). Signals were amplified with Axon Instruments (Foster City, CA) AxoClamp 2B amplifier, monitored and stored with a Millennia Plus computer (Micron Electronics, Nampa, ID) using a Digidata 1200A D/A - A/D converter (Axon Instruments, Foster City, CA). The software packages pClamp 6 and AxoScope 1 (Axon Instruments) were used for data acquisition and analysis. Records were digitized at 2–10 kHz and filtered with low-pass Bessel filter at 10-kHz cutoff frequency. To reduce the digitization noise high-resolution records with signal amplitude ≤5 mV were digitally low-pass filtered at 10 Hz if not otherwise stated. As currents >5 nA injected through one recording microelectrode induced changes in potential detected by a second microelectrode, we used a maximal stimulus of 3 nA to study synaptic connections between neurons. All experiments were performed at room temperature (23–24°C).

**Mass spectrometry**

As matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is not tolerant of high concentrations of cations, the salts associated with cells and nerve tissue were removed as previously described (Garden et al. 1996). Briefly, after pinning down the cerebral ganglia, the physiological saline was replaced by an aqueous MALDI matrix solution, 10 mg/ml of 2,5-dihydroxybenzoic acid (DHB; ICN Pharmaceuticals, Costa Mesa, CA). Individual F cells were isolated based on their position, size, and pigmentation. Tungsten needles were used to transfer each cell onto a MALDI sample probe containing 0.5 μL DHB solution. Peripheral nerve samples were prepared in a similar manner; a short section (<1 mm) of the interior of the nerve was isolated and placed on one of the sample spots with DHB matrix solution. After drying at the room temperature, samples either were inserted into a mass spectrometer for immediate analysis or stored in the freezer for future analysis. MALDI mass spectra were obtained using a Voyager Elite mass spectrometer equipped with delayed ion extraction (PerSeptive Biosystems, Framingham, MA). A pulsed nitrogen laser (337 nm) was used as the desorption/ionization source, and positive-ion mass spectra were acquired using reflectron mode with an accelerating voltage at 19–20 kV. Each unsmoothed mass spectrum is the average of 64–128 laser pulses. The peaks are listed below as present in a specific group of cells if 70% of the samples analyzed contain that peak. Mass calibration was performed externally using either bovine insulin (Sigma) and synthetic Aplysia α-bag cell peptide (BCP; American Peptide, Sunnyvale, CA) or a previously calibrated mass spectrum obtained from bag cells (Garden et al. 1998).

**RESULTS**

**Morphology of the F cluster**

As shown in Fig. 1, the two symmetrical F clusters of *A. californica* are located in lateral part of the each cerebral hemiganglion dorsal surface near the cerebral commissure and contain ~70 white neurons with cell body size >50 μm and nearly 100 neurons of <50 μm in diameter. Cell bodies are distributed in at least three layers (Fig. 2A). The top layer contains bright white CF₅₁ neurons of 50–100 μm in size that mechanically contact and sometimes are embedded partially in the nervous system sheath. Removal of the sheath without enzyme pretreatment results in these cells being destroyed or sticking to the sheath layer instead of the cerebral cluster. The second layer is made up of smaller F-cluster (CF₇) neurons (30–60 μm). Two classes of the CF₇ neurons can be observed depending on cytoplasm appearance; when viewed under ap-
appropriate lighting, some neurons contain granular material and others clear cytoplasm. The first type is likely the granular neurons described by Soinila and Mpitsos (1996). Generally the granular neurons are located in the lateral part of the F cluster opposite to the cerebral commissure. Interestingly, it appears that some granular neurons extend out of the F cluster. The clear CF_M neurons occupy the portion of the middle layer of the F-cluster located toward the cerebral commissure. Tiny (10–30 μm) neurons (CF_B) form a third layer located just above the neuropil. In some preparations, light-orange (80–120 μm) neurons are found in the caudal part of F cluster top layer, whereas in most preparations, such cells are only observed in other regions of the cerebral ganglion. We further characterize the CF_T neurons and the CF_M neurons of the F cluster in the following text.

Morphology of CF_T neurons

To examine the structure of axon processes, the neurons were filled electrophoretically with Lucifer yellow or biotin dextran amine. The CF_T neurons are generally unipolar and have wide range of somata diameters from 50 to 150 μm [92.6 ± 24.8 (SD), n = 34, Table 1]. The CF_T neuron somata are nonuniformly shaped. The axons travel throughout neuropil to the AT nerve (Fig. 2B) and have two morphologically distinct regions. Starting from the cell body and ending near of the AT nerve base (Fig. 2C), the first region is characterized by the absence of visible varicosities and arborization. The second region lies mainly in the AT nerve and displays multiple branching and varicosities, which often are places of sharp changes in the direction of the axon propagation. The diameter of the branches is most commonly two to three times smaller than the diameter of main axon. The axon forms many parallel thin branches at

![Image](https://via.placeholder.com/150)

**Table 1.** Electrophysiological characteristics of the CF_T and CF_M neurons

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<th>Cell</th>
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<th>CF_M Neurons</th>
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<tr>
<td>Diameter, μm</td>
<td>92.6 ± 24.8 (34)</td>
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<td>Resting potential, mV</td>
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<td>Input resistance, MΩ</td>
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<td>Spike amplitude, mV*</td>
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<td>Spike time of peak, ms</td>
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<td>Spike half-width, ms</td>
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<td>Max rise of spike, mV/ms</td>
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<td>Max fall of spike, mV/ms</td>
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<td>Spike time of fall slope, ms</td>
<td>18.1 ± 4.6 (8)</td>
<td>6.5 ± 2 (9)</td>
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Values are means ± SD; n values in parentheses. *Spike amplitude was measured from spike threshold to spike peak.

**Fig. 2.** Distribution and morphology of F-cluster neurons. A: 40-μm-thick cross-section of the left cerebral hemiganglia (magnification, ×160) through the F-cluster region (dorsal side up). ⌈, F-cluster layers: T, top layer; M, middle layer; B, bottom layer. ⊕, CF_T and *, CF_M granular neurons. Black zone on photo is a vascular system compartment filled by Indigo. Blank areas are not observed with thicker sections (50–60 μm). B: schematic representation of 2 CF_T neuron somata locations and their collateral projections through the neuropil to the AT nerves as determined by injection of biotin dextran amine (n = 6) or Lucifer yellow (n = 7) with C being an expanded view of the fine structure of CF_T neuron terminals in the AT nerve. ψ, putative region of signal molecule release (C).

**Fig. 3.** Heterogeneity of electrophysiological parameters of F-cluster neurons. CF_T neurons had significantly longer spike half-width and intensive broadening of action potential (dark, top) than the CF_M located in granular neuron region (light, bottom). Neurons were current clamped at -40 mV and stimulated using 5-nA, 25-ms square pulses. Interval between pulses was 175 ms.
the nerve surface (Fig. 2C) that may be the locations of neurotransmitter and/or neuromodulator release into the blood sinuses. It is difficult to make conclusions about the placement of the axon in the nerve periphery because of the protease treatment and potential mechanical disruptions due to removal of the nerve sheath.

Indigo was injected into the right pedal or parapodial artery to locate the vascular system within the cerebral ganglia and cerebral nerves. A well-developed vascular system is observed on both the dorsal and ventral sides of the cerebral ganglia, AT and upper labial (ULAB) nerves (ULAB, Jahan-Parwar and Fredman 1976; C1, Hoffmann 1939), with at least one artery contacting the F-cluster region. Sections of all three cerebral ganglia injected with Indigo reveal the presence of a blood sinus in the granular cell region (Fig. 2A). Generally the AT nerve has several major arteries and the ULAB a single artery. Fast green injection into the artery approaching the left cerebral ganglia induces a quick coloring of the left cerebral hemiganglion, the left AT, and ULAB nerves. No visible color changes in the right cerebral hemiganglion, right AT, and right ULAB nerves were observed for several minutes after the injection, indicating some level of vascular system separation of the

![Diagram](http://jn.physiology.org/)

**FIG. 4.** CF$_T$ neurons exhibited 3-phase spike adaptation to electrical stimulation. A: phase 1 contains fast action potentials; the absence of action potentials and membrane potential oscillations characterize phase 2, whereas phase 3 exhibits broad action potentials (bottom). B: some preparations do not exhibit phase 2. Action potentials were induced by brief 3-nA square pulse current injection.

**FIG. 5.** Synchronous spontaneous changes in membrane potential of the CF$_T$ neurons. A: simultaneous registration of somatic membrane potential of 2 CF$_T$ neurons. B: recordings of somatic membrane potential in CF$_T$ neuron (top) and A-cluster neuron (bottom). C: action potentials appearing in 1 CF$_T$ neuron (top) induced EPSP in target neuron (↓, bottom) but had little influence on membrane potentials (MP). D: depolarization of the neuron significantly ($P = 1.33 \times 10^{-9}$) increased spontaneous changes in MP (SCMP) amplitude. Recording shows the CF$_T$ neuron SCMP before and after steady depolarizing current injection. - - -, membrane potential. Spikes are manually truncated in C and D.
cerebral hemiganglia. The location of the CF_T neuron varicosities near these vascular structures may be important for the rapid distribution of signal molecules released from the CF_T and granular neurons.

**Morphology of CF_M neurons**

The CF_M neurons located in granular neuron region are unipolar and have smaller, round somata (46.1 ± 11.7 μm, n = 20) than CF_T neurons. The neurons are not disrupted to any great extent during the desheathing process due to their location in the second lower layer. The CF_M axons travel toward the space between the cerebral hemiganglia and there develop branches and varicosities (not shown).

**Electrophysiology**

The CF_T and CF_M neurons are not only topologically and morphologically distinct but have significantly different electrophysiological parameters. These properties are summarized in Table 1, with aspects of action potential (AP) broadening illustrated in Fig. 3. The CF_T neurons become spontaneously active generally after depolarization to −20 to −30 mV or immediately after the cell membrane are penetrated by the microelectrode. A three-phase adaptation of spike frequency to continuous current injection (3 nA) is found in the CF_T neurons (Fig. 4). The first phase is characterized by fast, high-amplitude action potentials. The second phase consists of increasing potential oscillations finally resulting in spike generation, which is the start of third phase including broad action potentials. Spike frequency in the first phase was higher than in the third phase mainly because of broadened spikes. The CF_M neurons predominantly had one or few fast action potentials at the start of continuous depolarization (3–5 nA).

The spontaneous changes occurred in somatic membrane potential of the CF_T neurons (Fig. 5). The spontaneous changes in membrane potential (SCMP) had different levels of synchronization in randomly selected CF_T neuron pairs. Some neuronal pairs had an almost one-to-one pattern of the SCMP (Fig. 5, A and C), whereas others had different patterns (not shown). Measurements did not show similar SCMP in neurons from A (Fig. 5B) and B clusters. Cell depolarization induced significant increase in amplitude of the SCMP (1.63 ± 0.73 mV at −50 mV and 3.64 ± 2.0 mV at −40 mV, P = 1.33 × 10^-3) and caused brief spike bursts with 10- to 120-s interburst intervals (Fig. 5D).

To determine the cause of the SCMP, we examined whether there are synaptic connections between CF_T neurons; as shown in Fig. 6, the CF_T neurons are electrically coupled. The coupling coefficient varied from <0.01 to 0.169 and was not significantly different for neuronal pairs in the left and right F...
that electrical coupling was not rectifying. Interactions between the CF T neurons of opposite F clusters: the left F cluster, 0.048 ± 0.045 (n = 49); the right F cluster, 0.049 ± 0.026 (n = 38). Careful measurements of interactions between the CF T neurons of opposite F clusters revealed seldom and weak electrical coupling with a coupling coefficient 0.012 ± 0.0046 (n = 3). The CF T neurons were electrically coupled to top layer neurons of C cluster (Fig. 6, A and B). It is, however, not clear how many neurons were electrically coupled. In one CNS, for example, we located 24 coupled neuronal pairs using two test neurons (Fig. 6, A and B), whereas in another CNS, only 6 coupled pairs were found among different neurons. As expected, some neurons had detectable coupling to a specific neuron but not to each other. We examined the effect of low-Ca2+ ASW on the electrical junction between the CF T neurons by gradually lowering the calcium concentration, which did not abolish the electrical coupling (not shown). No significant difference in coupling coefficient was found when two neurons current-clamped at −70 mV were injected with negative (−1 nA; coupling coefficient 0.023 ± 0.006, n = 5) or positive current (+1 nA; coupling coefficient 0.027 ± 0.003, n = 5) pulses, indicating that electrical coupling was not rectifying.

Presynaptic action potentials in pairs of the CF T neurons with high coupling coefficients evoked EPSPs, but they were of insufficient amplitude to elicit spike generation in postsynaptic neurons (bottom). Inset: full record with the position in the record (- - -). B: electrical coupling between 2 CF T neurons characterized by gradual increasing amplitude of EPSPs (top) through action potential train in presynaptic neuron (bottom). C: changes in action potential amplitude (■) and EPSP (□). Signals were filtered at 10 kHz, using Axoclamp 2B internal filter. Presynaptic neuron was injected with current 3 nA.

Electrical stimulation of the different regions of the AT nerve was performed to determine the location of the CF T terminals in the nerve. Stimulation of the AT nerve at the first branching area resulted in either an initial low amplitude depolarization followed by prolonged hyperpolarization (−4.1 ± 1.4 mV, n = 17) or just the hyperpolarization (Fig. 8). Low-Ca2+ ASW eliminated the hyperpolarization, indicating the involvement of chemical synapses in this effect. Surprisingly, stimulation of the posterior part of the left AT nerve produced excitation in both the left and right F-cluster CF T neurons. This effect indicated either strong electrical coupling between the CF T neurons or the presence of processes from the opposite F-cluster CF T neurons in the AT nerve that were not observed during our morphological investigation. Jahan-Parwar and Fredman (1976) demonstrated that the stimulation of the AT nerve produced depolarization in ~60–100% of the tested F- and C-cluster neurons, whereas hyperpolarization was detected only in 7–40% cases. As the sheath was removed without enzymatic pretreatment, CF T neuron damage was possible. We have not attempted to electrophysiologically characterize the CF B neurons because of the small size of the cell bodies and their location below the CF M neurons.

**Peptide profiling in F cluster by MALDI-TOF MS**

MALDI mass spectrometric peptide profiling of F-cluster cells isolated based on cell size and position reveals the presence of multiple peaks for each cell type. Essentially all peaks in cellular MALDI-TOF mass spectra correspond to the protonated molecular weights of peptides. Figure 9 shows the typical mass spectra of individual CF T, CF M and CF B neurons, with consistent, characteristic peak patterns for each type. As indicated in Table 2, a subset of putative peptides (mass peaks at 1,182, 1,600, 1,526, 2,999, 3,735, 4,137, 4,620, 6,053, 6,110, and 7,410) are present exclusively in CF T neurons. The characteristic masses of the peptides in small CF B neurons include 1,387, 1,433, 1,690, 3,389, and 3,775. The CF M neurons contained only one unique peptide 1247 and many molecules observed in the CF T and CF B cells. In addition, two distinct mass profiles are detected for these cells, indicating additional heterogeneity in the CF M cell population. Individual cellular mass spectra were obtained from 46 CF T cells, 40 CF M cells, and 44 CF B cells from 10 animals.

The MALDI analysis of short sections of the ULAB (C1) and AT nerve (C2) also revealed the presence of two reproducible and distinct spectra similar to those detected in the F-cluster cells (20 samples from 4 animals). As shown in Figs. 8.
9A and 10A, the peak profiles seen in the AT nerve are similar to the CF T neurons, whereas the ULAB nerve contains many peptides observed in the CF M neurons (Figs. 9B and 10B). Other tested nerves such as pleural-abdominal, cerebral-pleural, and cerebral-pedal do not show the presence of the F-cluster neuron peptides (Li et al. 1998).

**DISCUSSION**

Several authors have described complex structure of the *A. californica* ganglia (Frazier et al. 1967; Kandel 1979; Vigna et al. 1984). However, it has not been clear what is the functional role for the layered organization and whether the neurons in each layer are truly distinct. Using physiological, morphological, and biochemical approaches, we demonstrate that the neurons located in the top, middle, and bottom layers of the cerebral ganglion F cluster are distinct neuronal populations. Important implications for the vertical distribution of cell bodies within the cluster include the distance from the vascular system (or distance from the neuropil) and an increased number of neurons within a given area.

Several lines of evidence indicate the CF T neurons are a homogeneous group of cells. They have similar morphology and peptide profiles. Synchronized changes in somatic membrane potential of the CF T neurons are found in both F clusters. The SCMP can be explained partially by electrical coupling between the neurons. Stimulation of the AT nerve reveals a putative external source of coordination of the CF T neurons as the stimulation of the AT nerve induces prolonged hyperpolarization of the neurons. A possible functional role of the hyperpolarization and synchronized membrane potential changes is a shift of membrane potential below the potential of calcium channel activation. Thus low-threshold calcium currents can evoke action potential formation as in the case of GABA-induced hyperpolarization of a thalamocortical neurons (Crunelli and Leresche 1991). Interestingly, Jahan-Parwar (1972) found that application of the sperm sac content, food, and the sexual gland compounds to *A. californica* oral veil induced an increase the electrical activity in the AT nerve.

The morphology and physiology of the F-cluster cells suggests neuroendocrine roles for these cells. To confirm this, as well as substantiate the multiple distinct populations of F-cluster neurons, individual neurons have been assayed using MALDI-TOF MS. This technique has become a powerful method for single cell peptide profiling (Garden et al. 1996, 1998; Jimenez et al. 1998; Li et al. 1994). MALDI-TOF MS provides accurate mass (>0.01%) of the peptides present in individual cells. As described in Fig. 9, MALDI reveals the consistent presence of numerous peaks from 1,000–10,000 Da. We expect that most, if not all, of these peaks are from peptides based on the extensive prior studies on MALDI of invertebrates (Garden et al. 1996, 1998; Jimenez et al. 1998; Li et al. 1994). The masses of these peptides do not fit masses of known *Aplysia* peptides, these represent previously uncharacterized peptides. As shown in Fig. 9, at least three different patterns of peptide profiles are detected as a function of cell size and location. This result is in a good agreement with the morphological and electrophysiological data obtained from the F cluster.

Several peptides, which have molecular mass of 1,455, 2,985, 4,575, and 9,148 Da, are present in most F-cluster neurons.
neurons. Possible reasons for the same peptides to be observed in all cells include they are present in all neurons and, the most likely, the peaks may be due to axons, terminals from some neurons and pieces of neuropil being present in all layers/samples. For example, we have frequently observed bag cell peptides when examining both the left upper quadrant (LUQ) cells and the R3–14 neurons in the abdominal ganglion, which we have attributed to processes and terminals from the bag cells (Garden et al. 1998). This potentially explains why we observe many peaks in the CFM cells that also are observed with either the CF$_T$ or the CF$_B$ neurons. The CFM cells are located between the two other layers and so the possibility of contamination from the other layers is higher. However, as many peaks are only observed in a single cell type, the MALDI data substantiates observations that the F-cluster neurons contain at least three distinct neuron types and suggests that all types use peptides as extracellular signal molecules. Interestingly, on the basis of the peptide profile, the CF$_M$ neurons may consist of two subclasses, potentially the so-called granular neurons described by Soinila and Mpitsos (1996) and another set of undescribed neurons. The same peptide profiles observed in the CF$_T$ and CF$_M$ cells are observed in the cerebral AT and ULAB connectives; this provides evidence that the molecular masses detected in F-cluster neurons are likely a unique set of putative peptide transmitters that are transported out of the cerebral ganglion.

Although single-cell MALDI-TOF MS provides strong evidence for complex and different sets of peptides in each neuronal subclass, the list of peptide masses presented in Fig. 9 and Table 2 may not be complete. Peptides with a mass <900 Da are hard to identify or detect due to the presence of intense lipid peaks in cellular samples. Thus peptides such as APGWamide, FMRFamide, or other low-molecular-weight peptides were not detected in the observed mass range.

Given the large number of peptides observed, an important question is are these truly unknown peptides? Although peptides of these masses have not been previously described, they may closely resemble other known peptides with slight modifications. Several peptides have been suggested to be present in the F cluster. For example, Gonzalez et al. (1992) reported

\[ \text{TABLE 2. Putative neuropeptides in F cluster} \]

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\( n = 44 \) for CF$_B$, 40 for CF$_M$, and 46 for CF$_T$. The monoprotonated molecular masses are listed as present in a specific group of cells if 70% of the samples analyzed contain the peak. ++, molecular masses listed have relative intensity more than 20% of the most intense peak; +, molecular masses listed have relative intensity <20% of the most intense peak. *Only seen in granular cells.

FIG. 10. MALDI-TOF mass spectra from the AT (A) and ULAB (B) nerves showing a large number of similar peptide masses to the single-cell spectra shown in Fig. 9. Buccalins S and G (BucS, BucG) are neuropeptides described by Miller et al. (1993).
urotensin I and urotensin II immunoreactivities in the F cluster. The molecular weights of urotensin II varies from 1,364 to 1,526 in four different species (Conlon et al. 1992a,b; Pearson et al. 1980; Waugh et al. 1995), based on MALDI spectra of single F cells, we detected multiple peaks between 1,300 and 1,600, and one mass was 1,526. We expect that many of the F-cluster peptides will be similar to peptides isolated from related mollusks but with amino acid modifications producing the unknown masses. In other work, Chiu and Strumwasser (1984) demonstrated the presence of ELH immunoreactive neurons near the F cluster. However, we do not detect molecular weights corresponding to ELH nor atrial gland peptides [with the possible exception that the 6,053 peak is similar to calfin B molecular weight 6,072 (Rothman et al. 1986)]. However, the lack of all other peptides from the calfin B gene in the F cluster indicates this is unlikely a calfin.

Because CF_T neurons located in the cerebral ganglia clusters have similar appearance, position, and physiological properties to the A-type LGC-cluster neurons of L. stagnalis, we hypothesize that the CF_T neurons are homologous to the A-LGC cells of L. stagnalis. Several lines of evidence support this hypothesis. The A-LGC occupy the top position in the cluster (Smit et al. 1998), and both the CF_T and the A-LGC have neurohemal areas in the nerves of cerebral ganglia innervating sensory areas of skin in mollusks (Benjamin and Rose 1984; Smit et al. 1998). In addition, both the CF_T and the A-LGC are weakly electrically coupled, and synchronized changes in membrane potential are reported. Last, electrical stimulation of nerves containing terminals of the CF_T neurons or the A-LGC cells produced their long-term hyperpolarization. Both the A-LGC and the CF_T neurons are peptidergic, but, as expected from different species, the molecular weights of the peptides do not match.

Interestingly, other mollusks such as Planorbarius corneus and Helix aspersa have several clusters of light green neurons with a position on the dorsal surface of cerebral ganglia which is similar to the location of the F and C clusters of A. californica and the LGC clusters of L. stagnalis. Detailed study of the LGC of L. stagnalis show their neuroendocrine function including synthesis and release of molluskan insulin-related peptides (Smit et al. 1998). A similar distribution of immunoreactive cells was found in mollusks P. corneus (Sonetti et al. 1992) and H. aspersa (Gomot et al. 1992) that supports the idea of a conserved location of neurosecretory cells on cerebral ganglia dorsal surface of pulmonates and opistobranchs. To confirm the homology, the peptides in the F cluster are being isolated and characterized.

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