Distribution of Fine Neurites of Stomatogastric Neurons of the Crab Cancer borealis: Evidence for a Structured Neuropil

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ABSTRACT

The neuropil of the stomatogastric ganglion of the crab Cancer borealis contains many neuronal processes that may be arranged either at random or in some form of orderly structure. In this study, we provide evidence for two types of order in the neuropil, a segregation of the processes based on their size and a cell-specific distribution to the fine neurites. Identified neurons were injected with Lucifer yellow, fixed, and imaged as whole mounts with a confocal microscope. Four cell types were analyzed using the serial images, two pyloric neurons, one mixed pyloric/gastric neuron, and one gastric neuron. All of the neurons consisted of a ~60-μm-diameter soma, a ~20-μm-diameter primary neurite projecting into the center of the neuropil, a number of <10-μm-diameter medium-sized neurites radiating away from the center, and many <3-μm-diameter fine neurites around the periphery of the neuropil. The neuropil can, therefore, be divided into three layers, a central core containing the largest neurites, an intermediate region containing both medium-sized and fine neurites, and a peripheral neuropil containing mostly fine neurites. The distribution of the fine neurites was mapped using a three-dimensional grid. We found that the fine neurites were distributed not at random within the neuropil but in consistent, cell-specific patterns.

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The local neural circuit is one of the most widely studied of the many levels of the nervous system (ranging from behavior to synapses). Invertebrates have been useful experimental systems, in part because a specific local neural circuit can often be readily isolated by removing a single ganglion from the animal. In a review, Leise (1990) argues that invertebrate ganglia and their vertebrate counterparts (columns, barrels, etc.; see Shepard, 1980) represent a basic module used in the construction of nervous systems. In both vertebrates and invertebrates, the modules are often organized in a structured manner, with clear subdivisions existing within a module (Leise, 1990). One specific invertebrate example (for others, see Leise, 1990) is the difference within the locust thoracic ganglion between the dorsal neuropil, which contains neurites from neurons involved in flight (Tyror and Altman, 1974), and the lateral neuropil, which contains neurites from neurons involved in walking (Watkins et al., 1985). Although the neuropil of the crustacean stomatogastric ganglion (STG) is as large as the neuropil of many other ganglia, it has been thought to lack such functional substructure to its neuropil (Selverston, 1973; King, 1976a,b).

The STG contains ~30 neurons responsible for controlling the muscles of the foregut. Traditionally, these neurons are divided into two groups, the pyloric and the gastric mill (named for the part of the foregut that they control), which produce two distinct motor patterns with different periods (reviewed in Selverston and Moulins, 1987; Harris-Warrick et al., 1982). However, synaptic and electrotetric connections between cells of the two groups allow the two motor patterns to influence each other (Weimann et al., 1991). Additionally, a number of neurons within the STG have been shown to switch their activity from one motor pattern to the other (Hooper and Moulins, 1989; Katz and Harris-Warrick, 1991). Finally, the neurons are capable of regrouping to produce alternative motor patterns (Dickinson et al., 1990; Meyrand et al., 1991). Thus, it may be better to consider the two motor patterns as one commonly produced output of a single, plastic motor circuit (Dickinson and Moulins, 1992).

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Limited morphological data have revealed little substructure to the STG neuropil that might reflect a functional division of the motor circuit (Maynard, 1971; Selverston, 1973; King, 1976a,b). In studying the spiny lobster, Panulirus interruptus, King (1976a,b) used serial section electron microscopy to show that the neuropil is divided into a central core of large glial-wrapped neurites surrounded by an outer shell of fine neurites, where synaptic connections are located. This does represent a form of substructure to the STG neuropil, but no further segregation of the neurites was seen. Previous experiments examined very few ganglia and did not quantify the distribution of fine neurites. This prevented comparing the distribution of fine neurites for a specific neuron across many different animals.

The present study examines four cell types in the STG of the crab Cancer borealis for any indication of a consistent substructure to the fine neuropil. Weismann et al. (1991) found that the lateral pyloric (LP) and two pyloric dilator (PD) neurones fire exclusively with the pyloric pattern, that the dorsal gastric (DG) neuron fires exclusively with the gastric mill pattern, and that the ventricular dilator (VD) is a neuron with involvement in both patterns. VD is also one of the neurons found to switch between motor patterns in response to a modulatory input (Hooper and Moulin, 1989). Although the actual synaptic connections in the crab are not yet known (unlike the case in the lobster), PD, LP, and VD appear to share functional connections with each other, and DG appears to lack any functional connection to the other three cell types (for a review of both species, see Harris-Warrick et al., 1992). Any substructure to the STG neuropil might be seen as a consistent pattern in the distribution of fine neurites of these four cell types. In addition, comparing the four cell types may allow us to discern whether the substructure is correlated with the motor patterns.

We report here that the neuropil of the crab STG is not a randomly mixed collection of neurites but has a recognizable organization. First, the neuropil can be roughly divided into three regions based on the size of the neurites: a central core containing major neurites, an intermediate shell containing a mix of secondary and fine neurites, and an outer shell at the edge of the neuropil that consists almost exclusively of fine neurites. Second, the fine neurites of these STG neurones are not located at random within the outer shell. Instead, the fine neurites of each cell type project to specific regions of the fine neuropil. Comparisons of these preferred regions among the four cell types indicate that the fine neuropil of the STG may be subdivided in part according to motor pattern. This substructure adds another level of complexity to this "simple" nervous system. Preliminary results of this work have been reported in abstract form (Baldwin and Graubard, 1990, 1991).

**Materials and Methods**

**Preparation**

Joan crabs, C. borealis, were obtained from Neptune Lobster and Seafood (Boston, MA) and maintained in natural seawater at 10–12°C until use. Crabs were anaesthetized by packing in ice for ~20 minutes prior to dissection. The stomatogastric nervous system was dissected and pinned in a Sylgard-coated dish, and the ganglion was desheathed using standard techniques (Maynard and Selverston, 1975; Hooper et al., 1986). The preparation was bathed in crab saline (composition 440 mM NaCl, 11 mM KCl, 13 mM CaCl₂, 26 mM MgCl₂, 10 mM HEPES acid, adjusted to pH 7.4 with NaOH) and maintained at 13°C with a cooled microscope stage. Neuron somata within the desheathed STG were impaled with glass microelectrodes (3 M KCl, 10–30 MΩ) and were identified by bipolar suction electrode recordings from appropriate motor nerves (Hooper et al., 1986; Weismann et al., 1991).

**Labelling**

Once a desired neuron was located, the KCl microelectrode was replaced with a dye-filled microelectrode, and the cell was filled with dye using a combination of pressure, iontophoresis, and negative capacitance (to keep the electrode clear). Dye-filled microelectrodes were made by loading the tip with a solution of the dye in distilled water using capillary action, then filling the rest of the microelectrode with 3 M KCl or 2 M LiCl (if the dye was Lucifer yellow CH, leaving an air bubble to limit mixing. The dye used were Lucifer yellow CH or fluorescein-labeled dextran (10,000 or 30,000 MW). In some cases, a second cell was filled with Texas red (conjugated to a 10,000 MW labeled dextran) or tetramethylrhodamine (conjugated to a 10,000 or 30,000 MW labeled dextran). All the dyes were obtained from Molecular Probes (Eugene, OR). The progress of the dye fill was monitored intermittently with the epifluorescence illumination of the microscope.

**Fixation**

Once the portion of the cell within the STG was filled (~30 minutes for Lucifer yellow, longer for the other dyes), the STG was fixed for 30 minutes at 25°C in a solution of parformaldehyde, 140 mM NaCl, and 300 mM Millonig's phosphate-buffered saline (pH 7.6). It was then dehydrated in a series of ethanol solutions (50%, 70%, 80%, 100%) before clearing in methyl benzoate. The ganglion was mounted on a depression slide in methyl benzoate, covered, and sealed with clear nail polish (Sally Hansen's Hard-As-Nails).

**Imaging**

Fluorescent neurons were imaged using a Bio-Rad MRC 600 laser scanning confocal microscope with a 15 mW Argon laser using the BH3 filter set for Lucifer yellow and fluorescein and the GHS filter set for Texas red and tetramethylrhodamine. For each neuron, optical sections were acquired at 2.5, 5, or 4 μm intervals through the depth of the neuropil. Total neuropil thickness ranged from 90 to 130 μm, with 120 μm as an average, in agreement with the total neuropil depth for a few cells imaged from unfixed ganglia (data not shown). Images were acquired with a Nikon ×20 0.75 NA lens, Leitz ×25 0.6 NA water-immersion lens, Zeiss ×40 0.75 NA water-immersion lens, or Nikon ×60 1.2 NA oil-immersion lens. Pixel sizes for the

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DG</td>
<td>dorsal gastric neuron</td>
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<tr>
<td>dvr</td>
<td>dorsal ventricular nerve</td>
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<tr>
<td>LP</td>
<td>lateral pyloric neuron</td>
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<tr>
<td>PD</td>
<td>pyloric dilator neuron</td>
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<tr>
<td>STG</td>
<td>stomatogastric ganglion</td>
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<tr>
<td>stn</td>
<td>stomatogastric nerve</td>
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<td>VD</td>
<td>ventricular dilator neuron</td>
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confocal images ranged from 0.2 to 0.8 μm/pixel. Each series of optical sections was analyzed using either the Bio-Rad MRC 500 software and/or NIH Image software (for the Macintoch available via anonymous ftp from zippy.nih.gov). Qualitative analysis was done using the individual optical sections or by combining the sections into projections or stereo-pair images (Fig. 1). All projections (including stereo pairs) were done using either brightest projection in NIH Image or (the equivalent) maximum projection in the Bio-Rad software.

**Analysis**

The distribution of fine neurites for each neuron was analyzed by normalizing and mapping the neurites onto a radial grid. Figure 4A illustrates the process (though the actual grid used for each neuron was drawn on a transparency placed over the computer screen). The grid was constructed from an ellipse that was scaled to match the largest key dimension of the neuropil for the ganglion being mapped. Two additional, concentric ellipses were added to divide the distance from the center of the neuropil to the edge into equal one-thirds. This normalization was required in order to compare ganglia with different absolute dimensions. The three ellipses were then divided into 16 sectors by radial lines centered on the neuropil, spaced at 22.5° intervals and arranged such that the line separating sectors 1 and 16 was aligned with the stomatogastric nerve. This produced a grid with a manageable number of elements (48) that matched the general morphology of the neuron (e.g., the three concentric shells described in Results). The ganglion thickness was normalized by dividing the optical sections into ten bins (nine bins for five of the early cell files). For each bin, four to six optical sections were combined into a single projection image.

It is important to realize that the resulting grid uses a series of cross sections of a cylinder (of elliptical cross section) to map the location of fine neurites in a ganglion that is actually a flattened ellipsoid. This means that fine neurites near the dorsal and ventral surfaces of the neuropil will be mapped to the inner sectors of the cross sections. Fine neurites near the edge of the neuropil in the center of the depth of the ganglion will be mapped to the outer sectors of the middle cross sections. However, both sets of fine neurites are actually located at the periphery of the neuropil (treated as away from the center of the neuropil) in three dimensions.

To map the distribution of fine neurites, each element of the grid was scored for the presence of fine neurites (a subjective yes/no decision). This process produced a coarse representation of the three-dimensional distribution of fine neurites for the cell (Fig. 4B) that could be directly compared to those from other cells. In scoring the grid elements, care was taken to map each set of fine neurites only to the bin in which they were in best focus. Also, a grid element containing a few fine neurites along the border with an adjacent element that was scored “yes” would be scored “no” if it was otherwise empty. Because these considerations were important in scoring the grids, scoring Figure 4A (which shows a single bin with an illustrative grid) will not necessarily yield a grid identical to that shown in Figure 4B.

Composite grids for each cell type (Fig. 5) were made using NIH Image. Data for computer-generated composite grids, used to compare to the observed composite grids (Fig. 6), were obtained from simulations that were programmed using LabVIEW (National Instruments, Austin, TX) on the Macintosh.

**RESULTS**

**Cell morphology**

We analyzed repeated files of the following neurones: the pyloric LP (seven neurones), the pyloric PD (nine neurones), the mixed pyloric/gastric VD (five neurones), and the gastric DG (five neurones). For all of the neurones studied, the soma was large (>60 μm in diameter) and produced a single primary neurite (~20 μm in diameter) that projected towards the neuropil. In some cases, the primary neurite traveled over 120 μm past other somata before reaching the neuropil. Once there, the primary neurite curved through the central core of the neuropil before leaving the neuropil as one or more axons (Fig. 1). Smaller secondary neurites (<10 μm in diameter) branched from the primary neurite and radiated away from the core towards the periphery of the neuropil. Near the outer edge of the neuropil, these secondary neurites branched widely to form an outermost region of fine neurites (see, e.g., Fig. 1).

This morphology of the neuron divides the neuropil into three concentric shells. The primary neurites are largely confined to the central core of the neuropil. The neuropil core is similar to that reported for the spiny lobster by King (1976a,b) and is largely avoided by smaller neurites. The secondary neurites are found mainly in an intermediate shell, surrounding the core. Fine neurites occur almost exclusively in an outer shell surrounding the entire neuropil, although some fine neurites appear in the intermediate shell. The outer shell corresponds to the regions where King (1976a,b), using the electron microscope, observed discrete areas of synaptic connections among the fine neurites.

Fine neurites were 3 μm in diameter or less, with thicker swellings and smaller, knob-like projections occurring along their length (Fig. 2). Fine neurites from different cells or different regions of the same cell all showed this basic structure. The fine neurites are similar in both size and shape to the pre- and postsynaptic neurites reported for the lobster STG (Fried, 1978; King, 1976a,b).

For all four cell types, fine neurites were found in many areas of the outer region of the neuropil (Figs. 1, 3, 4). This made distinctions in the fine neurite distribution between different neurites difficult to detect by looking at one cell at a time. One distinction was clearly discernible with PD. A small clump of fine neurites was seen near the stomatogastric nerve (sta) for all of the PD neurones (Figs. 1, 3, 4). Only one LP neuron had any fine neurites near the sta; all other LP neurones were devoid of fine neurites in that region (Fig. 3). Both VD and DG consistently had a larger distribution of fine neurites in that region of the neuropil (Fig. 3).

**Fine neurite distribution**

In order to allow the distribution of fine neurites for one cell to be directly compared with the distribution from other cells, we mapped the distribution of fine neurites for each cell onto a grid (Fig. 4; see Materials and Methods). A composite grid for each cell type was constructed (Fig. 5) by combining the individual grids for each cell in an additive manner, such that the shade of gray indicates how many of the neurones of the cell type had fine neurites in that region of the neuropil. This technique loses the information about the behavior of any individual cell, but it highlights any
Fig. 1. Serial confocal images of a pyloric dilator (PD) neuron imaged at 50 sections taken at 2 μm intervals. A: Sections 1–8 show six single sections (13, 17, 21, 25, 29, and 33 of the 50). The number in the lower left of each section shows the absolute depth from the most dorsal section. The stomatogastric nerve (snt) is on the left, and the dorsal ventricular nerve (dvn) is on the right. The arrow highlights the region of fine neurites near the snt. B: Stereo-pair image formed by projecting all 50 sections at two slightly different angles (+5° on the left and +6° on the right). The arrow highlights the region of fine neurites near the snt.
consistent behavior within the cell type. For all four cell types, these composite grids showed distinctive patterns in the distribution of fine neurites. Some regions of neurite were seen to be preferred by fine neurites (areas of black and dark gray), whereas other regions were avoided (areas of white and light gray).

Fine neurites of PD neurons were found primarily in one region in the middle sectors (A in Fig. 6) and three regions in the outer sectors (B–D in Fig. 6) of the neuropil. Two of the outer regions (C and D in Fig. 6) overlap with the two regions in the outer sectors preferred by LPS fine neurites. Interestingly, these two regions may represent bilaterally symmetric locations. Note that the third outer region (B in Fig. 5) to which PD projected, near the stn, was avoided by LP. This region corresponds to the small clump of PD fine neurites mentioned above. VD neurones showed more variation in their distribution of fine neurites, but two regions in the outer sectors were still distinguishable, one broad area (E in Fig. 5) and one smaller region (F in Fig. 5). In addition to a number of small regions in the middle sectors, DGs fine neurites preferred two regions in the outer sectors, one around the stn (B and B’ in Fig. 5) and another near the dorsal ventricular nerve (dvn; G in Fig. 5). Note that the second area (G in Fig. 5) extends deeper than regions seen in the other cell types. This area of the neuropil corresponds to where the DG axon was usually seen to leave the neuropil en route to the dorsal gastric nerve (data not shown).

The regions in the outer sectors mentioned above are located in the center of the neuropil depth, which places any fine neurites in these regions in the peripheral shell (i.e., the fine neuropil). The inner sectors of the grid did not show a consistent pattern for any cell type. Fine neurites were seen in this part of the neuropil but almost exclusively in the top and bottom of the neuropil depth. In the inner sectors of the grid, the center of the neuropil depth is the central core (the coarse neuropil containing large processes), and the fine neurites generally avoid this area. The lack of any pattern at the top and bottom of the inner sectors may be due to the fact that these regions are the interior of the tectum and the central core. Fine neurites in the interior of the tectum are presented on the mapping in other sectors.

Is the fine neurite distribution random?

The patterns in the regions of neuropil occupied by fine neurites make it unlikely that the cells distribute their fine neurites at random. To test this hypothesis, a computer simulation was programmed to generate random composite grids. For each cell type, the simulation used the number of grid elements covered by each neuron (five Dcs, five Vds, seven Lps, and nine Fds) to generate a grid of randomly selected elements. A computer-generated composite grid was then produced that corresponded to each cell type. These simulated composite grids were equivalent (particularly in the number of grid elements covered) to the observed composite grids, except that they were known to be the result of a random process.

To compare the simulated composite grids to the observed composite grids, the fraction of total grid elements avoided by all of the cells was determined, as was the fraction sharing only one cell, showing an overlap of two cells, and so on up to the fraction of the grid elements preferred by all of the cells. A simulated composite grid could then be compared to an observed composite grid by plotting the fraction of grid elements vs. the number of cells. To minimize bias, a statistic in the simulated composite grid, 20 simulations were done for each cell type, and the mean ± S.D. was compared to the observed grids. The results of these comparisons are shown for the outer sectors in Figure 6. Regions in these outer sectors do not contain any neuropil in the outer sectors (due to the neuropil’s shape), the top and bottom rows of bins were excluded from the simulation and from the comparison.

The simulated random plots are almost identical to binomial distributions (Fig. 6). In contrast, the independent sorting of the distribution (Sokal and Rohlf, 1981). The binomial distributions plotted in Figure 6 showed the mean fraction of grid elements selected by each cell type (0.50 for DG and VD, 0.49 for LP, and 0.47 for PD) to determine the upper (95%) confidence limits of the observed plots for all four cell types differed significantly (Chi squared test, $P < 0.001$) from the randomly generated plots. Importantly, they differed in the direction expected for clamped distributions; i.e., the observed grids differed from the random grids in showing a larger percentage of the neuropil either containing no fine neurites or with fine neurites from all of the neurites. Conversely, the observed grids showed a smaller percentage of the neuropil with fine neurites from a few of the neurites than did the random grids. This indicates that the neurites do not distribute their fine neurites randomly. Instead, all neurites of the same cell type appear to send fine neurites into the same regions of neuropil.

The distribution of fine neurites between cell types was also compared using the composite grids. One striking feature of the composite grids was the significant overlap between PD and LP. Both cell types showed similarities in their fine neurite branching patterns and differed from the patterns seen for VD and DG. To highlight better the similarities and differences in the distribution of fine neurites of the four cell types, the composite maps shown in Figure 5 were combined pairwise (Fig. 7). A pseudocolor scheme was used, with red corresponding to one cell type and green to the other cell type. Yellow corresponds to a region of the neuropil with both cell types present. Using this approach, the two regions of overlap between PD and LP show up as yellow regions when the two cell types are directly compared. However, those yellow regions become red or green (little or no overlap) when either PD or LP is compared to DG. Thus, PD or LP does not overlap with DG in the regions that they prefer, and there is no overlap in the regions that DG prefers. VD differs from the other three cells, overlapping slightly with PD or LP in one region but showing a less restricted distribution. Although this description is only qualitative, it indicates that the fine neurite projections of each cell type are potentially unique.

DISCUSSION

Cell morphology

This study shows that the stomatogastric neurones of C. borealis consist of a soma that produces a large, major neurite that moves through the center of the neuropil and branches to form intermediate, secondary neurites that further branch to form small, fine neurites arranged in an outer shell surrounding the central neuropil. Thus, the neuropil is structured, not random. This structure presum-
Fig. 2. Projections of neurons imaged at 0.5 μm intervals using higher magnification to show details of the neurites. Note the progression towards smaller neurites.

A: Thirty sections (10 μm of depth) from a PD neuron.
B: Five sections (2.5 μm of depth) from a different PD neuron.
C: Twenty sections (10 μm of depth) from a third PD neuron.
D: Twenty sections (10 μm of depth) from the same PD as in B. Scales for the original confocal images were 0.28 μm/pixel (A,B) and 0.22 μm/pixel (C,D). Some features of synaptic processes can be seen; however, the light microscope imposes limits to the resolution of fine details that are greater than the scales listed above.
Fig. 2. Projections (left, made from all sections) and single sections (right, from the center of the neuropil) from four neurons. The stn is to the left, and the dvm is to the right. However, note that the long axis may be at a slight angle. The arrow highlights fine branches of the PD neuron in the region near the stn.
Fig 4. Method of scoring fine neurite location. A: Miniprojection of five sections of a PD neuron imaged at 50 sections at 2 μm intervals. The overlay illustrates how the grid used to score the presence of fine neurites was constructed. B: The resulting scoring for the cell, where each element in the scored grid corresponds to an element in the oval grid at a given depth in the neuropil. Care was taken to score fine neurites only at the depth at which they were clearly in focus, as some of the fine neurites visible in A would not have actually been scored at this depth. In addition, the grid in A is not necessarily positioned exactly as was the actual grid used for scoring.
Fig. 5. Composite grids for the cell types. A composite grid for each cell type was made by summing the individual grids for all cells of that type. Each location in the composite grid corresponds to a normalized location in the neuropil; the shade of gray corresponds to the number of cells of that type with fine neurites at that location. The letters label the preferred regions discussed in the text (see Results). In the PD grid, dashes highlight the first class of avoided regions (the top and bottom of the outer sectors), and circles highlight the second class of avoided regions (the central core).
ably results from developmental cues that guide the growth of the STG neurons.

Two features of this morphology are comparable to morphological features of the stomatogastric neurons of the spiny lobster, *P. interruptus* (King, 1976a,b). First, in both species, the neuropil is divided into a central core containing large neurites and an outer shell containing the fine neurites (King, 1976a). Second, the diameter, varicosities, and branching patterns of the fine neurites of the crab neurones are similar to those of the spiny lobster (King, 1976a). However, unlike the studies by King (1976a,b) that were performed with an electron microscope, this study cannot resolve synapses. Therefore, although the similarity in shape is suggestive, we cannot be certain that the fine neurites in crab are the sites of the synapses.

This similarity in neuropil organization occurs despite an obvious difference between the STG of the crab and that of the lobster. In the lobster STG, the somata are located primarily on the dorsal surface of the ganglion and must initially project their primary neurites in a ventral direction. In the crab STG, however, the somata are located alongside the neuropil, primarily at the posterior end, with some soma along the lateral edges of the neuropil. The primary neurites must, therefore, reach the neuropil by projecting either anteriorly or medially. If the development

tal mechanisms used by the crab and the lobster STG is similar, then it must be able to compensate for the difference in somata location. Preliminary work on the early development of the stomatogastric neuropil of the lobster *Homarus gammarus* (Garzino et al., 1993, personal communication) indicates that the extrinsic inputs may arrive at the ganglion before the intrinsic neurites develop and could serve as a framework to guide the growth of the STG neuropil.

**Fine neurite distribution**

We have found an additional level of substructure in the neuropil of *C. borealis*: the segregation of fine neurites into cell-specific regions. This means that the fine neurites are not only segregated into the outer shell of the neuropil, but they are arranged in a particular pattern within this shell. One important characteristic of these fine neurite regions is that they occur in fixed locations. If the fine neurite were segregated by cell type but the regions varied from animal to animal, then the substructure would not have been seen with our analysis techniques. Previous studies on the STG may not have been able to resolve a pattern to the distribution of fine neurites within the STG, because they examined neurons from only a few animals. By directly comparing the
distribution of fine neurites for many neurons of the same cell type, we were able to demonstrate that neurons of a given cell type project their fine neurites into specific regions of the fine neuropil, giving rise to a substructure to the fine neuropil.

Limitations. Our method of mapping does limit the resolution with which we can observe the fine neuropil substructure. The individual grid elements are relatively large and do not represent a consistent volume. In addition, no attempt was made to measure the density of fine neurites within a grid element. Figure 4 shows a typical example of the size and shape of a grid and the density of fine neurites. Therefore, the distribution of fine neurites seen in this study is only a coarse measurement. In particular, a large outer sector could contain patches of fine neurites from two neurons that are not close enough to be synaptically connected. The overlap seen between two cell types in Figure 7, therefore, in no way requires a synaptic connection to exist between the two cells. For reasons discussed below, this would be true even if fine neurites of the two cells were found within synaptic distance (~1 μm). Figure 7 is meant primarily to determine if any there is any “blueprint” to the distribution of fine neurites. Finally, we examined only four cell types of the approximately 13 within the STG. Taken together, this means that the results described in this paper are only an initial observation of the substructure of the fine neuropil, and there are many details yet to be resolved.

Avoided and prefered regions of fine neuropil. A general finding for all four cell types was the existence of regions of fine neuropil that were avoided and regions that were preferred (Fig. 5). The avoided regions can be divided into three classes. The first is at the top and bottom of the outer sectors, which may be devoid of neuropil as an artifact of the mapping procedure (i.e., regions outside the boundaries of the neuropil). The second class is most obvious at the center of the inner sectors, where the large neurites occupy most of the available space. The third and most interesting class of avoided regions occurs within the outer sectors of the fine neuropil. The location of avoided regions varies with cell type, because a region that is avoided by one cell type may be preferred by a different cell type. Avoided regions of this third class, along with the preferred regions of the outer sectors of neuropil, are part of the nonrandom (see Fig. 6) and cell-specific distribution of the fine neurites.

Implications for synaptic connectivity. A number of observations by King (1976a,b) of synaptic sites of identified neurons in the spiny lobster are important to consider here. First, pre- and post synaptic sites from both PD and DG were found in the same region of fine neuropil (within ~20 μm of each other; Figs. 10, 11 of King, 1976a). Second, for each neuron, synaptic sites were found distributed over all of the neuron’s fine neurites. For example, a single anterior median neuron had at least 32 presynaptic regions (each with several presynaptic sites) distributed throughout the fine neuropil (Fig. 8 of King, 1976b). Third, two presynaptic regions of a PD onto the LP (both the pre- and post synaptic neurons for a synaptic site were identified) were found on different neurites and were separated by hundreds of micrometers (Fig. 10 of King, 1976b). Using these last two observations, King (1976b) reasoned that a single functional synaptic connection between two neurons is the result of many synaptic sites distributed over several secondary processes of each neuron.

The data from the spiny lobster is consistent with our data from the crab and does not preclude the existence of a substructure to the fine neuropil (as described in this paper). In the crab, the four cell types project fine neurites to a number of large, widely spaced regions of fine neuropil. For the PD and DG, one of these regions was the same (the area of fine neuropil near the stn). For the PD and LP, two different regions of overlapping preference were seen. Assuming that the fine neuropil is the location of any potential synapses, the overlap between PD and LP could provide two separate areas for the location of the synaptic sites that produce the known connection between the two cell types. Given the lack of any functional connection between PD and DG, the overlap seen near the stn would serve an alternative function for these two cell types (e.g., establishing connections with other neurons). The existence of cell-specific patterns to the distribution of fine neurites may be only the beginning of the substructure to the fine neuropil. The manner in which the distribution patterns of the various cell types interact with each other and with the distribution of extrinsic inputs (Baldwin et al., 1992; Marder, 1987; Christie et al., 1993, 1994, 1995) is, potentially, the much more interesting aspect of the substructure to the fine neuropil.

Relationship of motor patterns to fine neurite distribution. The interactions between cell-specific distributions presented in this paper provide some data (albeit limited) on this more detailed substructure. The PD and LP were seen to overlap in two regions of the fine neuropil that were both avoided by the DG. In addition, the PD was seen to prefer a region avoided by the LP. This indicates that there may be multiple pyloric regions and that they may be used by different subsets of pyloric neurons. The same may be true of the gastric mill circuit, because the DG was seen to prefer more than one region of the neuropil that was avoided by the other cell types. The weak correlation of PD with the PD and LP in one region of the neuropil and with the DG in another region may reflect its nature as a neuron involved with both motor patterns. Therefore, the substructure of the fine neuropil may be divided partially based on motor pattern but may not be divided simply into a single pyloric region and a single gastric region.

However, the overlap near the stn between PD and DG means that even a complex substructure based solely on a pyloric vs. gastric dichotomy is unlikely, because this region of the neuropil is served by fine neurites from both a gastric neuron and a pyloric neuron. This result is not surprising given other known features of the STG. First, the STG is part of the larger stomatogastric nervous system that produces at least four motor patterns, including the pyloric and gastric mill rhythms, that interact in a number of ways that are poorly understood (Dickinson and Moulins, 1992). One example of such interaction is the recruitment of specific pyloric and gastric mill neurons in the STG into the swallowing motor pattern (Mohler et al., 1994). The fine neuropil may be divided into regions associated with all four motor patterns, not just the pyloric and gastric mill rhythms. Second, the synaptic connections in spiny lobster (King, 1976b) consist of triad synapses with one presynaptic site and two post synaptic sites. If the crab STG is similar and if the post synaptic sites belong to two different neurons, then connected neurons may form fine neurites in the same region in order to receive a common input. Taken together, these two features of the STG imply a substruct-
Fig. 7. Pseudocolor grids combining the composite grids from two cell types and allowing a direct comparison of the regional preferences. The smaller grids show the pseudocoloring scheme used for each pairing. Solid lines outline regions of consistent overlap or avoidance.
ture with a more complex nature than a simple dichotomy based on two motor patterns.

**Relationship of extrinsic inputs to fine neurite distribution.** A complex substructure of the fine neuropil may also reflect differences in the spatial distribution of the extrinsic inputs to the STG, although the data are limited. Synaptic profiles, probably those attributed by King (1976a) to extrinsic inputs, were reported by Friend (1978) to be distributed in a way that could indicate a preference for a subset of the fine neurite. In addition, the staining seen in immunocytochemical studies of putative transmitters have shown differences in spatial distribution within the STG (Grant, 1987). Recent confocal studies have shown that peptidergic innervation in *C. borealis* is found throughout the peripheral neuropil but avoids the core neuropil (Baldwin et al., 1992; Christie et al., 1996, 1996). In these studies, many input fibers were labeled by each antibody. Early results with protein (both intracellular labelling of a single, extrinsic modulatory neuron and immunocytochemistry of the STG) have shown that the afferent projections from the single neuron are spatially distinct subunits of all proteins containing processes (Christie et al., 1996). Therefore, the different extrinsic inputs may be subdivided into different regions of neuropil and the intrinsic neurites subdivided, in part, by whether they receive the extrinsic input specific to a given region of the neuropil.

**SUMMARY**

Two forms of substructure are present within the neuropil of the crab STG. One substructure is seen throughout the entire neuropil and segregates the neuropil based on the size of the process. The other substructure occurs within the fine neuropil and segregates the fine neurite based on the cell type. Although the functional significance to both of these substructures is still unclear, the cell-specific nature of the fine neurite distribution may provide one mechanism for a "simple" system, such as the crab STG, to possess a highly flexible, patterned output.

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**LITERATURE CITED**


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