Synapse Distribution of Olfactory Interneurons in the Procerebrum of the Snail *Helix aspersa*

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**ABSTRACT**

The procerebrum is believed to be important for processing olfactory information and storing olfactory memories in terrestrial pulmonate molluscs. Previous results have demonstrated that the procerebral cell population is morphologically heterogeneous. In the present study, serial sections and electron microscopy were used to investigate differences in synapse distributions. The results demonstrate that procerebral neurons with different sites of arborization have distinct patterns of synapse distribution that probably reflect different functional contributions to the olfactory pathway. Cells that have all their arborizations in the procerebrum, but none in the internal mass, have multiple large varicosities that are specialized for output. On the other hand, cells that arborize in the internal mass or outside the procerebrum have mostly input synapses proximal to the soma and mostly output synapses in the terminal region of the neurites. These cells appear to transmit information from the procerebral cell body mass to other central nervous system regions, e.g., the internal mass and the mesocerebrum. The implications of these data are twofold. Firstly, the procerebrum directly participates in distributing processed olfactory information to more central regions of the nervous system. Secondly, the procerebral neuronal population may be divisible into two subgroups: 1) intrinsically arborizing interneurons; and 2) projection neurons. This is significant because the neural organization of the procerebrum may now be compared with that of olfactory systems in other organisms. J. Comp. Neurol. 417: 366–384, 2000. © 2000 Wiley-Liss, Inc.

**Indexing terms:** ultrastructure; olfaction; gastropod mollusc; electron microscopy; biocytin

The procerebral lobe of terrestrial pulmonate molluscs is located at the entry site of the olfactory nerve (tentacle nerve) into the cerebral ganglion. It receives its input from the superior and inferior tentacles (Hanstro¨m, 1925), both of which house chemosensory organs (Chase and Croll, 1981). Morphological and physiological studies have demonstrated that the procerebrum is involved in olfaction (Chase and Tollozko, 1989; Gelperin and Tank, 1990; Kimura et al., 1998a). It is believed to process olfactory information (Delaney et al., 1994) and store olfactory memories (Kimura et al., 1998a). Hence the procerebral lobe is also referred to as the olfactory lobe and is analogous to other olfactory centers such as the antennal lobe in insects and the olfactory bulb in vertebrates (Chase and Tollozko, 1993).

The procerebrum is composed of very small neurons (5–8 μm diameter), numbering about 20,000 in *Achatina fulica* (Chase, 1986) and *Helix aspersa* (unpublished), and about 100,000 in *Limax maximus* (Gelperin and Tank, 1990). In contrast to standard invertebrate ganglionic organization, in which somata surround a central neuropil, the procerebral cell bodies are located laterally to the neuropil. In *Helix*, the olfactory nerve, bringing olfactory information, enters the cerebral ganglion at the distal end of the procerebral lobe. Some of the axons in this nerve may be olfactory receptor axons that project centrally, but most olfactory receptor cells terminate first in glomeruli present under the olfactory epithelium or in the tentacle ganglion at the tip of the tentacle (Chase, 1981; Chase and Kamil, 1983). Hence most olfactory fibers in the olfactory nerve are ganglionic interneurons originating peripherally. Some of the nerve afferents terminate in the procerebrum, whereas others project to more central regions of the cerebral ganglion (Chase and Tollozko, 1989). No

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structural organization such as laminae or glomeruli seem to be present in the procerebrum based on its histological appearance. Only the internal mass, a small region demarcated with glial cells, is present on the ventral side of the basal procerebral neuropil (Zs-Nagy and Sakharov, 1970). The internal mass is distinguished from the rest of the procerebral neuropil (referred to as the terminal mass) by a different texture and a different chemical content, as shown by immunocytochemistry studies (Cooke et al., 1994; Elekes et al., 1994). However, the functional significance of the internal mass is unknown.

Recent results demonstrated that the procerebral cell population is not morphologically homogeneous (Ratte´ and Chase, 1997), contrary to what was previously believed (Veratti, 1900; Hanström, 1925; Zaitseva, 1994). However, it was not possible to categorize procerebral neurons based on their morphologies, since the cells differ from one another in a graded manner. The neurons nevertheless vary considerably in the placement of their arborizations and the projection of their neurites. It was shown that, in addition to the intrinsic arborizations, some interneurons project and arborize in regions of the cerebral ganglion outside the procerebrum. Furthermore, for cells with only intrinsic arborizations, some project their neurites specifically to the internal mass. Because of this variability in patterns of arborization, it is likely that different procerebral cells contribute differently to the lobe's activity and function.

Strong evidence for output from the procerebrum exists for only two minor pathways: one going to a small group of cells in the pedal ganglion (Chase and Tollozcko, 1989) and another going to a pair of cells in the buccal ganglia (Gelperin and Flores, 1997). The cells that constitute these pathways send long dendrites to the procerebrum via connective nerves. We suggested (Ratte´ and Chase, 1997) that procerebral cells possibly provide a third output pathway for the procerebrum, since some of the cells arborize in various areas of the cerebral ganglia. This output pathway could allow the procerebrum to have a widespread influence over many regions of the snail's brain. It would constitute a more conventional way of distributing information, and it would permit closer comparisons of the procerebrum with other olfactory systems. There is, however, no direct evidence that procerebral cells carry information from the procerebrum to other regions of the cerebral ganglia, as these long neurites could alternatively provide a pathway by which other parts of the cerebral ganglion modulate procerebral activity.

The first goal of this study was to investigate differences in the synaptic connections of procerebral cells in different areas of their arborizations. Following from this, the second goal was to elucidate whether procerebral cells carry information into or out from the procerebrum, i.e., whether cells with long neurites exiting the procerebrum are in fact projection neurons. To address these questions, we proceeded with an electron microscopy study of single cells intracellularly labeled with biocytin. Serial sectioning and reconstructions of selected areas allowed us to determine the nature and position of synapses.

MATERIALS AND METHODS

Mature specimens of Helix aspersa (curved shell margin, 3–9 g with the shell) were obtained from California. During all procedures and unless specified otherwise, the tissue was kept cold by keeping the preparation either on ice or in the refrigerator. Details of our techniques for the dissection and intracellular injection of procerebral neurons have been described elsewhere (Ratte´ and Chase, 1997). Briefly, the cerebral ganglion was removed from the animal and placed in a Sylgard-coated dish filled with saline. All connective tissue was removed over the procerebrum so that the somata could be accessed directly. Under fluorescence in the blue spectrum (standard fluorescein isothiocyanate [FITC] pack on a Wild-Leitz M22 incident fluorescence microscope), individual neurons were penetrated and injected with a fresh solution of 2% biocytin and 1% Lucifer Yellow dissolved in 0.05 M phosphate buffer, pH 7.4. Initially, Lucifer Yellow was injected using short pulses of hyperpolarizing current to monitor the quality of the penetration. If this was successful, depolarizing current was then used to fill the cell with biocytin (0.6-second pulses at 1 Hz for 2–10 minutes). Although biocytin can be injected with both hyperpolarizing and depolarizing current, using depolarizing current prevented the continuous and simultaneous injection of Lucifer Yellow in the cell.

Once one or more successful injections had been achieved, the preparation was rinsed with cold saline and fixed by immersion in 2% paraformaldehyde, 1.25% glutaraldehyde, and 0.15% picric acid in 0.1 M phosphate buffer, pH 7.4, for 1–2 hours. Next, it was rinsed in phosphate buffer (pH 7.4) and sequentially transferred to 10% and 20% sucrose solutions. Permeabilization was achieved by taking the piece of tissue directly from the sucrose solution, immersing it in liquid nitrogen, and then thawing it in phosphate buffer (pH 7.4).

The tissue was left overnight at 4°C in a solution of avidin-biotin-horseradish peroxidase complex (Vectastain ABC solution) followed by a 2-hour period at room temperature. After multiple rinses, it was preincubated in a 0.06% solution of 3,3′-diaminobenzidine (DAB) with 1% CoCl2 for 10 minutes and subsequently reacted in a fresh solution of 0.06% DAB containing 0.0225% H2O2 for 2–5 minutes or until the background started to become dark. The reaction was terminated by multiple rinses with phosphate buffer (pH 7.4).

The tissue was serially sectioned at 75 μm on a Vibratome after embedding in 7% agar. Sections were post-fixed for 30 minutes in 0.75% OsO4 in 0.1 M phosphate buffer, pH 7.4, and subsequently stained en bloc in 1.5% aqueous uranyl acetate for 45 minutes. After dehydration in a graded series of ethanol dilutions, the sections were embedded in Epon between acetate sheets and polymerized at 55°C for 24 hours. Thick sections containing the stained neurons were examined with light microscopy. The morphology of the labeled cells was characterized, and camera lucida drawings were made at 1000× magnification under oil immersion to allow for later correlation with electron microscopy.

Selected thick sections were re-embedded in blocks, and 90-nm (gold) sections of the chosen regions were obtained on a Reichert Ultracut E ultramicrotome. Serial sections were collected on Formvar-coated single-slot copper grids, and alternate grids were stained with alcoholic uranyl acetate and Reynolds’ lead citrate (Reynolds, 1963). All cut sections were examined using a Philips 410 transmission electron microscope. Micrographs of labeled cells were taken from every third section at a magnification of...
Additional micrographs were taken with a magnification of 10,400×, and sometimes 20,000×, from any section on which the presence of a synapse was suspected. We examined a total of 8,040 micrographs.

The following criteria were used for the identification of synaptic contacts on electron micrographs: 1) straightening of apposing membranes, 2) increased electron density at the membranes, and 3) accumulation of vesicles against the presynaptic side of the membrane. Figure 1 shows a synapse involving unlabeled cells and identified using those criteria. As can be seen, synapses are sometimes small in Helix aspersa. Furthermore, all criteria were not always evident in a given section, but the observation of serial sections allowed us to recognize all three criteria and thus the presence of a synaptic specialization.

In rare instances the labeling was so dark that vesicles were hardly seen (criterion 3), but even then, the membrane specializations (criteria 1 and 2) were visible. Mostly, however, mitochondria and vesicles could be clearly detected, and all the criteria for the presence of a synapse could be identified. In other instances (15 of 166 output synapses observed), vesicles could be seen, but the dark label obscured their fine ultrastructure, e.g., whether or not they were dense-cored. These obscured output synapses were excluded from the data presented in Figures 4, 8, and 11.

Three-dimensional representations were achieved by tracing profiles of the labeled neuron, from electron micrographs, onto acetate sheets. Series of profiles were superimposed according to fiduciary marks placed apart from the labeled profile. The final image was obtained by drawing the contour of the neurite resulting from the stacked profiles.

Fig. 1. Example of a synaptic contact between unlabeled cells in the procerebrum. In this and subsequent figures, arrowheads delimit the synaptic specialization. Scale bar = 200 nm.

Fig. 2. A: Camera lucida drawing of two neurons with arborizations restricted to the procerebrum. Note the partial profile of one cell body located directly beneath the other. The shaded areas highlight the neurites that were serially sectioned and analyzed with electron microscopy. The box refers to the region reconstructed in Figure 5.

B: Placement of the cells within the procerebrum. The neurites occupy a small volume of the procerebrum and clearly do not enter the internal mass. cb, procerebrum cell body region; im, internal mass; np, procerebrum neuropil region; on, olfactory nerve.
SYNAPSES OF OLFACTORY INTERNEURONS IN THE SNAIL

Figure 3
RESULTS

Procerebral neurons cannot be characterized on the basis of quantitated morphological features but can be distinguished by the region in which their neurites arborize (Ratté and Chase, 1997). Some procerebral neurons have neurites that are completely intrinsic to the procerebral lobe. Among those neurons, some do not enter the internal mass, whereas some others have neurites with extensive convolutions specifically within the internal mass. On the other hand, some procerebral neurons project outside the procerebrum to various areas of the cerebral ganglion, and some even project to the contralateral cerebral ganglion.

Neurons were labeled, initially processed for electron microscopy, and reconstructed with light microscopy until a subset could be selected as representative of the several areas to which the neurites project. Four preparations were retained and processed further for electron microscopy. In some of the preparations, two neurons with comparable morphological features were identified and simultaneously analyzed. Two neurons with arborizations intrinsic to the procerebrum but not in the internal mass, two neurons with arborizations confined to the internal mass, one neuron with arborizations in the mesocerebral region (to represent areas outside of the procerebrum), and one neuron projecting to the contralateral cerebral ganglion. In the preparations in which two neurons were labeled, the neurite arborizations were overlapping. Although the two cells could be distinguished at the level of the light microscope, it was impossible to tell them apart at the level of the electron microscope. In those cases, all neurites looked the same, and the observations were pooled as if they came from a single neuron.

We found that the nature and distribution of synaptic interactions differ greatly depending on the site of arborization. All cells studied had distinct regions of preferred input and output.

Neurons with arborizations confined to the procerebrum but not in the internal mass

Procerebral neurons that are entirely intrinsic to the procerebral lobe are characterized by a large number of branches and prominent varicosities, and their arborizations are usually restricted to a small area of the procerebrum. Two neurons possessing those characteristics were simultaneously labeled (Fig. 2).

Electron microscopy revealed that the border between the procerebral cell body region and the neuropil region is not sharply defined; there is a gradual transition between a region with many cell bodies (the so-called cell body region) and a region where no cell bodies are found (the so-called neuropil, divided in two parts: the terminal mass, which constitutes most of the neuropil volume, and the internal mass, which is an inclusion near the ventral basal border of the procerebrum). Contrary to the situation in most invertebrate ganglia, our results show that synapses are commonly found in the cell body region of the procerebrum. Furthermore, although the representation in Figure 2 indicates that arborizations are almost all in the soma region, some of the illustrated branches are actually in the terminal mass of the neuropil region. The discrepancy results from the structure of the procerebrum. Because the flattened mass of cell bodies that constitutes the soma region wraps around the lateral side of the terminal mass of the neuropil, a portion of the neuropil is hidden when the preparation is viewed directly from the ventral or dorsal side. Parts of the procerebral neurons shown in Figure 2 arborize in this hidden region of the neuropil. We did not see any substantial differences in synapse frequency or ultrastructure in the cell body region compared with those in the terminal mass of the neuropil region. Collectively referring to these two regions, with the exclusion of the internal mass, as “the procerebrum” simplifies the presentation of the results and discussion.

Varicosities are numerous in intrinsic neurons, and the neurite diameter at a varicosity increases dramatically, sometimes up to an order of magnitude. Varicosities are thought to be preferred sites of synaptic contacts (Bailey et al., 1979). To verify whether the occurrence of varicosities along the procerebral neurites is indicative of specialized sites of synaptic interactions, we compared synapse distribution at varicosities and between varicosities.

Synapses at varicosities. Regions of neurites that were identified as varicosities were characterized by diameters of 0.8 μm. This would usually indicate at least a threefold increase in diameter, since between varicosities the diameter is only 0.1–0.3 μm.

Synapses were found in large numbers at varicosities, but they were not restricted to those areas and could also be seen along fine neurites between varicosities (Fig. 3). However, the density of synapses at varicosities was 10 times higher than between varicosities (Table 1). Varicosite profiles are typically filled with clear synaptic vesicles (Fig. 4A) and are involved in multiple output synapses. Each varicosite is presynaptic to multiple unidentified profiles, often to all immediately surrounding neurites (Fig. 3A), and sometimes even to cell bodies (Fig. 3B). Of 26 varicosities studied, 20 (77%) displayed synapses. The

### TABLE 1. Synapses on Neurons With Arborizations Confined to the Procerebrum but not in the Internal Mass

<table>
<thead>
<tr>
<th>Cellular region</th>
<th>Synaptic type</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>At varicosities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 μm sampled; 1.00</td>
<td>Input</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Output</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Reciprocal</td>
<td>5</td>
</tr>
<tr>
<td>Between varicosities; 658 μm sampled; 0.10 syn/μm</td>
<td>Input</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Output</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Reciprocal</td>
<td>2</td>
</tr>
</tbody>
</table>

* In all tables, asterisks indicate values that significantly deviate from a 1:1 output to input ratio (Chi-square test, P < 0.05).
number of synapses per varicosity ranged from 0 to 10 (2.6 ± 2.5, mean ± SD).

In contrast, input synapses are rare at varicosities (Table 1). The rarity of input synapses may be exaggerated by the fact that varicosities are usually filled with vesicles and, as mentioned above, they usually have output synapses. Consequently, when a profile is presynaptic to a varicosity, it often faces an output synapse, and the arrangement results in a reciprocal synapse.

Reciprocal synapses are commonly observed between unlabeled profiles in the cell body region, and a few were also observed on labeled profiles (Fig. 3D), both at and between varicosities (Table 1). Reciprocal synapses are not specific to intrinsically arborizing neurons; they are also present on all other neurons studied, and in all regions.

**Synapses between varicosities.** Although the ratio of output to input synapses in regions of neurite between varicosities is not as large as that at varicosities, it is significantly different from a 1:1 ratio (Chi-square test, \( P < 0.05 \)). The density of synapses, however, is lower, being only 0.10 synapse/μm. Thus, it should be noted that although a large number of synapses are reported between varicosities in Table 1, more than 600 μm of neurite was scanned for these observations.

The input synapses on these cells usually occur between varicosities (Table 1). The neurons receive synaptic inputs from profiles containing a variety of vesicles (Fig. 4). This suggests a variety of input sources to intrinsically arborizing procerebral cells. Similar to synapses at varicosities, output synapses between varicosities usually contain only clear vesicles.

The diameter of the neurites between varicosities can be quite small, as shown in Figure 3C, and we observed neurites as thin as 0.1 μm. This size challenges the resolution that can be obtained at the light microscope level and suggests that procerebral morphologies reported on the basis of light microscopy alone may sometimes be incomplete.

**Synapse distribution.** Figure 5 shows a reconstructed segment of a neurite belonging to an intrinsically arborizing cell. The prominence of varicosities and the high density of output synapses found at varicosities are obvious in this representation. The varicose regions contrast with the small diameter and sparsity of synapses characteristic of the neurites between varicosities.

**Neurons with arborizations confined to the internal mass**

Some of the neurons that have arborizations intrinsic to the procerbrium display a morphology that is intimately related to the internal mass. We simultaneously labeled two cells that unequivocally display this relationship (Fig. 6). Each cell's neurite branches only minimally before it reaches the internal mass. In the internal mass, the neurites still do not display many branches but rather meander extensively. Thanks to these meanders, the neurites possess a large surface area with which to make synaptic contacts in the internal mass.

Our observations focused on two regions: 1) the proximal part of the neurites in the cell body region, and 2) the meandering neurites in the internal mass region. We sampled 200 μm of neurite in the soma region, and 90 μm of neurite in the internal mass region.

**Synapses in the somatic region of the procerbrium**

Very few synapses are present on the proximal part of the...
neurite (Table 2). A majority of the synaptic contacts are inputs, but some output synapses also occur. Input synapses on the labeled cells are sometimes particularly small (Fig. 7C). The vesicle ultrastructure at input synapses varies greatly (Fig. 8A), suggesting that a diversity of neurons are presynaptic to this type of procerebral cell. On the other hand, three of the four output synapses in this area were characterized by dense vesicles. Although the sample size for these synapses is small, the data contrast with the clear vesicles that constitute most output synapses of intrinsically arborizing neurons that do not enter the internal mass (Fig. 4).

**Synapses in the internal mass region.** The density of synapses on the labeled neurons in the internal mass is relatively high, 0.57 synapses/μm, or eight times higher than in the cell body region. In the internal mass the output-to-input ratio is large (Table 2) and significantly deviates from a 1:1 ratio (Chi-square test, $P \leq 0.001$). The labeled profiles typically contain a large number of vesicles (Fig. 7A), but the vesicles nevertheless remain clustered around areas of synaptic specialization. Synapses are characterized by dense vesicles, as in the cell body region, but a larger proportion contain a mix of other vesicles in addition to the dense vesicles (50% of all synapses; see Fig. 8). In this region, vesicles involved in input synapses are similar to those at output synapses, suggesting that procerebral neurons interact with each other in the internal mass.

A few synapses showed a dyadic arrangement (Fig. 7B). Although this arrangement is common in some other invertebrates (leech, Muller and McMahan [1976]; cockroach, Malun [1991]), it is unusual in terrestrial molluscs. In dyadic arrangements in the internal mass, the labeled procerebral neuron is not always the presynaptic component; sometimes it is one of the two postsynaptic targets (not shown). These observations imply that the procerebral neurons participate in patterns of divergence as well as convergence within the internal mass.

**Synapse distribution.** The initial part of a neurite in the cell body region and portions of meandering neurites in the internal mass were reconstructed from serial thin sections. Figure 9 shows some reconstructed segments. The predominance of input synapses in the cell body region contrasts with the predominance of output synapses in the internal mass. Using all the data, the ratio of input to output synapses is significantly different in the two regions (Fisher’s exact test; $P \leq 0.001$), indicating a polarization of the neuron with respect to its input/output connections.

In contrast to the clustering of synapses observed in intrinsic neurons not arborizing in the internal mass (Fig. 5), internal mass-specific cells have input, output, and reciprocal synapses intermingled and evenly distributed along the neurite. In the cell body region, there are varicosities, but they are not necessarily sites of synaptic contacts (Fig. 9A). In the internal mass, the meandering neurite is exceptionally constant in diameter (around 0.5 μm) (Fig. 9B). Very few varicosities are present, but the data clearly show that the internal mass is a region of output for procerebral cells.

**Neuron with arborizations outside the procerebrum**

Some procerebral cells possess neurites that exit the procerebrum and terminate in various regions of the cerebral ganglion. One such cell, with neurites in the mesocerebral region, was labeled (Fig. 10). Our observations focused on four areas of the cell: 1) the initial part of the...
neurite in the procerebrum, 2) the branches and terminals of the neuron in the mesocerebrum, 3) the transition area as the neurite exits the procerebrum, and 4) the transition area as the neurite approaches the mesocerebrum. In total, 473 μm of neurite was serially sectioned and observed. **Synapses in the procerebrum.** Only input synapses were found on the neuron in the procerebrum. Although the synapse frequency is not particularly high (0.13 synapses/μm; Table 3), it is twice that observed on internal mass-specific cells in the same region (Table 2). Half of all input synapses had clear vesicles, and the remaining half contained dense, dense-core, or mixed vesicles (Fig. 11A). Thus input to these cells seems to be heterogeneous, with clear vesicles playing a dominant role.

**Synapses in the mesocerebral region.** A larger number of synapses were observed in the mesocerebral region (0.19 synapses/μm). Seventy percent of all synapses are output synapses (Table 3; Fig. 12A,C); the data significantly deviate from a 1:1 ratio of output to input (Chi-square test, $P < 0.01$). Vesicle populations contained in the unlabeled presynaptic profile at input synapses are strikingly similar to those of output synapses (Fig. 11B), suggesting that, as for internal mass-specific cells, neurons of this type interact with each other.

**Synapses in transitional areas.** To determine whether the long neurite’s only function is to bridge the distance to the projection site (in this case, the mesocerebrum), or if there are also cellular contacts along the way, we looked at the neurite in the transition area, i.e., as it exits the procerebrum and before it reaches the mesocerebral region. The density of synapses is low in these regions. However, the ratios of output to input synapses in the transition areas resemble the ratios found in the adjacent regions of denser interactions (Table 3). Thus, in the two segments observed in the transition area close to the procerebrum, all synapses are input synapses, whereas in the transition area close to the mesocerebrum, output synapses predominate. Hence the change from the area of dense input in the procerebrum to the area of dense output outside the procerebrum occurs gradually along the length of the neuron, in terms of both quantity and nature of synapses.

The ultrastructure of the neuropil through which the neurite travels as it transits between the procerebrum and the mesocerebrum may partly explain the rarity of synapses in this region because the neurite follows a tract (Fig. 12D). As the neurite approaches the more medial area of the mesocerebrum, the tract diffuses and broadens, and the fiber orientation becomes more variable (not shown). The texture begins to resemble that of a standard neuropil. When the neurite reaches the mesocerebrum, it arborizes in the adjacent neuropil. In this area of denser synaptic interactions, the neuropil no longer has the appearance of a tract. Although the site of the procerebral cell arborization was seen only ambiguously with light microscopy, it is clear with electron microscopy that the procerebral cell does not enter the cell body region of the mesocerebrum.
Fig. 7. Electron micrographs of synapses on neurons with arborizations confined to the internal mass. **A:** Electron micrograph showing three output synapses from the labeled procerebral neurons (stars). In the internal mass, the density of synapses on the labeled neurons is high, and the ratio of output to input synapses is large. **B:** Output synapse in the internal mass, displaying divergence through a dyadic arrangement. Dyadic synapses were only observed in the internal mass. **C:** Two labeled profiles in the cell body region corresponding to the two labeled neurons. A small input synapse on one of the labeled cells (arrow) is shown at higher magnification in the inset. Cb, neuronal cell body. Scale bar = 0.4 μm in A; 0.3 μm in B; 1.0 μm in C.
Synapse distribution.

The ratio of output synapses to input synapses on the labeled cell in the mesocerebral region is reversed from that in the procerebral region. This reversal of proportions, indicating cellular polarity, is significant (Fisher exact test, \( P \leq 0.001 \)) and similar to what was observed on the internal mass-specific cell (see section above and Table 2).

In the procerebrum, synapses are scattered along the neurite, and the synapse distribution is similar to that which was observed on the internal mass-specific cell in the same region (Fig. 9A). Although output synapses sometimes occur at varicosities, they are not restricted to these sites.

In the mesocerebral region, the distribution of synapses on the branching neurites is represented by the reconstruction in Figure 13. Synapses are scattered along the several branches, but it is noticeable that input synapses are often located close to output synapses (Fig. 12B,C, corresponding to synapses 2,3 in Fig. 13).

A neuron projecting to the contralateral cerebral ganglion

Some procerebral neurons project a long neurite through the cerebral commissure, presumably to arborize in the contralateral cerebral ganglion. One of our labeled neurons had a neurite that could be followed to the cerebral commissure (Fig. 14). However, our procedures required that only one cerebral ganglion be kept for processing, and the cerebral commissure was transected before we could visualize the morphology of the labeled neurons. Hence the contralateral cerebral ganglion was discarded without prior microscopic examination. However, because the labeled cell displays varicosities (possibly indicating synaptic interactions) as it exits the procerebrum and as it passes along the mesocerebral region, it was of interest to proceed with electron microscopy even if the cell was incomplete.

Our observations on this cell are reported in Table 4. The procerebral portion of the cell is comparable to the procerebral portion of other procerebral cells with projective neurites, i.e., the internal mass-specific cells and the extrinsically arborizing cell (Fig. 9A). All synapses observed on the proximal neurite were input synapses, but the possibility that output synapses are present outside the 50-μm region sampled is not to be excluded. The density of synapses is comparable to that of other projecting cells in the same region.

In the mesocerebrum, we looked at an area that extends over 154 μm of neurite and includes six of the seven varicosities seen with light microscopy (Fig. 14). To our surprise, no synapses could be seen either at the varicosities or between them. We did not see any synapses in the transition regions either. Hence, the absence of any ipsilateral output synapses suggests that the neuron’s output occurs exclusively in the contralateral ganglion.

DISCUSSION

Early morphological studies on the procerebrum suggested that the population of procerebral cells was homogeneous and that the cells’ processes were confined to the procerebral lobe (Veratti, 1900; Hanström, 1925; Zaitseva, 1994). More recently, a study using intracellular labeling of the neurons demonstrated that procerebral cells are not homogeneous (Ratte and Chase, 1997).

The main difference between the cells is the placement of their neurites, some of which exit the procerebrum. The present electron microscopic study demonstrates that procerebral neurons with different sites of arborizations have distinct patterns of synapse distribution that probably reflect different functional contributions to the olfactory...
pathway. These electron microscopy results have important implications for comparative studies on olfactory systems. The procerebrum was previously seen as a unique olfactory structure partly because of the homogeneity of its components. However, the data presented in this paper contradict the earlier view and suggest that the procerebrum, with a population of intrinsic interneurons and a population of projection neurons, has a relatively standard neural organization and may now be compared with the olfactory lobes of other animals.

Our observations are summarized in Figure 15. On the basis of these ultrastructural data, the procerebral population of neurons may in fact be divisible into two groups. The first group consists of intrinsic neurons with localized arborizations. These cells have multiple large varicosities that are specialized for output. Given the local action of these cells in the procerebrum, they are typical interneurons. The second group includes neurons that possess an input region at one extremity of the cell and an output region at the other extremity. The internal mass-specific diameter and the scattered distribution of output synapses contrast with the observations made on cells that are intrinsic to the procerebrum but do not enter the internal mass (see Fig. 5). Synapses numbered 1 and 2 correspond to synapses illustrated in Figure 7A; synapse 3 corresponds to the synapse seen in 7B. The star indicates the part of the neurite that is proximal to the soma. Scale bar = 2 μm.

Fig. 9. Reconstructions of two areas from a neuron arborizing in the internal mass. A: Neurite in the cell body region. Input synapses predominate and are distributed along the neurite. Synapse 4 corresponds to the electron micrograph shown in Figure 7C. B: Neurite in the internal mass. Most synapses are output synapses, and here again they are distributed along the neurite. The constancy in neurite
cells and the extrinsically arborizing cell described in this paper are examples of such polarized neurons. These cells appear to transmit information from the cell body mass and terminal mass to other central nervous system regions, e.g., the internal mass and the mesocerebrum, and thus can be considered projection neurons.

In earlier studies, two populations of procerebral neurons were identified based on physiology (Kleinfeld et al., 1994) and soma diameter (Rhines et al., 1993). We were unable to correlate morphology with physiology by recording intracellularly from the cells before we injected them with label, probably because the soma of procerebral neurons is so small that the membrane is usually badly damaged when penetrated with a sharp electrode. Using the perforated patch recording technique, Watanabe et al. (1998) recently managed to correlate cell physiology with morphology, but, given our findings, their labeling was probably incomplete. Nonetheless, their results suggest that the procerebral cells we identify as interneurons correspond to bursting cells and that the projection neurons correspond to non-bursting cells.

Although cells projecting to the internal mass have a synapse distribution comparable to that of cells projecting outside the procerebrum, and are similarly polarized, processing in the internal mass may be different. The precise morphology, the microcircuitry details, and the vesicle ultrastructure of internal mass-specific cells are slightly different than in cells projecting outside the procerebrum. The importance of the internal mass is undeniable given the number of procerebral cells that project specifically to it (Ratte´ and Chase, 1997). Understanding the significance of the internal mass in the olfactory pathway, however, still requires further investigations.

### Ultrastructure of local interneurons

The most striking characteristic of the local interneurons is the varicosities, which are usually marked by multiple output synapses. It is noticeable that varicosities are not indicative of output synapses in all procerebral cells. As shown by the reconstructions of projection neurons (Figs. 9, 13), input synapses and output synapses are evenly distributed along the neurites, regardless of the neurite diameter, and varicosities often lack synaptic specializations. In local interneurons, by contrast, we found that 77% of varicosities have at least one synapse, and most have multiple synapses. This makes for an average of 2.6 synapses per varicosity, which is considerable given the general sparsity of synapses in which procerebral cells participate. It is also 13 times more synapses per varicosity than reported by Bailey and Chen (1989) for *Aplysia* sensory neurons. These groupings of output synapses not only allow for a considerable divergence of information, but their compact localization provides for a synchrony of activation and, consequently, synchronized activation of multiple postsynaptic targets. The local interneurons may
therefore have an important role in the physiological oscillation of field potentials first described by Gelperin and Tank (1990) in the procerebrum of the slug *Limax*.

Whereas output synapses are clustered, input synapses are scattered along neurites, and the distance between input synapses can be large. The large inter-synapse distance and small neurite diameter raise the question of whether sufficient spatial summation can occur for spike threshold to be reached. However, because clusters of output synapses are also distributed along the neurites (the average distance between varicosities is 11 μm), it is likely that action potential generation is not necessary for these cells to release neurotransmitter and communicate with postsynaptic neurons. The synaptic arrangement is such that output synapses are always located in proximity to input synapses. Hence, although all procerebral neurons are apparently able to spike (Kleinfeld et al., 1994), release of transmitter may also occur with graded potentials, as is known to be the case in many other systems (Roberts and Bush, 1981), including molluscs (Shimahara and Peretz, 1978). The role of the action potentials recorded at the cell body may have a significance other than that of activating output synapses in distal neurites.

Based on its histological appearance, the procerebrum does not seem to have any internal divisions such as laminae or glomeruli. However, recent experiments using Lucifer Yellow uptake as an indicator of cellular activity revealed a compartmentalization of procerebral activity in relation to olfactory memories (Kimura et al., 1998b). In another study, preliminary observations suggested that discrete groups of procerebral cells briefly decouple from the overall field potential oscillation when specific odours are presented (Gervais et al., 1996). The varicosities, with their multiple outputs, could provide the morphological substrate for such a functional compartmentalization of the procerebrum.

The synapses from local interneuron varicosities are large. The varicose structure is usually filled with vesicles, and the membrane specializations are extensive. These features are likely to underlie reliable transmission. It is impossible to infer from our results whether transmission is inhibitory or excitatory since, in molluscan tissue, inhibitory synapses cannot be identified by the flattening of their synaptic vesicles during fixation. In the procerebrum, the only neurotransmitter thought to have an inhibitory role is glutamate (Rhines et al., 1993; Gelperin et al., 1993). Many studies in a variety of tissues have shown that glutamate vesicles are usually small, round and clear, just like those present in varicosities of local procerebral interneurons. However, many other neurotransmitters are also contained in round, clear vesicles (for review, see Peters et al., 1991); immunocytochemistry will be necessary in order to identify the transmitter content of the procerebral vesicles confidently.

What components of the olfactory pathway are activated by procerebral interneuronal varicosities? A large proportion of the vesicles at output synapses of local interneurons are clear (Fig. 4). Our ultrastructural results for synapses on projection neurons in the procerebrum, and especially on extrinsically projecting cells (Fig. 11), show that many of these input synapses also contain clear vesicles. These findings suggest that projection neurons are likely candidates for postsynaptic targets of local interneurons.

Based on the heterogeneity of their synaptic vesicles (Fig. 4), input synapses on local intrinsic neurons seem to be from a variety of sources. It is likely that one of the important input sources to these cells (and possibly, to projection cells as well) consists of olfactory afferents. Kawahara et al. (1997) showed that most fibers from the olfactory nerve arborize and end in the terminal mass of the neuropil (the main procerebral neuropil, outside the internal mass). However, nerve backfills in *Helix aspersa*...
Figure 12
show that at least some olfactory nerve fibers terminate in the cell body region of the procerebrum (N. Gill and R. Chase, unpublished observations). We found many synaptic contacts in that region. Given the olfactory nature of the input to the procerebrum, it is expected that some of the input terminals in this region of dense interactions belong to the main input fibers. In an early electron microscopy study of the procerebrum in a different species, Zs-Nagy and Sakharov (1969) also presented some evidence for synapses in the cell mass. The presence of synapses in the soma region is unusual for invertebrate tissues, as synaptic contacts are normally restricted to a centrally located neuropil surrounded by somata, but the procerebrum’s laterally located cell mass is itself non-standard for invertebrate ganglia.

Ultrastructure of projection neurons

Although the cells that project to the internal mass and to regions outside the procerebrum are significantly polarized, with a region of input in the procerebrum and a region of output at the distal extremity of the cell, they should not be considered as simple relays. Our results show that, except for the procerebral region of the extrinsically arborizing neuron, none of the regions are 100% input or output. The proximity of input and output synapses implies that even in projection neurons, there must be some local processing of information. Output synapses in the region dominated by inputs, i.e., in the procerebrum, may recruit neighboring neurons or contribute to lateral inhibition. Input synapses in regions dominated by outputs, whether they mediate depolarization or hyperpolarization, could significantly modulate output synapses. Alternatively, these synapses could depolarize the projection neuron terminals and activate output synapses independently of signals travelling down the neurite from the procerebrum.

The long unbranching neurite that connects input and output regions in projection cells contrasts with the many branches seen in intrinsic local interneurons. The regularity in neurite diameter also differs from local interneurons. These characteristics probably facilitate the transfer of information between the input and output regions. However, ironically, the small size of some of the input and output synapses raises doubts as to the reliability of transmission. For example, the synapse on the proximal part of an internal mass-specific cell in Figure 7C is remarkably small (only 5 vesicles and 0.3 \( \mu \)m long). Furthermore, synapses are scarce on the proximal part of projecting neurites (on average, 0.1 synapse/\( \mu \)m; see Tables 2–4). Can such fine neurites sustain enough passive propagation for small synaptic potentials generated at distant synapses to spatially summate and reach threshold? Synapses are often separated by 10–15 \( \mu \)m of fine, constant diameter neurite (diameter, 0.5 \( \mu \)m). Using the cable equation, we estimated the passive properties of the neurites. If we assume the specific membrane resistance (\( R_m \)) and axoplasm resistance (\( R_i \)) to be typical of neurons in other terrestrial snails (\( R_m = 4 \times 10^3 \Omega \cdot \text{cm}^2 \), averaged from
Maiskii, 1963; Magura, 1967; Meves, 1968; Pawson and Chase, 1985; and Vázquez, 1980, averaged from Graubard, 1975; Kandel, 1976; Nicholls et al., 1992), the space constant for those neurites is 274 m. This is more than sufficient to allow, in any region, the spatial summation of a large number of synaptic potentials, even if synapses are relatively sparse. Even synaptic potentials in the transition region of projection neurons could passively propagate and be sectioned and analyzed with electron microscopy. B: Placement of the cell in the cerebral ganglion. For abbreviations, see Figure 2 and 10 legends.

**TABLE 4. Synapses on a Neuron Projecting to the Contralateral Cerebral Ganglion**

<table>
<thead>
<tr>
<th>Region</th>
<th>Synaptic type</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inside the procerebrum (50 μm sampled, 0.1 sym/μm)</td>
<td>Input</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Output</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reciprocal</td>
<td>0</td>
</tr>
<tr>
<td>Outside the procerebrum, ipsilaterally¹ (154 μm sampled)</td>
<td>Input</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Output</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reciprocal</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ No observations were made in the contralateral cerebral ganglion.
summate with potentials generated in distant areas of denser synaptic interactions. If we substitute values for $R_m$ and $R_i$ from one extreme of the reported range, the space constant value is reduced to $91 \mu m$, but this is still large enough to accommodate spatial summation. If, on the other hand, we substitute values from the opposite extreme of the reported range, the space constant reaches $612 \mu m$, which would often allow passive propagation along the entire neurite, as procerebral projection neurons only rarely exceed 1 mm in length (Ratte´ and Chase, 1997).

A proportion of procerebral cells regularly generate bursts of action potentials (Kleinfeld et al., 1994). So in addition to the spatial summation discussed above, bursting inputs may allow temporal summation of postsynaptic potentials. Because they can elicit facilitation, for instance, bursts could allow even small synapses to have a substantial effect on the postsynaptic cell potential (reviewed by Lisman, 1997).

**Limits of interpretation**

The results presented in this study were gathered from a serial sections analysis of one or two neurons from each of several areas of arborization. Because of the technical difficulties and time-consuming nature of the work, it was not possible to repeat the observations with multiple cells. Our goal was to map the sites of arborization identified previously with light microscopy (Ratte and Chase, 1997) for differences in synapse distributions, and we have done this for the selected cells. Our interpretations are based on the assumption that procerebral cells with similar sites of arborization will display similar patterns of synapse distribution.

We chose not to analyze a cell that arborizes both inside and outside the procerebrum. In fact, the extrinsically arborizing neuron on which we performed the electron microscopic analysis does possess branches in the procerebrum (Fig. 10A), although they are very short and so...
fine that they were not initially detected with light microscopy. Input synapses were present on these small branches. Hence for neurons that arborize both inside and outside the procerebrum, branches in the procerebrum may simply increase the spatial extent from which inputs can be gathered, and, based on the results of the present study, we suggest that those neurons would also be polarized, with input in the procerebrum and output outside the procerebrum.

One of the limitations of electron microscopy studies is that while classical chemical and electrical synapses can be identified, other types of cellular communication cannot be detected. For example, nitric oxide, which has been shown to be present in the procerebrum and to have a significant effect on its activity (Gelperin, 1994), is a gaseous messenger and is not contained in vesicles. Because no ultrastructural features allow the identification of its sites of production and action in neurons, it is impossible to demonstrate where nitric oxide may be used by procerebral neurons. Similarly, we observed large dense-core vesicles, which may contain peptidergic compounds acting as neuromodulators, at both synaptic and non-synaptic sites. It is impossible to identify, in our study, the non-synaptic sites of release and action of these neuromodulators. Finally, the activity of neurons can be affected by changes in excitability due to increases in extracellular potassium (Eng and Kocsis, 1987). Because cell bodies and fibers are both densely packed in the procerebrum, a cell’s activity may change when the surrounding neurons are active, but this cannot be perceived ultrastructurally.

Many electrical synapses are evident in the procerebrum from the appearance of gap junctions (data not shown), but none were localized on labeled cells. It is possible that intracellular labeling procedures and the formation of a reaction product disrupted gap junctions. As electrical synapses could be involved in the synchronization of the field potential oscillation, it would be interesting to study them in more detail.

**Procerebrum in the olfactory pathway**

The present data provide evidence that the procerebrum directly participates in distributing olfactory information to more central regions of the nervous system. Because of the large proportion of neurons that project outside the procerebrum (Ratté and Chase, 1997) and the present demonstration that output synapses predominate at the terminals of such projecting cells, there is considerable divergence of the information at the level of the procerebrum. Furthermore, procerebral output is not restricted to the areas of the cerebral ganglion reached by projection neurons but is also distributed to the pedal and buccal ganglia (Chase and Toltloczko, 1989; Gelperin and Flores, 1997). This dissemination of information suggests that the procerebral lobe is the final processing step of the olfactory pathway before the appropriate behavior is generated. The procerebral projection cells could activate or modulate the activity of neurons involved in motor pathways. It would be interesting, in future studies, to identify the postsynaptic targets of procerebral projection neurons. Mesocerebral cells, around which one of the studied cells arborizes, are known to be important mediators of mating behavior and reproduction (Chase and Li, 1994; Koene et al., 1999).

In conjunction with the morphological study of procerebral cells published earlier (Ratté and Chase, 1997), the results presented here have important implications for comparative studies on olfactory systems. Our data refute the early view that the procerebrum is composed of a morphologically homogeneous population of neurons, all of which are arborizing entirely within the procerebral lobe. The procerebrum seems in fact to have a relatively standard, triadic neural organization for a sensory lobe or nucleus. The present study demonstrates that different sites of arborization correlate with different synapse distributions on the cells. Hence, as in other olfactory systems, the procerebral cell population can be divided into two groups: 1) intrinsic interneurons that mediate mostly local interactions, and 2) projection neurons that participate in local interactions but are also important for the distribution of the olfactory information to other areas of the brain. However, an important difference remains between the procerebral lobe and the antennal lobe in insects or the olfactory bulb in mammals. Glomeruli are not present in the procerebrum. Hence, the manner by which olfactory information is coded, and whether specific subsets of projection neurons can be activated with different odours, as seems to occur in other olfactory systems, remain unclear in the procerebrum.

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**LITERATURE CITED**


