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# Morphology of Interneurons in the Procerebrum of the Snail *Helix aspersa*

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ABSTRACT

Terrestrial snails have a highly developed sense of olfaction. Because the procerebrum has a large number of cells and is located at the entry site of the olfactory nerve into the brain, the structure is thought to have a significant role in the processing of olfactory stimuli. The morphology of the procerebral neurons in the snail *Helix aspersa* was investigated through intracellular injections of biocytin. No formal categorization of neuronal types was possible, but some cells were seen to have neurites entirely intrinsic to the procerebrum, whereas others had both intrinsic and extrinsic arborizations, and still others had only extrinsic arborizations. These interneurons were previously thought to have arborizations restricted to the procerebral lobe. We demonstrated the extent of the neurite projections outside of the procerebral lobe by making focal injections of biocytin or Neurobiotin into various regions of the cerebral ganglion. This technique revealed subsets of cells that send neurites not only in the ipsilateral ganglion but also through the cerebral commissure into the contralateral cerebral ganglion. Our results demonstrate not only that the procerebral cell population is heterogeneous but also that the procerebrum interacts more directly with the rest of the central nervous system than was formerly believed. J. Comp. Neurol. 384:359–372, 1997. © 1997 Wiley-Liss, Inc.

**Indexing terms: olfaction; cerebral ganglion; gastropod mollusc; biocytin**

Terrestrial snails have a high sensitivity for odors and sophisticated learning abilities in the olfactory domain (Croll and Chase, 1980; Chase, 1982; Gelperin et al., 1985). Because they rely almost entirely on their sense of smell to find food and conspecifics, it is reasonable to suppose that the olfactory system is evolved to meet exacting requirements. Van Mol (1974) argued that the procerebral lobe of the cerebral ganglion first appeared in the semiterrestrial and terrestrial snails as these organisms left the aquatic environment. The procerebrum, therefore, may be viewed as the product of an evolutionary process for the successful adaptation of snails to a terrestrial environment, where olfaction is important for their survival.

Anatomical and physiological data indicate a role for the procerebrum in olfactory processing. The anatomy of the olfactory pathway in *Achatina fulica* has been reviewed by Chase and Tolloczko (1993). Briefly, olfactory stimuli generate activity in the receptor cells of the olfactory epithelium at the tip of the superior tentacles. Most of these cells have axons that terminate in glomerular structures under the epithelium or in the tentacle ganglion. The remaining sensory cells and the ganglionic interneurons then carry the signal directly to the central nervous system through pathways that have been characterized by electrophysiological (Chase, 1981) and morphological (Chase and Kamil, 1983) methods. The procerebrum is located at the entry site of the olfactory nerve into the cerebral ganglion (Fig. 1). Nerve backfills with hexamminecobalt demonstrate that a large proportion of the olfactory nerve fibers branch and terminate in the procerebrum (Chase and Tolloczko, 1989). Electrophysiological studies in *Limax maximus* and *Helix pomatia* have identified an oscillating field potential that is spontaneously active at a constant frequency and that continuously propagates as a wave across the procerebrum (Gelperin and Tank, 1990; Schütt and Basar, 1994). By recording intracellularly from procerebral cells, Kleinfeld et al. (1994) observed that some neurons produce periodic bursts of spikes, whereas the majority receive periodic inhibitory inputs and fire only infrequently. The continuous field potential oscillation evidently emerges from the coordinated activity of these two subpopulations of cells. Olfactory stimulation causes a collapse of the procerebral wave of depolarization and subtle changes in the waveform of the resulting standing

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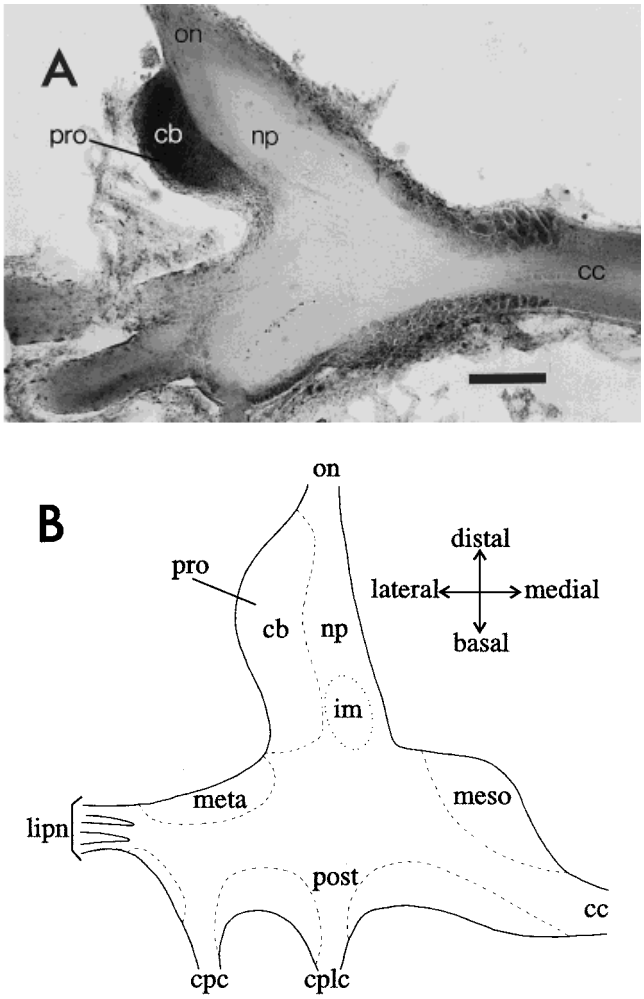


Fig. 1. **A:** Photomicrograph of a longitudinal section of the left cerebral ganglion. The procerebral lobe is located at the base of the olfactory nerve. The dense cell body region of the procerebrum is positioned lateral to a fine neuropil. **B:** Schematic drawing of the ganglion identifying the main landmarks that are used in subsequent figures. cb, cell bodies; cc, cerebral commissure; cpc, cerebropedal connective; cplc, cerebropleural connective; im, internal mass; lipn, lip nerves; meso, mesocerebrum; meta, metacerebrum; np, neuropil; on, olfactory nerve; post, postcerebrum; pro, procerebrum. Scale bar = 200 µm.

oscillation (Gelperin and Tank, 1990; Delaney et al., 1994; Kleinfeld et al., 1994).

The number of neurons in each procerebral lobe has been estimated to be 20,000 in *Achatina fulica* (Chase, 1986) and 100,000 in *Limax maximus* (Gelperin and Tank, 1990). In both cases, this is more than the total number of neurons in the remainder of the animal's nervous system. The somata of procerebral neurons are small (5–8 µm), and the cell population appears to be homogeneous. The cell bodies are located laterally to a fine and dense neuropil (Fig. 1) in which there seems to be no structural organization (e.g., glomeruli). With the exception of the olfactory nerve entering at the distal end and running as medial and lateral tracts on each side of the neuropil toward the core of the cerebral ganglion, no other tract is discernable in the procerebrum. Only the internal mass, a small region of the neuropil that is marked by surrounding somata (probably glial cells), can be distinguished in the ventral portion of the basal procerebrum (Zs-Nagy and Sakharov, 1970).

Known inputs to the procerebrum are sensory in nature; they originate in the superior and inferior tentacles and travel via the olfactory nerve and the medial lip nerve, respectively (Hanström, 1925; Chase and Tolloczko, 1989). Only two outputs are known, and they have been described through morphological and electrophysiological studies: Cells in the pedal ganglia send long, receptive processes into the procerebral neuropil via the cerebropedal connectives (Chase and Tolloczko, 1989), and a pair of cells in the buccal ganglia innervate the procerebrum via the cerebrobuccal connectives (Gelperin and Flores, 1997). Previous morphological studies using the Golgi silver-impregnation method have characterized the procerebral cells as interneurons whose processes are restricted to the procerebrum (Veratti, 1900; Haller, 1913; Hanström, 1925; Zaitseva, 1992). However, given the delicacy of the cells and the uncertainties associated with the Golgi silver stain, it is possible that these descriptions are incomplete. Therefore, we decided to use intracellular injections of biocytin to better characterize procerebral neurons. Our results show that a subset of procerebral neurons provides a putative output pathway from the lobe.

## MATERIALS AND METHODS

Mature specimens of the terrestrial snail *Helix aspersa* (curved shell margin; 3–9 g with the shell) were obtained from Santa Barbara and Long Beach, California. The circumoesophageal ring of ganglia was removed from the animal and immediately placed in a Sylgard-coated dish filled with cold saline solution (80 mM NaCl, 4 mM KCl, 7 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.8). The cerebral ganglion was pinned to the dish, and the connective tissue was removed from its surface. The last layer of connective tissue was left intact, except over the procerebrum, where it was delicately ripped and removed with fine forceps, so that the cell bodies could be accessed directly.

### Intracellular labelling

Fine glass pipettes (<1-µm tip diameter) were filled with a fresh solution of 2% biocytin and 1% Lucifer yellow dissolved in 0.05 M phosphate buffer, pH 7.4. The micropipette was mounted on a piezoelectric driver (Model PZ-501, Burleigh Instruments, Fishers, NY), and its position was controlled electronically. All manipulations were carried out and observed under epifluorescence in the blue spectrum (Leitz fluorescence microscope equipped with a 100 Watt mercury lamp). Cell penetrations were achieved by quick, short, downward movements of the micropipette. Individual cells were not targeted. Initially, Lucifer yellow was injected by using short pulses of hyperpolarizing current to confirm that an adequate penetration had been achieved. If this was successful, then depolarizing current was used to fill the cell with biocytin (0.6 second pulses at 1 Hz for 2–5 minutes). Intermittently during the biocytin injection, Lucifer yellow was briefly injected to verify the quality of the seal around the microelectrode.

Once one or more successful injections had been completed, the preparation was rinsed with saline and fixed by immersion in 2.5% paraformaldehyde, 1.25% glutaraldehyde, and 0.15% picric acid in 0.1 M phosphate buffer, pH

7.4, for 1–16 hours at 4°C. Next, it was rinsed in phosphate buffer 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.

The tissue was incubated overnight at 4°C in an avidin-biotin and hydrogen peroxidase complex (Vectastain ABC solution; Vector Laboratories, Burlingame, CA) followed by 3–6 hours at room temperature. It was then preincubated in a 0.06% solution of 3,3′-diaminobenzidine (DAB) with 1% $CoCl_2$ for 10 minutes and was subsequently reacted in a fresh solution of 0.06% DAB, 0.0225% $H_2O_2$ for 10 minutes or until the background started to become dark. The reaction was terminated by multiple rinses with phosphate buffer.

The tissue was serially sectioned at 60 µm after embedding in 7% agar. The sections were dehydrated in a graded series of ethanol dilutions, cleared in methyl salicylate, and mounted on slides with Permount. Neurons were reconstructed from camera lucida drawings of sections viewed at ×1,000 magnification under oil immersion. Terminals were identified by an abrupt end of the label as opposed to a gradual fading, which would be indicative of incomplete diffusion. Although results were obtained from injections in both the left and the right ganglia, all drawings are standardized to portray the left cerebral ganglion; as far as we can tell, the left and right procerebra are completely symmetrical. The camera lucida drawings were captured and digitized with a video camera by using Video Blaster software (Creative Labs, Milpitas, CA) to permit computer-assisted measurements.

## Retrograde labelling

Dissections were carried out as described above, except that the procerebrum was not desheathed; instead, the region of the ganglion that was to receive the focal injection was desheathed. Alternatively, pronase crystals were applied for about 2 minutes to the region that was to be penetrated. A micropipette was filled with either 2% biocytin or 2% Neurobiotin plus 1% Lucifer yellow in 0.05 M phosphate buffer or 0.1 M Tris buffer. The site of injection was visually localized by the ejection of Lucifer yellow using hyperpolarizing current. The labelling was done by positive iontophoresis of biocytin or Neurobiotin in the targeted neuropil for 10–20 minutes (0.5 second pulses of 0.2–0.45 µA at 1 Hz). The brain was then rinsed twice in saline and stored at 4°C to allow retrograde diffusion of the label to the procerebral cell bodies (1–20 hours). Tissue was fixed either with the same fixative solution as for intracellular labelling or with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Subsequent processing was the same as for intracellular injections. Vibratome sections were cut at 80 µm. Photomicrographs and camera lucida drawings were obtained by using low-magnification (×250) light microscopy.

# RESULTS

## Morphological characterizations

In total, 43 neurons were successfully visualized and reconstructed from 166 procerebra that were dissected and probed. The low yield resulted from the difficulty of obtaining good penetrations without cell damage and the frequent blockage of our finely tipped micropipettes. The spatial distribution of somata from the 43 successfully labelled cells is shown in Figure 2. Random sampling resulted in a good coverage of all parts of the procerebral lobe, from distal to basal ends, from lateral to medial edges, and from dorsal to ventral sides; therefore, we can assume that the cells are representative of the neuronal population in the procerebrum.

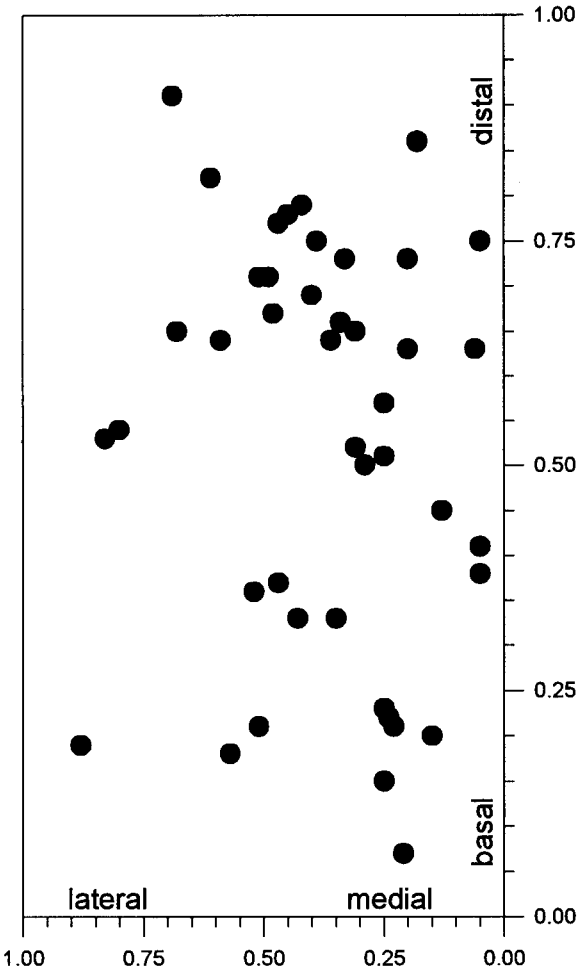


Fig. 2. Distribution of labelled procerebral neurons. Each circle represents the cell body of a labelled neuron. Axes give positions relative to the total length and width of the procerebrum cell body region. A value of 1.0 on the x-axis designates the most lateral position (the farthest from the neuropil); a value of 1.0 on the y-axis designates the most distal position (the closest to the olfactory nerve entry site).

A selection of cells is illustrated in Figure 3, in which certain extreme forms can be seen. For example, whereas neuron C carries a long neurite that lacks any branches in the procerebrum, the neurites in neuron H are short but display profuse and dense arborizations. The morphology of the two cells clearly contrasts, and it is tempting to assign the cells to different subpopulations. However, intermediate forms were also observed. Neurons B and G, for example, have neurites that extend far from the soma but that also arborize along their lengths, even though the branching is not as profuse as in neuron H.

The characterization of cell subpopulations was also investigated through quantitative analysis of the data. A

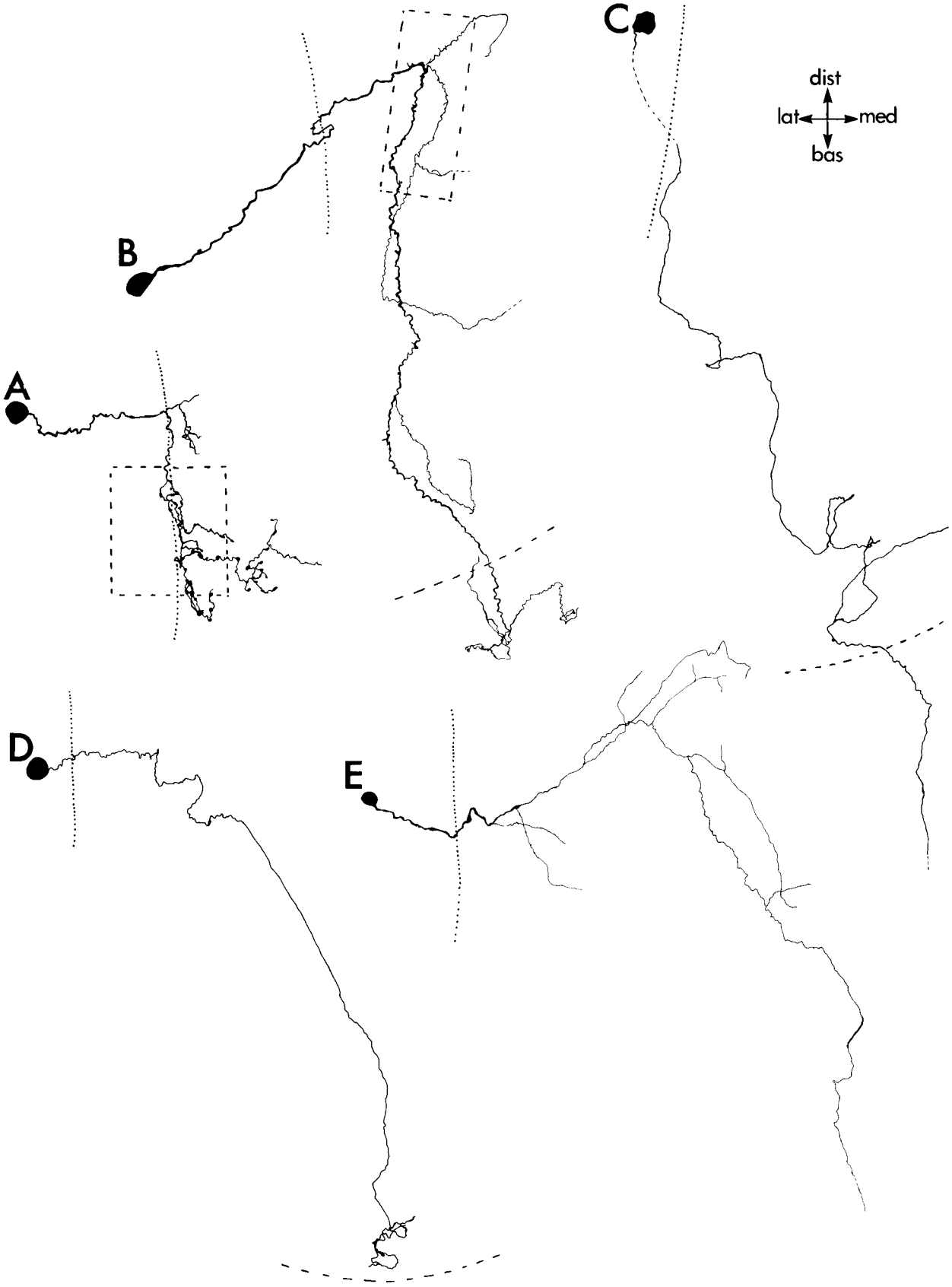


Fig. 3. **A–K:** Camera lucida drawings of a representative sample of procerebral cells. Dashed lengths along the neurites represent areas that were difficult to visualize. The orientation of each drawing is standardized to portray placement in the left cerebral ganglion (as in Fig. 1). Dotted arcs designate the boundary between the procerebrum cell body and neuropil regions; dashed arcs indicate the boundary between the procerebrum and the rest of the cerebral ganglion. Dashed boxes demarcate the areas shown in subsequent figures (Figs. 4, 6, 7). Scale bar = 40 µm.

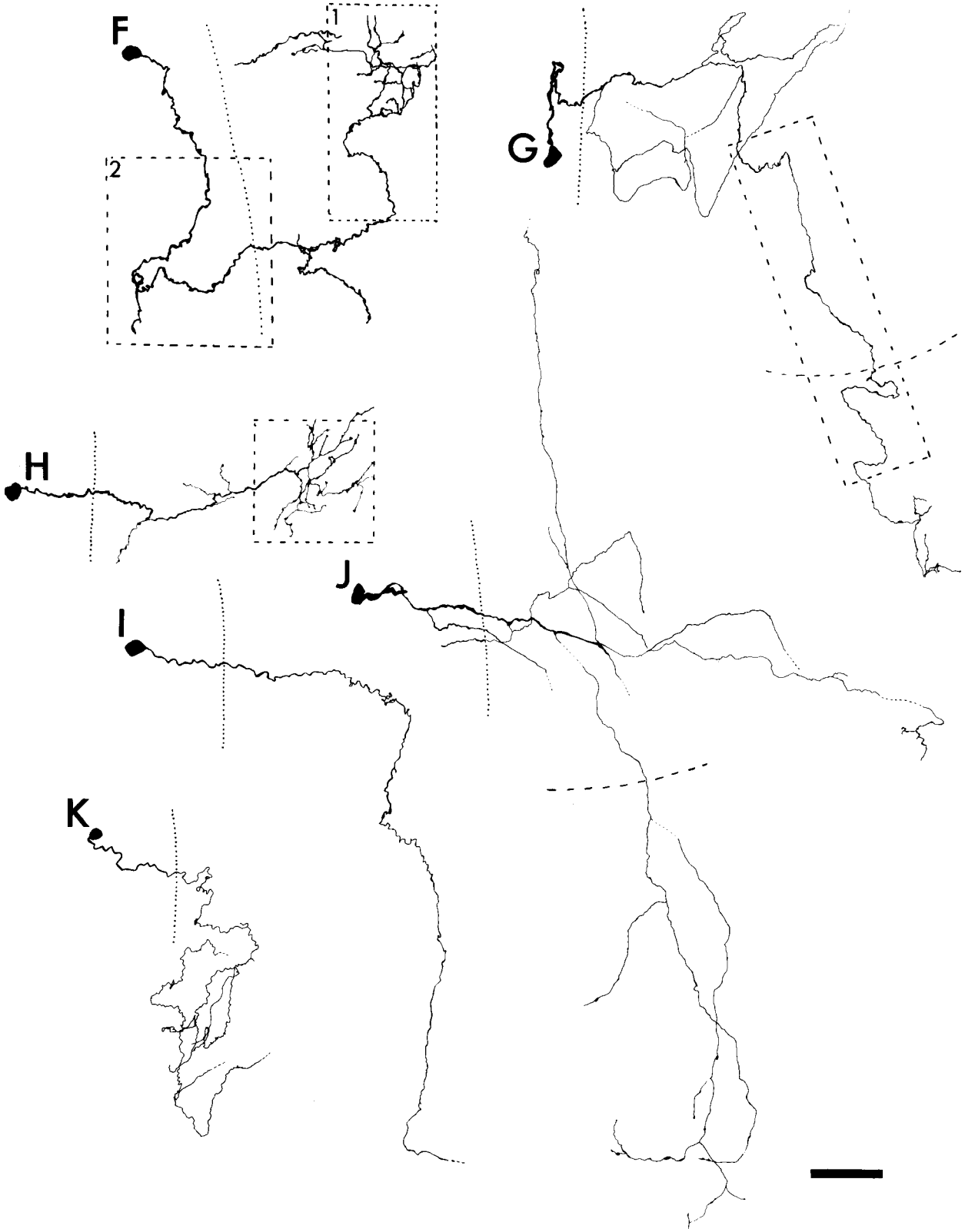


Figure 3 (Continued.)

TABLE 1. Summary of Neuron Morphologies

| Site of arborization | Number of labelled cells | Percentage of sample |
|---|---|---|
| Only in the procerebrum | | |
| Mainly in the neuropil | 21 | 49 |
| Mainly in the internal mass | 7 | 16 |
| Inside and outside the procerebrum | 7 | 16 |
| Only outside the procerebrum | 8 | 19 |

number of measurements were carried out on the reconstructed neurons, namely, 1) the length of the longest neurite (distance), 2) the radial extent of the neurites (displacement), 3) the branching density, 4) the frequency of varicose structures, 5) the percentage of branch points with varicosities, and 6) the percentage of varicosities at branch points. None of the measures yielded an obvious multimodal distribution (data not shown). Thus, neither qualitative nor quantitative assessments allow a rigid categorization of the labelled neurons.

However, the cells vary significantly in the placement of their arborizations and the projection of their neurites (Fig. 3). Whereas some neurons seem to be entirely intrinsic to the procerebrum, others clearly send processes to adjacent or even distant regions of the cerebral ganglion. To facilitate reporting of the intracellular labelling data, cells are described hereafter based on the localization of their arborizations: 1) neurons with only intrinsic arborizations, 2) neurons with both intrinsic and extrinsic arborizations, and 3) neurons with only extrinsic arborizations. Table 1 summarizes the distribution of the sample of labelled cells based on these distinctions.

***Neurons with only intrinsic arborizations.*** In Figure 3, neurons A, F, and H are examples of cells whose neurites are restricted to the procerebrum. Dense arborizations are frequently displayed by such neurons. The branching is usually restricted to a small volume in the neuropil region (Fig. 4A,B), but it may also be observed along the border between the cell body mass and the neuropil (Fig. 4C). Because ultrastructural studies have demonstrated that varicosities are preferred sites of synaptic contacts (Bailey et al., 1979), the presence of varicosities on the cell illustrated in Figure 4C suggests synaptic contacts in the lateral part of the neuropil, which is where the afferent fibers from the olfactory nerve emerge. Figure 4D shows the initial part of cell F's neurite, which also displays varicosities in the soma region, before the neurite reaches the neuropil and arborizes.

Seven of the intrinsic neurons displayed a morphology that was influenced by the structure of the internal mass within the procerebral neuropil. In Figure 5, cells A–C are examples of such cells; they have long neurites that travel almost the entire length of the procerebrum and display very few branches. Only when the neurites enter the internal mass do they start arborizing, looping, and displaying varicosities, suggesting that their interactions and final destinations are targeted to this region. Because these neurons do not seem to project farther than the internal mass, they can be considered as intrinsic cells. The internal mass region of the neuropil was also outlined in a preparation in which a group of three neurons was labelled; in contrast to the neurons described above, which specifically arborize in the internal mass, the neurites of these three cells go around the internal mass in such a manner that they seem to specifically avoid this part of the neuropil (Fig. 5D).

***Neurons with both intrinsic and extrinsic arborizations.*** Neurons B, G, and J in Figure 3 are examples of cells that arborize in the procerebrum but that also have some neurites projecting outside the procerebrum. In this sense, the cells can be considered as intermediates between the extremes described in the preceding and following subsections. In our sample of 43 cells, seven neurons displayed such arborizations. Usually, one neurite was much longer than the others and projected outside the procerebral lobe. For example, Figure 6A,B shows the projecting neurite of cell G (Fig. 3) displaying varicosities as it passes near the internal mass to reach the core of the cerebral ganglion.

When varicose structures occur on neurites of cells that project outside the procerebrum, they are sparsely distributed along the fibers and rarely appear at branch points. Also, as seen in the drawings of neurons B, G, and J (Fig. 3), branch points are widely separated along the length of the neurites; thus, the density of arborization is less than for cells that do not project outside the procerebrum (Fig. 6C). Overall, the neurons with both intrinsic and extrinsic arborizations seem to have intermediate characteristics with regard to the number of varicosities, the density of branch points, and the length of branches compared with cells that have only intrinsic arborizations or cells that have only extrinsic arborizations.

***Neurons with only extrinsic arborizations.*** Among the cells that showed neurites projecting outside of the procerebral lobe, eight showed no arborizations in the procerebrum. Neuron C in Figure 3 is one example. Three additional examples are shown in Figure 7A, where it can be seen that the most distally located cell body has a single neurite that travels, without branching, to the basal procerebrum before branching in the postcerebral neuropil.

Often in such projecting cells, the neurite initially orients medially, but then, once in the neuropil, it abruptly reorients basally and exits the procerebrum (Fig. 7B). Other neurons send their projecting process directly toward the central core of the cerebral ganglion without entering the procerebral neuropil (not shown). Five of the 43 cells showed this latter feature, and they were all cells that did not arborize in the procerebrum.

Many of the unadorned fibers distant from the soma were as small as 0.5 μm. Even near the soma, neurite diameter was rarely greater than 1 μm. Very fine projecting fibers could sometimes be followed far into the cerebral ganglion (Fig. 7C). The farthest any neurite projected from the cell body was 967 μm. However, because, in some cases, the labelling of the cells was clearly incomplete as the density of the label was seen to taper toward the visible end of the fiber, the full extent of some of the neurites was not discernable using intracellular injections.

## Orientation of neurites

In light of the fact that the procerebral cells were thought to be anaxonal and intrinsically arborizing (Chase and Tolloczko, 1993), the identification of substantially long fibers is a novel and consequential observation. The morphological data seem to indicate that the majority of these lengthy neurites are oriented basally, regardless of the position of the cell soma in the procerebrum or the occurrence of extrinsic arborizations. However, the extent to which the neurites arborize externally might have been underestimated because of incomplete diffusion of label.

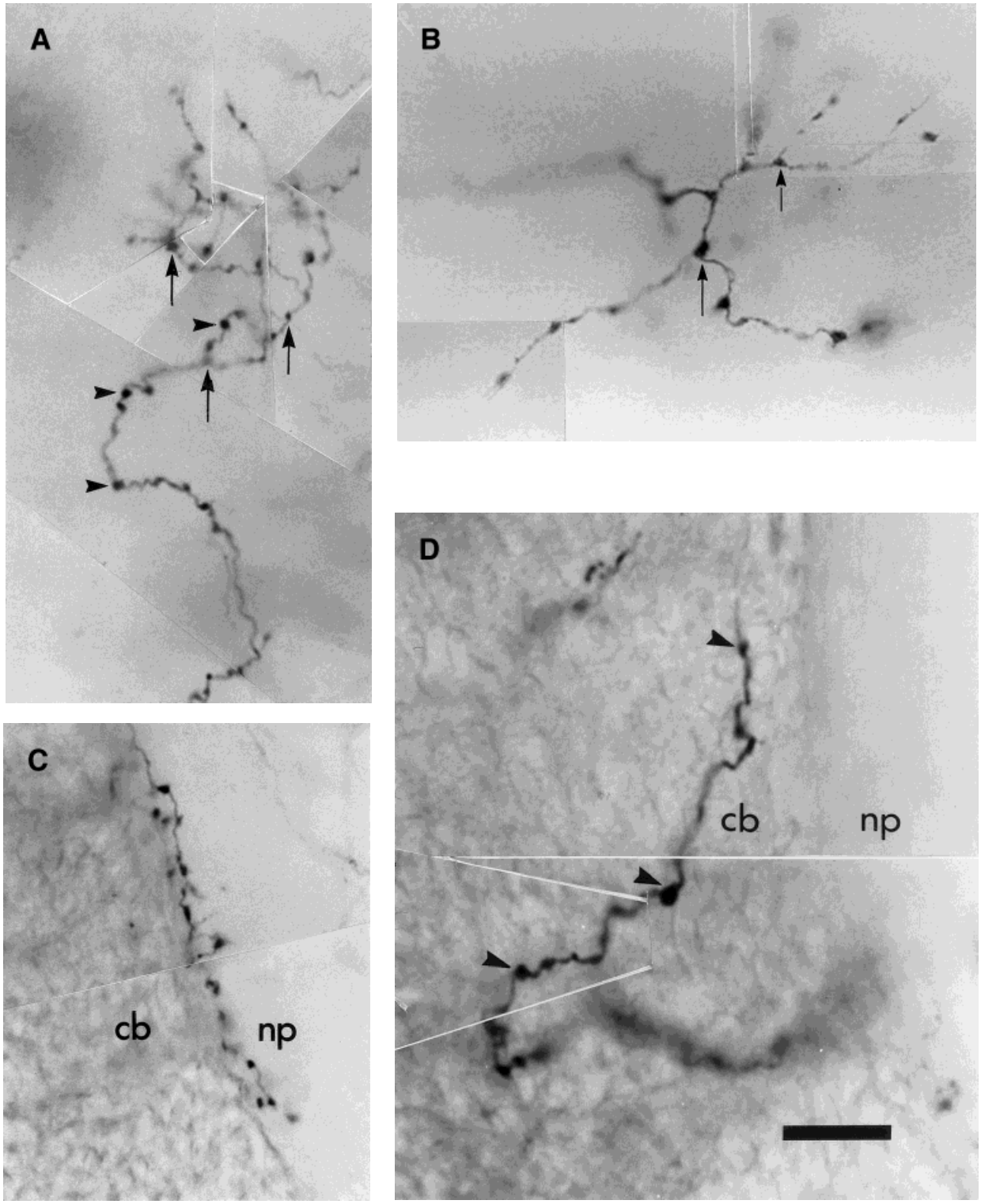


Fig. 4. Neurons with arborizations restricted to the procerebrum. The photographic fields are indicated by boxes in Figure 3, which should be referred to for complete drawings of the cells illustrated here. Letters refer to cell identification labels A–K in Figure 3. Varicosities can be observed along neurites (arrowheads) and at branch points (arrows). **A:** Cell F (box 1, Fig. 3). Note the profuse arborization with numerous varicosities. **B:** Cell H. Branch point varicosities can be clearly seen at high magnification. **C:** Cell A. This neuron displays fibers that closely follow the border between the cell body mass and the neuropil. The neurite possesses varicosities, indicating possible synaptic interactions in this area. **D:** Cell F (box 2, Fig. 3). The neurite displays varicosities while it is still in the cell body region. Scale bars = 20 μm in A, 15 μm in B,D, 25 μm in C.

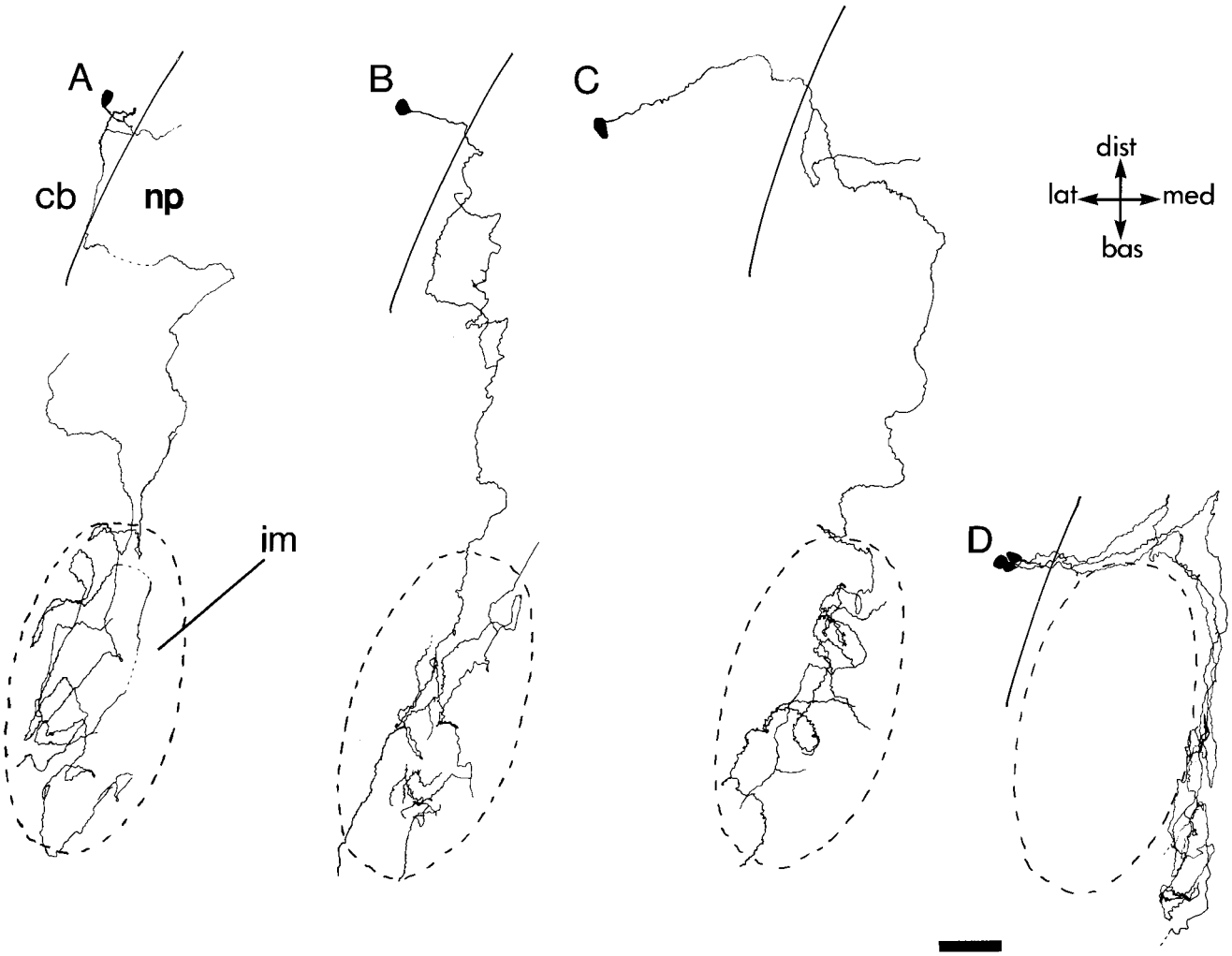


Fig. 5. Procerebral neurons that arborize in the internal mass. Solid arcs indicate the boundary between cell bodies and neuropil, and dashed ovals demarcate the internal mass. **A–C:** Neurons with branches largely in the internal mass of the neuropil. **D:** Three neurons filled from a single injection. The neurites follow a path around the perimeter of the internal mass. Scale bar = 40 μm.

We reasoned that, because a neurite that is to exit the procerebrum will necessarily be oriented basally, the proportion of procerebral cells possessing basally oriented neurites will be an indication of the proportion of neurites that is putatively exiting the procerebrum. To quantify this basal directionality, for each labelled neuron, we calculated a mean vector value representing the mean neurite orientation. The results presented in the histogram of Figure 8 show that, as expected from qualitative observations, there is a definite tendency toward negative values for the mean vector. Although this does not directly reflect the extent to which cells have neurites that actually do exit the procerebrum, it nevertheless indicates an overall tendency for neurites to orient basally.

For most of the cells, the mean vector length was above 0.9. Because the maximum possible length value is 1.0, which would indicate that all the neurites of a cell project in the same direction, values above 0.9 indicate cells that have neurites clustered closely around the mean vector. Because most of the vectors are long, it may be inferred that the neurites of a given cell generally show little variation in their orientation and that the values given in the histogram of Figure 8 are reliable estimates of the orientation of the neurites of procerebral cells. There were only a few exceptions to this, an example of which is neuron G in Figure 3. For this neuron, many processes arborize and terminate near the soma, but the remaining neurites terminate far from the soma and at a large negative angle. Such a dispersion in neurite terminations resulted in a small vector length for only three cells.

## Retrograde labelling

Focal injections of label reveal subsets of procerebral neurons that have a neurite either terminating in or passing through the region into which the label is injected. We injected biocytin and Neurobiotin into various regions of the cerebral ganglion and observed labelled cells not only in the ipsilateral procerebrum but also in the contralateral procerebrum (Fig. 9). Figure 9A shows a site of injection into the left postcerebrum and labelled cells in the basal part of the right procerebrum. Because only one

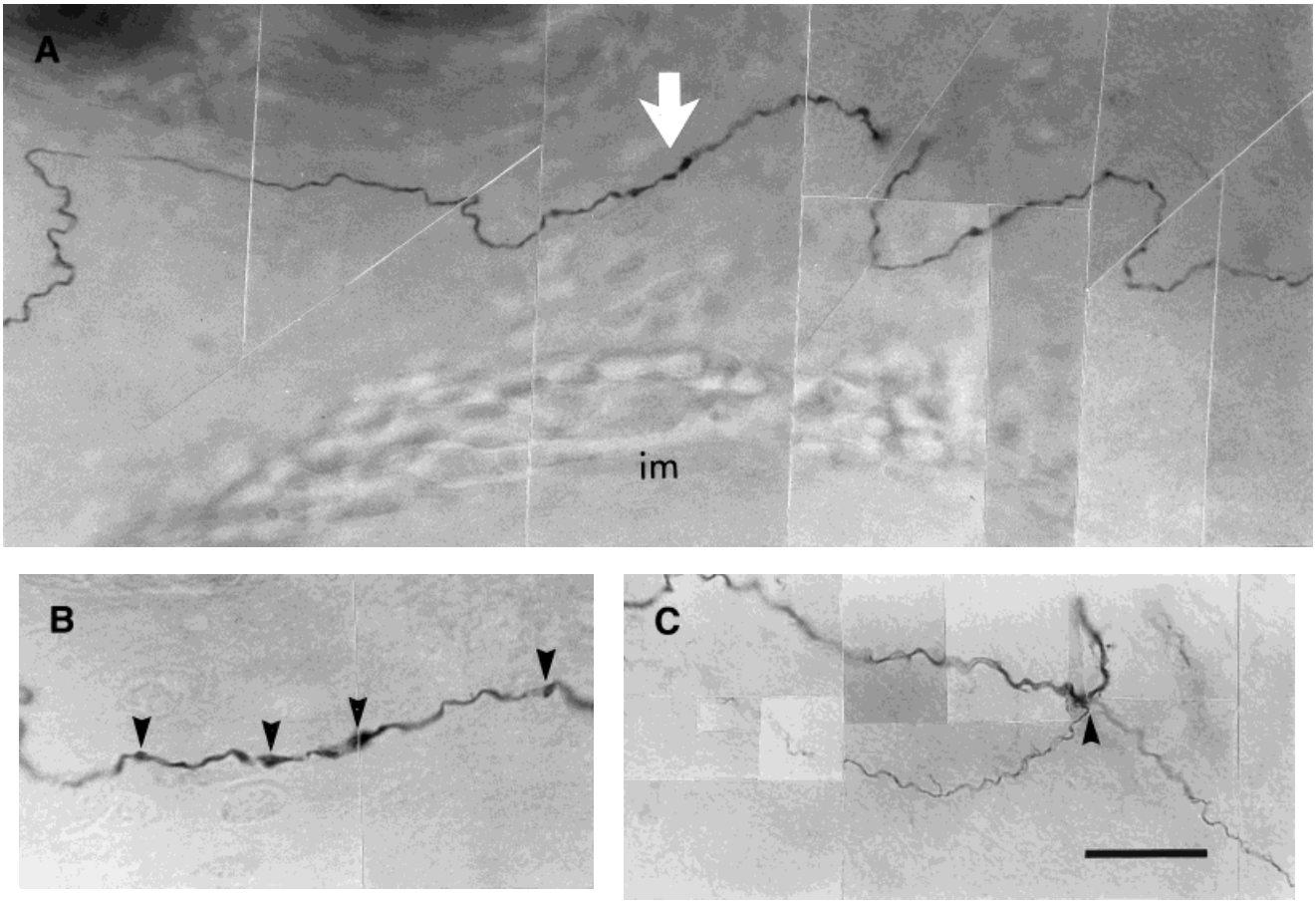


Fig. 6. Neurons that arborize both within the procerebrum and outside of it. Each photographic field corresponds to a box on the whole cell drawings of Figure 3. **A:** Cell G. The projecting fiber meanders in the neuropil before it exits the procerebrum. The neurite, as it passes near the internal mass, exhibits several varicosities. The arrow points to the area shown at higher magnification in B. **B:** Varicosities (arrowheads) of cell G shown at higher magnification. **C:** Three branches emerging from a single point (arrowhead) on neuron B. Note the sparsity of branch points and the length of the branches relative to the neurons shown in Figure 4. Scale bars = 20 μm in A, 10 μm in B, 35 μm in C.

preparation revealed contralateral projections, there is not enough evidence to conclude that all contralaterally projecting neurons are located basally; however, from the intracellular labelling experiments, all of the cells that had neurites extending in a contralateral direction also had cell bodies located in the basal region of the procerebrum.

A summary of the results from the focally injected preparations is presented in Figure 10. Injections into the mesocerebrum and the postcerebrum resulted in labelled somata located mostly in the basal half of the procerebrum cell body region. Although this could indicate a topographical segregation, we believe, instead, that the pattern of labelling resulted from a failure of the label to reach the distally located somata in the procerebrum due to limitations of diffusion. In fact, intracellular injections did reveal some distally located somata that possessed arborizations in the postcerebrum (Fig. 7A). Data combined from both techniques thus suggest that the somata of neurons with arborizations in the mesocerebrum and the postcerebrum are distributed throughout the procerebrum cell body region. With metacerebrum injections, labelled cell bodies could be observed from the distal end to the basal end of the procerebrum, but there was a tendency for basally located cells to be in a ventral position (Fig. 10B).

## DISCUSSION

By using intracellular injection techniques, our study reveals that, in addition to the known intrinsic arborizations, procerebral interneurons project outside of the procerebrum and possess extrinsic arborizations (Table 1). These extrinsic arborizations were not revealed in previous studies using Golgi impregnation techniques (Veratti, 1900; Haller, 1913, Hanström, 1925; Zaitseva, 1992). Although Golgi impregnations often yield excellent results, the method is capricious and unpredictable in many nervous tissues (Strausfeld, 1980). Thus, the results previously obtained with this method in the snail procerebrum seem to have been incomplete.

The intracellular labelling technique, however, does not exclude all problems of interpretation. In many of the filled cells, the exact site of a neurite's termination often remained uncertain despite the fact that neurites were shown to be longer than seen in previous studies. In some cases, it was clear that the neuron had only intrinsically arborizing neurites, because terminals could be clearly identified (10 of 21 cells; Table 1). In the remaining 11 cells with neurites apparently limited to the procerebrum, the lack of clear identification of terminals raises doubts about

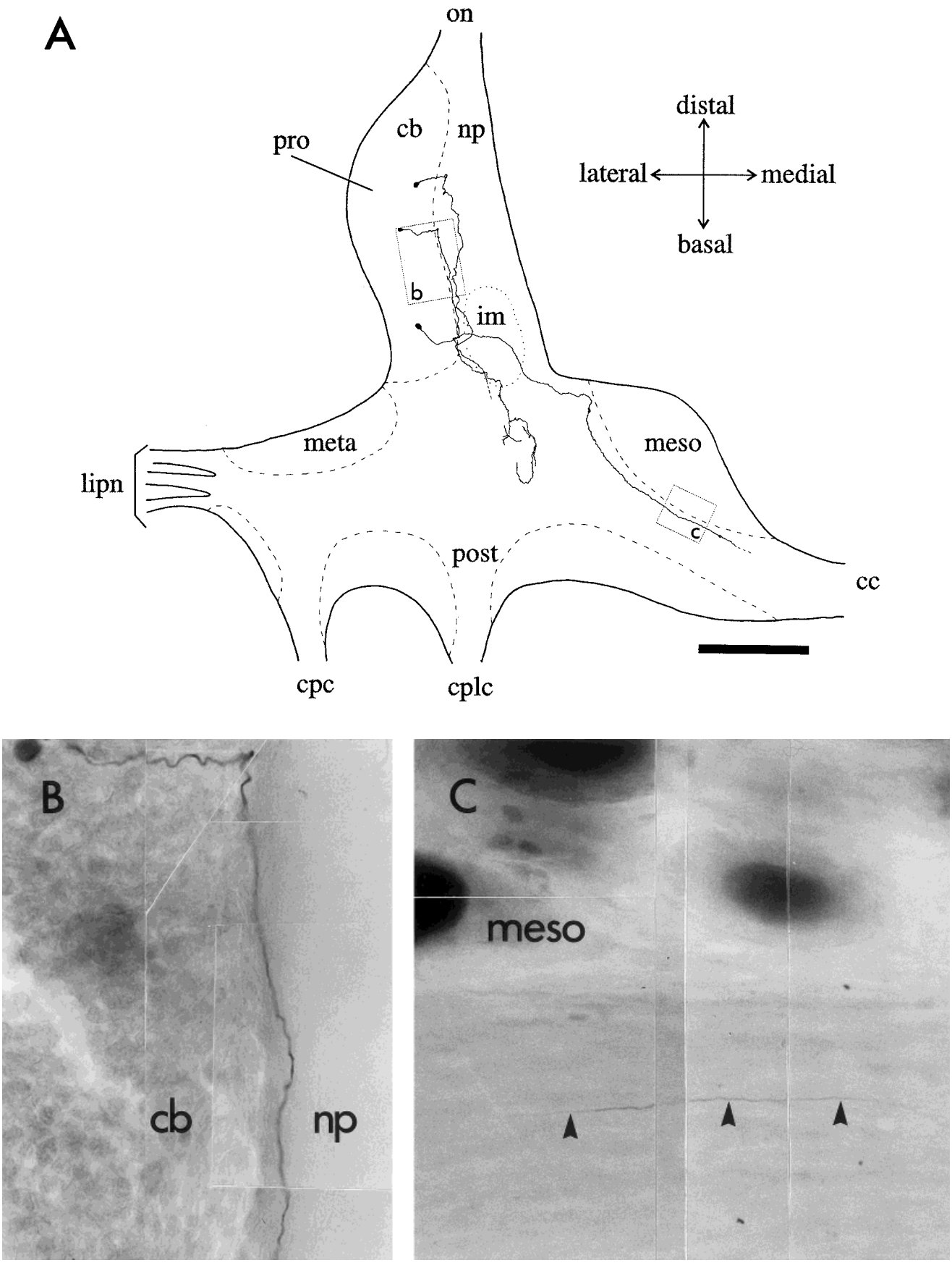


Fig. 7. Neurons with only extrinsic arborizations. **A:** Neurite extensions of three projecting neurons. Boxes refer to the illustrations in B and C. **B:** A cell whose neurite travels directly toward the neuropil (top of figure) and then reorients toward the basal limit of the procerebrum. It eventually leaves the procerebrum to enter other regions of the brain. **C:** A neurite (arrowheads) that is seen travelling far outside the procerebral lobe. The fiber passes through the mesocerebrum and toward the cerebral commissure. For abbreviations, see Figure 1. Scale bars = 225 µm in A, 40 µm in B, 30 µm in C.

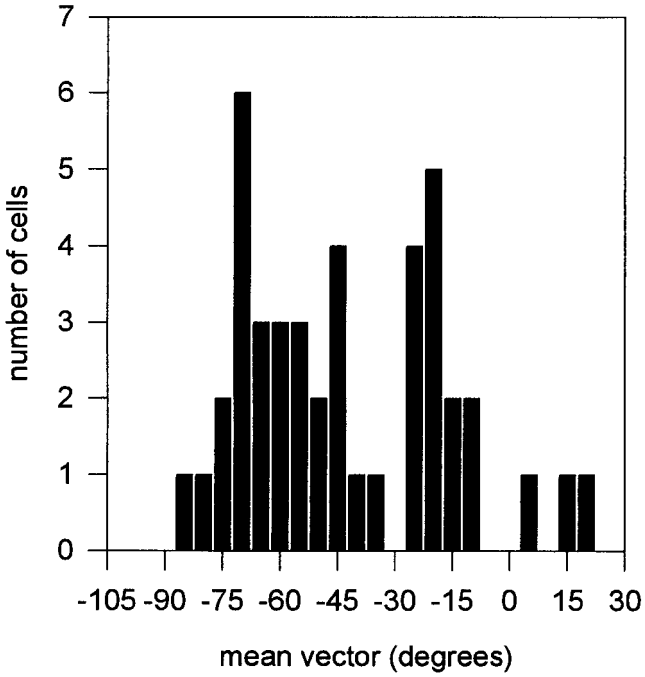


Fig. 8. Orientation of neurites. A mean vector was calculated for each neuron by using a Cartesian coordinate system, in which the 0° defining vector has its origin in the soma and passes perpendicularly through the medial edge of the procerebrum (Batschelet, 1981). From this defining vector, positive rotation was counterclockwise. Vectors for the neurites belonging to a cell were determined by drawing straight lines from the axial origin to the end of the neurites. The vector length was normalized to 1 for every neurite to avoid an influence of neurite length on the mean vector measurement. In light of this normalization, cosine and sine values of each measured angle determined the unitless x and y components, respectively. The components of the multiple vectors of each cell were added and averaged, resulting in the coordinate values of a cell's mean vector. By using the Pythagorean theorem, the length of the mean vector was calculated, and the angle from the 0° defining vector was determined. Cells with neurites oriented toward the olfactory nerve consequently have positive values, whereas those with neurites oriented toward the base of the procerebrum have negative values. The histogram demonstrates the propensity of neurites to be oriented basally. The few cells that have a positive mean orientation are those that have numerous small neurites of no preferred direction, for example, cell H in Figure 3; none of the labelled cells project into the olfactory nerve.

the completeness of the fill. It is therefore possible that the projecting neurites of some cells were not detected, in which case, the numbers given in Table 1 would underestimate the actual number of procerebral neurons with extrinsic arborizations. This idea is consistent with the observation that the calculated mean orientation of neurites indicated an overall tendency for procerebral cells neurites to orient basally. Even in the cases where neurites were seen to exit the procerebrum, the exact site of termination was often uncertain, and the extent of projection is probably underestimated.

The cellular morphology did not reveal any gross structural organization in the procerebrum, such as tracts, glomeruli, or lamination. In the neuropil, neurites were not predisposed to run at any particular position along any of the axes, and the neurites exiting the procerebrum seemed to do so in a random fashion. Nor do the sites of arborization suggest any spatial organization, with the exception of the internal mass, whose presence was made apparent by some of the cells' morphologies (Fig. 5). Several immunohistochemical studies have demonstrated that the neurochemical content of the internal mass is different from the rest of the procerebral neuropil (Cooke et al., 1994; Elekes et al., 1994). However, the functional significance of this structure and, thus, that of the cells that arborize in it remains uncertain.

It may be suggested that the differences in the neurons' morphologies and the extent of their arborizations reflect a developmental process. However, neurogenesis in the procerebrum is limited to the distal end (Zakharov and Ierusalimski, 1995); therefore, if our labelled neurons were at different developmental stages, then they would be differentially distributed, such that the less developed cells would be distally located, and the more developed ones would be proximally located, but this is not the case. Somata of neurons with various sites of arborizations are distributed indifferently along the procerebrum length, width, and depth. Differences in neuronal development, therefore, cannot account for differences in the extent of arborization and projection.

Although a subtle variation in the distribution of procerebral neurons, based on physiological criteria, was observed by Kleinfeld et al. (1994), the morphological data argue against a strong compartmentalization of the procerebral cell population. Previous studies using 2-deoxyglucose autoradiography support the absence of discrete spatial compartments, because they demonstrated that no focus of activity can be observed from stimulation with different odors (Chase, 1985). However, some preliminary observations recently made by Gervais et al. (1996) in *Limax* suggest that discrete groups of procerebral cells might decouple for a few seconds from the overall oscillation when specific odors are presented. The question of compartmentalization may have to be reconsidered in light of such results, but, for now, the evidence is not sufficiently strong to prove either a structural compartmentalization or a spatial coding of odors in the procerebrum.

The varicosities displayed on the neurites, especially on those of intrinsically arborizing cells, suggest that synaptic interactions occur not only in the core of the procerebral neuropil but also at the edge between the cell body region and the neuropil, where one of the tracts formed by the olfactory nerve travels. Varicosities are also present in the cell body region. Zs-Nagy and Sakharov (1970) reported, both in *Limax* and in *Helix*, the presence of axosomatic synapses, but Chase and Tolloczko (1989) later reported an absence of synaptic specializations in the soma region, despite the presence of large numbers of vesicles in fiber profiles. Our data suggest that the question needs to be examined once again. Finally, varicosities could be observed along projecting fibers while they were still in the procerebrum as well as on such fibers outside the procerebrum and at presumed termination sites. Because such varicosities could signify either unilateral output sites or symmetrical input and output sites (Bailey et al., 1979), the information flow in the procerebral cells and the circuitry that emerges from the distribution of input and output sites remains unknown.

Our results demonstrate that the procerebral cell population is not morphologically homogeneous, as most previous studies had suggested (Zs-Nagy and Sakharov, 1970; Chase and Tolloczko, 1989; Zaitseva, 1992). Although the extremes of the spectrum are strikingly different from

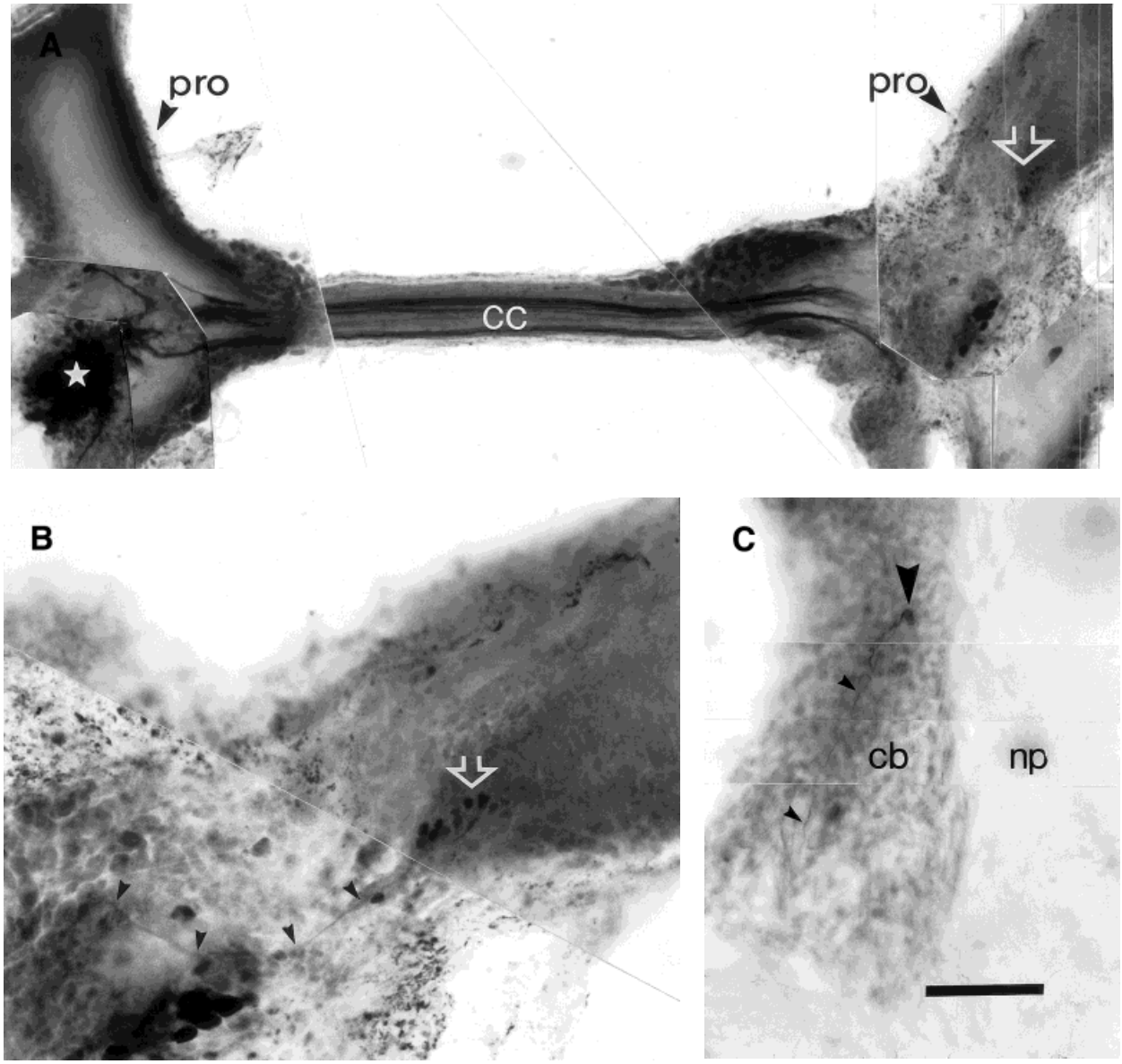


Fig. 9. Retrogradely labelled procerebral neurons resulting from focal injections of biocytin in various regions of the cerebral ganglion. **A:** A group of cells (arrow) labelled in the right procerebral lobe by an injection into the left cerebral ganglion (star). **B:** Higher magnification view of the cells labelled in A (arrow). The arrowheads indicate a small bundle of labelled fibers that presumably belong to the same group of labelled cell bodies. **C:** Representative example of a retrogradely labelled cell body (large arrowhead) resulting from an injection of label in the ipsilateral cerebral ganglion. The small arrowheads indicate the cell's neurite. Scale bars = 170 μm in A, 100 μm in B, 65 μm in C.

each other (Fig. 3), no definite categorization can yet be made, because the cells differ from one another in a graded manner. Hence, the physiological characteristics of two classes of cells, identified in *Limax* by Kleinfeld et al. (1994) and defined as bursting and nonbursting, cannot be attributed to the neurons described by our morphological data. Our own attempts to record electrophysiologically the activity patterns of the neurons before they were injected with the label were unsuccessful probably because of the very high electrode resistance. Differences in the arborization patterns and projection extents, however, must underlie different contributions to the lobe's functional activity. Whereas intrinsically arborizing neurons probably mediate local interactions, projecting neurites are likely to exchange information between the procerebrum and other central elements of the nervous system. The fine projecting neurites are surprisingly long (more than 1.5 mm in some cases), especially because procerebral cells were initially thought to be anaxonal. Action potential generation and propagation is probably necessary in these neurites for any signal to be conducted to distant sites. Passive conductance in the procerebral neurites would presumably not be sufficient for anything other than local interactions, because the space constant for such fibers (diameter approximately 0.5 μm) is expected to be small. In fact, intracellular recordings in *Limax* indicate that all procerebral cells can generate spikes (Kleinfeld et al., 1994). Hence, even though the projecting neurites do

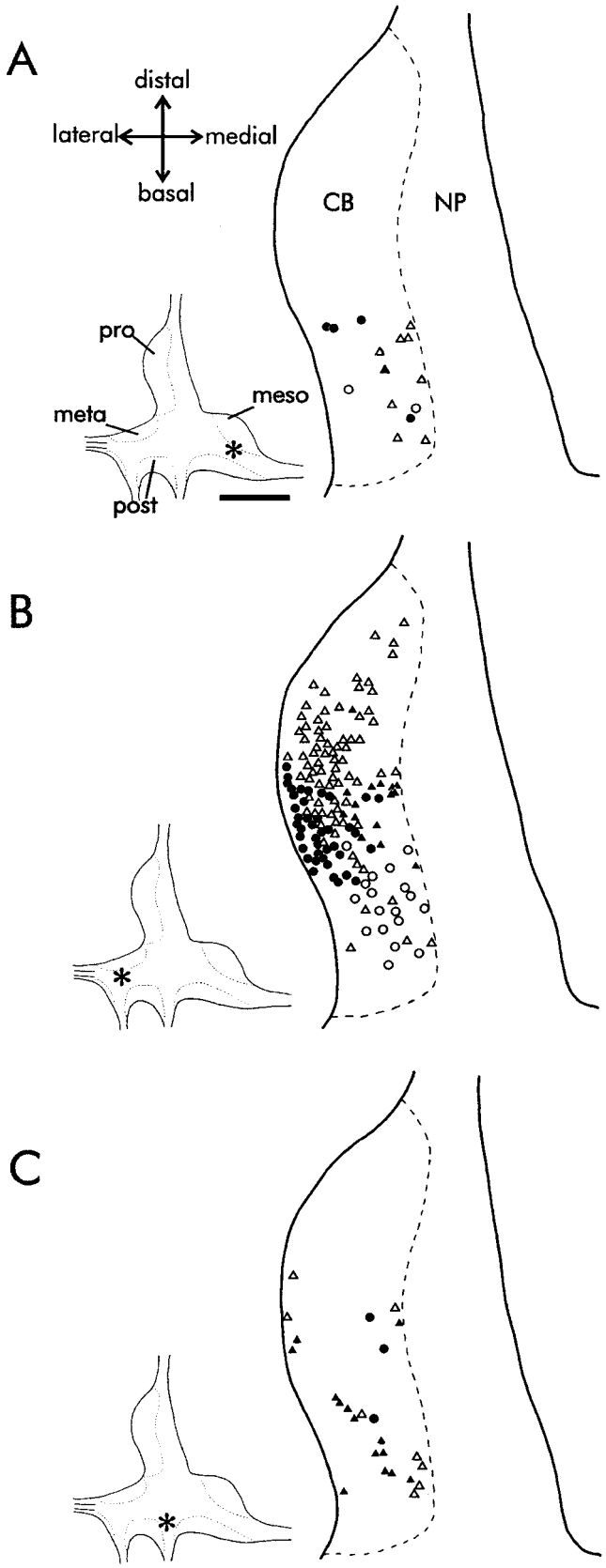


Fig. 10. Retrograde fills observed after iontophoresis of biocytin in selected regions of the ipsilateral cerebral ganglion. The asterisks mark the injection sites. Each symbol indicates the position of a retrogradely labelled cell body in the procerebrum. The different symbols indicate the position of the cells in relation to the dorsal/ventral axis (in order, from the most dorsal to the most ventral, solid triangles, open triangles, solid circles, open circles). Results from several preparations are shown collectively (n = 4 in A; n = 2 in B; n = 3 in C). For abbreviations, see Figure 1. Scale bar = 525 μm for the small drawings; 100 μm for the large drawings.

not display typical axonal features (e.g., a straight process with a constant diameter and few branches), it is likely that they propagate signals actively.

The novel observations in our study allow new comparisons between the snail's olfactory pathway and other model systems for olfactory processing. In the insect olfactory system, for instance, the mushroom body is possibly analogous to the procerebrum. After integration is achieved within the mushroom bodies, multiple channels send the resulting output to various regions of the brain (Mobbs, 1982; Rybak and Menzel, 1993). Our results suggest that the processed information is similarly diverged at the level of the procerebrum in the snail olfactory system. However, the organization of the procerebrum and the mushroom body differs, in that procerebral neurons project and arborize outside of the lobe, whereas Kenyon cell fibers are restricted to the mushroom body structure (Mobbs, 1982). A comparison can also be made with the vertebrate system, because the variability in arborizations and projections in the procerebrum invites an analogy to the olfactory bulb. In the bulb, locally arborizing granule cells synapse with mitral cells and tufted cells. The latter cells have both local outputs (i.e., back onto the granule cells) as well as projections to more central regions of the brain. A similar arrangement might occur in the snail procerebrum, where some neurons that have only local interactions (granule cell analogues) might synapse onto others that project outwardly (mitral and tufted cell analogues). Chase and Tolloczko (1989) observed symmetrical synapses in the procerebrum of *Achatina fulica* that are comparable to the granule cell-mitral cell reciprocal synaptic arrangements in the olfactory bulb (Rall et al., 1966). Investigations need to be carried out to identify which neurons these symmetrical synapses are distributed on in order to elucidate the underlying circuitry in the procerebrum and further pursue the analogy between the two systems.

If it was true, as previously thought, that the procerebral interneurons had only intrinsic arborizations, then their interactions would be limited to cells sending processes into the procerebrum. The only central elements that have been identified as possessing such neurites are two groups of cells in each pedal ganglion (Chase and Tolloczko, 1989) and a pair of cells in the buccal ganglia (Gelperin and Flores, 1997), all of which have been shown to be receptive to the procerebrum. Because our data show that procerebral cells themselves send neurites to various regions of the cerebral ganglion, it is possible for the activity of these neurons to have a widespread influence on the brain's activity. Alternatively, the described projections might provide a pathway by which extraprocerebral regions of the cerebral ganglion could modulate procerebral activity. The cerebral ganglion is the major neural structure where sensory information is integrated and motor

actions are triggered and modulated. The precise status of the ensemble of procerebral interneurons, placed somewhere between the olfactory sensory input and the motor elements of the central nervous system, is still unclear. However, the morphology of these interneurons suggests that they play a major role in the circuitry that influences appropriate behaviors as a result of olfactory stimulation.

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## LITERATURE CITED

Bailey, C.H., E.B. Thompson, V.F. Castellucci, and E.R. Kandel (1979) Ultrastructure of the synapses of sensory neurons that mediate the gill-withdrawal reflex in *Aplysia*. J. Neurocytol. *8:*415–444.

Batschelet, E. (1981) Circular Statistics in Biology. New York: Academic Press, pp. 7–18.

Chase, R. (1981) Electrical responses of snail tentacle ganglion to stimulation of the epithelium with wind and odors. Comp. Biochem. Physiol. *70A:*149–155.

Chase, R. (1982) The olfactory sensitivity of snails, *Achatina fulica*. J. Comp. Physiol. *148A:*225–235.

Chase, R. (1985) Responses to odors mapped in snail tentacle and brain by 2-deoxyglucose autoradiography. J. Neurosci. *5:*2930–2939.

Chase, R. (1986) Lessons from snail tentacles. Chem. Senses *11:*411–426.

Chase, R., and R. Kamil (1983) Neuronal elements in snail tentacles as revealed by horseradish peroxidase backfilling. J. Neurobiol. *14(1):*29–42.

Chase, R., and B. Tolloczko (1989) Interganglionic dendrites constitute an output pathway from the procerebrum of the snail *Achatina fulica*. J. Comp. Neurol. *283:*143–152.

Chase, R., and B. Tolloczko (1993) Tracing neural pathways in snail olfaction: From the tip of the tentacles to the brain and beyond. Microsc. Res. Tech. *24:*214–230.

Cooke, I.R.C., S.L. Edward, and C.R. Anderson (1994) The distribution of NADPH-diaphorase activity and immunoreactivity to nitric oxide synthase in the nervous system of the pulmonate mollusc *Helix aspersa*. Cell Tissue Res. *277:*565–572.

Croll, R., and R. Chase (1980) Plasticity of olfactory orientation to foods in the snail *Achatina fulica*. J. Comp. Physiol. *136:*267–277.

Delaney, K.R., A. Gelperin, M.S. Fee, J.A. Flores, R. Gervais, D.W. Tank, and D. Kleinfeld (1994) Waves and stimulus-modulated dynamics in an oscillating olfactory network. Proc. Natl. Acad. Sci. USA *91:*669–673.

Elekes, K., L. Hernádi, J.E. Muren, and D.R. Nässel (1994) Peptidergic neurons in the snail *Helix pomatia*: Distribution of neurons in the central and peripheral nervous systems that react with an antibody raised to the insect neuropeptide, leucokinin I. J. Comp. Neurol. *341:*257–272.

Gelperin, A., and J. Flores (1997) Vital staining from dye-coated microprobes identifies new olfactory interneurons for optical and electrical recording. J. Neurosci. Methods (in press).

Gelperin, A., and D.W. Tank (1990) Odour-modulated collective network oscillations of olfactory interneurons in a terrestrial mollusk. Nature *345:*437–440.

Gelperin, A., J.J. Hopfield, and D.W. Tank (1985) The logic of *Limax* learning. In A.I. Selverston (ed): Model Neural Networks and Behavior. New York: Plenum Press, pp. 237–261.

Gervais, R., D. Kleinfeld, K.R. Delaney, and A. Gelperin (1996) Central and reflex neuronal responses elicited by odor in a terrestrial mollusc. J. Neurophysiol. *76(2):*1327–1339.

Haller, B. (1913) Die Intelligenzphären des Molluskengehirns. Arch. Mikrosk. Anat. *81:*233–322.

Hanström, B. (1925) Uber die sogenannten Intelligenzsphären des Molluskengehirns und die Innervation des Tentakels von *Helix*. Acta Zoologica *6:*183–215.

Kleinfeld, D., K.R. Delaney, M.S. Fee, J.A. Flores, D.W. Tank, and A. Gelperin (1994) Dynamics of propagating waves in the olfactory network of a terrestrial mollusk: An electrical and optical study. J. Neurophysiol. *72(3):*1402–1419.

Mobbs, P.G. (1982) The brain of the honeybee *Apis Mellifera*. I. The connections and spatial organization of the mushroom bodies. Phil. Trans. R. Soc. London B *298:*309–354.

Rall, W., G.M. Shepherd, T.S. Reese, and M.W. Brightman (1966) Dendrodendritic synaptic pathway for inhibition in the olfactory bulb. Exp. Neurol. *14:*44–56.

Rybak, J., and R. Menzel (1993) Anatomy of mushroom bodies in the honey bee brain: The neural connections of the α-lobe. J. Comp. Neurol. *334:*444–465.

Schütt, A., and E. Basar (1994) Olfactory field potential oscillations in the isolated *Helix* brain: Different odors induce different rhythmicities (Abstract). Proceedings of the 39th Annual Meeting of the German Electroencephalography Society.

Strausfeld, N.J. (1980) The Golgi method: Its application to the insect nervous system and the phenomenon of stochastic impregnation. In N.J. Strausfeld and T.A. Miller (eds): Neuroanatomical Techniques, Insect Nervous System. New York: Springer-Verlag, pp. 131–203.

Van Mol, J.J. (1974) Evolution phylogénétique du ganglion cérébroïde chez les gastéropodes pulmonés. Haliotis *4:*77–86.

Veratti, E. (1900) Ricerche sul sistema nervoso dei *Limax*. Mem. Ist. Lombardo *18:*163–179.

Zaitseva, O.V. (1992) Structural organization of the snail sensory systems [in Russian; translation in Neurosci. Behav. Physiol. *24(1),* 1994]. Zhurnal Vysshei Nervnoi Deiatelnosti Imeni I.P. Pavlova *42:*1132–1149.

Zakharov, I.S., and V.N. Ierusalimski (1995) Postembryonic neurogenesis of the olfactory analyzer in the snail. Doklady Akad. Nauk *342(3):*418–420.

Zs-Nagy, I., and D.A. Sakharov (1970) The fine structure of the procerebrum of pulmonate molluscs, *Helix* and *Limax*. Tissue Cell *2:*399–411.