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Interneurons of the subesophageal ganglion of *Sarcophaga bullata* responding to gustatory and mechanosensory stimuli

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Summary. Intracellular recordings were made from interneurons in the subesophageal ganglion (SEG) of *Sarcophaga bullata* while stimulating the labellar lobes with solutions of sucrose, NaCl and with distilled water. Neurons that responded to sucrose did not respond to NaCl and vice versa, while sucrose-sensitive neurons often responded weakly to water. Several of the recorded neurons were filled with Lucifer Yellow, and their morphology was reconstructed. Most showed extensive arborizations within the SEG, suggesting that they were local interneurons involved in the early stages of gustatory processing. Some of the filled neurons had extensive projections to the brain, in addition to arborizations in the SEG. This is the first published record of gustatory interneurons in the higher flies.

Key words: Taste – Gustatory coding – Subesophageal ganglion – Local interneurons – Labellar taste hairs – *Sarcophaga* – Feeding behavior – *Phormia* – *Calliphora*

Introduction

Blowflies and flesh flies are model organisms for studying the physiology of gustation, mechanoreception and the regulation of feeding behavior by chemical and mechanical stimuli. It has been known for some time that, with suitably starved flies, stimulation of a single labellar sensillum with sucrose can lead to activity in muscles involved in feeding (Getting 1971). The sugar-sensitive cells convey information which is primarily responsible for initiating feeding behavior. The physiology of these sugar-sensitive cells, in both tarsal and labellar sensilla, has been thoroughly studied (see Morita and Shiraishi 1985), and the large body of information on fly feeding behavior and its mediation by sugars and other compounds is summarized in Dethier (1976). The role of the central nervous system in the initial stages of feeding behavior has been indirectly explored by simultaneously recording afferent input and motor output, via muscle recording, to selected muscles of the proboscis (Getting 1971; Fredman and Steinhardt 1973; Fredman 1975).

The morphology of the sensory and motor neurons of this system has received less attention than their physiology, but recent work shows that cells of the labellar sensilla project exclusively to specific regions of the subesophageal ganglion (SEG) (Yetman and Pollack 1986; Edgecomb 1986; Edgecomb and Murdock 1992). The motor neurons of the various proboscal muscles, including the protractor of the furca, extensor of the haustellum, retractor muscles and the cibarial dilators (sucking pump) also have their cell bodies and extensive arborizations exclusively in the SEG (van Mier et al. 1985; Sudlow 1991).

The interneurons involved in proboscal movements are virtually unknown. Using delay time calculations from simultaneously recorded sensory and motor activity, Getting (1971) proposed that two levels of interneuron integration may be involved in processing sugar cell input, while van Mier et al. (1985), reported an apparent overlap of sensory and motor cell arborizations in the SEG, and suggested that some taste afferents may synapse directly with motor neurons. The rapid proboscis extension response of these flies is compatible with relatively simple integration within the SEG neuropil. Altman and Kien (1987) also suggested that much of the integration related to the regulation of feeding could occur within the SEG.

This study had two primary objectives. First, to develop a preparation that could be used to record directly from SEG neurons while stimulating the labellum with various chemical solutions and second, to record from and mark with fluorescent dye, interneurons which responded to mechanical, sugar, salt and water stimulation of the labellar sensilla. We report the first physiological and morphological characterization of such interneurons.

Abbreviations: *LY* lucifer yellow; *SEG* subesophageal ganglion

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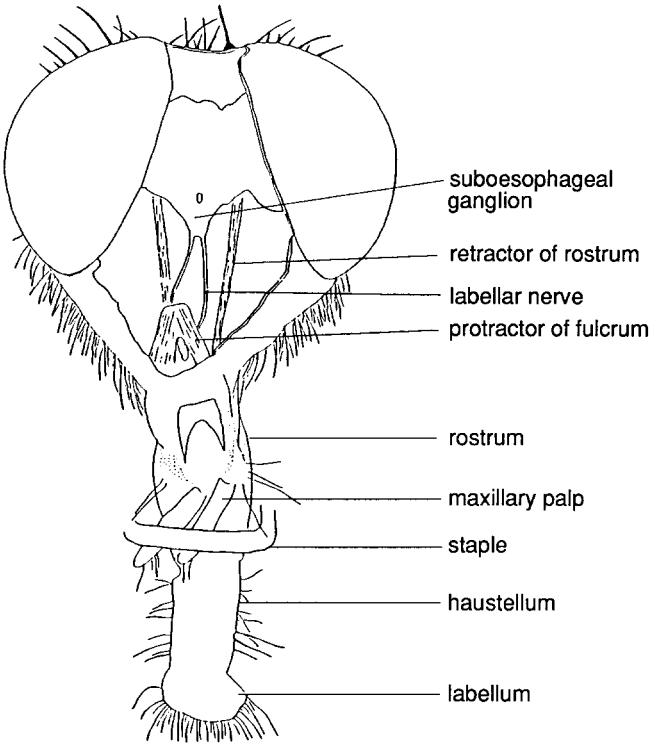
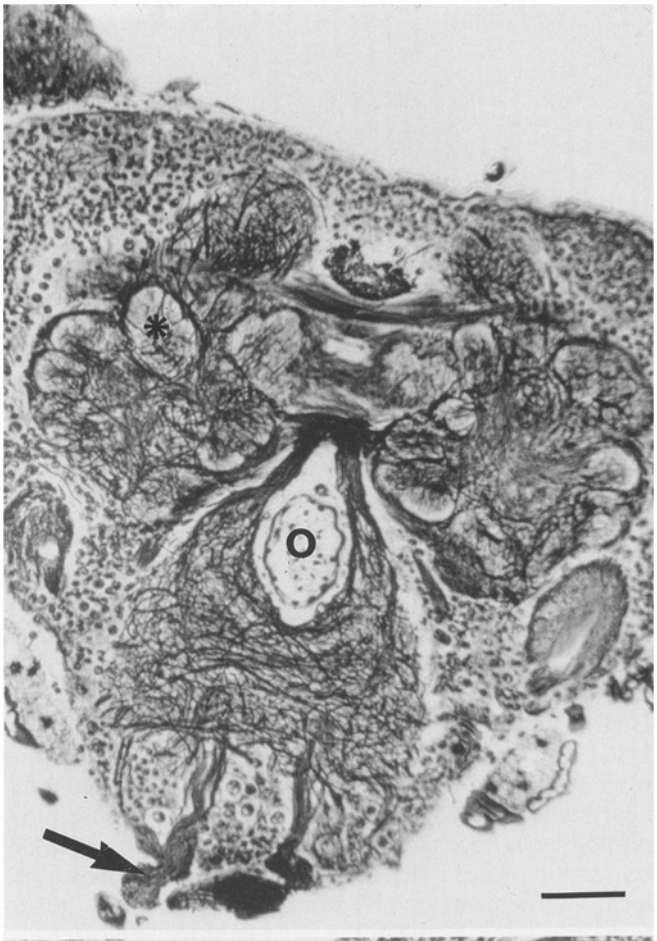


Fig. 1. Drawing of the head of a typical preparation dissected for recording. The proboscis was secured to the dental wax cylinder with a small staple such that the labellar lobes projected beyond the edge of the dental wax (see text for details). The brain was stabilized by a metal platform

Materials and methods

Animals. Sarcophaga bullata were obtained from Carolina Biological Supply Co. as prepupae and allowed to pupate in jars in an incubator held at 22 °C. Developed pupae were placed into cages at 22 °C, and emerging adults were fed sucrose and milk powder ad lib. Only young flies between 3 to 14 days after eclosion were used for recording, and they were starved (no food or water) overnight in high humidity before use.

Flies were anesthetized by chilling on ice, and their legs and wings were removed with scissors. They were then placed in a V-shaped trough cut longitudinally in a cylindrically shaped piece of soft dental wax, and initially restrained by inserting the neck region into a smaller V-shaped notch cut in a thin sheet of dental wax that was attached to one end of the larger cylinder. Then, a strip of wax was placed across the top of the neck, securing the head more firmly, while a second strip was placed across the thorax. Flies thus restrained were held in a vertical orientation with the mouthparts



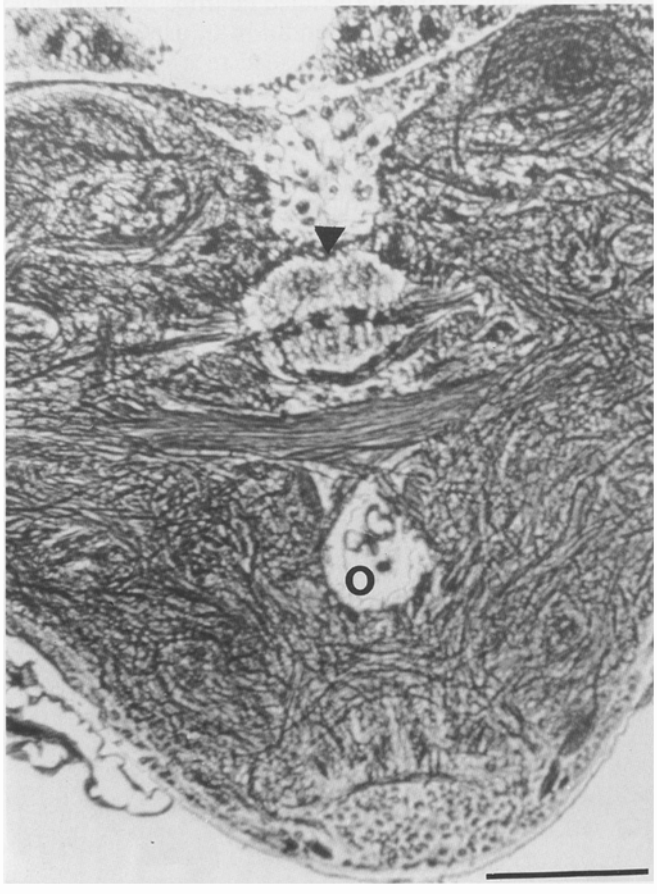


Fig. 2. Bodian stained frontal sections through two levels of the brain and SEG of *S. bullata*. The *top section* is the more anterior of the two and shows the olfactory lobes with their glomerular organization as well as the most frontal part of the SEG neuropil. Tracts of the maxillary/labellar nerve can also be seen, together with numerous cell bodies near the surface of the SEG. The *bottom section* is deeper in the SEG, approximately mid-way through the ganglion. The central body of the brain is visible. In both sections, the stratified nature of the SEG neuropil is evident. * glomerulus of the olfactory lobe; *arrow* labial-maxillary nerve; *arrowhead*, central body; *O* oesophageal foramen. Scale bar = 100 μm; top is ×110, bottom is ×210

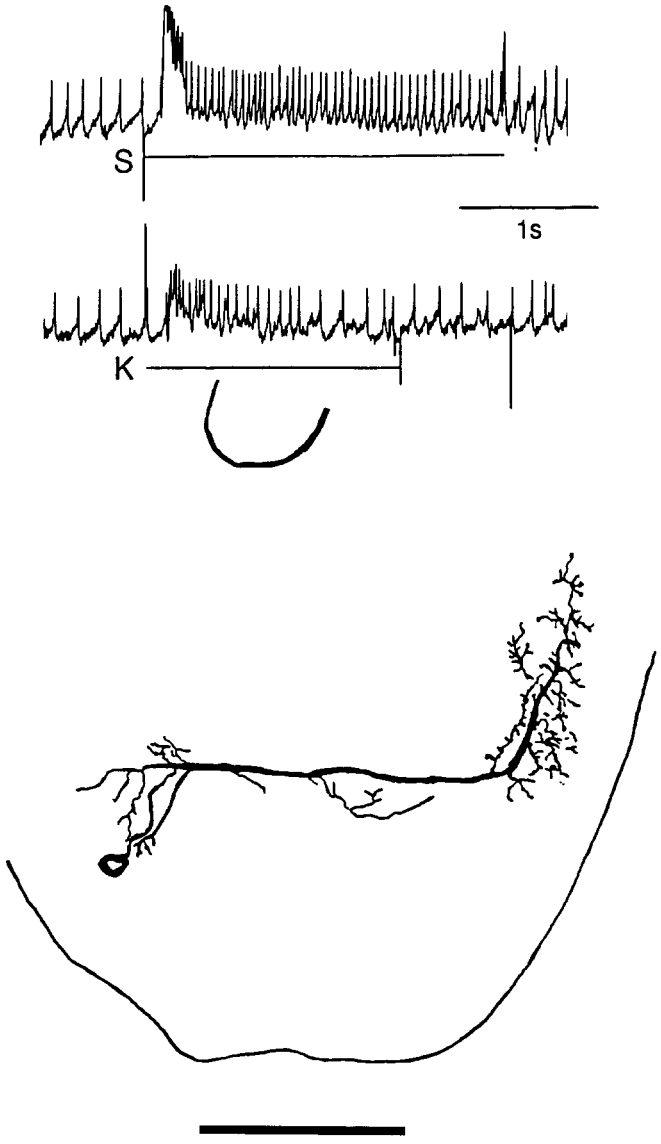


Fig. 3. Reconstruction and physiological response of cell 64. The view is frontal and dorsal is up, as is true for all reconstructions presented here. This cell had a small cell body located laterally about mid-depth in the SEG. The major arborizations innervated a region of lateral neuropil approximately 48 μm in depth, lying some 100 μm below the frontal surface of the SEG (estimate from incomplete serial sections). Reconstruction was made from the whole mount. The solid line below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 *M* KCl). Stimuli appear in the order presented. Scale bar, 100 μm

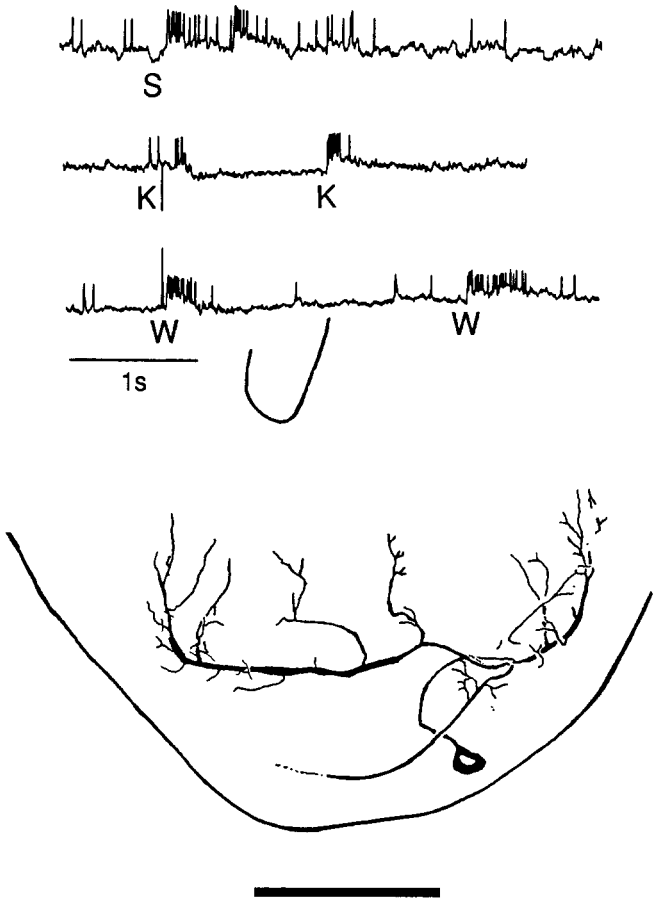


Fig. 4. Reconstruction and physiological response of cell 59. This was a strongly bilateral neuron with a cell body located close to the periphery in the posterior SEG. The cell body lies posterior to most of the arborizations. Overall, it covers approximately 150 μm in depth with the arborizations not reaching the most anterior neuropil. The processes on the left of the drawing lay slightly deeper in the SEG than those on the right. The segment traversing the ganglion lies in a different tract (more ventral) than the corresponding segment in cell 64 (Fig. 3). Reconstruction was from the whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

at the same level as the end of the wax cylinder. The proboscis was gently extended with forceps by pulling on one of the maxillary palpi, and stapled in this position to the top of the wax, using small staples made from stainless steel minuten pins. The wax block was then trimmed so that the labellar lobes were fully exposed beyond the edge of the supporting wax. All of these steps are more easily accomplished with chilled blocks and flies, so additional periods on ice were given as necessary.

Using fine scissors and forceps, the frons and vertex were removed from the head, a procedure in which both antennal nerves were carefully severed. This revealed the underlying musculature, tracheae and fat body. The fat body on top of the brain and the air sacs under the frons were removed, exposing the SEG. The retractors of the rostrum and the fulcrum often had to be severed to stabilize the preparation (Fig. 1), and the esophagus was cut at the point where it passes into the esophageal foramen. A small metal platform, notched at the center to accommodate the ventral nerve cord, was fabricated from a flattened hypodermic needle and placed under the brain/SEG from the top of the head for stability. The brain was manually bathed with saline (in m*M*): 149.9 NaCl, 3.0 KCl, 3.0 $CaCl_2$, 10 TES buffer, 25.0 sucrose, pH 6.9 as needed. Preparations remained viable for 2 to 3 h without continuous perfusion. To ease impalements, a portion of the neural sheath covering the SEG was removed with fine, sharpened forceps.

Gustatory stimulation. The preparation was stimulated manually by applying small drops of stimulating fluids to the labellar hairs via one of 3 small diameter intermedic tubes held together at the delivery end by dental wax. The ends of the tubes, embedded in the wax, were cut evenly with a razor blade. This provided a suitably small hydrophobic surface to receive fluid from any one of the 3 tubes. Because of the hydrophobic surface, the expressed fluid rounded into a drop which was used to stimulate the labellum. When the fluid was withdrawn, the hydrophobicity of the surface insured that virtually all of the fluid returned to the tube from

whence it came. The tubes were connected to 1 ml, tuberculin syringes filled with one of the 3 stimulating fluids (1.0 *M* KCl, 100 m*M* sucrose, distilled water). During a stimulus, the plunger on one of the syringes was pushed lightly to produce a droplet of fluid at the common output. The expressed droplet contacted the chemosensory hairs on the labellum, spreading apart the two lobes as the droplet expanded. After 1 to 2 s, the plunger was withdrawn, sucking the stimulating fluid back into the tubing. This was repeated with each stimulus in turn. Several drops of fluid were expressed from each tube between impalements of different cells to reduce contamination. The times of application and removal of the stimulus were indicated on the voice channel of the data recorder by the observing experimenter. The application and removal of the stimulus also generated a stimulus artifact that was visible on the physiological recording (usually for stimulus onset, often for stimulus offset).

Intracellular recording and staining. Intracellular electrodes were pulled on a horizontal puller (Sutter Instruments model P–80 PC) and filled with Lucifer Yellow CH (LY, Aldrich, 4% solution in dH_2O (Stewart 1978)). Typical electrode resistances were 100–200 MΩ. The signal from the electrode was amplified (Getting Model 5A), viewed on a storage oscilloscope (Tektronix model 5113), and recorded on magnetic tape (Vetter model B FM tape recorder). The Getting electrometer was modified to allow a short (2–3 ms) "buzz" of capacitance overcompensation. The buzz was triggered by a timed pulse from a microcomputer-based stimulator running a software package programmed in ASYST, which closed a relay (Magnacraft model 545) to activate the compensation circuit (Itagaki and Hildebrand 1990). Analysis of taped physiological records was performed on data transcribed by a pen recorder (Gould model 2200).

LY was iontophoresed into impaled cells with a DC hyperpolarizing current (10 nA for 4 to 10 min). Brains/SEGs were then dissected out and fixed in 2.5% formaldehyde in 0.1 *M* Sorensen's phosphate buffer (pH 7.4) with 3% sucrose, dehydrated by passage through a graded ethanol series, cleared in methyl salicylate (Aldrich), and viewed in whole mount with an epifluorescence microscope (Marder and Eisen 1984). Optical sections of whole mounts with filled cells were photographed for subsequent viewing and reconstruction.

Preparations with dye filled cells were further processed by washing in absolute ethanol, transferring to acetone and embedding in Spurr's medium by standard procedures. The embedded preparations were then sectioned on a sliding microtome at 24 μm and mounted on glass slides with Fluoromount (Gurr). Sections were photographed under epifluorescence, with serial photographs of each 24 μm microtome section being taken on Ektachrome 160 (Kodak) at 3 to 8 μm intervals, as indicated by the focus knob of the microscope. Where possible, a full reconstruction of the cell was made from the photographic slides of the sectioned preparation using a carousel projector and an angled mirror projecting the image onto a desk (Kanzaki et. al. 1989). The same method was used to make reconstructions from whole mount serial photographs in cases where the sections were poor or where the fluorescence had faded due to photographing.

Histology. Bodian's protargol technique was used to visualize anatomical features of the brain/SEGs of *Sarcophaga bullata.* After fixation in alcoholic Bouin's fixative, the brain/SEGs were em-

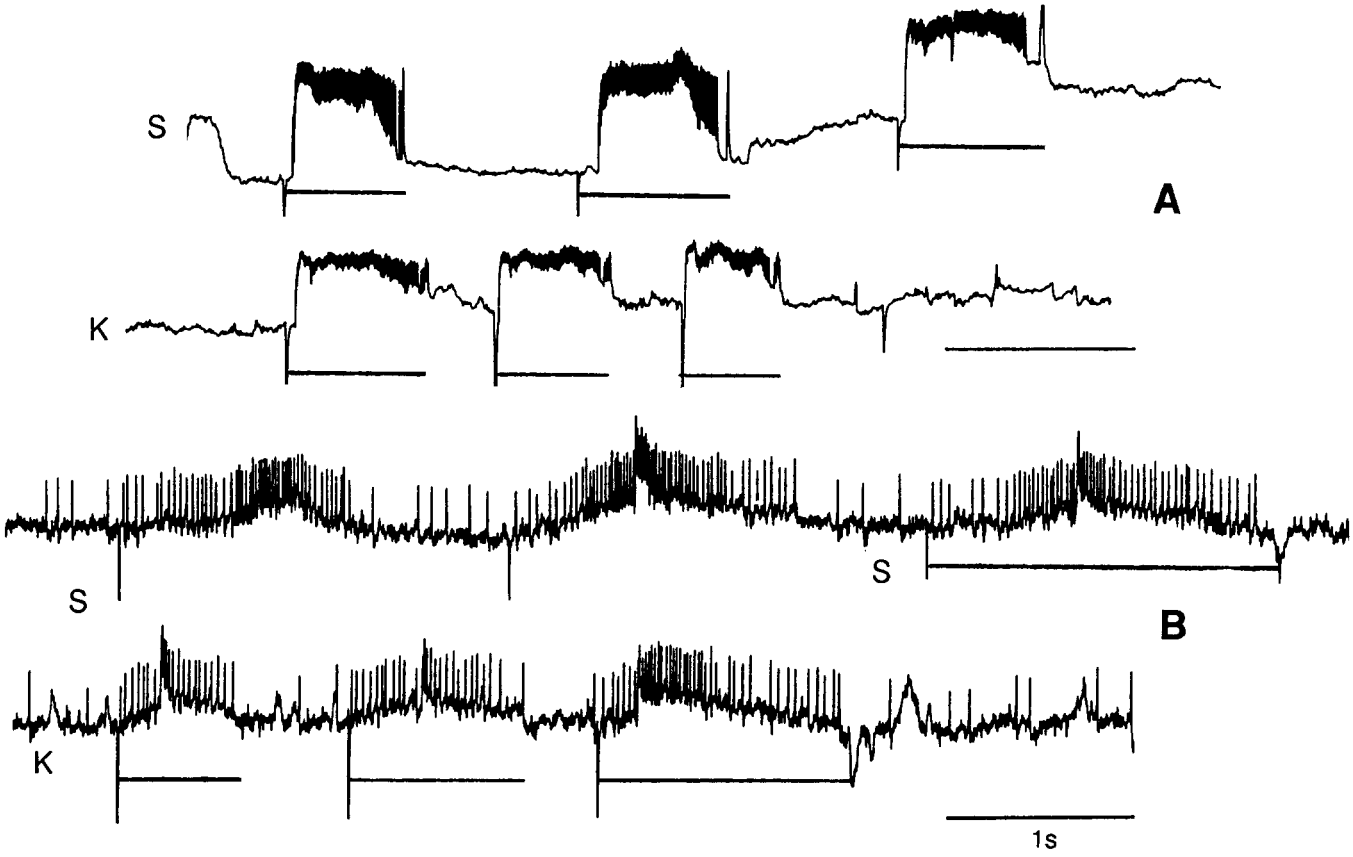


Fig. 5A and B. Physiological records from two cells (**A** and **B**) that received mechanosensory input from the labellum. In both cases responses to movement tended to be augmented by sucrose suggesting that the cell received input from both the sugar cell and mechanosensitive afferents. Note the large depolarization of cell (**A**) when sucrose was applied. The solid line below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; K 1 *M* KCl). Stimuli appear in the order presented

bedded in Paraplast, sectioned at 8 μm, and placed on slides. The sections were hydrated, impregnated with 2% protargol (pH 8.4, titrated with 10% NH_4OH) in the presence of copper shot, and developed in 2.5% sodium sulfite with 1% hydroquinone, further developed in 1% gold chloride, reduced in 2% oxalic acid, and then treated with sodium thiosulfate before dehydration and coverslipping with Permount (Gregory 1980).

Cobalt staining. For retrograde staining of a population of labellar chemosensory afferents with cobalt, flies were restrained and the proboscis extended as in the physiological preparation. The maxillary-labellar nerve was dissected about two-thirds of the way along the proboscis and the cut end placed in a small well composed of Vaseline/petroleum jelly mixture and containing distilled water. After allowing time for the distilled water to dilate the cut axons, the water was replaced with 0.1 *M* $CoCl_2$. The well was then covered with petroleum jelly/Vaseline mix and the preparation was placed in a humidified chamber at 4 °C for 18–72 h to allow diffusion of the cobalt into the CNS. After the diffusion time, the brain/SEGs were dissected out in TES saline (Itagaki and Hildebrand 1990) and the cobalt precipitated by bubbling H_2S gas through the saline for 15 s. The preparations were fixed in alcoholic Bouin's solution (Humason 1967), then silver-intensified by Timm's method (Bacon and Altman 1977). The brain/SEGs were dehydrated in a graded ethanol series, cleared in methyl salicylate, and embedded in Canada Balsam for viewing in whole mount and photography.

Results

General comments on recording success

Fifty of the recorded neurons responded primarily to sucrose, 23 responded to all three test stimuli (considered mechanosensitive) and 3 responded primarily to KCl. None responded primarily to water. 82% of the cells tested did not respond to any of the stimuli. The success rate for filling with LY was 20%–30%.

General histology of SEG neuropil

The SEG receives sensory input from the mandibular, maxillary and labial segments of the head. Chemosensory input comes from both olfactory and gustatory sensilla on these mouthparts, though the relative distribution of mouthpart olfactory inputs between the SEG and the olfactory lobes of the brain is poorly known. Figure 2 shows typical Bodian stained frontal sections from two levels of the brain-SEG complex of *S. bullata*. The top section is at the level of the olfactory lobes, which are quite superficial in this frontal aspect, while the second section represents deeper tissue. Note the clear

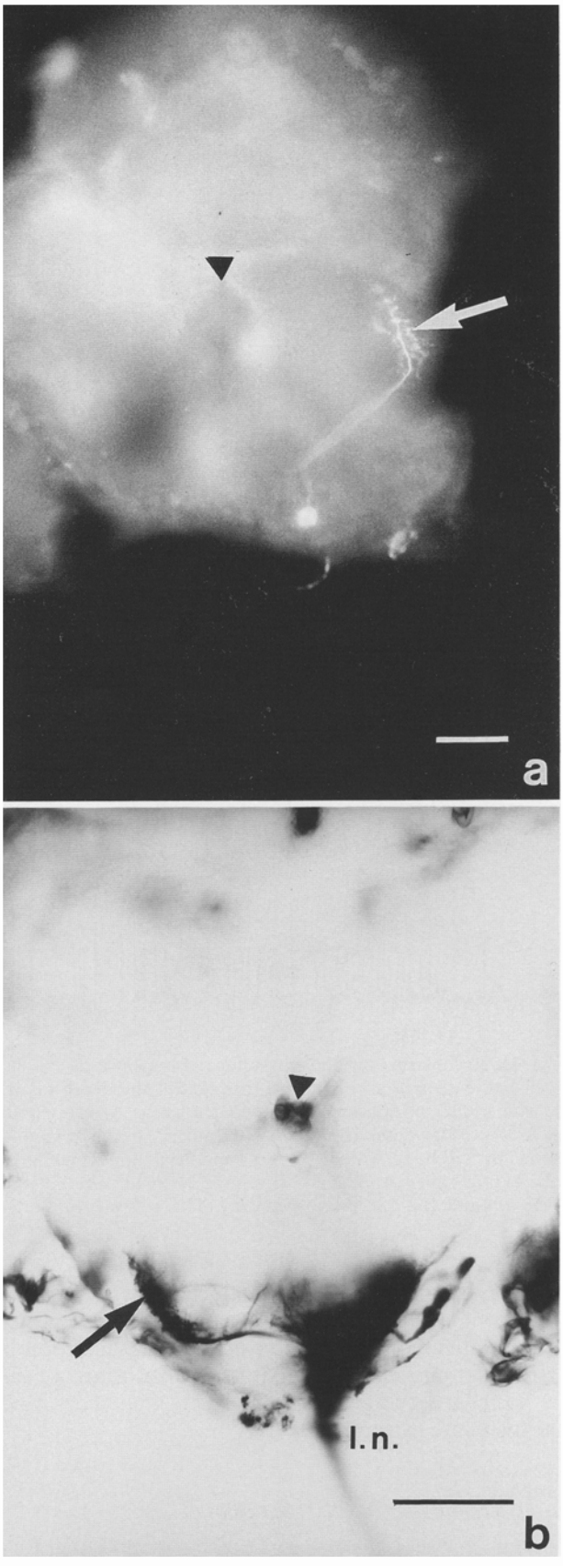


Fig. 6a and b. a LY fill of cell 64 (Fig. 3) showing extensive arborization in presumptive mechanosensory region. The view is frontal and dorsal is up. *Arrow* mechanosensitive region; *arrowhead* oesophageal foramen. **b** Co backfill of labellar nerve showing major arborization of chemosensory afferents *(arrow)* and the lateral mechanosensory region contralateral to the filled nerve *(arrowhead)*. Orientation as in **a**. *Arrow*, presumptive mechanosensitive region; *arrowhead*, oesophageal foramen; *l.n.* labellar nerve. Compare the lateral arborizations of the presumed mechanosensory fibers in preparation **b** with the lateral arborizations of the interneuron receiving mechanosensory input in preparation **a** *(arrows)*. The heavily filled triangular area near the root of the labellar nerve contains arborizations of motor nerves and primary afferents that run in the labellar nerve. Scale bars = 100 μm **a** ×100; **b** ×170

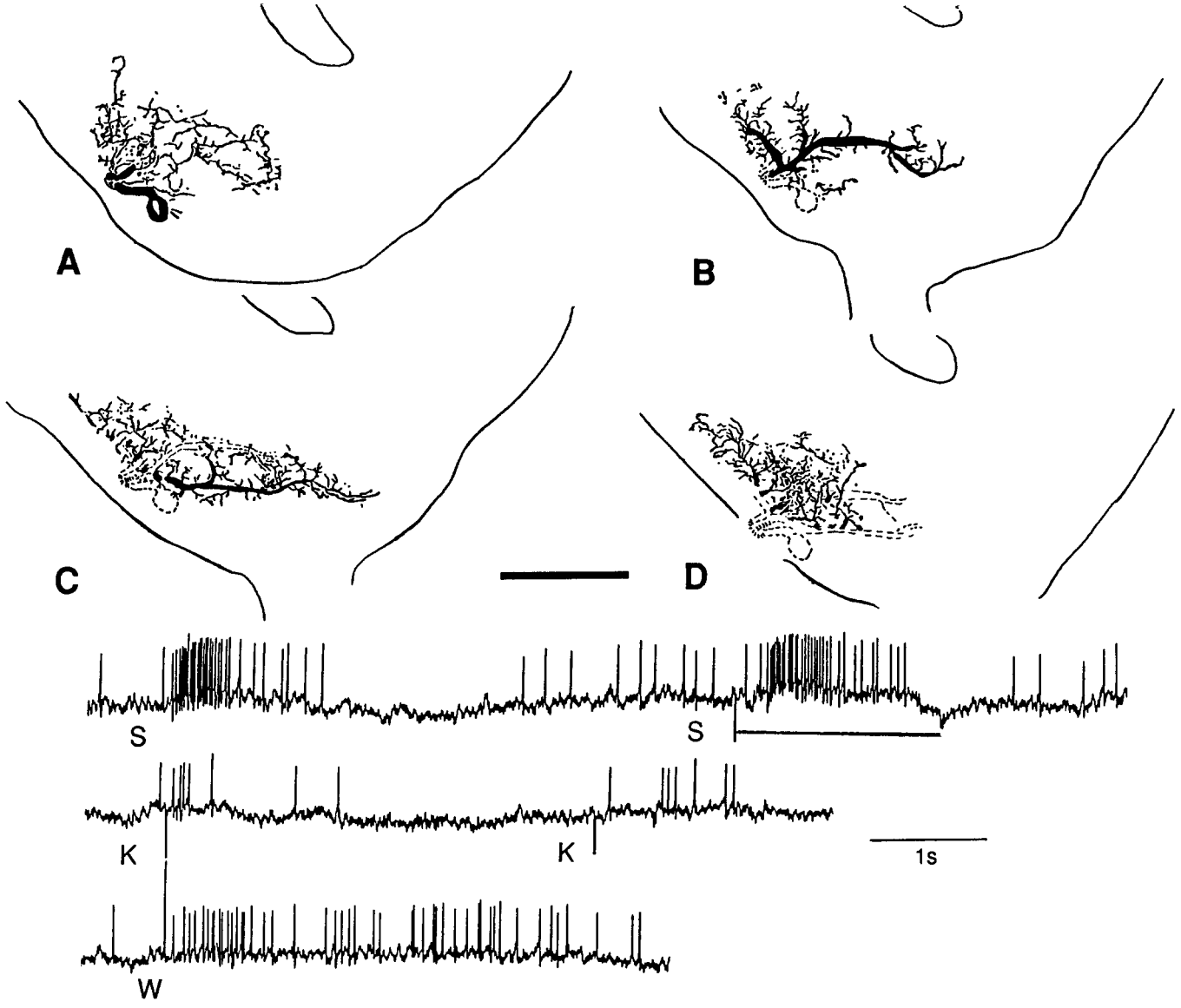


Fig. 7A–D. Reconstruction and physiological response of cell 88. The cell had extensive arborizations throughout the depth of the SEG, with a distinct division into layers shown as **A** through **D**. Layer **A** covered 0–96 μm, from the frontal surface of the SEG, and contained the cell body, which was very superficial, appearing in the first 24–48 μm. Layers **A, B, C** and **D** were at 24–96 μm, 96–120 μm, 120–168 μm and 168–288 μm respectively. This cell was primarily unilateral, with some processes extending to the other hemiganglion in level **C** (120–168 μm). The processes found in **B** and **C**, as well as the cell body and axon, were the largest found among cells reported here. Reconstructed from sections. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

glomerular arrangement of the olfactory neuropil, and the apparent lack of glomerular organization in the SEG. The most obvious organization of the SEG neuropil is the stratification reflecting in part the contribution of the three ganglia serving the mandibular, maxillary, and labial segments (Strausfeld 1976).

Neurons responding to all 3 test stimuli

Often, all 3 stimuli (sugar, salt and water) caused significant spiking in the test neuron. Since the presentation of the stimulus droplet unavoidably stimulated mechanoreceptors (in the sensilla, and, at least in some cases, chordotonal organs of the labellar lobes and the proboscis), neurons that responded to presentation of all 3 stimuli were considered to be potentially mechanosensitive. In most cases, we did not attempt to fill such cells, however, we did obtain two fills (Figs. 3 and 4). These cells showed some common physiological and morphological features. Two other cells in this category that were not filled showed additional features of this type of response (Fig. 5A, B). The responses were often vigorous and closely tied to visible movements of the proboscis caused by the applied droplet. Removal of the droplet usually caused immediate termination of action potentials (Fig. 5A, B). Responses to sucrose were often more vigorous than those to KCl (9 of 11 cases), as is par-

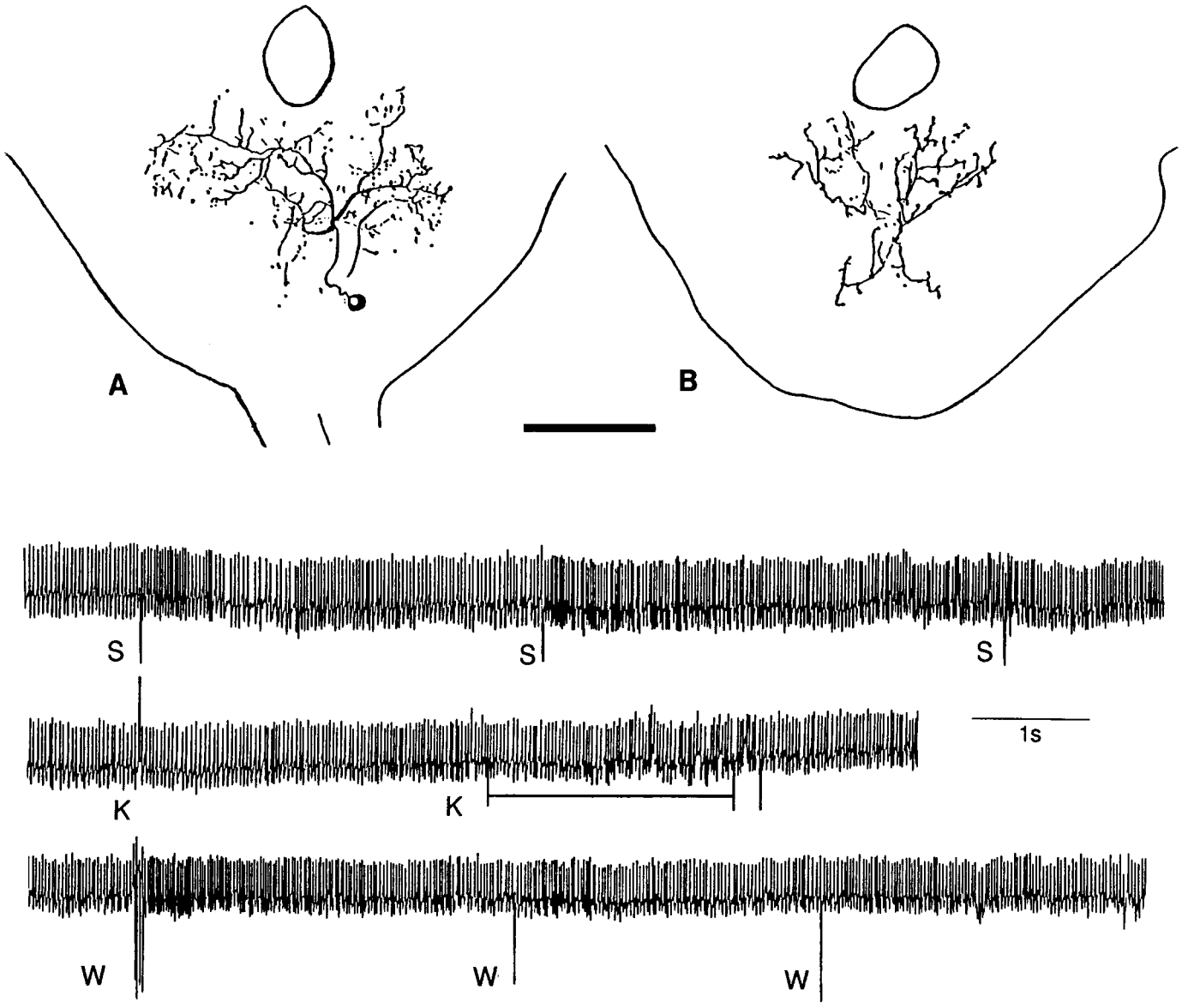


Fig. 8A and B. Reconstruction and physiological response of cell 104. The cell body was superficial, appearing 24 to 48 μm from the anterior surface of the SEG. The cell's processes were strongly bilateral, and were arranged in two distinct layers (left and right reconstructions). Varicosities were much more obvious in the deeper layer B. Layers A and B covered depths of 48–72 μm and 120–164 μm respectively, with respect to the frontal surface. Reconstruction from the whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; K 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm. Background activity was high and no attempt was made to reduce it with hyperpolarization during experiments

ticularly evident in Figs. 4 and 5B. (Note the much larger depolarization upon stimulus onset with sucrose in Fig. 5B). It may be that these interneurons received both chemosensory and mechanosensory inputs.

The most striking feature of the two neurons, and of a third partially filled neuron in this category (not shown) was a clear projection to the lateral SEG, which could be unilateral (Fig. 3) or bilateral (Fig. 4). Cobalt fills of the labellar nerve also revealed extensive arborization of afferents in this region (Fig. 6b). Comparison of the general cobalt fill and the LY fill of cell 64 (Fig. 6a) suggests that the contralateral branches of the afferent cells innervate the same region as the major arborization of cell 64. This region, near where the tritocerebrum and SEG meet, appears to lie just below the antennal mechanosensory region (Strausfeld 1976, p 103) and may also be involved in mechanosensory processing, perhaps with connections to the antennal region. It is the most obvious gross organizational feature of the SEG neuropil discovered in this study. As our experimental protocol did not use an exclusively mechanical stimulus, we cannot be sure that these neurons receive primarily mechanosensory input rather than input from all 3 chemosensory afferents (salt, water and sugar).

Neurons receiving "sugar-cell" input

Reconstructions of 7 cells and their physiology are illustrated in Fig. 7–13. In all cases, the most obvious physio-

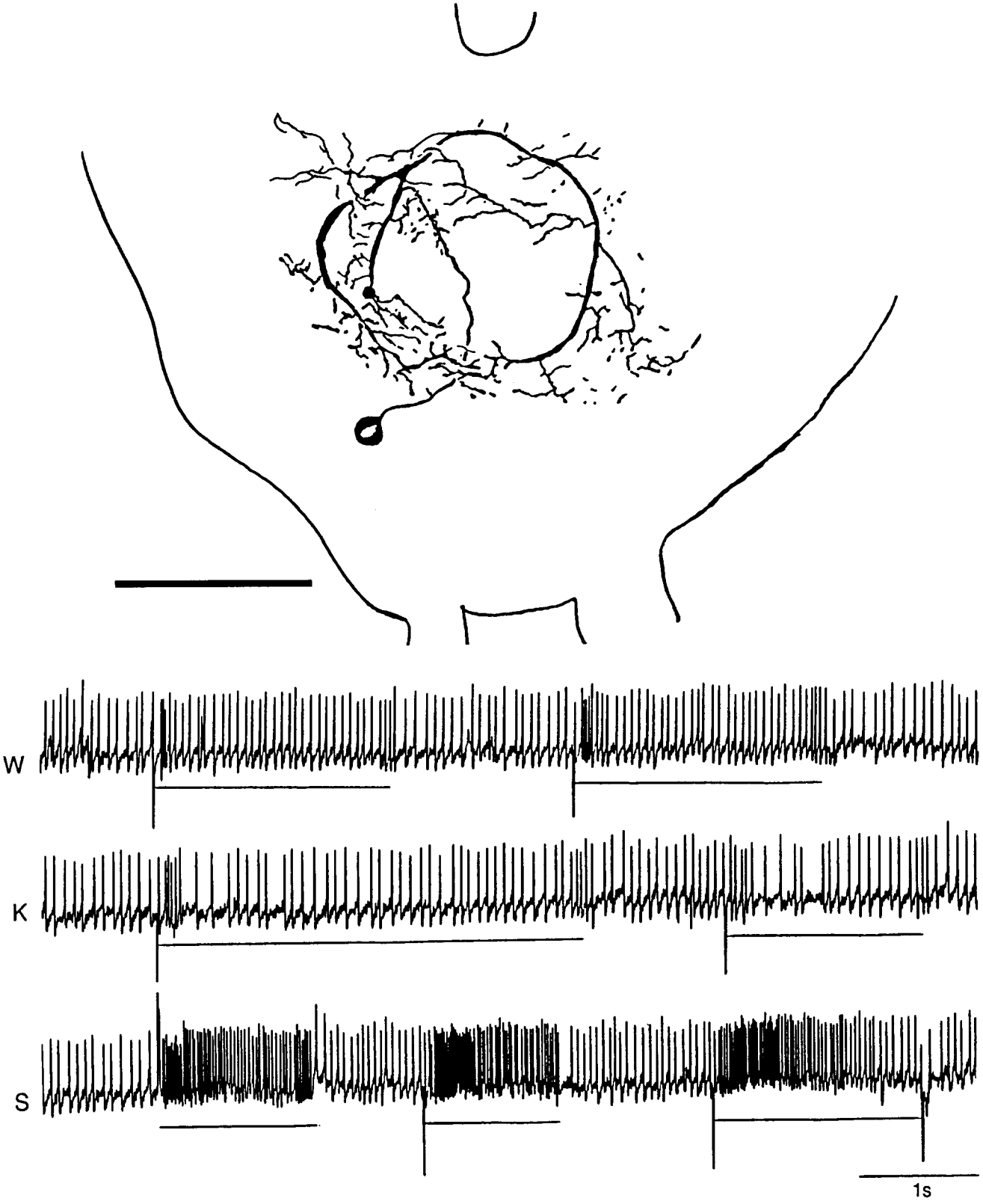


Fig. 9. Reconstruction and physiological response of cell 85. From the cell body, which lay very deep near the level of the ventral connective, the axon ascended to produce 3 major branches. Two of these form an almost complete circle, with the dorsal part being most superficial relative to the frontal surface. The third branch traveled through the ganglion, with a particularly straight antero-postero portion *(large black dot)* before producing a number of branches that innervate approximately the same area as the other two branches. The area where the 3 major branches met (bottom part of the circle in the reconstruction) was much deeper in the ganglion than the dorsal portion. The arborizations in this deep region were very dense, more so than depicted here. The axon from the cell body ran deeper than this arborization, before ascending to connect with the more superficial part of the cell. Reconstruction from whole mount. The *solid line* below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

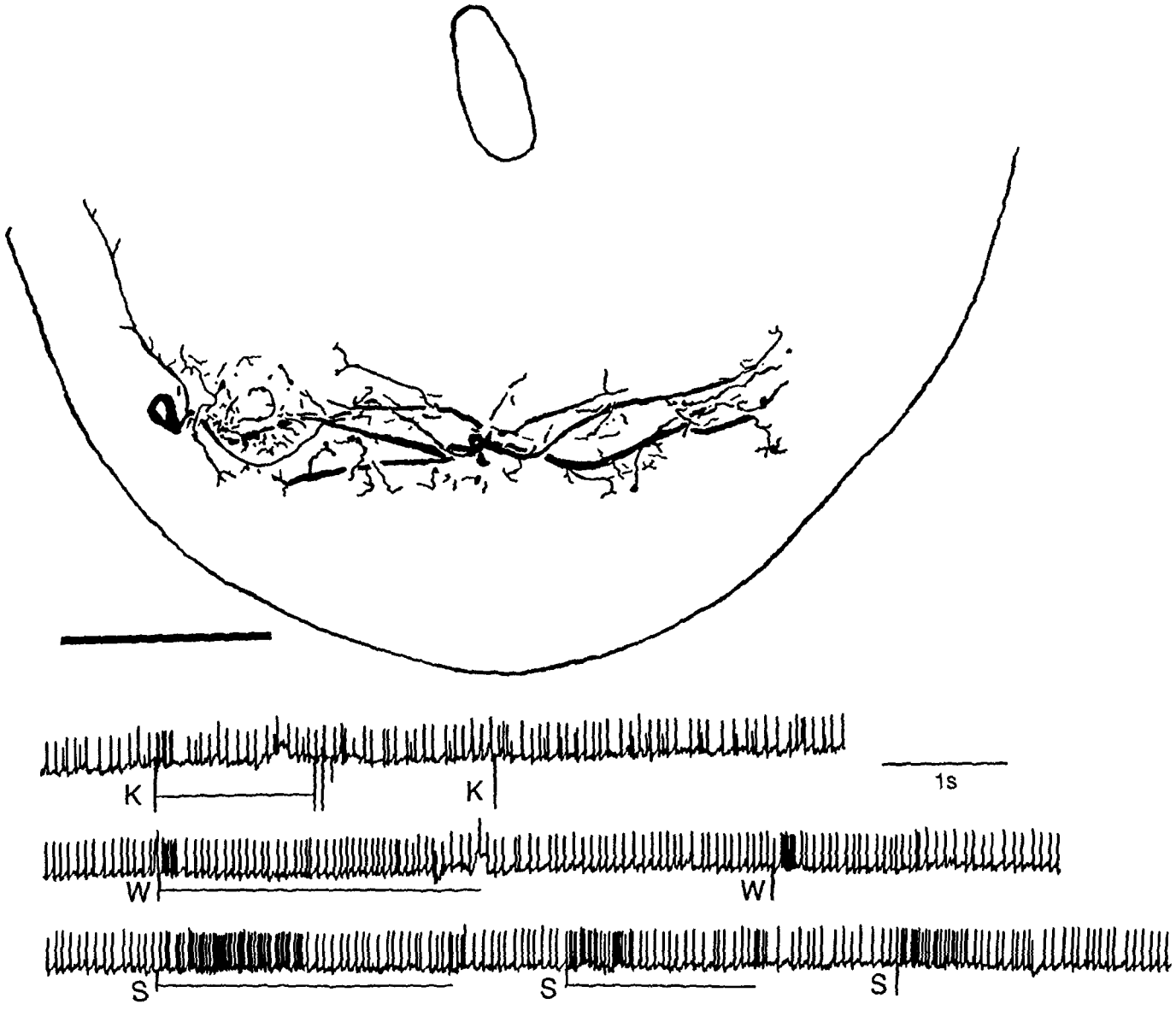


Fig. 10. Reconstruction and physiological response of cell 112. The cell body was superficial, appearing in the first 24 μm. Arborizations were strongly bilateral, and spread in the shape of a shallow basket through most of the depth of the SEG. A small branch projected unilaterally into the putative mechanosensory region. The largest fibers of the cell penetrated through the SEG near its center (into the plane of the drawing). These large fibers appeared in sections between 42 μm and 114 μm, relative to the frontal surface. Reconstruction from the whole mount. The onset of the second salt stimulus and the second water stimulus are indicated by *K* and *W* respectively. In other cases, the solid line below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

logical characteristic was a clear response to sucrose together with no response, or comparatively weak response to KCl. As can be seen from the reconstructions, and accompanying anatomical descriptions, there were very few common morphological features to go with this common physiological feature.

When strong responses to sucrose were encountered, we attempted to apply the stimulus droplet several times within a period of about 10 s. This confirmed the response and provided an estimate of adaptation in the system. Individual labellar chemoreceptive cells respond well to repeated stimulation with 100 m*M* sucrose over 10 s (personal unpublished observation). In most cases, the neurons were able to follow repeated stimulations of sucrose with a few second interstimulus interval, usually with little obvious decrement (Figs. 7–11). All of these cells had extensive arborizations in the SEG, several had bilateral arborizations, and most of them penetrated deeply into the SEG.

Two neurons that responded well to sucrose were not able to follow quickly repeated sugar stimulation (Figs. 12 and 13). This was most obvious with cell 83 (Fig. 12), where a 4 min disadaptation period was required to restore a vigorous response. Both of these cells had extensive arborizations, and there were no obvious morphological features distinguishing them from more slow-

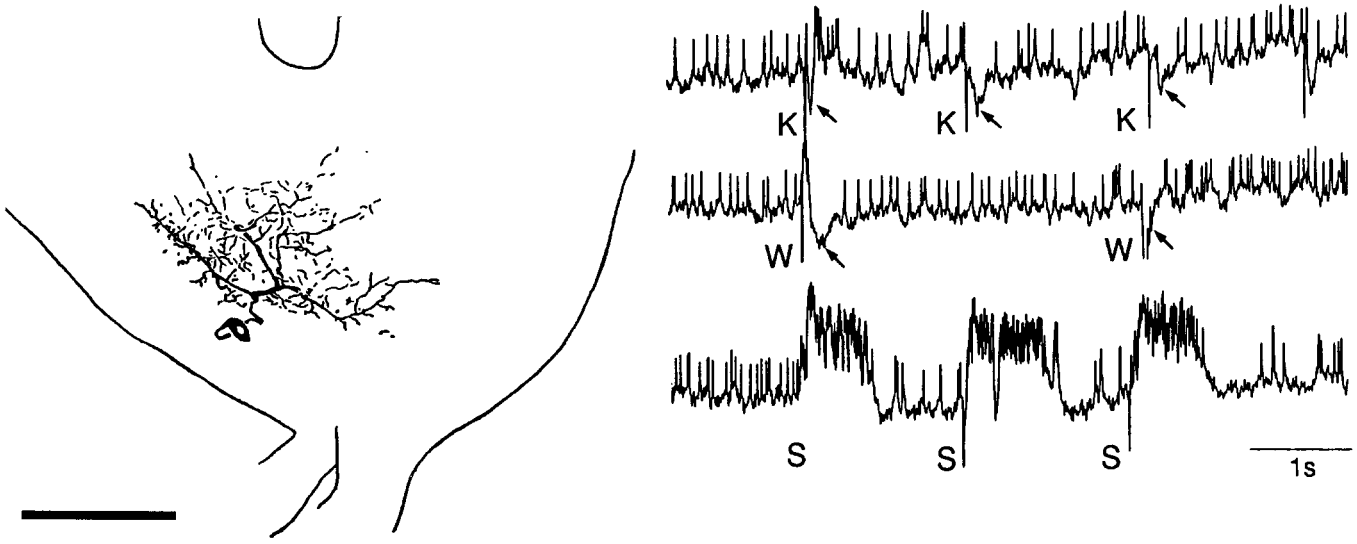


Fig. 11. Reconstruction and physiological response of cell 110. This cell had fairly restricted arborizations primarily in one hemi-ganglion of the SEG. Its cell body was near the frontal-lateral surface of the SEG, with major arborizations in the shape of a shallow basket penetrating into the frontal half of the SEG. These arborizations covered a depth of 150 μm, and did not penetrate deeper than the middle of the SEG. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Note the depolarizations associated with sucrose stimulation and the short hyperpolarizations associated with stimulation with KCl and water *(arrows)*. Stimuli appear in the order presented. Scale bar, 100 μm

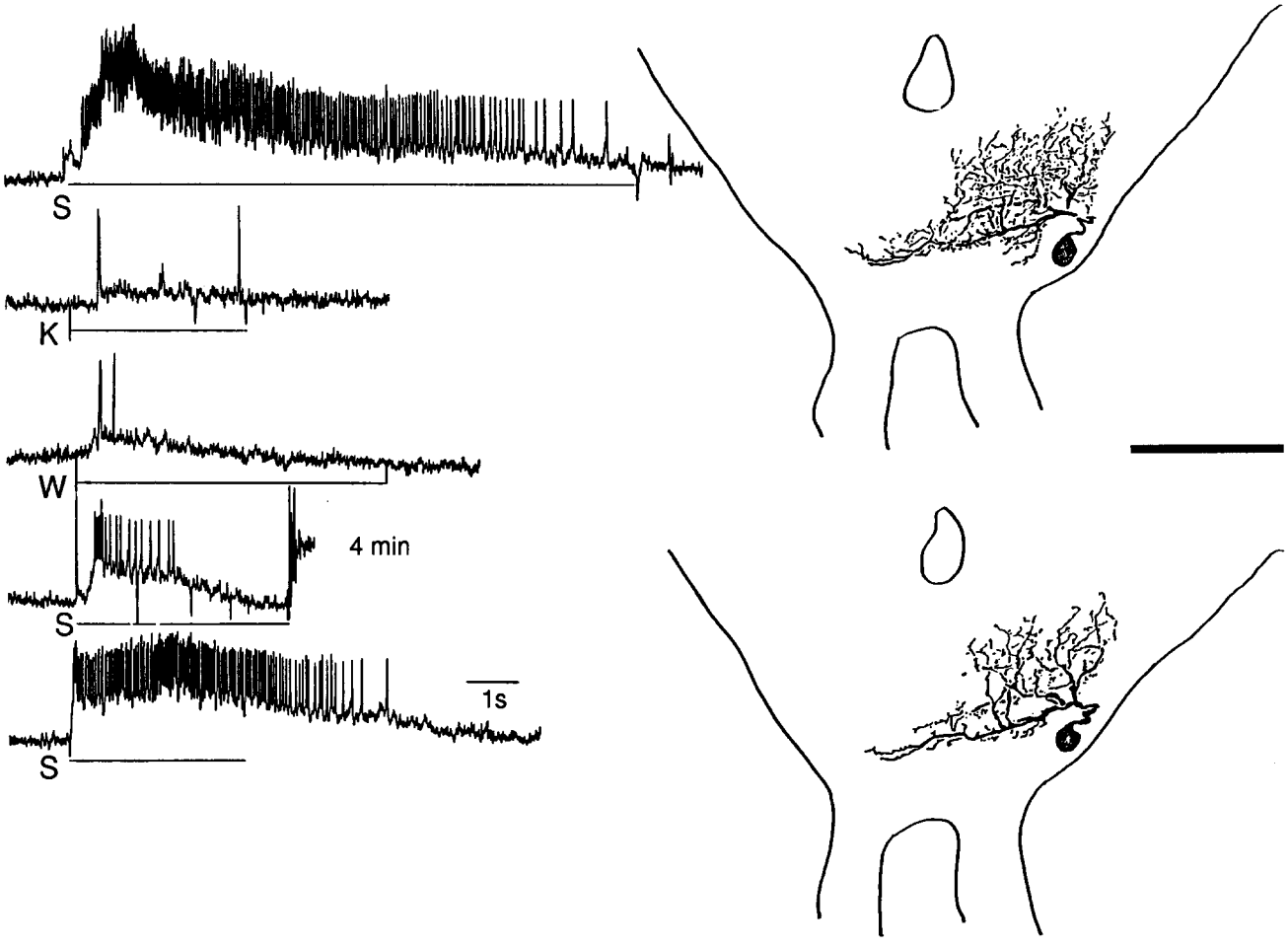


Fig. 12. Reconstruction and physiological response of cell 83. The cell body of this two layered cell was very near the frontal surface of the SEG, within the first 24 μm. Arborizations were largely unilateral, and the bi-layered organization was distinct. Varicosities were not as obvious as in, for example, Cell 49 (Fig. 13), but they did appear in the deeper layer (B). Reconstructed from whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Four min without stimulation elapsed between the last two stimulations with sucrose. Stimuli appear in the order presented. Scale bar, 100 μm

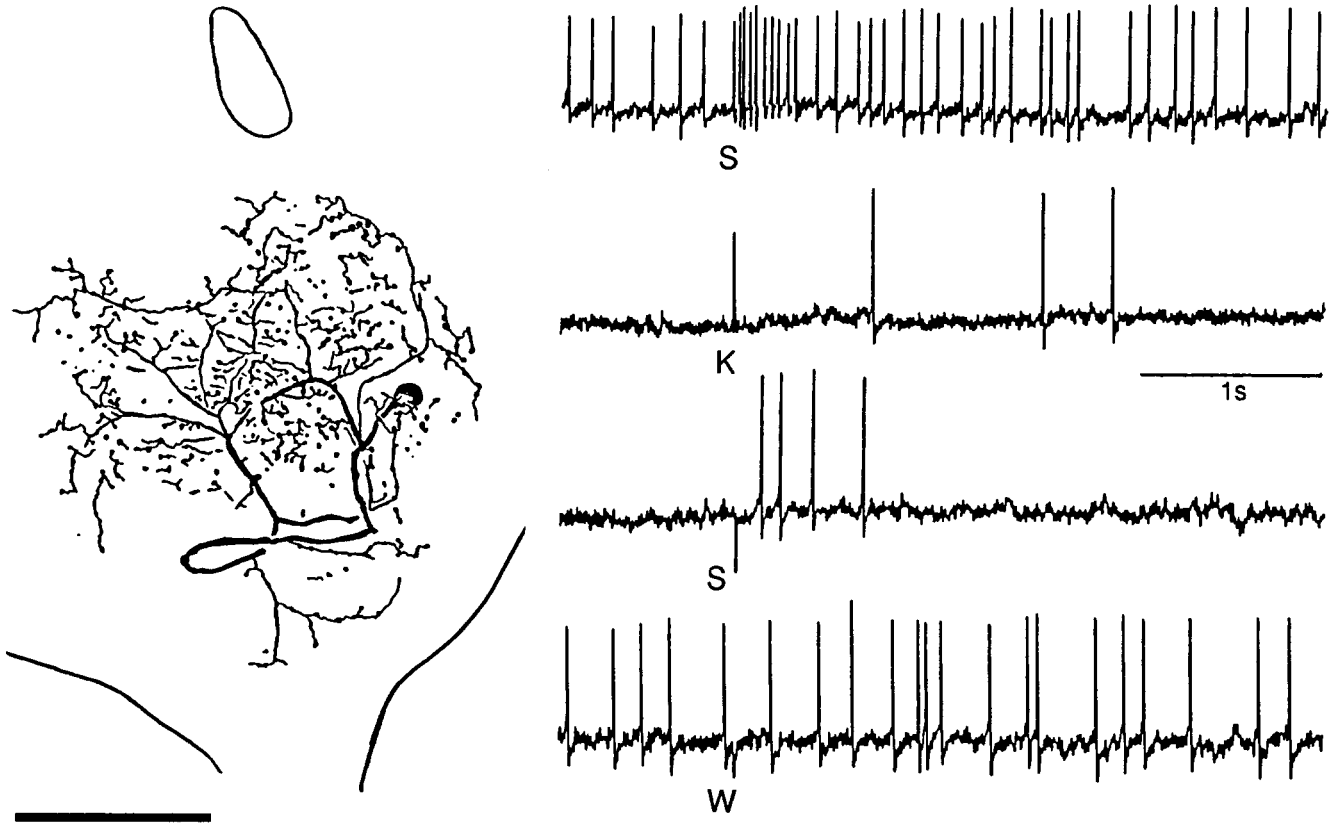


Fig. 13. Reconstruction and physiological response of cell 49. The cell body lay fairly deep in the ganglion, but since sections were not available, it was not possible to locate it more precisely. All arborizations were above the cell body. They ascended through the entire depth of the SEG, and were bilateral. Though this cell penetrated through the ganglion, there was no obvious division into layers, as in cells 83 and 88. Reconstruction from the whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

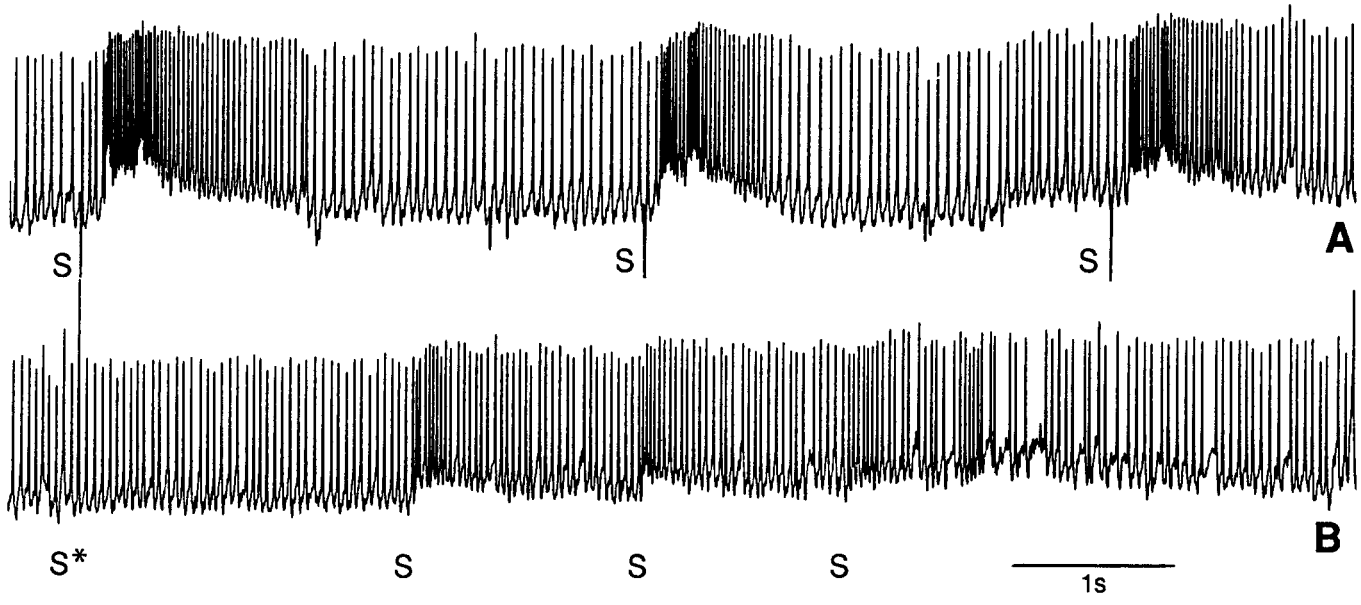


Fig. 14A and B. Recordings from a SEG neuron which was a good "follower" of sugar cell input **A**. In trace **B**, the drop of sucrose was applied to a single "largest" labellar sensillum (*S**), and subsequently to numerous medium and long sensilla (3 other *S* in trace **B**). The onset of the sugar stimulus is indicated by the appropriate letter (*S* 100 m*M* sucrose). Stimuli appear in the order presented

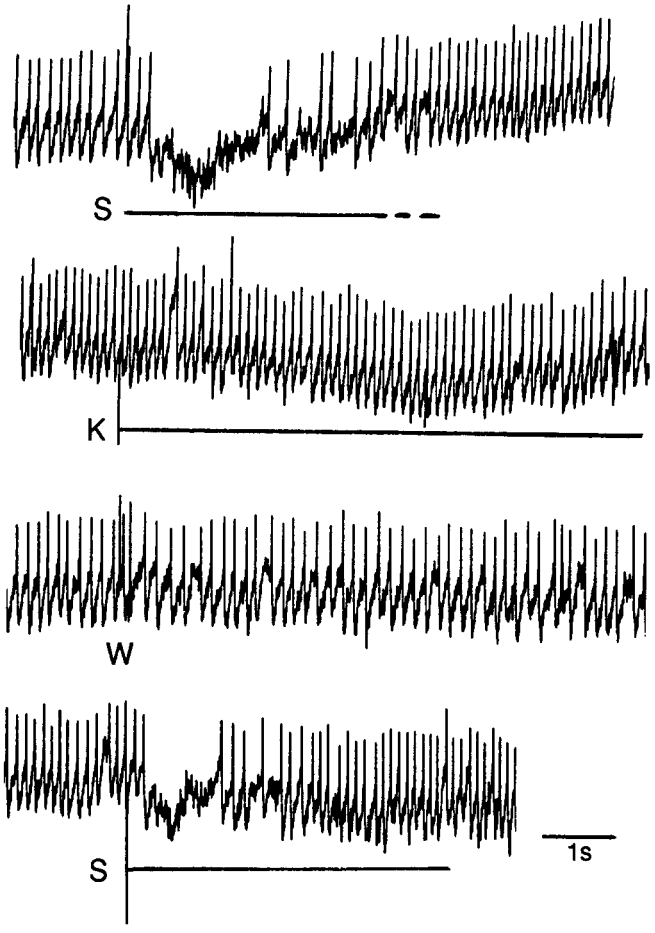


Fig. 15. Recordings from a SEG neuron that was inhibited following stimulation of the labellum with 100 m*M* sucrose. Note the delay in onset of the inhibition. The onset of the water stimulation is indicated by *W*. In other cases, the solid line below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented

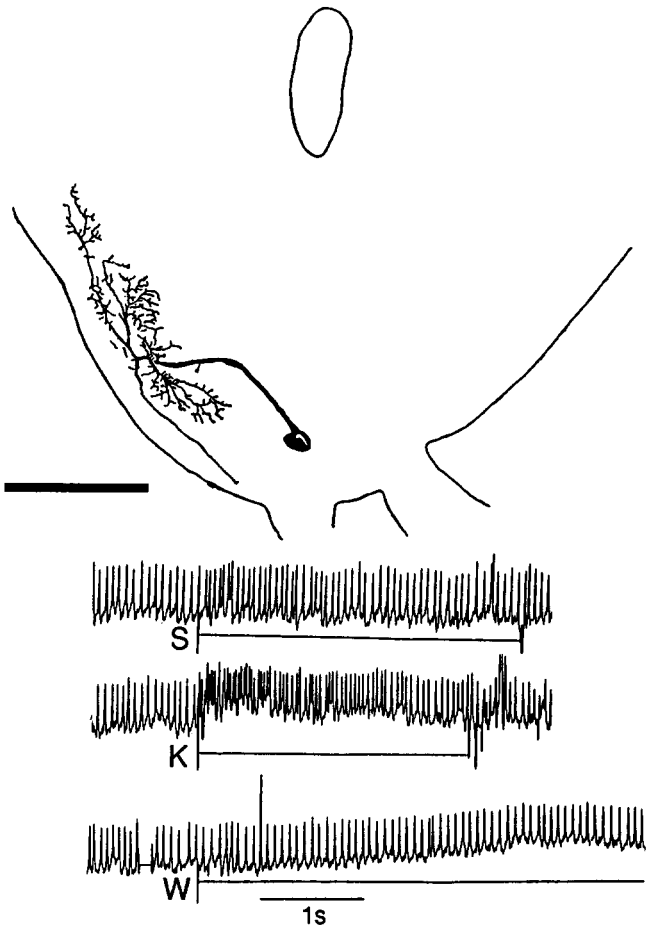


Fig. 16. Reconstruction and physiological response of cell 80. The cell body was near the frontal surface of the SEG. The axon ran along the lateral SEG neuropil, penetrating through 120 μm before the first arborizations were found. Thus, the arborizations shown in the reconstruction lay between 140 μm and 260 μm from the frontal surface of the SEG. Arborizations were unilateral. The solid line below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

ly adapting cells. If our sample was random, slowly adapting cells are more numerous than fast-adapting cells.

Cells that responded well to 100 m*M* sucrose often responded to distilled water (Figs. 7, 8 and 9). In all cases, the response to distilled water was much weaker than the response to sucrose. No cells responded exclusively, or preferentially, to water.

Three of the sugar-responsive neurons responded weakly to KCl; this could represent salt-sensitive or mechanosensory input (Figs. 7, 9 and 10). Similarly for the phasic response of cell 112 to water (Fig. 10). Note that in cell 112, removal of the sugar drop, a potential mechanosensory stimulus, also caused a small burst. Cell 112 provided one of the few examples where the response to sucrose clearly ended before the removal of the sucrose droplet, while the cell remained responsive to sucrose stimulation a few s later. (Cells 83 and 49, Figs. 12 and 13, also adapted quickly, but they failed to respond to subsequent stimulation presented a few s later). Cell 112 also had a distinct branch into the putative mechanosensory region. It is possible that this cell received relatively strong input from both sugar and mechanosensory afferents, and that the strong response to sucrose was really a composite chemo-mechano response. This suggests that interneurons processing chemosensory input, or at least input from sugar-sensitive cells, have a range of mechanosensory input from near zero to as strong as the input from sugar-sensitive cells.

All of the responses discussed so far were elicited by massive stimulation of the labellum with a drop of fluid containing the stimulus. In a few favorable cases, it was possible to stimulate a single "longest" labellar sensillum with the drop before advancing to stimulate many of the medium length sensilla. Figure 14 shows the result of one such experiment. The cell in question (not stained) followed repeated sucrose stimuli (Fig. 14A). When the droplet was advanced to cover the tip of a single "longest" labellar sensillum (Fig. 14B S*), there was no increase in background firing. Further advances of the

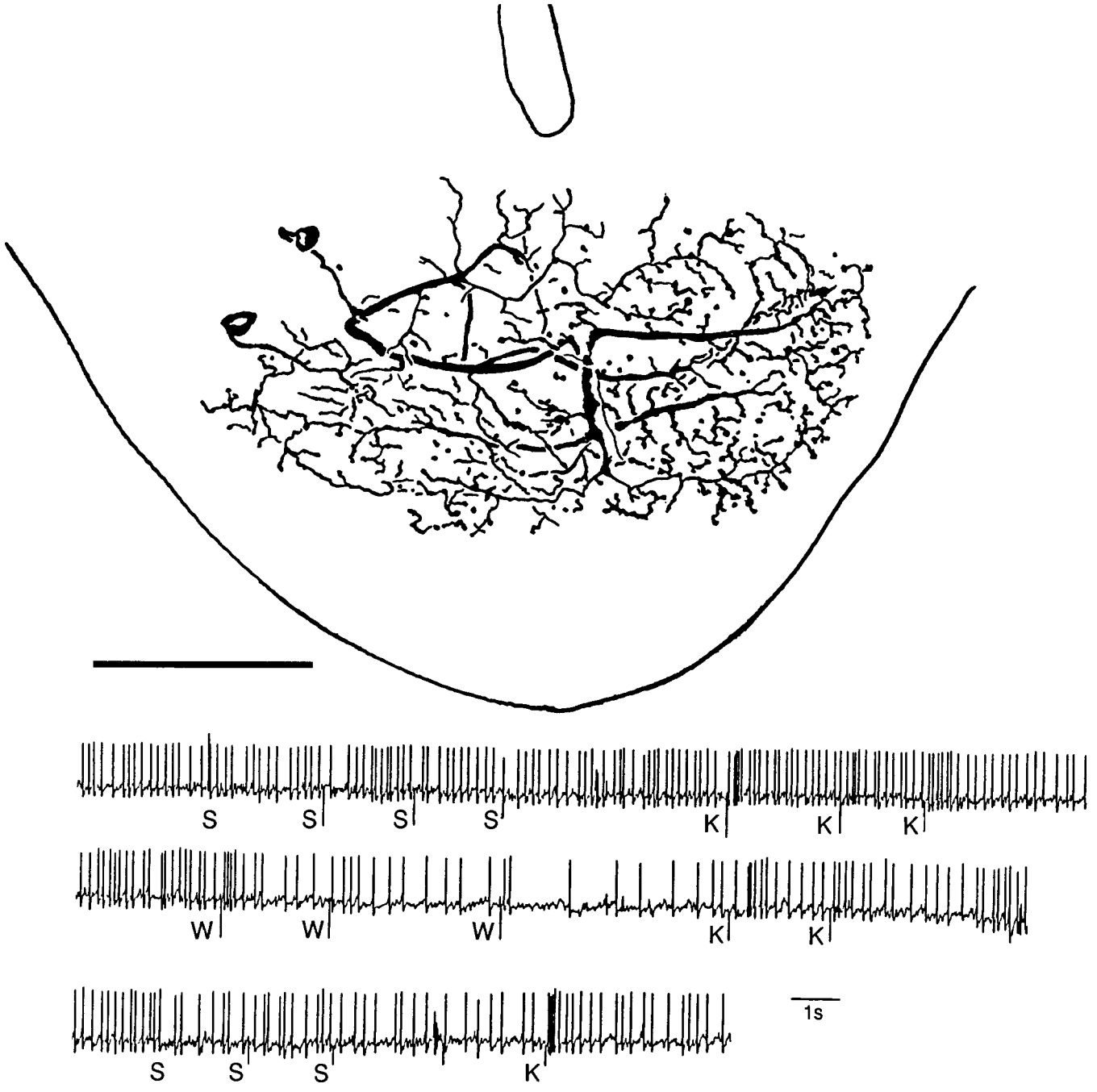


Fig. 17. Reconstruction and physiological response of cell 113. Two dye coupled cells with arborizations throughout the SEG, but with the most extensive branching in the first 120 μm from the frontal surface. The dorsal cell body in the drawing was the more peripheral of the pair, lying near the frontal surface. The second cell body was 24 μm deeper. Reconstruction was made from the whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W*, distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

drop to stimulate a number of medium length sensilla resulted in a series of responses.

In 5 cases, presentation of the sugar stimulus inhibited spontaneous firing of a neuron (Fig. 15). None of these penetrations produced a usable LY fill. That the inhibition was specific to the sucrose input is clear from the lack of response to KCl and to water. The inhibition usually did not last for the duration of the stimulus. The delay of approximately (500 ms) for sucrose inhibition (Fig. 15) suggests that these may have been at least second order interneurons.

Neurons receiving salt cell input

While 73 cells responded to sucrose and/or mechanical stimulation of the labella, only 3 responded primarily to KCl. Two of the 3 neurons receiving salt input were filled with LY and the physiological and morphological results

are shown in Figs. 16 and 17. Cell 80 (Fig. 16) was strongly unilateral and included arborizations in the putative mechanosensory region (see above). This may account for the small response to sucrose in this cell, since this would provide a mechanical stimulus as well. The strongest response of this cell was to KCl.

Cell 113 (Fig. 17) also responded to KCl, as shown in the continuous electrophysiological record. This cell was also inhibited by the application of water, and possibly sucrose, to the labellum. Clearly, cells 80 and 113 showed different response characteristics than the larger group of sucrose responders discussed above. Morphological differences between the cells were also apparent. While cell 80 was strongly unilateral with limited arborizations in one hemi-ganglion, cell 113 arborized extensively throughout the SEG. This cell was also dye-coupled to a cell with similar morphology. The complex physiological response of these coupled cells suggests that they may be second order or higher interneurons in the taste processing system. There were no ascending or descending processes from these cells apparent in the LY fill. However, given the nature of LY and the fact that these cells were filled for only 3 to 4 min, it is possible that ascending or descending fibres were not filled (see cell 73 Fig. 18).

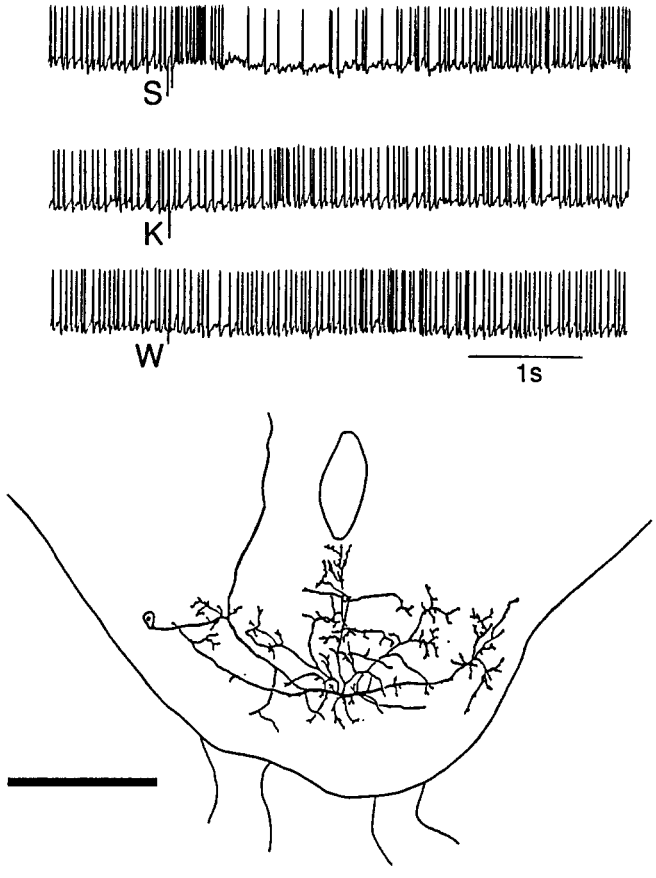


Fig. 18. Reconstruction and physiological response of cell 73. This was an incompletely filled projection neuron with a cell body midlateral in the SEG and with bilateral arborizations. Reconstructed from whole mount. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

Projection neurons that responded to sugar stimuli

A total of 4 cells were filled sufficiently to determine that they sent projections to the brain. Three of these are shown, with their physiological traces, in Figs. 18–20. Cell 73 (Fig. 18) gave a complex response when sucrose was applied to the labellum; brief excitation, followed by a longer inhibition. No change from the relatively high spontaneous background was seen with applications of KCl or water. The process running toward the brain was clearly filled. Cells 107 and 19 (Figs. 19 and 20) were the most morphologically similar pair of cells filled in this study. However, as can be seen from differences in their physiology, and some major differences in arborization patterns in the brain (see detailed descriptions in the figure captions), it is unlikely that these cells represent the same cell type from two preparations. Cell 107 (Fig. 19) showed a clear sugar-sensitivity. Cell 19 (Fig. 20) clearly responded to sucrose and to water, and not at all to KCl. Background activity was also very low to non-existent. There were delays of a second or more before spikes were seen following stimulation.

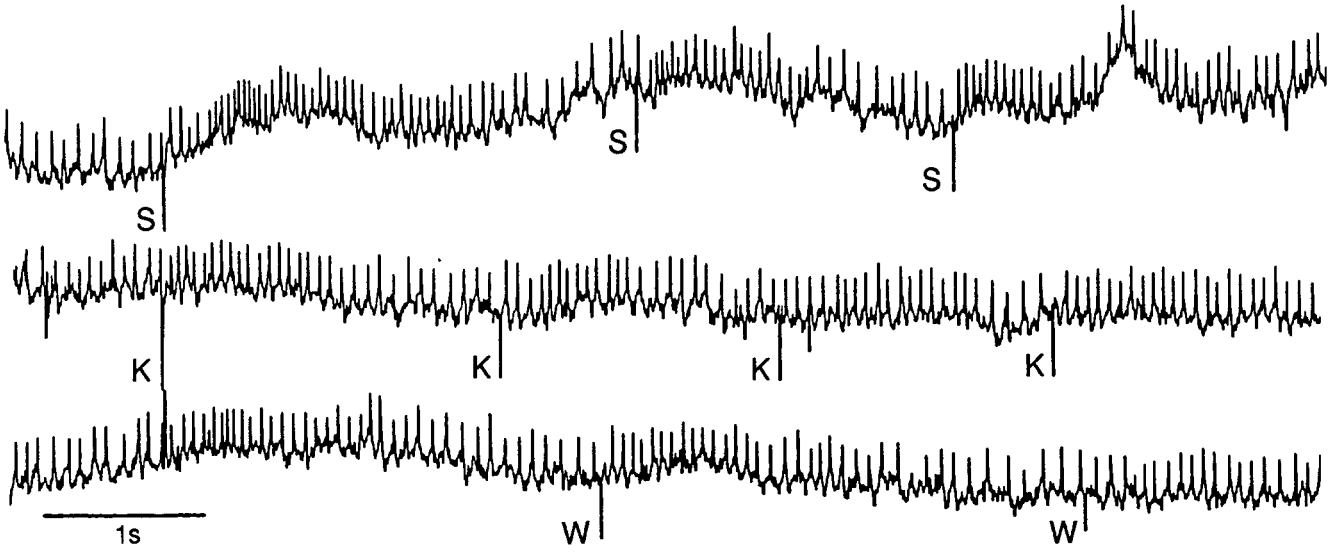
Both of these projection neurons had extensive arborizations in the brain, and were presumably involved in relaying taste information from the SEG to higher centers. As with the local interneurons responding to sugar cell input (above) these projection neurons had extensive arborizations in the SEG.

Discussion

Comparative morphology of sensory, inter- and motor neurons

The data presented here, together with the data from Yetman and Pollack (1986), Edgecomb (1986) and Edge-

Fig. 19. Reconstruction and physiological response of cell 107. This was a large projection neuron with extensive arborizations in the SEG and in the brain. Its cell body lay on the ventral surface of the SEG, in the posterior half viewed from the frontal aspect. This cell was bilateral in both the SEG and the brain. The asymmetry apparent in the reconstruction of the projections into the brain may have been due to incomplete filling. Varicosities were found throughout, but were much clearer and more abundant in the projections to the brain. Part of this cell penetrated deeply into the brain, SEG and tritocerebrum, with many varicose fibers forming a field surrounding the esophagous. The first long fibers penetrating into the brain appeared at the level of the dorsal separation of the two halves of the protocerebrum (see Strausfeld 1976, p 77). At this level, the most central fiber ran around the central body neuropil. Some small branches appeared anterior to this, but none appeared to be associated with the mushroom bodies. The major SEG arborizations were found much deeper. The deepest fibers of this cell penetrated into the most posterior neuropil of the SEG, just before the ventral connective proper. No process was seen descending into the connective. The cell body lies ventrolateral in the SEG about 120–160 μm from the frontal surface. Reconstruction from sections. The onset of the various stimuli is indicated by the appropriate letter (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm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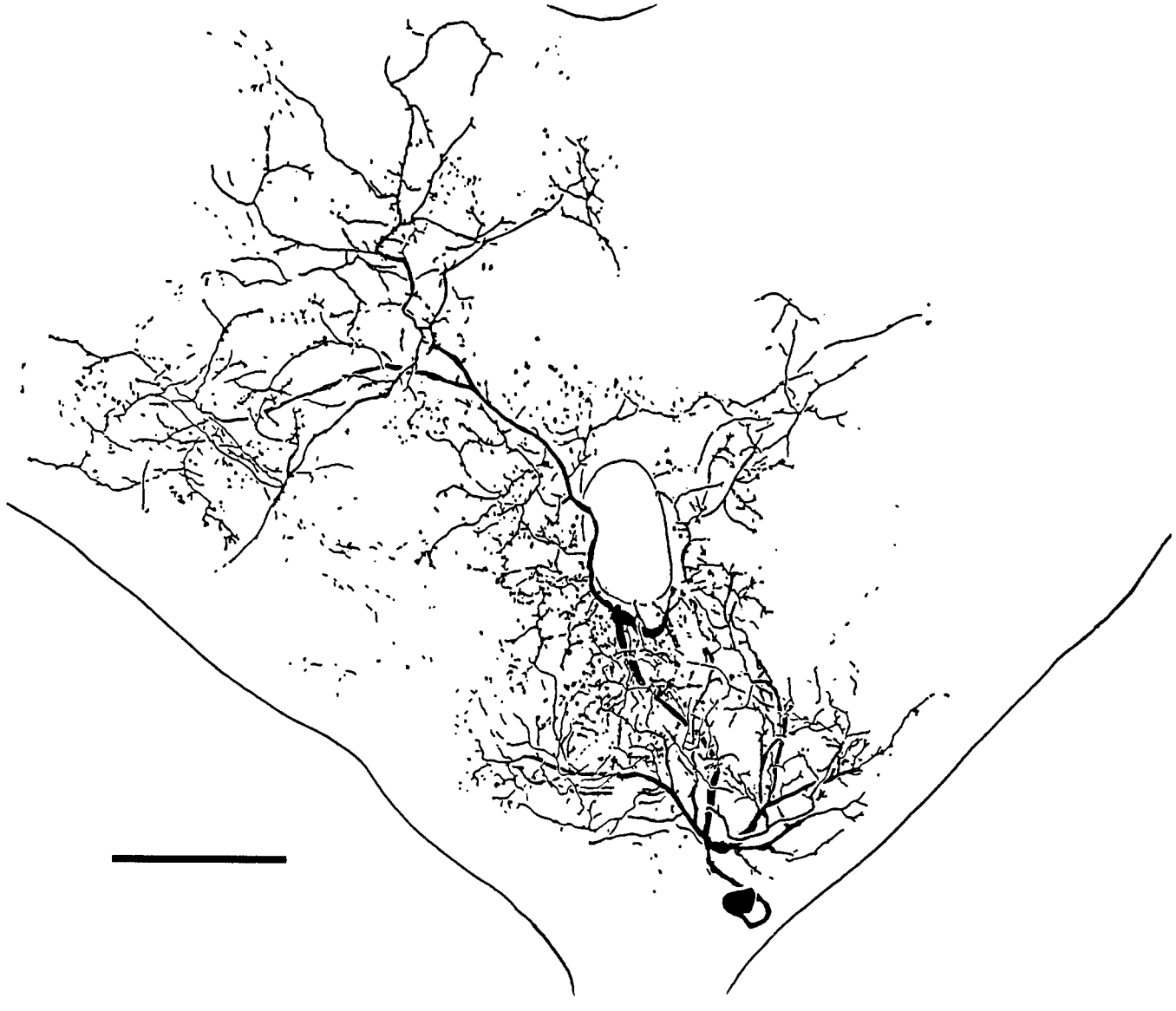

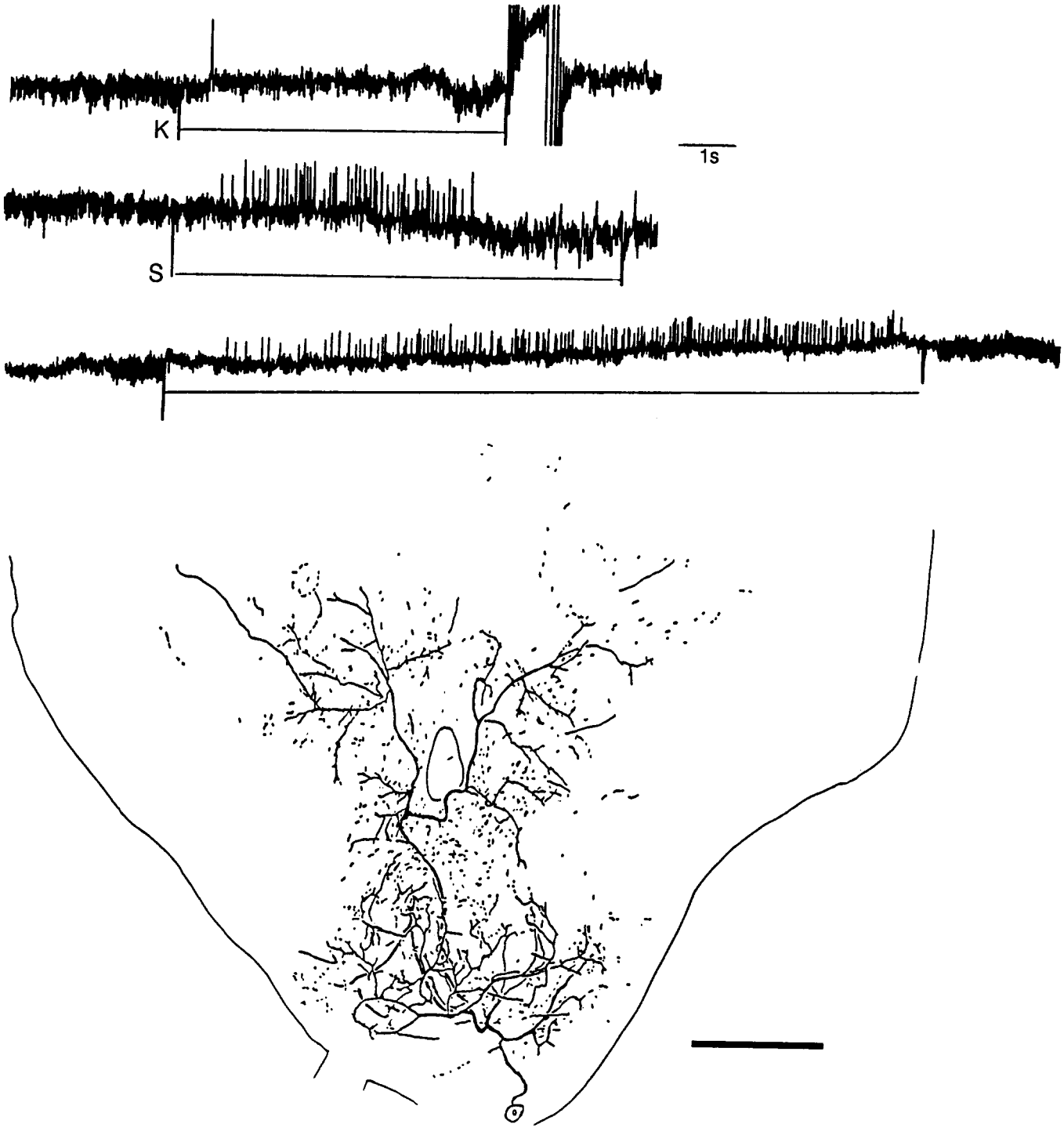

Fig. 20. Reconstruction and physiological response of cell 19. The view is frontal and dorsal is up. This was a large projection neuron with extensive bilateral arborizations in the SEG and the brain. The branches to the brain arose from processes which were in the first half of the SEG, and most of the processes appeared in sections before the central body was reached. The processes in the SEG lay deeper, in the posterior half of the ganglion. The cell body was ventro-lateral, approximately 170 μm from the frontal surface of the SEG. Reconstruction was made from sections. The *solid line* below the physiological records shows onset and duration of stimuli. (*S* 100 m*M* sucrose; *K* 1 *M* KCl; *W* distilled water). Stimuli appear in the order presented. Scale bar, 100 μm

comb and Murdock (1992) on sensory neurons and van Mier et al. (1985) and Sudlow (1991) on motor neurons, allow a general reconstruction of the integrative region for taste in the fly SEG. Though the data are drawn from 3 fly species (*Phormia regina, Calliphora vicina* and *Sarcophaga bullata*), we feel that there are enough similarities among the species to allow useful comparisons.

There is no evidence of distinct foci of gustatory integration in the fly SEG, such as the glomeruli of the insect olfactory lobe. The sensory afferents project to a

region near the root of the maxillary-labellar nerve and cover a rather limited depth of neuropil from the frontal aspect. From this same aspect, these fibers have extensive projections between the esophagous and the maxillary-labellar nerve and between the roots of the labro-frontal nerves (Edgecomb 1986). The motor nerves have projections throughout a much greater volume of the SEG neuropil. Some cells have bilateral projections and/or projections from near the frontal surface to deep in the SEG near the cervical connective (Sudlow 1991). The interneurons described here with extensive projections throughout the SEG cover at least the same volume of neuropil as the most widely arborizing motor neurons. Sudlow (1991) compared the dendritic fields of the sensory and motor cells and concluded that there is little opportunity for overlap, and therefore, of direct sensory to motor synapses. The interneurons generally have some arborizations in the regions to which the sensory afferents project, supporting Getting's (1971) suggestion that many of the first level synapses are on interneurons. This is in contrast with the suggestion by van Mier et al. (1985) that direct afferent to motor synapses may exist in flies. Altman and Kien (1987) also described some overlap between mouthpart sensory afferents and motor nerves in the orthopteran SEG, and concluded that direct synapses between the two were possible.

Of the cells reconstructed in this paper, only cell 83 (Fig. 12) remotely resembles any of the motor neurons described to date. There are general similarities in form and projection regions between cell 80 and the motor neuron to the retractor of the furca of *P. regina* drawn by Sudlow (1991). The furca retractor neuron sends its axon out the maxillary-labellar nerve, while there is no evidence that cell 80 does. It is also possible that some neurons were incompletely filled. For instance, Figs. 4 and 16 show cells with a process that could be an axon leading out a peripheral nerve. We therefore cannot be sure that these two cells are not motor neurons. Interestingly, these two cells both also have projections in the putative mechanosensory region, though only the cell in Fig. 4 responded to all stimuli (i.e. was potentially mechanosensitive). The putative mechanosensory region also appears in the general cobalt backfill of the labellar nerve (Fig. 6b) and it is impossible to determine from those preparations if motor neurons project there. However, none of the motor neurons filled by Sudlow (1991) had obvious projections to this region. The weight of available evidence suggests that this obvious lateral neuropil processes mechanosensory information, though a role as a motor center cannot be excluded.

Gustatory processing

Given the overwhelming importance of sugar, water and amino acids to nutrition in these flies, and their well known "sweet tooth", it is not surprising to find a number of interneurons responding to this input. The classical "sugar-sensitive cell" in fly labellar hairs responds to various sugars and to some amino acids (Morita and Shiraishi 1985) and in *S. bullata* is moderately sensitive to 100 m*M* sucrose, the test stimulus used (Mitchell et al. 1990). The sugar receptor cells in many labellar sensilla should have been firing at an average rate of 37.8 imp/s when the drop of 100 m*M* sucrose touched the proboscis. Such stimulation should have caused additional cells to fire (including the salt receptor cell and probably the water receptor cell) at a mean total rate of 19.5 imp/s (values taken from Tables 1–3 in Mitchell et al. 1990). Thus, the labellar nerve input to the SEG during 100 m*M* sucrose application would have come from 3, physiologically distinct chemosensory afferents, and, as such, was the most complex afferent input of the 3 used here. The major, and least variable, sensory cell activity during 100 m*M* sucrose application (see Mitchell et al. 1990) would have been from the classical sugar receptor cell. In the present work it is impossible to determine the actual contribution from each of the 3 inputs to driving any of the "sugar" interneurons. The sugar receptor cell presumably played a major role, since all of these interneurons responded better to 100 m*M* sucrose than they did to 1 *M* KCl or to distilled water. Sucrose dose-response characteristics for such interneurons could provide further evidence that the sugar cell is primarily responsible for driving them.

The experiment to determine how many sugar cells need to be stimulated to drive a sugar-sensitive interneuron raised some interesting points. Advancing the sucrose droplet to stimulate a single "longest" labellar sensillum was not effective, and more sensilla (cells) had to be recruited before a response was obtained. However, advancing the stimulus droplet to recruit more cells led to a smaller response than the control stimulus (compare Fig. 14A, B). We are not certain that fewer sensilla were stimulated in the second part of Fig. 14B than in Fig. 14A. Since the stimulus droplet was presented more slowly in trace B than in trace A, it is possible that temporal differences in stimulus presentation and stimulation of fewer sensilla contributed to the much reduced responses in trace B. Proboscis extension, and related motor activity has been measured in hungry flies when stimulating single sensilla (Fredman and Steinhardt 1973). Our results suggest that activity in gustatory interneurons may be influenced by the number of sensilla stimulated and by the rate of stimulus presentation.

Cells 16 and 113 (Figs. 16 and 17) have different responses than the other 7 sugar-sensitive interneurons (88, 104, 85, 112, 110, 83, 49) in that they appear to be concerned with input from the classic "salt receptor". This sensory cell has been associated with both proboscis extension (low concentrations) and retraction (high concentrations) (Dethier 1976) and it is in the latter context that we have studied it here. The 1 *M* KCl used probably stimulated a large number of salt receptors to fire at a rapid rate, thus sending a qualitatively and quantitatively different afferent response to the SEG compared to the 100 m*M* sucrose or water stimuli. The discovery of different cells for handling the salt vs sugar/water inputs, suggests that these inputs remain separate through the first stage of central integration. The fact that we found only 3 of 50 chemosensitive cells connected to salt afferents suggests that there are fewer interneurons of this physiological type in the system.

The sensitivity of many interneurons to both sucrose and water (eg. cells 88, 104, 85; Figs. 7, 8 and 9) probably relates to the similar behavioral effects of these stimuli on hungry and thirsty flies. Proboscis extension responses can be elicited in such flies by either water or sucrose, and feeding/drinking occurs on both (Dethier 1976). Interestingly, no interneuron was found that predominantly responded to water. There is a receptor cell sensitive to water in various flies (Evans and Mellon 1962; Dethier 1976), and the same cell probably responds to sugar as well (Wieczorek et al. 1988). The distilled water stimulus used here should have stimulated the water-sensitive cell in each labellar sensillum to a significant degree, though nothing is known about this type of cell in *S. bullata* per se. Assuming a similar situation to *P. regina,* this water input should have been qualitatively different from the 100 m*M* sucrose and 1 *M* KCL inputs, and this is borne out by the interneuron responses. It would not be expected that water and 1 *M* KCL would stimulate the same interneurons, and indeed, this was not the case. The clear separation of the sugar/water inputs and the KCl inputs at the level of SEG interneurons agrees with the known physiology of gustatory afferents and associated behavior, at the stimulus concentrations tested here. The greater effectiveness of sucrose as compared to water would not necessarily be predicted from earlier work. However, the close link between water and sugar inputs is expected.

The SEG of the fleshfly appears to use a large number of local interneurons for the processing of basic gustatory input (sugar, salt and water). The fact that we found no repetition of morphological features in the 9 local interneurons that responded to salt or sugar, suggests that further study will uncover other morphologically distinct gustatory interneurons. In the most extensively studied insect chemosensory neuropil, the antennal lobe of the male sphinx moth, *Manduca sexta,* it is also difficult to find identified local interneurons despite thousands of fills (TC Christensen, personal communication). Only further experimentation will reveal whether it is possible to identify local gustatory interneurons, in the classical sense, in the fly SEG.

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References

Altman JS, Kien J (1987) Functional organization of the subesophageal ganglion in arthropods. In: Gupta A (ed) Arthropod brain; its evolution and development, structure and function. John Wiley & Sons, New York, pp 265–301

Bacon JP, Altman JS (1977) A silver intensification method for cobalt-filled neurons in wholemount preparations. Brain Res 138:359–363

Dethier VG (1976) The hungry fly. A physiological study of the behavior associated with feeding. Harvard University Press, Cambridge, MA, 489 pp

Edgecomb RS (1986) The proboscis extension response in the black blowfly, *Phormia regina* Meigen: neural correlates and regulation of tarsal taste. PhD Thesis, Dep Entomology, Purdue University

Edgecomb RS, Murdock LL (1992) Central projections of axons from taste hairs on the labellum and tarsi of the blowfly, *Phormia regina* Meigen. J Comp Neurol 315:431–444

Evans DR, Mellon deF (1962) Electrophysiological studies of a water receptor associated with the taste sensilla of the blowfly. J Gen Physiol 45:487–500

Fredman SM, Steinhardt RA (1973) Mechanisms of inhibitory action by salts in the feeding behaviour of the blowfly, *Phormia regina.* J Insect Physiol 19:781–790

Getting PA (1971) The sensory control of motor output in fly proboscis extension. J Comp Physiol 74:103–120

Gregory GE (1980) The Bodian protargol technique. In: Strausfeld NJ, Miller TA (eds) Neuroanatomical techniques; insect nervous system. Springer, New York Heidelberg Berlin, pp 75–95

Humason GL (1967) Animal tissue techniques, 2nd ed. Freeman, San Francisco

Itagaki H, Hildebrand JG (1990) Olfactory interneurons in the brain of the larval sphinx moth *Manduca sexta.* J Comp Physiol A 167:309–320

Kanzaki R, Arbas EA, Strausfeld NJ, Hildebrand JG (1989) Physiology and morphology of protocerebral olfactory neurons in the antennal lobe of the male moth *Manduca sexta.* J Comp Physiol A 168:281–298

Marder E, Eisen JS (1984) Transmitter identification of pyloric neurons: electrically coupled neurons use different transmitters. J Neurophysiol 51: 1345–1361

Mitchell BK, Smith JJB, Albert PJ, Whitehead AT (1990) Variance: a possible coding mechanism for the gustatory sensilla on the labellum of the fleshfly *Sarcophaga bullata.* J Exp Biol 150:19–36

Morita H, Shiraishi A (1985) Chemoreception physiology. In: Kerkut GA, Gilbert L (eds) Comparative insect biochemistry, physiology and parmacology, Vol 6. Pergamon Press, pp 133–170

van Mier P, van der Molen L, van der Starre H (1985) The innervation of some proboscis structures involved in feeding behavior of the blowfly *(Calliphora vicina).* J Morphology 186: 279–287

Strausfeld NJ (1976) Atlas of an insect brain. Springer, Berlin Heidelberg New York, 214 pp

Sudlow LC (1991) Neuronal organization of the feeding behavior of the black blowfly *Phormia regina* Meigen. PhD Thesis, Dep Entomol, Purdue University

Wieczorek H, Shimada I, Hopperdietzel C (1988) Treatment with pronase uncouples water and sugar reception in the labellar water receptor of the blowfly. J Comp Physiol A 163:413–419

Yetman S, Pollack GS (1986) Central projections of labellar taste hairs in the blowfly, *Phormia regina* Meigen. Cell Tissue Res 245:555–561