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CHARACTERISTICS OF NEURONES PROJECTING FROM THE SUPRAOESOPHAGEAL GANGLION IN THE SHADOW REFLEX PATHWAY OF THE BARNACLE

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Summary

Barnacles respond to decreases in light intensity (shadows). Previous evidence indicates that the first stages of the visual pathway within the supraoesophageal ganglion comprise a limited number of neurones: a single pair of second-order neurones (I-cells), a pair of third-order projection neurones (A-cells), and at least five other projection neurones (four A-like shadow-excited cells and one shadow-inhibited cell) whose spikes may be recorded extracellularly from the circumoesophageal connective nerve. Here we present evidence that information in this pathway diverges at the A-cell stage. Destruction of both A-cells by intense illumination of cells injected with carboxyfluorescein abolishes all shadow-evoked activity in the connectives and also the ability of shadows to influence motor neurones in the ventral ganglion.

In addition, we have located the somata of these previously unidentified projection neurones, injected them with carboxyfluorescein, and observed that they have distinct branching patterns different from that of the A-cell. We name these cells 'alpha' (α), 'beta' (β) and 'gamma' (γ) cells. Simultaneous impalements show that A-cells drive at least one of the β-cells but not *vice versa*. The same observation has not yet been made with regard to α- or γ-cells. Depolarizing an I-cell does not readily drive a β-cell impaled simultaneously. These observations support our conclusion that information diverges from the A-cells and not from the second-order I-cells to the other projection neurones in this ganglion.

Introduction

Many aquatic animals defend themselves by withdrawing into a tube or shell when potentially dangerous conditions arise. An early warning of possible danger, the sudden dimming of light that may accompany the approach of a predator, often triggers a reflex withdrawal, or 'shadow reflex', that is a common part of the behavioural repertoire of many animals. It is especially well developed in those

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animals that have a sessile or sedentary mode of life, such as barnacles, because other escape procedures are not available to them.

In barnacles, the shadow reflex pathway is now known in some detail (Gwilliam, 1963, 1965; Fahrenbach, 1965; Shaw, 1972; Millecchia and Gwilliam, 1972; Hudspeth *et al.* 1977; Stuart and Oertel, 1978; Oertel and Stuart, 1981; Oland *et al.* 1983; Oland and Stuart, 1986). A limited number of neurones are involved in the early stages of the visual pathway, suggesting that an analogous simplicity may be found at later stages, rendering a synapse-by-synapse analysis possible. In this way one may discover the general rules that govern the processing of sensory information beyond the first few interneurones.

The photoreceptors, the pair of second-order cells to which they project, and a pair of third-order cells, all located in the supraoesophageal ganglion (SEG), have been studied in some detail (for reviews see Stuart, 1983; Gwilliam, 1987). The second-order cells, non spiking local interneurones, hyperpolarize when the photoreceptors are depolarized by illumination and depolarize when there is a sudden reduction in the light level. Consequently, they invert the signal and have been called 'inverting cells' (I-cells). The third-order cells project from the SEG to the ventral ganglion (VG) and have been called 'amplifying cells' (A-cells).

Previous work on A-cells has shown that they are important in passing information that the light has dimmed to motor neurones (Gwilliam, 1987). Depolarizing a single A-cell with an intracellular electrode (to mimic a shadow) hyperpolarizes extensor motor neurones and depolarizes retractor motor neurones in the VG. But other neurones in the SEG (A-like cells) respond to shadows and project to the VG (Millecchia and McIntyre, 1978). Whether these A-like cells are third-order, whether they project to the same population of motor neurones as does the A-cell, and whether they respond to the same visual information as does the A-cell is not known.

This paper is concerned with identifying the stage at which the visual information diverges in the shadow reflex pathway. We present evidence that the A-cells are the only third-order cells in this visual pathway, other shadow-responding cells being of higher order. We have also located the somata of the higher-order projection neurones and describe the branching pattern of their major processes.

Materials and methods

The isolated nervous system of the giant barnacle *Balanus nubilus* Darwin was the principal experimental material, but *Semibalanus cariosus* (Pallas) was also used. *B. nubilus* were obtained periodically from Mr Charles Eaton, Seattle, WA, and maintained in refrigerated marine aquaria until needed. *S. cariosus* were collected from the central Oregon coast as required. Following dissection (Gwilliam, 1965; Gwilliam and Bradbury, 1971), the preparation was pinned in a Sylgard-lined chamber either dorsal side up or with the supraoesophageal ganglion (SEG) dorsal side up and the ventral ganglion (VG) ventral side up, to allow

simultaneous penetration of VG motor neurones and SEG projection neurones (the 'flipped brain' configuration). The whole preparation was treated with Pronase (Sigma, type XIV or Calbiochem, B grade), 1–3 mg ml^{-1} for 1–4 min, and one or both hemiganglia of the SEG was then desheathed with fine forceps. This procedure exposed the dorsal group of small cells (Fig. 1 in Schnapp and Stuart, 1983; and Fig. 1), which includes both the I- and A-cells, for penetration with microelectrodes. The chamber was mounted so that the preparation could be illuminated by a substage darkfield condenser and viewed with a dissecting microscope. The preparation was superfused with chilled (10–14°C) barnacle saline (Hudspeth and Stuart, 1977) or with chilled Millipore-filtered (0.22 μm) artificial sea water (Instant Ocean).

Intracellular recording was accomplished with fibre-bearing microelectrodes containing (a) 4 mol l^{-1} potassium acetate (40–80 MΩ resistance), (b) cobaltic hexamine chloride (>100 MΩ) or (c) 5,6-carboxyfluorescein (3 % w/v; Kodak) in the tips and 4 mol l^{-1} potassium acetate in the shafts (80–150 MΩ). All extracellular recordings were accomplished with suction electrodes.

The ionophoretic injection of carboxyfluorescein has been used to identify and to photoinactivate single cells (Miller and Selverston, 1979; Cohen *et al.* 1983; Davis and Miller, 1985). Both viewing and photoinactivation were accomplished with the output of a Zeiss 100-W super-pressure mercury lamp focused on the preparation by the substage condenser. The illuminated portion of the preparation consisted of the entire SEG and about 100 μm of the CECs. This ensured the photoinactivation of all the arborizations of the A-cell and the initial part of the axon. The light beam passed, in turn, through a heat-absorbing filter, a BG 38 red cut-off filter, a BG 12 exciter filter, the preparation, and a yellow barrier filter and then entered the microscope objective. Up to 30 min exposure of the preparation, in the absence of dye, had no discernible effect on membrane potentials or on the activity of a wide variety of neurones in the SEG. Similarly, cells that were near neighbours of other cells injected with dye were unaffected by the photoinactivation of the injected cell (G. F. Gwilliam, unpublished results; Miller and Selverston, 1979). Dye was injected by 50 ms, 10–20 nA hyperpolarizing current pulses at 10 Hz for 2–20 min, depending on the purpose of the dye injection and the size of the cell. Reversal of polarity of the injecting pulse often unblocked a blocked electrode. More recently we have used dye-filled electrodes bevelled to resistances of 50–80 MΩ, which usually prevented electrode blockage and permitted the passage of larger amounts of current. Current was monitored by a virtual ground circuit.

In any given experiment, we determined whether the shadow reflex pathway was functioning by recording either extracellularly or intracellularly from motor neurones influenced by shadows, or extracellularly from the circumoesophageal connectives (CEC). Extracellular recordings were also made from a branch of the great splanchnic (GS) nerve, the mid-dorsal (MD) nerve or one of the cirral (CN) nerves. These nerves contain the axons of motor neurones which give prolonged regular bursts of spikes that are abruptly truncated by shadows (see Fig. 5A–C) or

excited by shadows (see Fig. 5D). Recordings from the CECs showed increased activity at light offset if the pathway was intact (Figs 7 and 9). The intracellular recordings were made from a neurone called the 'ventral responding cell' (VRC) (Fig. 4; Fig. 5C) or from one of the motor neurones innervating muscles of the cirri (CMN) (Fig. 5).

Originally, voltage traces were stored on a four-channel Racal FM magnetic tape recorder and printed out on a Gould two-channel chart recorder or photographed from the oscilloscope face with a kymograph camera. More recently, the traces were digitized with the aid of locally developed A/D hardware (Metaresearch, Inc., Portland, Oregon) and a data acquisition program (developed at Reed College) which has a 7 KHz/channel sampling rate and signal-averaging capability. The sampling rate is more than adequate to define accurately the spikes generated by barnacle neurones. Such records were stored on an Apple Macintosh computer and printed out on a laser writer.

Results

Location, morphology and physiology of A-cells

Locations of A-cells in the SEG

Fig. 1 is a *camera lucida* tracing of the neuronal cell bodies that are visible on the dorsal side of a typical SEG of *B. nubilus*. The only cell types that are readily identifiable are two large cell pairs, one indicated by a triangle on the anterior margin (the 'cross commissural' cells of Ross *et al.* 1986; see also Fig. 1 of Davis and Stuart, 1988), and the other, indicated by an asterisk, on the apex of the thickest part of the ganglion (the 'dorsal bulge'). The group of small cells that surrounds the cross commissural cell in a hemiganglion and lies between it and the dorsal bulge cell consists of approximately 45 somata in both *B. nubilus* and *S. cariosus* (Schnapp and Stuart, 1983; Moskowitz, 1986).

A-cells and I-cells are always found medial to the cross commissural cell, and the I-cell very close and almost always medial to the A-cell. They are usually located on the anterior margin and are sometimes only to be found by 'rolling' the ganglion posteriorly to rotate the anterior edge in the dorsal direction. With experience, one learns to increase the chances of locating the A-cell on the first penetration by choosing one of the larger cells near the medial margin of the cross commissural cell. The A-like cells discussed below are usually found near the A-cell but none of them are topographically identifiable in the living preparation.

Morphology of complete A-cells

Fig. 2 presents a composite diagram of a complete A-cell (Fig. 2A) as well as *camera lucida* tracings of two cobalt-injected A-cells from *B. nubilus* (Fig. 2B,C) and one from *S. cariosus* (Fig. 2D). Although A-cells have been filled with horseradish peroxidase and their synapses investigated by electron microscopy (Schnapp and Stuart, 1983), only a preliminary description of the gross morphology has appeared (Gwilliam, 1987). We here present a detailed description of

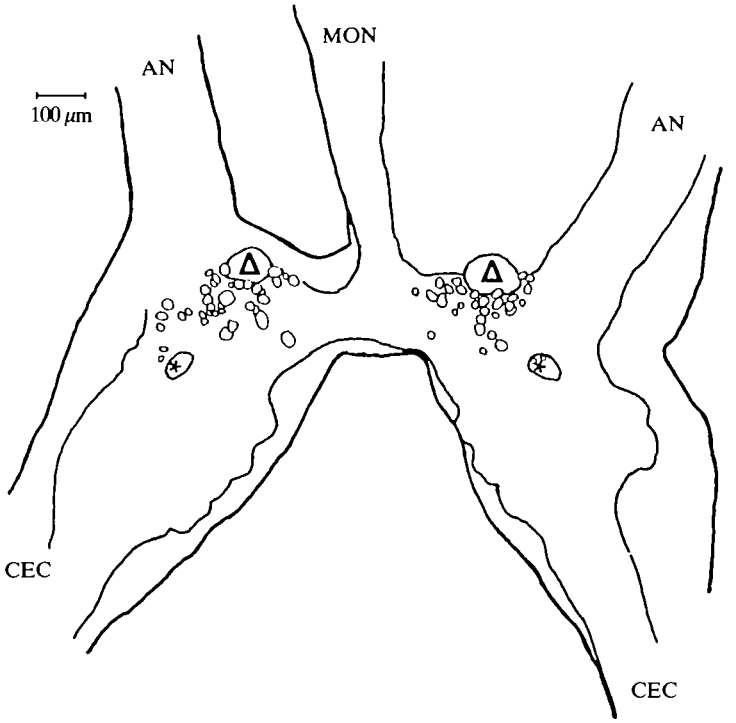


Fig. 1. *Camera lucida* tracing of the dorsal aspect of the supraoesophageal ganglion of *Balanus nubilus* to show the disposition of the group of dorsal small cells. The antero-medial cell cluster includes the I-cell, A-cell and other projection neurones, none of which may be identified simply by size and/or position. The preparation was fresh and unstained, and the right side had been desheathed. Two identifiable cells, the cross commissural (CC) neurone (△) and the dorsal bulge cell (*) are indicated. CEC, circumoesophageal connective; MON, median ocellar nerve; AN, antennular nerve.

the morphology of the cell and demonstrate that the two genera have essentially the same A-cell morphology and that there is little individual variation. In both species the A-cell was identified by its characteristic hyperpolarizing response to the onset of light and a large and prolonged depolarization at light offset (Stuart and Oertel, 1978, and see below). The A-cell has a cell body 10–15 μm in diameter in the SEG. A short stalk proceeds posteriorly from the soma and branches into an ipsilateral neuropilar arborization relatively close to the cell body (Fig. 2B,C). The process then bends almost at a right angle, crosses the commissure, and arborizes in the contralateral hemiganglion in about the same position as that of the ipsilateral arborization. The distance of these two arborizations from the midline suggests that they are the sites of I-cell to A-cell synapses (Schnapp and Stuart, 1983). Contralaterally, in addition to this medial arborization, two or three separate arborizations branch from the major neurite into the main part of the neuropile.

The neurite then continues unbranched through the contralateral circumoeso-

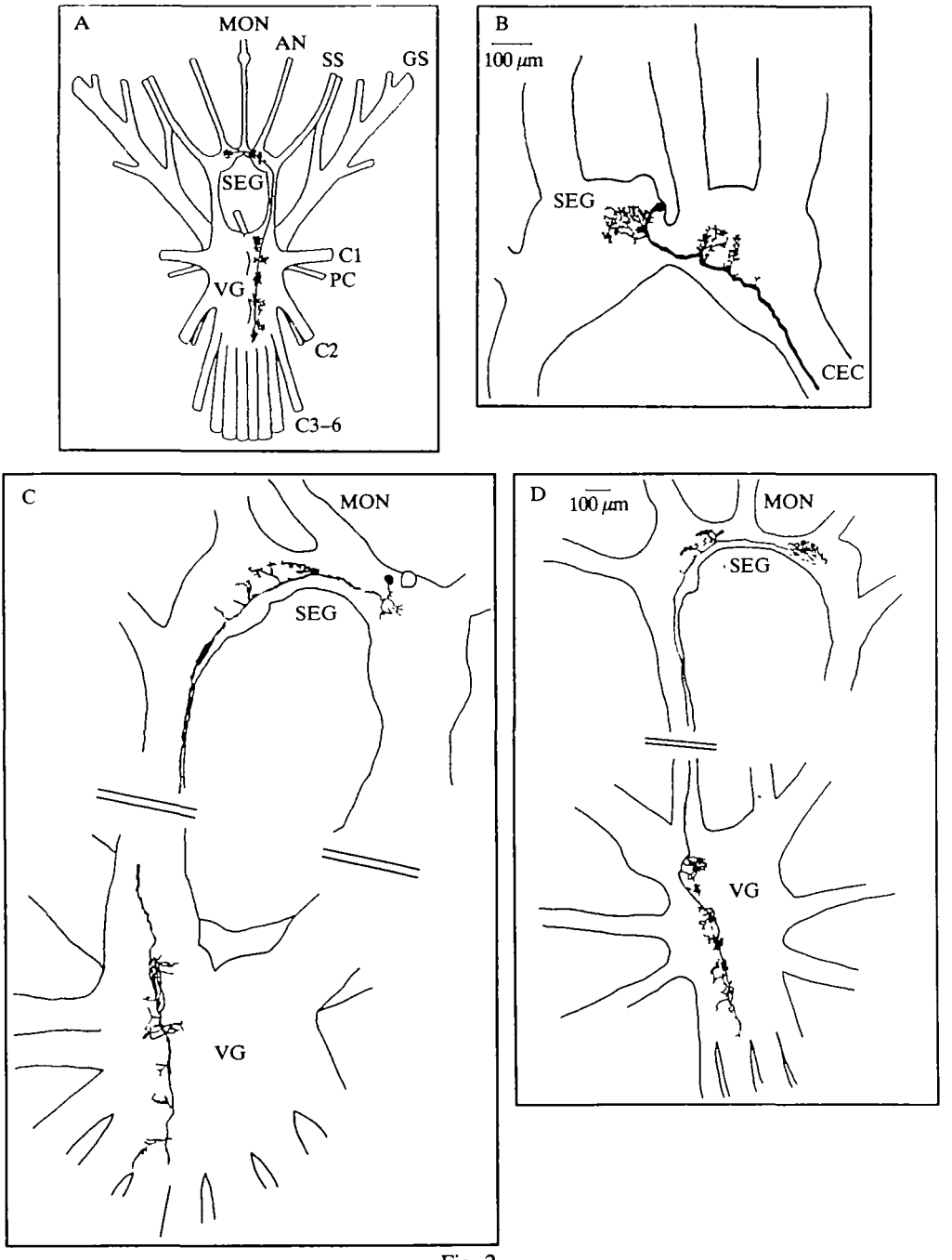


Fig. 2

phageal connective (CEC) to the VG (Fig. 2A). Upon entering the VG towards the ventral side (Fig. 2C,D), the neurite immediately executes two almost right-angled bends, coursing first dorsally then posteriorly. As it travels posteriorly, it arborizes first in the suboesophageal region, and then periodically five or six times

Fig. 2. *Camera lucida* tracings of cobalt-injected A-cells in *Balanus nubilus* and *Semibalanus cariosus*. (A) Composite diagram drawn from four injected cells in *B. nubilus* and two injected cells in *S. cariosus* to illustrate the extent of the A-cell in the CNS. (B) The soma and arborizations of an A-cell within the supraoesophageal ganglion (SEG) and its projection into the circumoesophageal connective (CEC) in *B. nubilus*. (C) An entire A-cell from *B. nubilus*. (D) An entire A-cell from *S. cariosus*. SS, suprasplanchnic nerve; GS, great splanchnic nerve; C1–6, cirral nerves; PC, paracirral nerve; VG, ventral ganglion; other abbreviations as in Fig. 1. Scale in B also applies to C.

in regions appearing to correspond to the locations of cirral motor neurones (Gwilliam, 1987). This single A-cell pair, then, extends essentially the entire length of the central nervous system and potentially could exert widespread influence on its activity.

Fig. 2D shows the SEG arborizations of one of the two injected A-cells from *S. cariosus*. The detailed similarities of the branching pattern of this cell with that of *B. nubilus*, in addition to their responses to light onset and offset, strongly suggest that these cells are homologous.

Activity of A-cells and input to them from the VG

A-cells show a striking amount of spontaneous synaptic and impulse activity in the dark or the light (Stuart and Oertel, 1978; Fig. 3). The dimming of light (Fig. 3A, arrow; Fig. 5A,B) causes a large transient depolarization, generating a burst of spikes in the cell, followed by an increase in the frequency of existing spikes riding on a prolonged underlying depolarization. In preparations such as this one where the VG is still attached to the SEG, the background activity is interrupted occasionally by 'spontaneous' hyperpolarizations (Fig. 3A,B, asterisks). Curiously, dimming has no effect on the cell during these events (Fig. 3A). This hyperpolarization is coincident with activity in neurones in the VG and projecting to the SEG, as may be shown by recording from the cut VG end of the circumoesophageal connective that does not contain the axon of the impaled cell (Fig. 3B, arrow on diagram). While we have no evidence that these units projecting from the VG to the SEG caused the A-cell's hyperpolarization, complex postsynaptic potentials may be generated in the A-cell by stimulating the connective either ipsilateral (arrow, Fig. 3C) or contralateral to its soma. Clearly, the A-cells do not simply signal shadows to other neurones, but integrate information derived from several sources.

A-cell activity affects motor neurones

Depolarization of the A-cell with shadows or injected current influences the activity of motor neurones in the VG. Fig. 4 shows intracellular recordings from a cell in the VG that we call the VRC (ventral responding cell); this projects to the periphery and thus is likely to be a motor neurone. Action potentials in the A-cell elicited either by the offset of light (Fig. 4A) or depolarizing current (Fig. 4B) are followed one-for-one by depolarizing synaptic potentials in the VRC, which is

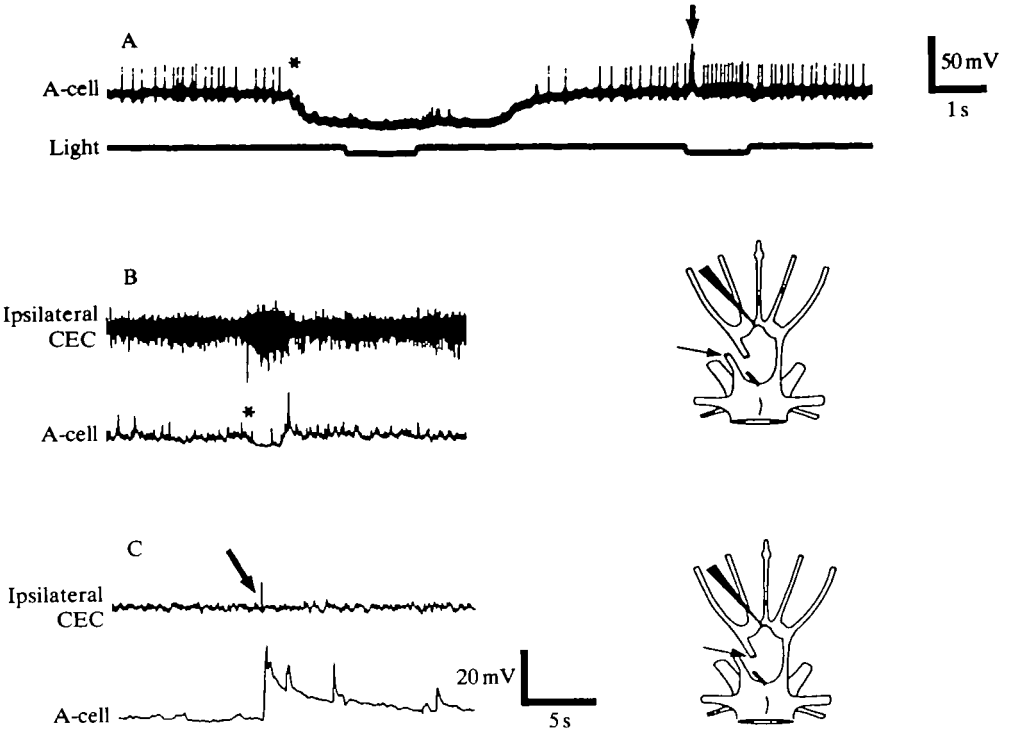


Fig. 3. Characteristic spontaneous and evoked activity in A-cells in preparations with VG attached to SEG. (A) Shadows (downward deflection of light trace) delivered to an A-cell during and after a spontaneous wave of hyperpolarization (*). Shadows normally evoke a depolarization (arrow) but are ineffective during the hyperpolarization. (B) Extracellular recording from the VG end of a severed CEC (small arrow on diagram) shows that a spontaneous burst in this CEC is coincident with a hyperpolarization (*) of the A-cell (large arrowhead on diagram). VG has been cut in half in the diagram. (C) Stimulation of the SEG end (small arrow on diagram) of this severed CEC evokes a large, prolonged depolarization of the A-cell. The stimulus was a single, supramaximal shock to the nerve (arrow). Calibration bar in C also applies to A-cell trace in B. All traces are from the same preparation.

consistent with the connection being monosynaptic. This pathway, then, would appear to consist of only four stages: photoreceptor, I-cell, A-cell and VRC. Other motor neurones that are excited or inhibited by shadows have been described as units in VG nerves (Gwilliam and Bradbury, 1971, and see below), but their cell bodies have for the most part not been found; thus the A-cell's connections to these motor neurones are not yet known.

Selective removal of A-cells from the visual pathway

Effect of shadows on motor neurones

Shadows excite A-cells but also excite several other units in the SEG that project to the VG (Millecchia and McIntyre, 1978). These units could be third-order, like

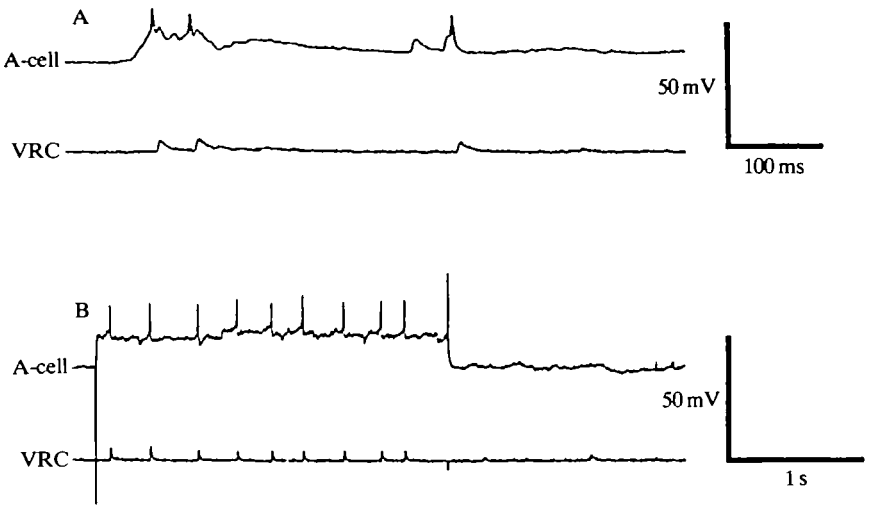


Fig. 4. Evidence that the A-cell is connected monosynaptically to a presumptive motor neurone (VRC: ventral responding cell) of the VG. (A) Spikes elicited in the A-cell by the offset of light, which triggers the sweep, elicit depolarizing synaptic potentials in the VRC. (B) Spikes elicited in the A-cell by a depolarizing current pulse are followed one-for-one by synaptic potentials in the VRC.

A-cells, or could be higher-order and driven by A-cells. One of the aims of the present work was to determine whether and at what stage the visual information diverged in the pathway. Specifically, we attempted to destroy the A-cells and see if the shadow reflex disappeared. If the reflex were to remain intact, it would indicate that other third-order cells exist in the pathway. As an assay of whether the reflex was intact, we recorded the effect of shadows on motor neurone somata in the VG or on motor units in the VG nerves.

Fig. 5 shows motor units recorded extracellulaly from certain nerves leaving the VG and from two somata, in four different, normal preparations. In such preparations where the SEG and VG are left connected to one another (Fig. 5, diagram) there exists a regular bursting pattern of action potentials in motor units within these nerves (Gwilliam and Bradbury, 1971; Gwilliam, 1976). The offset of light (a 'shadow') depolarizes the A-cell (upper traces of each set) and alters this pattern. In most cases, when light offset occurs during a burst it abruptly terminates the burst (Fig. 5A–C). Fig. 5 illustrates this truncation for units in a branch of the great splanchnic nerve (GS, Fig. 5A), the mid-dorsal nerve (MD, Fig. 5B) and one of the cirral nerves (CN, Fig. 5C; the VRC was also impaled during this experiment). Note that bursting can resume even when the light is kept off, but the interruption of a burst by light offset is clear and complete (see also Figs 6A,B; 8A,C). Thus, these motor units act as if they or their antecedents have been transiently inhibited. They are units which would serve to extend cirri; a shadow causes them to pause.

Conversely, certain VG motor neurone somata and cirral motor units are

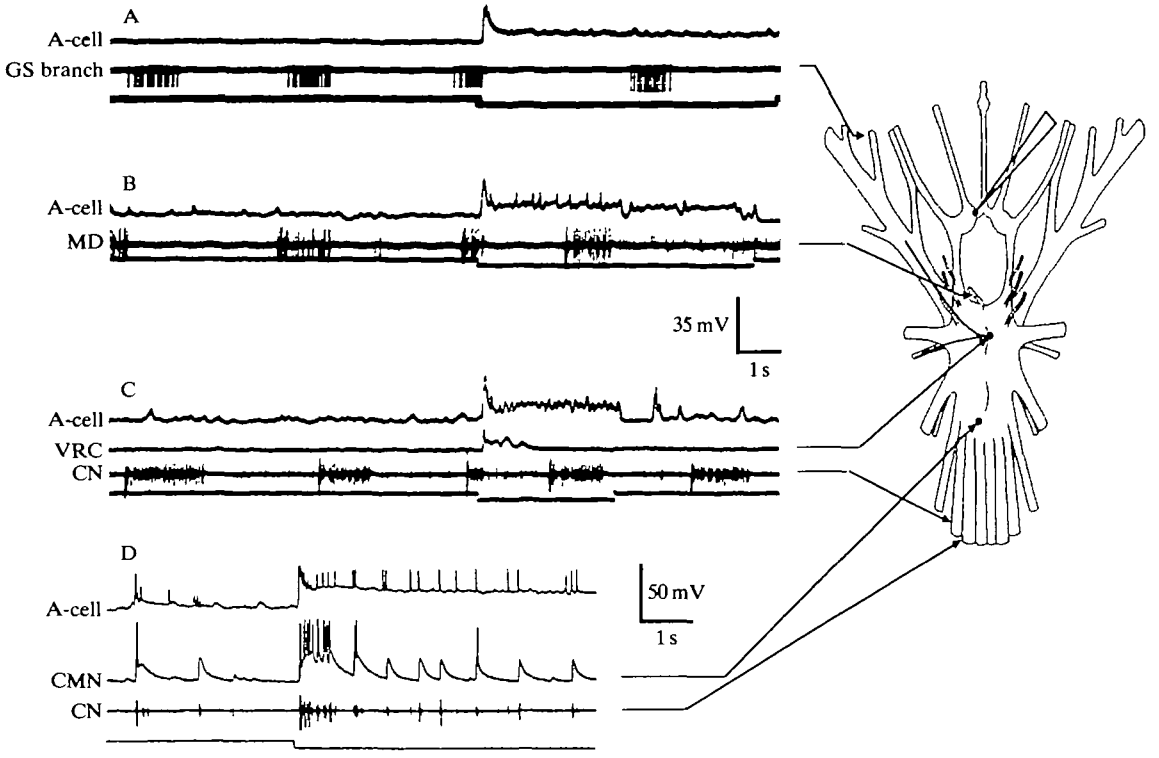


Fig. 5. The effect of the offset of light on the firing of the VG motor neurones. Recordings were made in four different preparations (A–D) from various somata and nerves indicated on the diagram. In A–D, the uppermost trace is an intracellular recording from the A-cell's soma (open arrowhead on diagram) and the lowermost trace indicates the light (downward deflection=offset). (A,B,C) Light offset depolarizes the A-cell and interrupts bursting in motor units of, respectively, a branch of the great splanchnic nerve (GS), the mid-dorsal nerve (MD) and a cirral nerve (CN). Light offset also excites certain motor neurones, among them, shown in C, the ventral responding cell (VRC). (D) A cirral motor neurone (CMN) and units in another of the cirral nerves (CN) are excited by light offset. Voltage calibration applies only to the intracellular records.

excited by shadows (Fig. 5D). These units participate in retracting the cirri. The excitation or inhibition of motor neurones by shadows occurs in both nerves of a pair, even when one connective is severed (not shown); thus, information from the SEG invades the VG bilaterally.

Killing A-cells with carboxyfluorescein

We killed the A-cells by injecting them with the dye carboxyfluorescein, which diffuses quickly throughout the cell and is lethal to it when brightly illuminated with light of the appropriate wavelength (approx. 425 nm) (Miller and Selverston, 1979; Cohen *et al.* 1983; Davis and Miller, 1985). Fig. 6 shows the effect of injected dye on an A-cell and on motor units in the MD nerve. Responses of the A-cell and

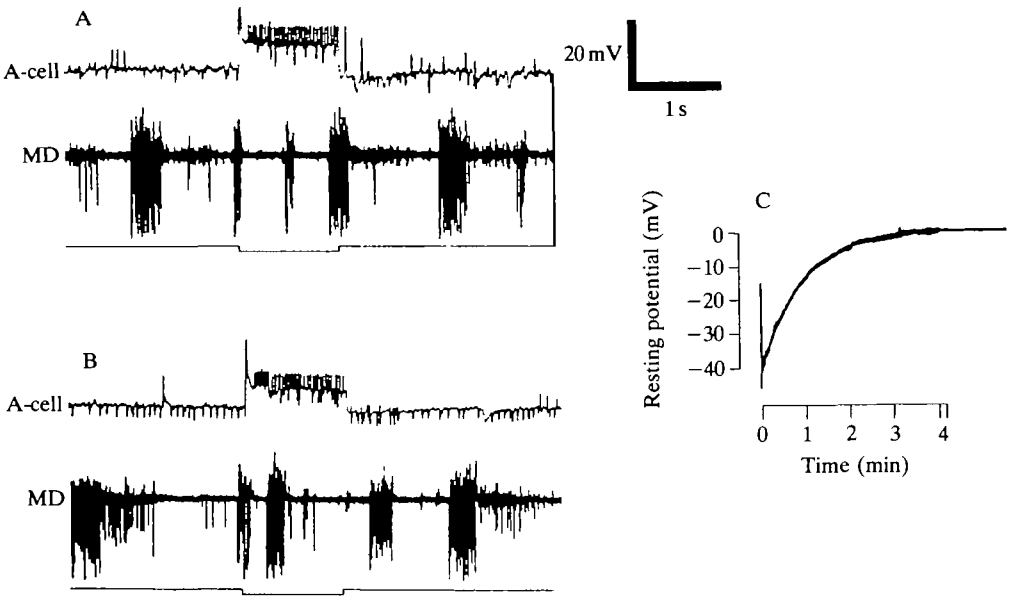


Fig. 6. Injected dye does not affect the A-cell until it is illuminated (see also Fig. 9). (A,B) The responses of an A-cell and the mid-dorsal nerve (MD) to a 1-s shadow (lowermost trace) were recorded before (A) and after (B) injection of the cell with carboxyfluorescein but before intense illumination. The dye has no obvious effect on the responses of the A-cell or the nerve to the shadow (offset of light). (C) Recording from another dye-filled A-cell showing the time course of photoinactivation of the cell. The resting potential (ordinate) falls to zero over about 4 min after illumination of the cell with the intense blue light.

MD units to the offset of light were observed before (Fig. 6A) and immediately after (Fig. 6B) injection of dye into the cell. As long as the preparation was not illuminated, the dye did not significantly alter the response of the A-cell or motor units to the offset of light.

Upon illumination of the entire SEG with the appropriate wavelength (Fig. 6C), dye-filled A-cells depolarized slowly to 0 mV and never recovered. (This process is essentially identical to photoinactivation with Lucifer Yellow, but needed to be established independently for carboxyfluorescein.) Fig. 7 shows that the A-cell's spike in the contralateral connective also disappeared after photoinactivation, indicating that the lethal effects of the dye had extended beyond the cell's soma. Before dye injection, action potentials in the cell's soma were followed one-to-one by spikes in the contralateral connectives (Fig. 7A) and the offset of light elicited a burst dominated by this unit (Fig. 7B). After photoinactivation, the unit no longer appeared in the recordings or responded to the offset of light (Fig. 7C). The short burst in smaller, more delayed units probably represents activity in higher-order shadow-activated units driven by the other A-cell of the pair. It must be said, however, that Fig. 7 is not entirely convincing on its own because there are two spikes in Fig. 7C (the third in the initial burst and the large spike in the middle of

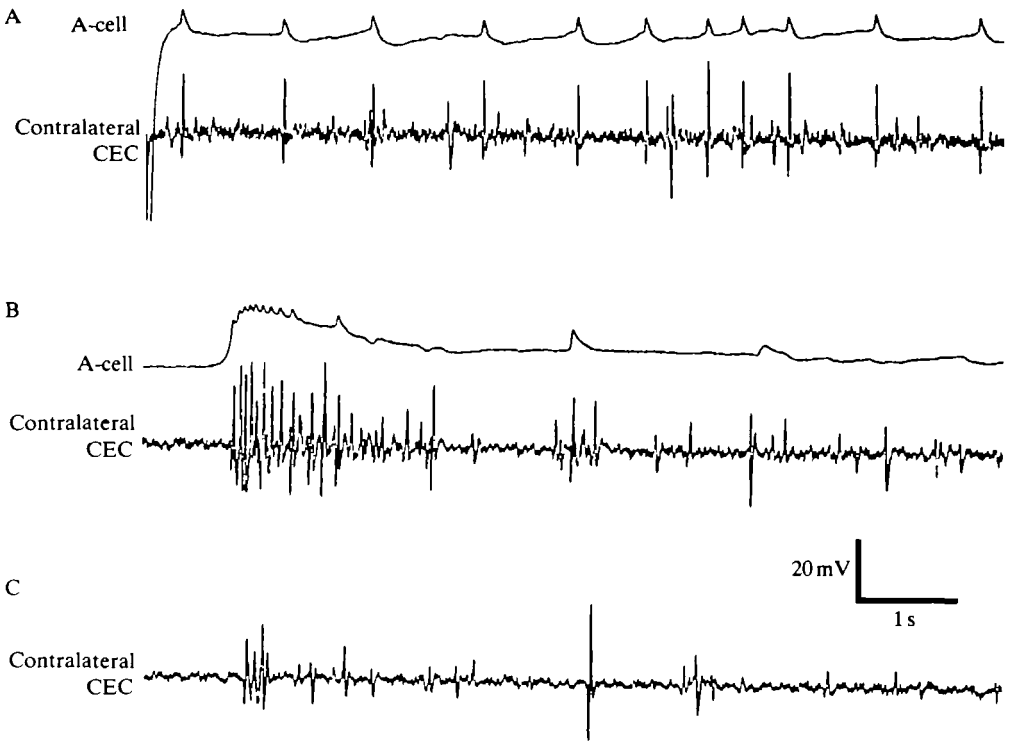


Fig. 7. Photoinactivation of an A-cell results in the disappearance of the cell's action potential from the contralateral CEC. In A and B, the upper trace is an intracellular recording from the A-cell's soma and the lower trace an extracellular recording from the contralateral CEC. In B and C the sudden offset of light triggers the sweep. (A) Driving the A-cell with steadily injected depolarizing current results in spikes in the CEC that follow those in the A-cell one-for-one. (B) The offset of light results in a characteristic depolarization of the A-cell on top of which ride small action potentials that do not invade the soma. It also elicits a burst in the CEC comprising several units. The large or intermediate spikes in the CEC are from the A-cell's axon, and the smaller spikes are other shadow-activated units. (C) Following photoinactivation of the A-cell, the sudden offset of light no longer evokes obvious A-cell spikes but does evoke other shadow-activated units of at least three different amplitudes. Note: the CEC has many axons and displays spontaneous activity. The judgment that the spikes in C are not A-cell spikes is based on latency and spikes of similar size not associated with A-cell spikes in A and B. See text for further comment.

the record) that are similar in size to the A-cell spikes and might represent some residual activity in a 'damaged' A-cell. In an extracellular recording of a multi-axon nerve there is no way absolutely to assign similar spike amplitudes to the same axon in the absence of a correlated event (such as soma spikes as in Fig. 7A,B). This figure, however, in conjunction with, especially, Fig. 9, provides strong evidence that the A-cell is indeed rendered inoperable by this photoinactivation procedure. Even though the ventral ganglion projections and a part of the CEC axon would not be affected by the killing process (see Materials and

methods), the photoinactivation of the entire SEG part of the cell (soma and the bilateral neuropilar segments) and the initial CEC length of the axon, would effectively remove any possibility of light-related input to the A-cell and, thus, transmission of that kind of information.

Injecting one A-cell with dye and cutting the other's axon

To remove both A-cells from the visual pathway while retaining connections of other (potentially third-order) cells between the SEG and VG, we attempted to kill one A-cell in its entirety, and then severed the connective containing the axon of the other. With one connective left intact (containing the dead axon of the A-cell), we expected to leave operative other possible shadow-activated pathways from SEG to VG. Fig. 8 presents the results of destroying one A-cell in a

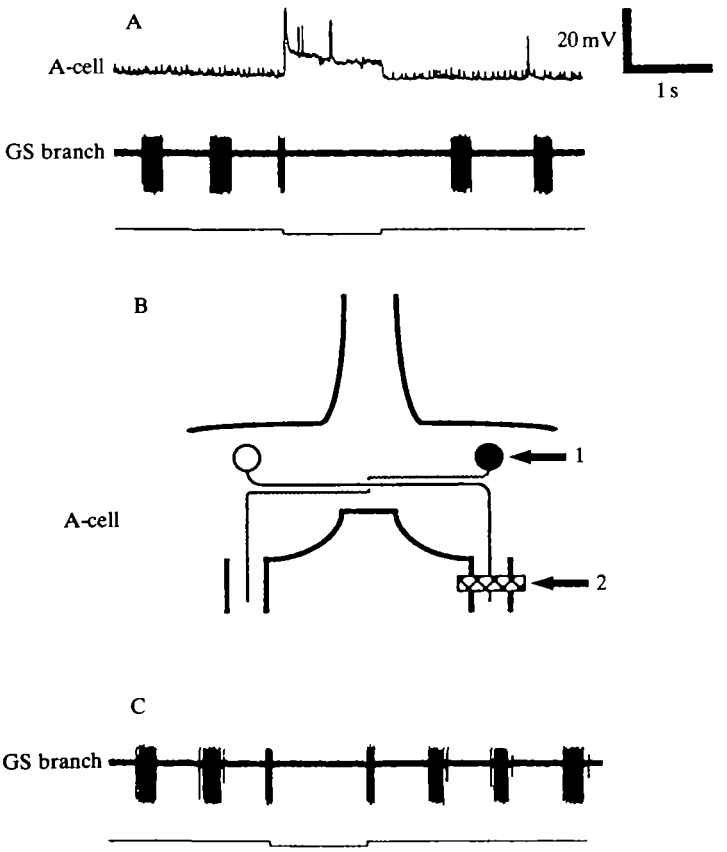


Fig. 8. Photoinactivation of only one A-cell does not disrupt the shadow reflex pathway. (A) Response to a shadow (lower trace) of an A-cell and of a motor unit in a branch of the great splanchnic nerve (GS) before dye injection. (B) Diagrammatic summary of the experiment. Step 1, inject and kill the impaled A-cell (shaded); step 2, sever ipsilateral CEC, thus removing the other A-cell from direct communication with the VG. (C) After photoinactivation of the A-cell and severing the ipsilateral CEC, the GS unit still responds to a shadow.

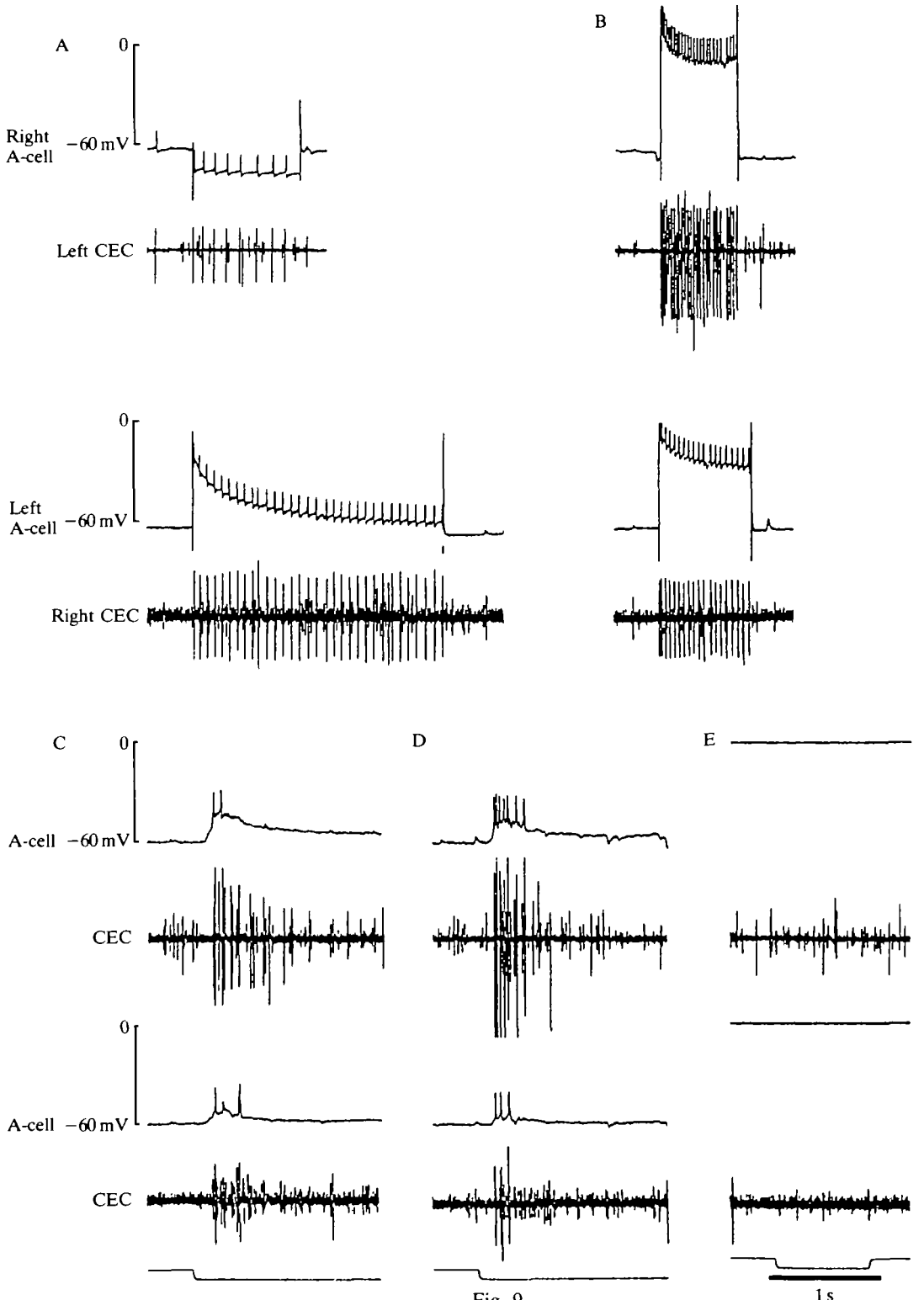
preparation by photoinactivation and interrupting the projection to the VG of the other A-cell by cutting the CEC (see diagram, Fig. 8B). In the intact preparation (Fig. 8A), the offset of light depolarized the A-cell and interrupted bursting in a nerve of the VG. The A-cell was then injected with dye and killed by illumination (Fig. 8B, step 1), and the connective containing the axon of the other A-cell was cut (Fig. 8B, step 2). In this and three other similar experiments, a shadow still interrupted bursting in the VG nerve (Fig. 8C). Thus, information that the light has dimmed must be conveyed from SEG to VG by cells in addition to the A-cells. Such cells might be other third-order visual interneurones, or higher-order cells driven within the SEG by the uninjected A-cell.

Injecting both A-cells with dye

To test the hypothesis that the A-cell is the only third-order neurone and that visual information must diverge from A-cells to higher-order cells, both A-cells were successfully injected with dye and killed simultaneously on three occasions. The results of this extraordinarily difficult experiment are presented in Figs 9 and 10. In the experiment illustrated in Fig. 9, we recorded intracellularly from both A-cells and monitored their action potentials in both CECs simultaneously. Since each A-cell projects into the contralateral CEC, the right A-cell's axon projects into the left CEC and *vice versa*. In normal saline, a depolarizing current pulse injected into each A-cell (Fig. 9A,B), or the offset of light (Fig. 9C,D), elicited spikes in the soma followed one-for-one by large spikes in the contralateral connective. The offset of light also elicited activity in other smaller units in each CEC (Fig. 9C,D). After injection of carboxyfluorescein but before illumination (Fig. 9B,D), there was no significant change in the response of either cell to a current pulse or to the offset of light. Intense illumination of the dye-filled cells with blue light killed both A-cells, and their resting potentials fell to zero (Fig. 9E). The offset of light then caused no response in either CEC, neither from the A-cell's axon nor from lower-amplitude units which are presumably the α- and β-cells described below. This result provides evidence that the A-cell is the sole shadow-excited third-order cell of the visual pathway.

In the experiment illustrated in Fig. 10, simultaneously impaled A-cells were

Fig. 9. Simultaneous photoinactivation of both A-cells disrupts the shadow reflex pathway. A–E all show simultaneous intracellular recordings from both A-cells (first and third traces) and from both CECs (second and fourth traces). The trace from each A-cell is displayed above the trace of the contralateral CEC containing its axon. (A) Depolarization of each A-cell with current sets up spikes in the cell, followed by an action potential in the cell's axon in the connective. In both CECs the largest unit is that from the A-cell's axon. The left CEC trace in this panel is at half its gain in the other panels. (B) Injection of each cell with carboxyfluorescein does not alter the ability of the cell to spike or to conduct them into the nerve. C–E show the responses of the cells to the offset of light: (C) before dye injection, (D) after injection of carboxyfluorescein but before illumination and (E) after photoinactivation with intense illumination. Photoinactivation causes each A-cell's resting potential to fall to zero; the offset of light elicits no activity above background in any units of the CEC.


A
0
Right
A-cell
−60 mV
Left CEC
B
0
Left
A-cell
−60 mV
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CEC
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CEC
D
E
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Fig. 9

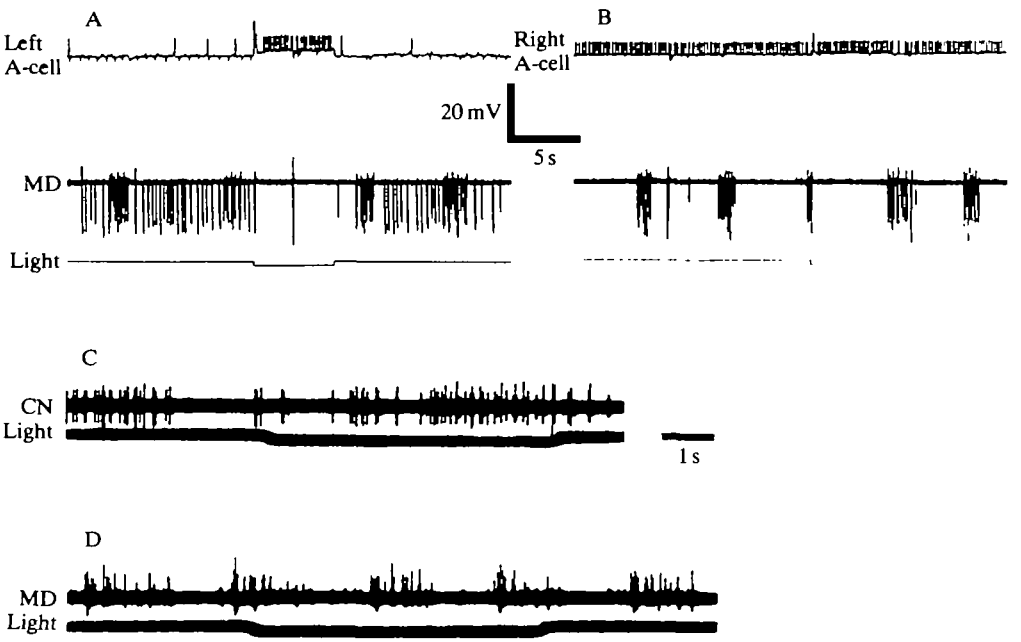

Fig. 10. Photoinactivation of both A-cells also removes the influence of shadows on motor neurones in the ventral ganglion in the intact preparation. (A,B) Responses of right and left A-cells displayed with those of the mid-dorsal nerve (MD) showing that, after filling with carboxyfluorescein but before illumination, shadow-evoked interruption in the mid-dorsal nerve is still operative. (C) Shadows have no effect on cirral nerve motor neurones (CN) after the A-cells have been illuminated. (D) The same for the mid-dorsal nerve.

killed in preparations still connected to the VG. VG motor nerve units in these preparations (in cirral and mid-dorsal nerves) then lost the ability to respond to shadows. This evidence corroborates the conclusion that all excitation which signals the offset of light passes through the A-cell and thence to higher-order cells.

Other shadow-activated neurones projecting from the SEG to the VG

We systematically explored the dorsal small cell group to find the somata of the additional units activated by shadows. Millecchia and McIntyre (1978), using a computer spike-recognition program, found six shadow-detecting units in *S. cariosus*: the A-cell, four A-like cells excited by dimming and one cell inhibited by dimming. If it is true that A-cells are the only third-order cells in the pathway, they must be driving these other units either directly or through other interneurones. Electrical stimulation of an A-cell does indeed result in increased activity of other units in the ipsilateral connective (92 spikes during stimulation *versus* 55 spikes in a comparable period without A-cell stimulation). Incidental observations during searches for A-cells revealed cells among the dorsal small cells that resembled A-cells in giving a large, transient depolarization at the offset of light. One cell gave a

large hyperpolarization at light offset. Injection of these cells with carboxyfluorescein showed that they could be distinguished from one another by the characteristic pattern of branching of their major processes. This morphology could be visualized during physiological recording without damage to the cells. Cells were injected with carboxyfluorescein and viewed immediately with a fluorescent light source. Although the dye is lethal when intensely illuminated, cells survive and function normally if they are viewed for short periods with low light levels. The dye then leaks from the cell within 15–20 min, permitting successive cells to be impaled, dye-filled and identified. Typically, each cell was injected following observations of its characteristic spontaneous activity and responses to the offset of light, then viewed very briefly for identification purposes to avoid photo-damaging the cell. Here we present a preliminary account of the known characteristics of these cells that can be shown to have quite profound effects on motor neurones in the ventral ganglion.

Characteristic activity and branching patterns of A-like cells

A-like cell pairs project into the *ipsilateral* connective, rather than the contralateral connective, as the A-cell does. Fig. 11 shows the morphology of these two neurones. One cell type, called the α-cell (Fig. 11A) arborizes bilaterally in the SEG, as does the A-cell; the other, called β (Fig. 11B), arborizes only unilaterally. We have observed nine α-cells and 24 β-cells. Three β-cells have

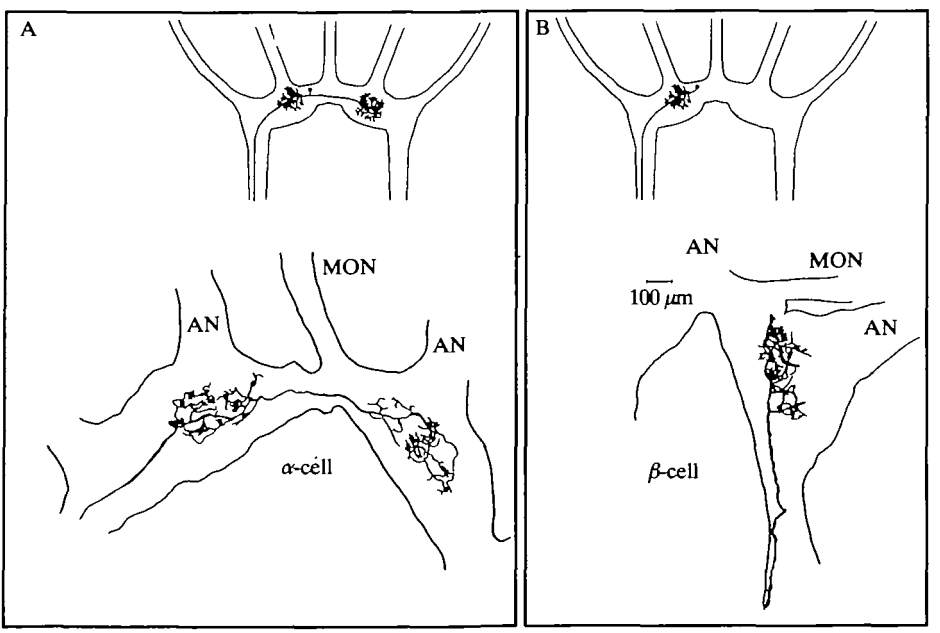

Fig. 11. Morphology of the α- and β-cells in the SEG. Each cell has been drawn with a *camera lucida* from a cell injected with cobalt and each is accompanied by a diagram to clarify the distribution of the neuropilar segments. Abbreviations are as in Fig. 1. The scale bar applies to A and B. Superficially, the γ-cell is indistinguishable from a β-cell.

been filled with dye in one hemiganglion in the same preparation, suggesting that they form a subgroup rather than a single pair. We have never observed more than one α-cell in a hemiganglion.

Fig. 12 shows the responses of the two types of ipsilaterally projecting neurones

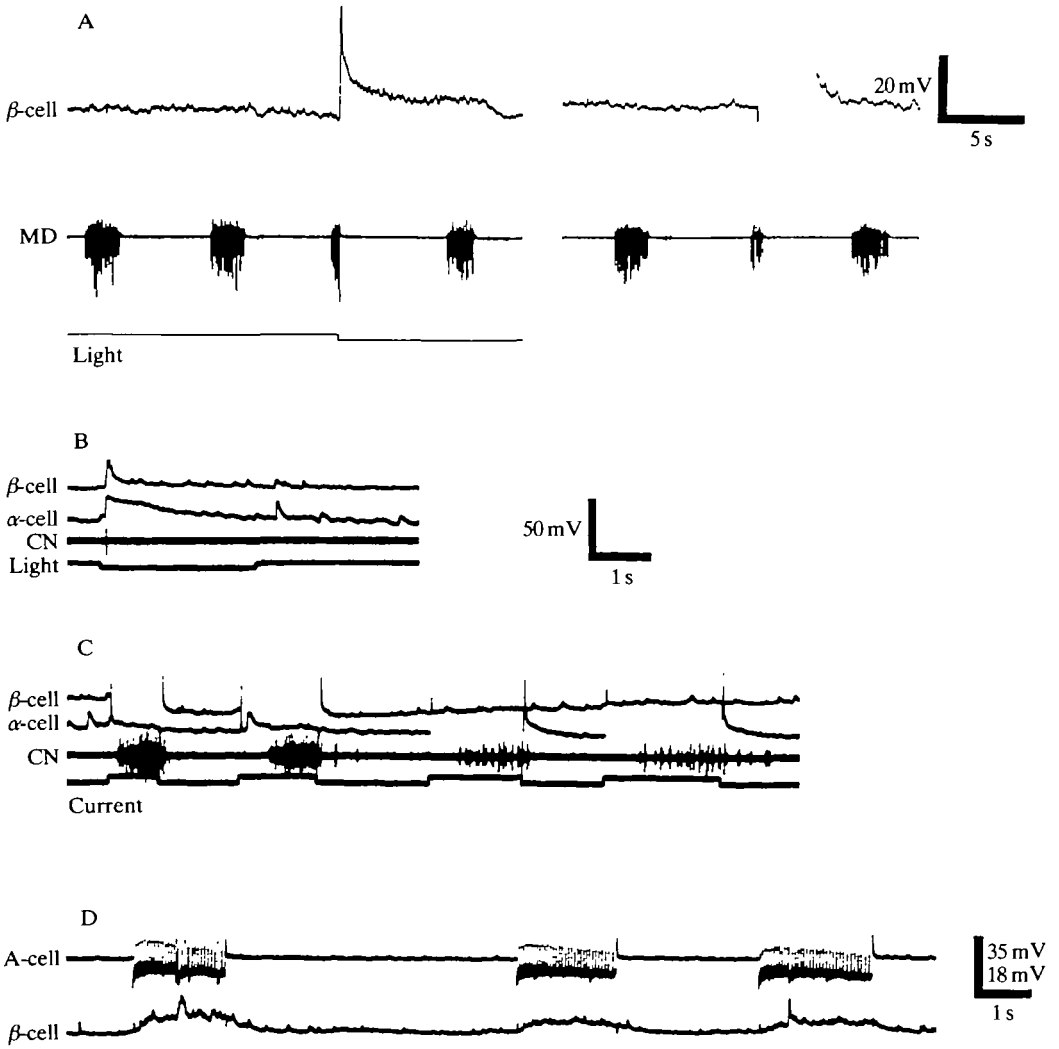

Fig. 12. Responses of the α- and β-cells to light and their effect on motor units. (A) Left, responses of a β-cell and a unit of the mid-dorsal nerve (MD) to the offset of light. Right, depolarizing current injected into the β-cell inhibits the burst in the MD unit. (B) Responses of β- and α-cells (and of a cirral nerve, CN, motor unit) to the offset of light. (C) Current passed into either the β- or the α-cell drives units in the CN nerve. (D) Depolarizing current injected into an A-cell sets up impulses in that cell and causes depolarizations in a β-cell, impaled simultaneously. Onset and offset of depolarizing current is obvious from the intracellular trace in the absence of a current monitor. B and C are from the same preparation; A and D from two other preparations.

to shadows and depolarizing current pulses. Both cell types depolarize at the offset of light (Fig. 12A,B). Although in this figure the off-response of the α-cell is longer than that of the β-cell, we have not so far found a repeatable difference between the two. They are usually distinguishable from A-cells on the basis of their activity (as recorded intracellularly), but are not clearly separable from each other (compare Figs 3, 5, 6, 7 and 9 with Figs 12 and 13). Both the A-like cells truncate motor neurone bursts when depolarized with current, as shown for a β-cell in Fig. 12A, and excite motor units in one of the CN nerves (Fig. 12C). It is not yet known if the different projection cell types excite and inhibit the same or different motor neurones.

The principal evidence that α- and β-cells project to the VG and influence motor neurones there is derived from the morphology (an axon courses through the ipsilateral CEC) and from the observations recorded in Fig. 12. It should also be possible to demonstrate that spikes from these neurones are detectable in the CECs and Fig. 13 illustrates this for a dye-confirmed β-cell. The nature of the preparation makes it impossible to demonstrate the effect of shadows or depolarizing current on neurones in the VG and then show directly that the same cell's activity is detectable in the CECs. This would be theoretically possible with *en passant* recording, but several attempts to do that have not been successful.

In one simultaneous impalement of an A-cell and a β-cell (Fig. 12D), firing the A-cell with current is correlated with depolarization of the β-cell, but not *vice versa*. While this particular record does not demonstrate one-to-one postsynaptic potentials in the β-cell, the observation is consistent with the conclusion that the A-cell precedes the other shadow-excited projection neurones in the pathway. In other dual recordings of dye-confirmed β-cells and A-cells, there is no clear evidence of a connection. It is possible that the three β-cells occupy different positions in the circuit. In addition, in several simultaneous impalements of I-cells and β-cells we were unable to drive the β-cell by depolarizing the I-cell (or *vice versa*). Had the β-cell been directly postsynaptic to the I-cell, we would have expected to be able to drive it, as is possible when the postsynaptic cell is the A-cell (Oertel and Stuart, 1981). It is not clear just how direct the connection is from A-cells to A-like cells. Dual penetration of the two types of cells is technically very difficult (the cells are small and very close to each other) and it will be some time before we can accumulate enough evidence to settle this issue. A complete understanding of the role of the A-like cells will also depend upon being able to distinguish between the three β-cells. Experiments are currently underway to resolve this.

A cell that hyperpolarizes in response to shadows

In the vicinity of the A- and A-like cells lies a cell soma of similar size but which hyperpolarizes at the offset of light (γ-cell, Fig. 14). We have often impaled these cells when looking for the other cell types. Injection of carboxyfluorescein and cobalt shows that the γ-cell, like the β-cell, arborizes only ipsilaterally and projects into the ipsilateral connective, and on superficial morphological grounds is

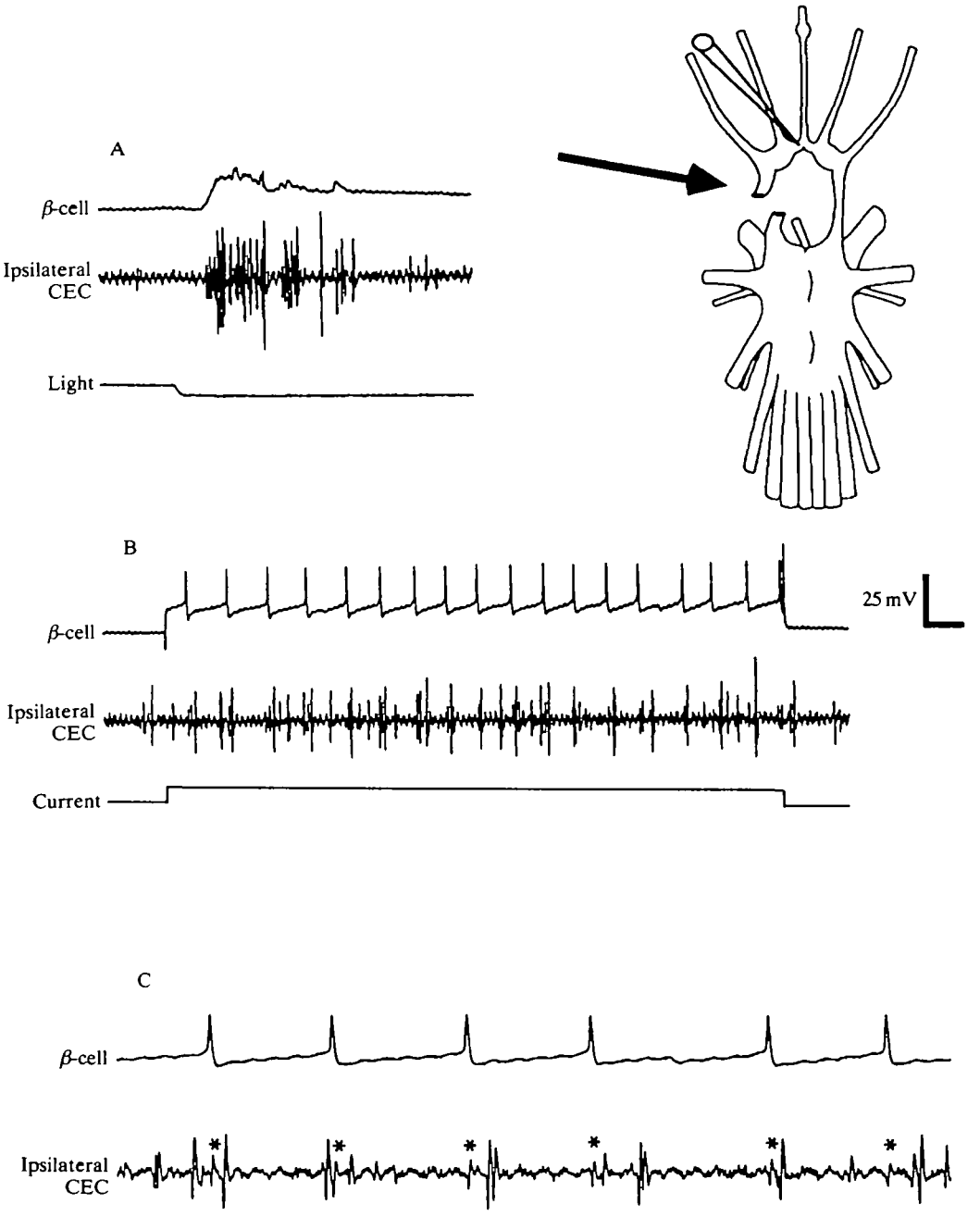

Fig. 13

indistinguishable from a β-cell. Depolarizing γ-cells (with current) does not affect activity in VG nerves, and so their role in the shadow reflex is not immediately obvious. Typically, when well penetrated, these cells are silent, although they may

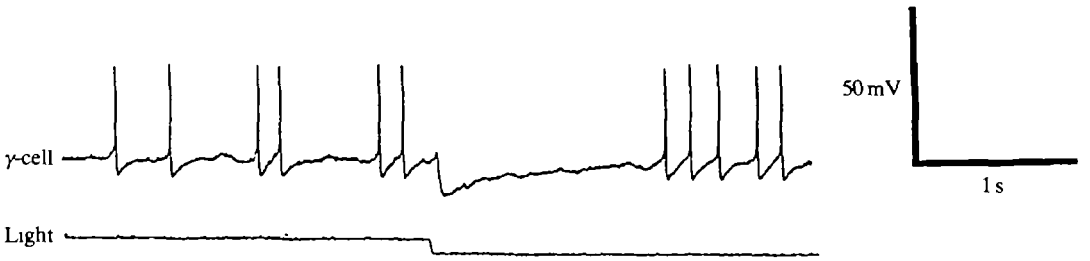

Fig. 14. Response of a γ-cell to the offset of light. Light offset elicits a marked, prolonged hyperpolarization in the cell.

also fire slowly (Fig. 14). The γ-cell is obviously a candidate for the single shadow-inhibited unit described by Millecchia and McIntyre (1978).

Discussion

Many of the neurones within the two ganglia that constitute the barnacle's nervous system are responsive to changes in light intensity. As might be expected, cells of the VG controlling body extension, retraction and fishing movements are affected by shadows (Gwilliam and Bradbury, 1971). Within the smaller SEG, to which the photoreceptors project, attention has previously focused on the second-order I-cells and the third-order A-cells. But many other neurones within this ganglion also respond to shadows (Oland *et al.* 1983), including the large presumptive motor neurones of the ventral side and a group of neurones in addition to the A-cell that project to the VG (Millecchia and McIntyre, 1978).

The major finding of this paper is that information in this shadow-detecting pathway diverges from the unique, single pair of third-order A-cells and not before. Photoinactivation of both A-cells abolishes all shadow-evoked activity in the connectives and prevents shadows from influencing motor neurones in the VG. In addition, we have located the somata of the higher-order visual projection neurones and described their branching patterns. Anatomically, these cells are distinct from the A-cells and from each other.

Divergence within the visual pathway

Extending from the region of the photoreceptor terminals to arborize in each of the fused segments of the VG, the third-order A-cells are in a position to link the

Fig. 13. Evidence that the ipsilaterally projecting neurones can be detected in the CEC. (A) Response to light offset. (B) Response to depolarizing current. (C) Sweep speed in B increased to show that the 1:1 spike (indicated by asterisks) is a small-amplitude event and not the larger spike that in a compressed time scale it may appear to be. Voltage calibration applies to all intracellular records; time calibration, A, 100 ms; B, 137 ms; C, 33 ms.

sensory limb of this visual pathway (the photoreceptors and the I-cells) with the motor cells of the VG. It has been known for some time, however, that the A-cells are not the only visual projection from the SEG to the VG: Millecchia and McIntyre (1978) estimated that four other units in the CEC were excited and one was inhibited by shadows. It is natural to ask whether they carry redundant information, and whether they influence the same set of motor cells.

The present experiment, in which both A-cells were photoinactivated, argues that information diverges not from the I-cell but from the A-cell to the other projection neurones. Destruction of both A-cells abolished activity in all the units normally responding to the offset of light in both CECs and in nerves of the VG. Since we have now located the cell bodies of these CEC units, it will eventually be possible to confirm their proposed higher-order position in the pathway by recording from them simultaneously with an I- or A-cell. Simultaneous recordings made in the present work from β-cells and I- or A-cells showed that depolarization of the A-cell but not the I-cell drove the β-cell. Depolarization of the β-cell did not drive either the I-cell or the A-cell. From this sort of experiment one can argue that the β-cell must be higher-order than either the I- or A-cell, and that it is closer to the A-cell in the hierarchy. Present evidence does not allow us to say whether these other projection neurones are immediately postsynaptic to the A-cell or if other cells (presumably local interneurones) are interposed between them.

One would, of course, expect depolarizations of the I-cell to drive all the visual cells if they mimicked those evoked by light. Probably the current passed into the I-cell's soma does not spread well into its arborizations (Oland *et al.* 1987) and thus the voltage change there is too weak to mimic the light-evoked events.

Backfills of the CEC with cobalt tracer reveal 8–10 somata in the contralateral dorsal small cell group (Gwilliam and Cole, 1979, Fig. 4D) and certainly the A-cell must be among this group. The other cells are unlikely to be visual cells because the newly identified visual interneurones all project out of the ipsilateral CEC. Further, it is unlikely that more than one A-cell exists, since the disappearance of all shadow-evoked activity upon photoinactivation of both A-cells argues that this pair of cells is unique in the ganglion. This finding supports the belief of its singularity, developed over years of searching through this group of cells without finding more than one A-cell per hemiganglion.

While the evidence indicates that A-cells are the only third-order projection neurones *excited* by shadows, we do not know the position of the shadow-*inhibited* (γ) cell in the pathway. This cell must be spontaneously firing in order to detect inhibition of its spike in the CEC, and intracellular recordings show that it is often silent. Whether this cell is third-order will have to be determined by recording from I- and A-cell/γ-cell pairs.

The precise number of visual projection neurones

In each hemiganglion we have found only one A-cell, one α-cell and one γ-cell, but as many as three β-cells. This collection of projection neurones could entirely account for the six shadow-respónsive projection neurones described by Millecchia

and McIntyre (1978). These authors extracted six units from extracellular recordings with a computer program using 20 templates, based on arbitrary designation of parameters. Their number of five shadow-excited units agrees with counts of spike heights from oscilloscope traces of CEC off-responses (G. F. Gwilliam, unpublished results); this agreement, together with the number of α- and β-cells that we located, and the fact that their computer program unexpectedly located an inhibited unit that we now confirm as the γ-cell, suggests that this program is effective.

Clearly α- and β-cells are distinct from the A-cell, since their axons project ipsilaterally, but are they distinct from one another? The sole evidence that they are distinct types is their branching patterns within the SEG: α-cells arborize bilaterally and β-cells unilaterally. There is always the possibility that β-cells are actually α-cells whose commissural process has been damaged during dissection so that dye does not spread to the contralateral arborization. We think this unlikely because dye spreads across the commissure in processes of other cells in the same preparation. Physiological characterization of these cells may point out differences in the cells' activity and resolve this issue.

The central role of the A-cell in the visual pathway

Of all the projection neurones, the A-cell is the most obvious. Its action potential is consistently associated with the largest unit in the contralateral CEC, indicating that its axon is the largest of the visual projection neurones. Typically, in a given neuronal population, those neurones with larger axons also have larger somata; indeed, the A-cell's soma, more prominent than its neighbours in the dorsal cell cluster, attracted attention before the others. One would also expect information to travel more quickly along its axon than along those of the other projection neurones. The A-cell would appear to be the alarm system for the visual pathway, aided by the other projection neurones in a manner yet to be understood.

In contrast to the I-cell, the A-cell's activity suggests that it has a complex integrative function in the pathway and is not simply an 'amplifier' of I-cell voltages, as its name implies (Stuart and Oertel, 1978). In particular, our evidence indicates that A-cells receive both depolarizing and hyperpolarizing input from neurones of the VG. One particularly intriguing observation was that, during a period of hyperpolarizing input, shadows could not elicit a response from the cell. The A-cell might provide a point at which the shadow-reflex could be 'turned-off'; for example, when shadows are cast by the animal's own cirri during fishing movements.

It is also possible that the A-cells receive other sensory input (e.g. touch) that provokes withdrawal. Many of the spontaneous synaptic potentials on the A-cell, even in a preparation with the VG attached to the SEG, are not related in frequency to the onset or offset of light. These potentials might be governed by other sensory inputs that have been destroyed in the dissection. In fact, any mechanical stimulus to the cirri, the mantle edge or the shell will cause a closure

response that is indistinguishable from that provoked by shadows (Gwilliam, 1965). Because the same muscles are ultimately involved, the visual and mechanosensory pathways must come together at some point. It will be of interest to see if this point is the A-cell.

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