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LOCALIZATION AND STIMULATION OF CHROMATOPHORE MOTONEURONES IN THE BRAIN OF THE SQUID, *LOLLIGUNCULA BREVIS*

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SUMMARY

The relatively simple chromatophore system of the squid, *Lolliguncula brevis*, was studied with combined behavioural, morphological and electrophysiological methods in order to understand how the chromatophore patterns in the skin are organized at the level of the posterior chromatophore lobes (PCL). There are nine simple chromatic components of patterning in *L. brevis*. Retrograde transport of horseradish-peroxidase from chromatophores in the mantle skin established that the chromatophore motoneurones are located in the PCL. Focal threshold stimulation of the PCL in perfused, semi-intact preparations showed that the motor fields of individual chromatophore motoneurones are compact, including 2–60 chromatophores, generally of the same colour. Adjacent motoneurones in the lobe do not necessarily have adjacent motor fields in the skin.

INTRODUCTION

A unique feature of cephalopod behaviour is the repertoire of chromatophore patterns in the skin that serve as intra- and interspecific signals (Holmes, 1940; Packard, 1963; Packard & Sanders, 1971; Hanlon, 1982). These patterns are generated by neuronal activity that coordinates chromatophore expansion in different regions of the body. Unlike the chromatophores of other animals (notably crustaceans, fishes and amphibians, see Bagnara & Hadley, 1973), cephalopod chromatophores are true organs comprising a pigment-containing cell surrounded by 10–30 radially arranged muscle fibres whose contraction and relaxation, under direct nervous control, cause expansion and retraction of the pigment cell (Hofmann, 1907; Cloney & Florey, 1968). As a result, the chromatophore patterns can change instantly in response to environmental or endogenous stimuli, and provide a remarkable two-dimensional display in the skin of neural activity in the brain. In crustaceans and amphibians, chromatophore expansion is hormonally controlled, whereas in flounders it is neurally controlled (Fujii, 1969) but does not involve

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muscle contraction. Cephalopod chromatophores form the only colour change system in the animal kingdom that relies upon the contraction of independent muscle fibres to expand the pigment cells and is controlled directly by the nervous system.

Four levels of organization have been distinguished for chromatophore patterns (Packard & Sanders, 1971; Packard, 1982): (1) elements: individual chromatophores; (2) units: groups of chromatophores defined either morphologically by their repeated arrangement in the skin (chromatic units) or functionally as those chromatophores that are innervated by a single motoneurone (motor units); (3) chromatic components: including several motor units and representing distinctive and repeatable features of the patterns; and (4) whole chromatophore patterns: composed of the coordinated appearance of several components. In this paper, we describe the chromatic components in *Lolliguncula brevis* and we combine morphological and electrophysiological techniques to understand how the motor units are organized at the level of the lower motor centres.

Evidence from different cephalopod species suggests that several brain lobes are involved in the control of chromatophore activity (Fig. 1). A central role seems to be played by the lateral basal lobes of the supraoesophageal brain mass. These lobes receive inputs from the optic, peduncle, olfactory, medial basal and probably also

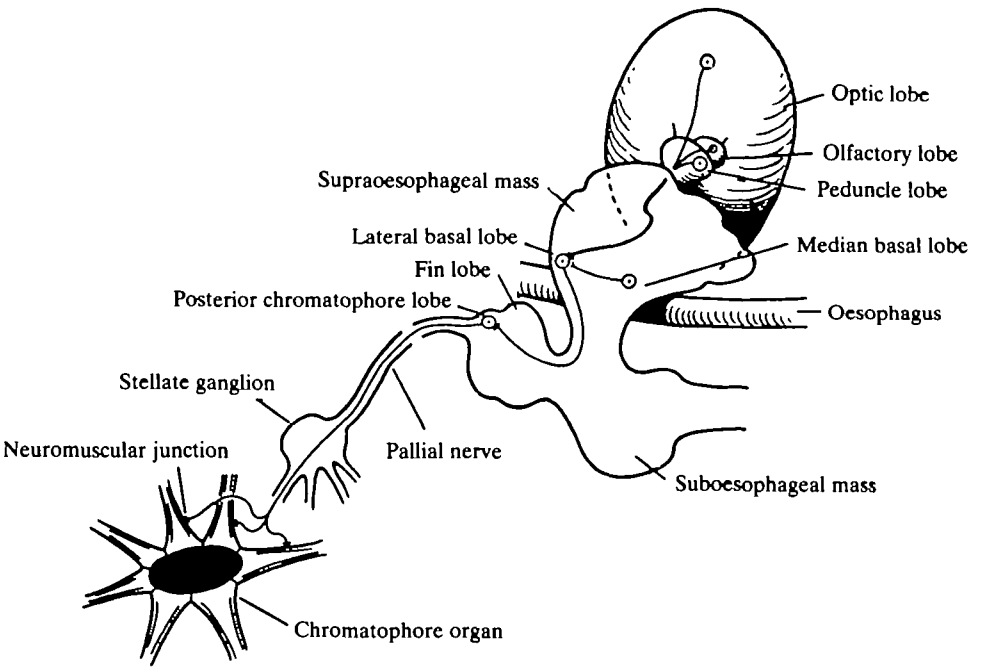


Fig. 1. Diagrammatic representation of lobes in the cephalopod CNS that are thought to control chromatophore patterning. Only one side of the brain is represented and the anterior chromatophore lobe is omitted for the sake of clarity. The main pathway is considered to be: optic lobes to lateral basal lobes to chromatophore lobes to chromatophores.

from other lobes integrating sensory or motor inputs (Young, 1971, 1974, 1976; Boycott, 1961). Extracellular stimulation of the lateral basal and peduncle lobes in *Sepia* (Boycott, 1961) and of the peduncle lobes in *Octopus* (Messenger, 1965) produces chromatophore expansion in all parts of the body, whereas stimulation of the pedal lobes of *Sepia* (Boycott, 1961) results in chromatophore retraction. Stimulation of the medial basal lobes in *Sepia* produces expansion and retraction of chromatophores, sometimes giving crude patterned responses (Boycott, 1961). However, features resembling normal patterning have only been obtained by stimulation of the optic lobes (Boycott, 1961; Chichery & Chanelet, 1976).

Morphological evidence in *Octopus* (Young, 1971), *Sepia* (Boycott, 1961) and *Loligo* (Young, 1976) has shown that the main efferent fibres of the lateral basal lobes run to the chromatophore lobes in the suboesophageal mass. The paired posterior chromatophore lobes (PCL) control chromatophores in the skin of the mantle and fins, and the paired anterior chromatophore lobes control those in the arms and head region. Degeneration studies suggest that axons originating in the chromatophore lobes travel *via* the pallial nerve, without synapse, to chromatophores in the skin (Sereni & Young, 1932; Young, 1971, 1976), except perhaps in the arms of *Octopus* (Rowell, 1963). The pallial nerve is a mixed nerve that is the main pathway from the lower motor centres to the stellate ganglia in the mantle. Electrical or mechanical stimulation of the chromatophore lobes in *Sepia* (Boycott, 1961) results in ipsilateral chromatophore expansion, but no recognizable patterning.

The motor fields of chromatophore motoneurones have been studied by stimulating peripheral nerve branches in octopods (Packard, 1973, 1982; Dubas & Boyle, 1985), *Sepia* (Maynard, 1967) and *Loligo* (Hofmann, 1910; Bozler, 1931; Florey, 1966). There is extensive overlap of motor fields of adjacent nerve branches. In *Loligo* (Florey, 1969), motoneurones activate several radial muscles of different chromatophores, generally of the same colour. Chromatophores of different colours are innervated independently. In *Sepia* (Maynard, 1967), *Octopus* (Packard, 1973, 1982) and *Eledone* (Dubas & Boyle, 1985) there is evidence that the distribution of the motor units follows the distribution of the morphological chromatic units. In *Sepia* (Maynard, 1967), individual motor units clearly represent recognizable parts of certain chromatic components of patterning.

The neuromuscular junction of the chromatophore organ has been studied in detail in squids (Florey, 1966; Cloney & Florey, 1968; Florey & Kriebel, 1969; Florey, 1969; Weber, 1968, 1970, 1973) and in the octopus *Eledone* (Dubas, 1982). Each radial muscle of a chromatophore is innervated, sometimes by several different motoneurones. Intracellular recordings of electrical activity in the muscle fibres (Florey & Kriebel, 1969) show that nerve stimulation produces non-propagating excitatory postsynaptic potentials (EPSPs) of graded amplitude. Several size classes of EPSPs are found, indicating polyneuronal innervation. In addition, there are gap junctions linking adjacent muscles (Cloney & Florey, 1968), permitting electrical interaction between muscle fibres (Florey & Kriebel, 1969). All evidence indicates that the muscle fibres are innervated by excitatory motor fibres only, with no direct inhibitory innervation (Florey & Kriebel, 1969; Dubas, 1982). Facilitation and

summation of the electrical response are minimal (Florey & Kriebel, 1969; Florey, 1969).

Chromatophore patterning has been studied mostly in species with complex patterning and behaviour (e.g. *Octopus vulgaris, Sepia officinalis*). Various aspects of pattern repertoire and behaviour have been described in octopods (Packard & Sanders, 1971; Packard & Hochberg, 1977; Hanlon & Hixon, 1980; Boyle & Dubas, 1981), cuttlefishes (Holmes, 1940) and squids (Boycott, 1965; Hanlon, 1978, 1980, 1982; McConathy, Hanlon & Hixon, 1979; Moynihan & Rodaniche, 1982). We have studied the relatively simple chromatophore patterns of the squid, *Lolliguncula brevis*, in an attempt to understand how the motor units are organized in the posterior chromatophore lobes (PCL). In this paper, we describe the chromatic components of patterning in *L. brevis*, establish that the chromatophore motoneurones are located in the PCL by retrograde transport of horseradish peroxidase (HRP), and characterize the motor fields of chromatophore motoneurones by focal threshold extracellular stimulation of individual somata in the PCL in a perfused, semi-intact preparation. Preliminary reports of some of these findings have appeared (Dubas, Ferguson, Hanlon & Pinsker, 1984; Hanlon *et al.* 1984).

METHODS

Male and female *Lolliguncula brevis* were caught in the Gulf of Mexico and maintained in the laboratory for several months in recirculating artificial sea water (Instant Ocean) systems (Hanlon, Hixon & Hulet, 1983). They were available in the laboratory throughout the year (Hulet, Hanlon & Hixon, 1980).

Behavioural observations

Extensive observations of *L. brevis* were made over a 5-year period. Observations were made (1) rarely in the field due to the turbid water that these squids commonly inhabit, (2) during transport aboard ship when they were in large tanks, and (3) during long-term laboratory maintenance in 2-m diameter round tanks or 10-m long raceway systems (Hanlon *et al.* 1983). In the tank systems, squids were observed from above the tanks or, more commonly, through glass viewing ports built into the walls. Behavioural notes and photographs were taken primarily during feeding and when squids were especially active (e.g. when mating). Particular attention was paid to chromatic components of patterning, to postures and to general aspects of behaviour associated with temperature or salinity shock, fin damage, feeding, intraspecific aggression, courtship and mating.

HRP injection in the skin

Horseradish peroxidase (HRP) is taken up at the axon terminal and travels retrogradely to the cell body where it accumulates (LaVail & LaVail, 1972; LaVail, 1975). Since it does not cross neuro–neuronal synaptic junctions, only nerve cells terminating in the injection area are labelled. This technique was used to localize the chromatophore motoneurones in the brain. *L. brevis* with mantle lengths between 30

and 70 mm were anaesthetized in 1·5 % ethanol in sea water. HRP crystals (Sigma type VI) were pushed under the skin with an insect pin. Injections were considered successful when a small quantity of HRP was clearly visible under the skin. A single injection was made in a total of 16 animals. Six injection sites were chosen (see Fig. 5): (A) mantle rim, (B) dorsal mantle, (C) ventral mantle, (D) mantle tip, (E) fin, and mantle muscle. Injections were always made on the left side, the uninjected contralateral side serving as a control. Animals recovered within minutes when returned to their tanks, and were kept alive at water temperatures ranging from 18 to 23 °C for 7–10 days. The experimental animal was then re-anaesthetized, its brain and stellate ganglia were surgically exposed and fixed in a solution of 2·5 % glutaraldehyde and 0·5 % paraformaldehyde in 0·1 mol l^{-1} phosphate buffer. The tissues were then removed and stored overnight in phosphate buffer containing 15 % sucrose and were later embedded in gelatin (Crane & Goldman, 1979). Frozen sections 40–50 μm thick were prepared with a freezing microtome and reacted with tetramethyl benzidine (TMB: Mesulam, 1978), which, in octopus, seems to be more sensitive than diamino benzidine (Saidel, 1982). The sections were mounted on subbed slides, air dried, stained with neutral red (Ramon-Moliner, Vane & Fletcher, 1964), coverslipped and examined under phase contrast illumination with a compound microscope. Sections obtained in this way were compared to a series of brain sections stained by the Cajal method (Stephens, 1971) and the positions of HRP-filled cells were determined using the reference figures in Young (1976).

General procedure for semi-intact squid preparations

The semi-intact preparation had the PCL exposed surgically with the mantle attached so that acute physiological experiments could be conducted with functionally intact chromatophores (Fig. 2). The experimental animal was anaesthetized for as short a time as possible (typically 1–3 min) in 2–3 % ethanol in artificial sea water. The mantle was then cut along the ventral midline and the animal pinned, dorsal side down, in a Lucite chamber with circulating, cooled (15 °C) and aerated artificial sea water. Transillumination of the internal organs revealed the heart and its blood vessels. A thin plastic cannula was placed in the aorta at its exit from the heart, and secured with a double ligature. The perfusion medium consisted of artificial sea water buffered with 10 mmol l^{-1} HEPES (pH adjusted to 7·4 with 10 mol l^{-1} NaOH) and was placed in a plastic perfusion bag. Pure oxygen was blown into the bag until a slight pressure existed to produce supersaturation of the medium. During surgery, the perfusion medium contained 1 % ethanol. Regular flow (about 1·5 ml min^{-1}) was assured by a peristaltic pump. The vena cava was then cut open, the ink sac ligatured and the animal turned and pinned ventral side down. The skin, the muscle layers and the salivary glands overlying the posterior part of the brain were removed to expose the cephalic aorta and the cephalic hearts, which are situated just above the PCL. To expose the PCL, the oesophagus was removed and the cephalic aorta displaced sideways. Transillumination of the suboesophageal mass revealed clearly the location of the PCL neurones. In good preparations, the PCL remained excitable, and activity could be recorded in the pallial nerve, for up to 4 h.

Extracellular stimulation of the PCL

Neurones in the PCL were stimulated in three different ways to afford the most localized stimulation: (1) monopolarly with a glass microelectrode (tip diameter 75–100 μm) filled with artificial sea water and referenced to an indifferent lead in the bath, (2) bipolarly with a microconcentric metal electrode (diameter of the outer contact, 150 μm; inner contact, 25 μm), and (3) bipolarly with a double-barrelled glass microelectrode (tip diameter 50 μm) filled with artificial sea water or 3 mol l^{-1} KCl. The chromatophore responses were recorded with a still camera (Nikon) or a photocell mounted in a black container fitted over one of the microscope eyepieces (Florey, 1966). Focal threshold stimulation is defined as the lowest intensity stimulus that causes one-for-one expansion of one or more chromatophores. A motor unit (i.e. presumably the motor field of a single chromatophore motoneurone) is defined as the smallest number of chromatophores that always respond synchronously to a threshold stimulus.

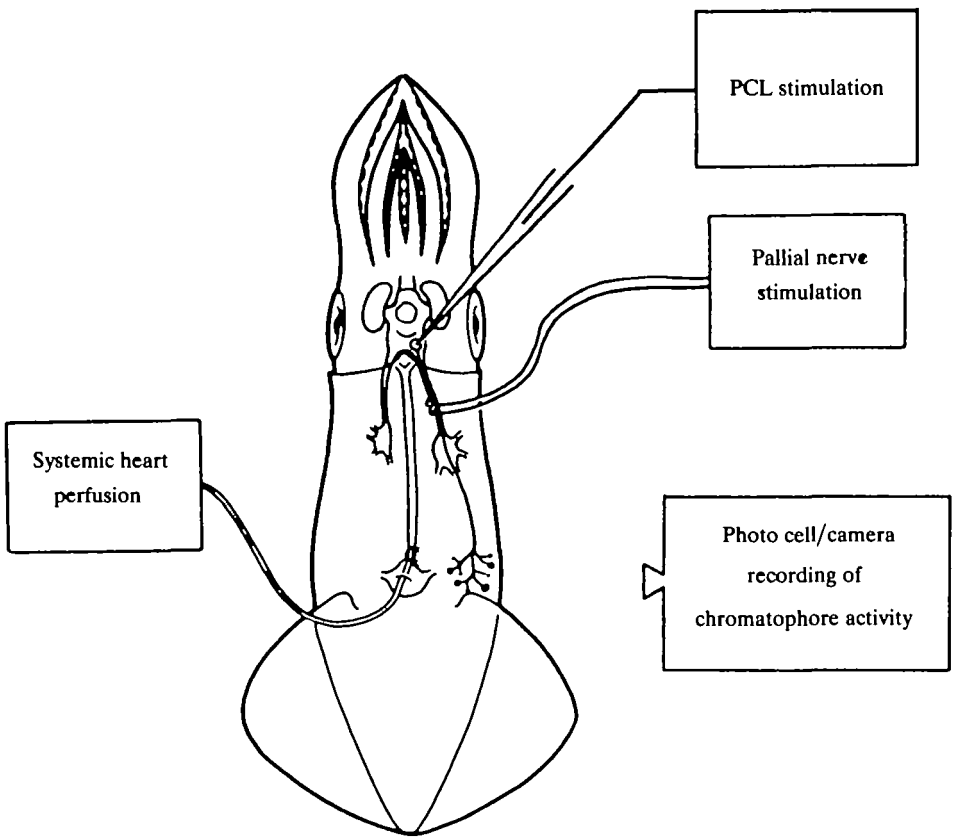


Fig. 2. Diagrammatic representation of the semi-intact preparation. A cannula is inserted into the systemic heart to perfuse the brain and the body. The PCL neurones are stimulated with single or double barrelled glass electrodes or bipolar microconcentric metal electrodes. The motor axons in the pallial nerve are stimulated *via* a cuff electrode. Chromatophore responses are monitored with a photocell or camera.

Pallial nerve recording and stimulation

Activity in the pallial nerve was monitored *en passant* with a cuff electrode (Pinsker & Eberly, 1982), connected to an extracellular amplifier (WPI DAM 5A). The amplifier could also be used to pass current to stimulate the chromatophore motor axons in the pallial nerve *via* the cuff electrode. Recorded signals were displayed on an oscilloscope and stored on an FM tape recorder. To obtain a hard copy, the spike trains were played back at low speed onto a chart recorder.

RESULTS

Behaviour and the chromatic components of patterning

Lolliguncula brevis is a social, schooling species that spends most of its time in shallow water near the bottom. In the laboratory, these squids survive for long periods (up to 4 months) due partly to their hardiness, but also to their relatively greater intraspecific compatibility compared to the squid *Loligo*. Social behaviour is less complex than in related squids (e.g. *Loligo, Sepioteuthis*), there is little intraspecific aggression and most chromatic components of patterning are used in an interspecific context.

L. brevis has a very simple and limited pattern repertoire (Fig. 3) that is made up from about three postural, three locomotor and nine simple chromatic components. When the squids are undisturbed and swimming calmly they usually maintain a clear body pattern in which few or no chromatophores are expanded (i.e. no chromatic components expressed). At times, squids may show the chromatic component Eye Shade (Fig. 3A) to camouflage the brightly iridescent eyeballs when viewed from above. When disturbed by a human observer or a predatory fish, the squids react quickly with a graded response that is roughly proportional to the stimulus strength. The first response to a mild stimulus is to slowly swim away; a slightly stronger response is to swim more quickly in combination with one of the following chromatic components: Dark Arm Tips (Fig. 3E), Mantle Margin Stripe (Fig. 3G) or Dark Fin Line (Fig. 3H). A typical strong response is to suddenly turn All Dark (Fig. 3I), then blanch and jet away very quickly. Another response to a strong stimulus, especially when the animal is approached closely or cornered, is to show the common threat pattern (see Fig. 3A–C) in which the squid hovers in the water showing simultaneously the postural component Upward-V Curl and the chromatic components Eye Shade, Dark Third Arms (Fig. 3B) and Ring (Fig. 3C). The chromatic component Lateral Mantle Spot (Fig. 3D) is shown unilaterally by females during courtship when males are attempting to mate them. Males and females may briefly show Dark Arm Tips, Dark First Arms (Fig. 3F), Mantle Margin Stripe or Dark Fin Line when chasing shrimps or food fishes.

Morphological arrangement and distribution of chromatophores over the mantle and fins

The elements of patterning in *L. brevis* include chromatophores and iridophores. Iridophores are not dealt with in this report. There are two colour classes of

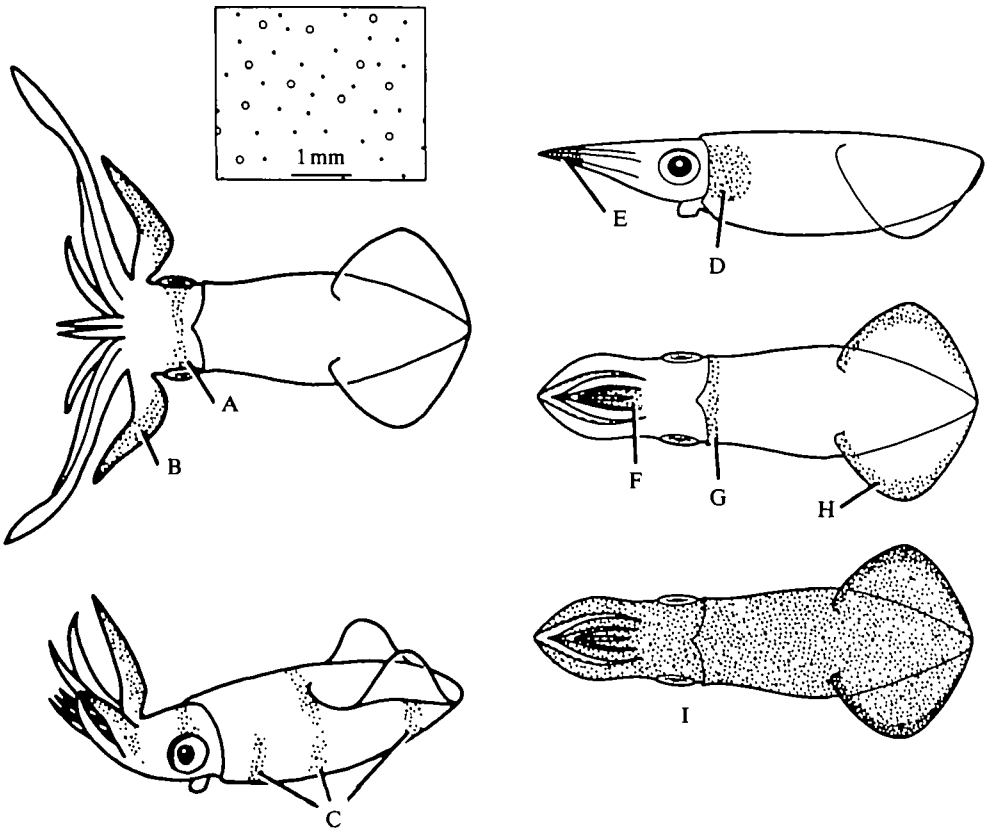


Fig. 3. Body patterning in *Lolliguncula brevis*. Drawings represent the following chromatic components: (A) Eye Shade, (B) Dark Third Arms, (C) Ring, (D) Lateral Mantle Spot, (E) Dark Arm Tips, (F) Dark First Arms, (G) Mantle Margin Stripe, (H) Dark Fin Line, (I) All Dark. Left set of drawings represent a common threat pattern that includes: Ring, Eye Shade, Dark Third Arms plus the postural component Upward-V Curl. Inset: typical chromatophore arrangement of dorsal mantle skin (chromatic unit called yellow-brown discoid unit) in which smaller yellow chromatophores (dots) are regularly interspersed among larger brown ones (open circles).

chromatophores in *L. brevis*: yellow and brown. The yellow chromatophores are located more superficially in the dermis and are smaller than the brown ones. The typical static morphological array of chromatophores is depicted in Fig. 3 (inset). Brown chromatophores are spaced rather evenly with respect to other browns, and yellows are interspersed. This arrangement has been termed the yellow-brown discoid unit (Hanlon, 1982). The spacing of chromatophores is relatively constant across the body surface, except at the periphery of the fins where the chromatic component Dark Fin Line (Fig. 3H) occurs. There, the chromatophores are smaller and denser, with only brown chromatophores along the outer 2 mm. Elsewhere on the fin the yellow-brown discoid unit prevails.

The total number of chromatophores on the mantle and fins increases with the size of the squid (Table 1). The counts are based on photographs of animals of different sizes. Since the PCL innervate the mantle and fin exclusively, these values indicate

the approximate number of chromatophores whose muscle fibres are directly controlled by PCL motoneurones.

Localization of chromatophore motoneurones with HRP injections

The morphology of the PCL and fin lobes (FL), as demonstrated by Cajal stain, is illustrated in Fig. 4A. Several rows of cell bodies surround a central neuropile. The largest cell bodies (40 to 45 μm) are generally on the surface of the lobes, whereas the smallest ones are adjacent to the neuropile. Ventrally, the neuropile of the PCL and FL are incompletely separated from the neuropile of the magnocellular and palliovisceral lobes. Similarly, the somata of the PCL and the FL are not clearly separated from one another.

Peripheral injections of HRP were used to identify the chromatophore motoneurones in the brain and distinguish them from the mantle muscle motoneurones which were thought to synapse in the stellate ganglion. Fig. 4B shows photographs of sections of the PCL and the stellate ganglion (SG) of animals in which HRP was injected either in the mantle muscle or in the skin close to chromatophore muscles. Injection of HRP crystals next to the chromatophore muscles labelled cells only in the posterior suboesophageal mass. In contrast, HRP injection in the mantle muscle (from the internal surface of the mantle where there are no chromatophores) labelled cells in the SG as well as in the posterior suboesophageal mass. This indicates clearly that the chromatophore motoneurones are situated in the suboesophageal mass, whereas at least some of the mantle muscle motoneurones are located in the SG.

For all injections, marked cells were found mainly in the ipsilateral lobes with occasional cells in the contralateral lobes. A single, localized injection in the mantle skin labelled up to 50 cells either isolated or clustered. Labelled cells were found primarily in the PCL and FL, but also in the magnocellular and the palliovisceral lobes. Since skin muscle motoneurones and sensory neurones could also be labelled, it is not certain that all the marked cells (especially those outside the FL and PCL) are chromatophore motoneurones. Cells labelled in the PCL had an apparent diameter of about 30–40 μm whereas those labelled in the FL appeared to be larger (about 50–60 μm). Only the cell bodies were labelled; neither axons nor neurites were visible.

To determine the topographical relationship between the location of the motoneurones within the PCL or the FL and the location of their motor fields on the mantle, HRP was injected in different areas of the mantle skin and the position of the labelled cells recorded precisely. The diagram summarizing the results of these

Table 1. *Total chromatophores on mantle and fins of* Lolliguncula brevis

Mantle length (mm)	2	27	40	50	80	100
Total chromatophores	53	1800	5400	7400	14 000	27 000

Squids with a mantle length of 2 mm were hatchlings and those with a mantle length of 100 mm were adults.

Actual counts were obtained from photographs of anaesthetized or freshly dead squids.

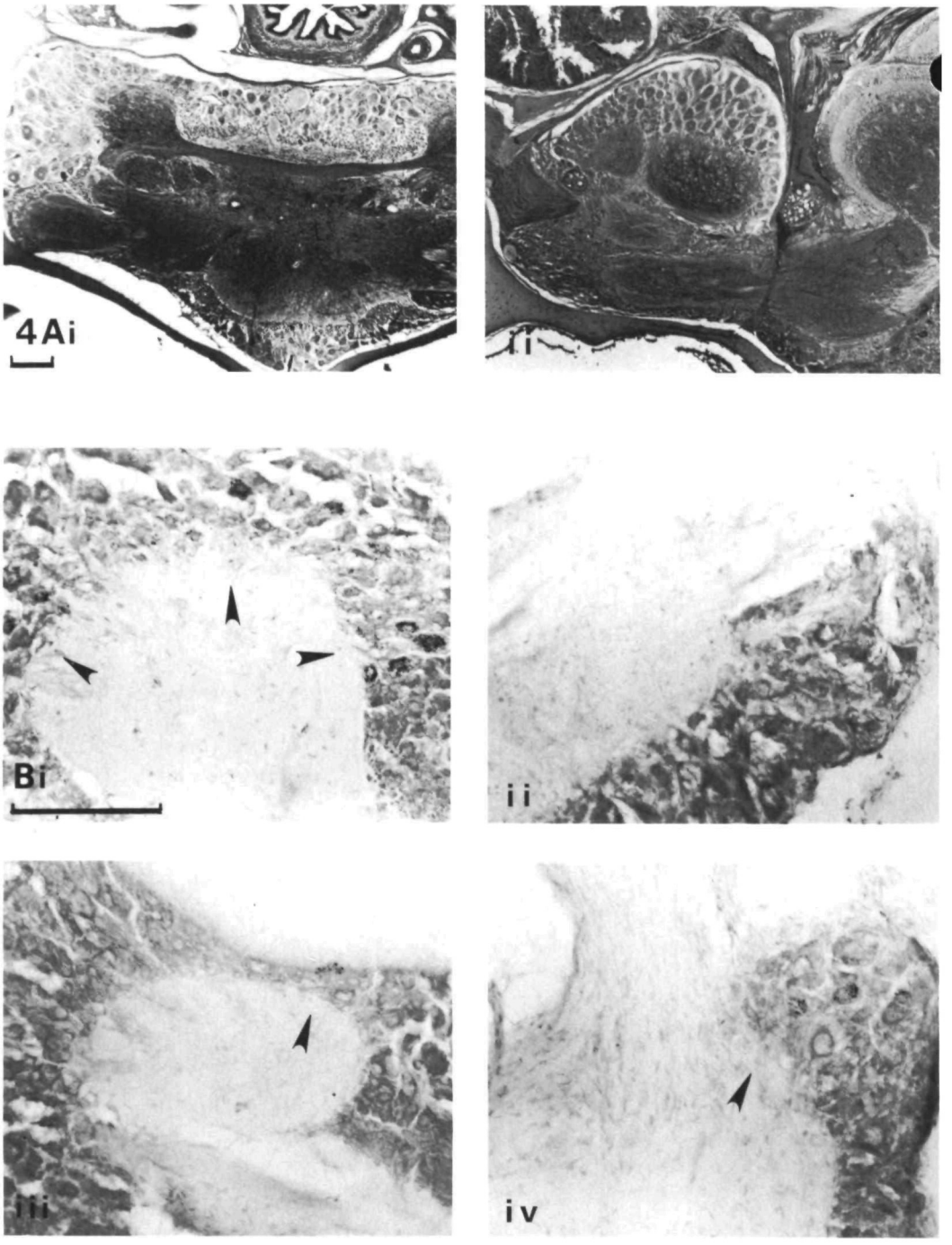


Fig. 4. (A) Sections of the posterior suboesophageal mass in the brain of *Lolliguncula brevis* (Cajal stain) to show the morphological arrangement of the posterior chromatophore lobes (PCL) and fin lobes (FL). Transverse (i) and sagittal sections (ii). In sagittal section, the lateral basal lobe (LBL) of the supraoesophageal mass is visible. Scale bar: 200 μm (courtesy of J. Z. Young). (B) Sections through the posterior chromatophore lobe (PCL: i,iii) and stellate ganglion (SG: ii,iv) of two representative animals. One animal (i,ii) had HRP injected in the mantle skin close to the chromatophores, and the other (iii,iv) had HRP injected in the mantle muscle. Labelled cells (arrowheads) are visible in both the SG and the PCL after injection in the mantle muscle, whereas labelled cells are found only in the PCL after injection in the skin. TMB reacted and neutral red stained sections. Scale bar: 200 μm.

experiments (Fig. 5) suggests that there is no clear topographical relationship. Indeed, cells marked by a localized injection in any part of the mantle skin were scattered over the whole PCL. Furthermore, marked cells in the FL could be found not only in animals injected in the skin of the fins but also in animals injected in different areas of the mantle skin.

Extracellular stimulation of PCL neurones

Single motoneurones in the PCL were stimulated at focal threshold voltage to study the properties of individual motor units. It was clear that the cell bodies in the FL or PCL were stimulated rather than the axons in the pallial nerve for the following reasons: (1) chromatophore responses could be elicited with the same voltage whether or not the stimulating electrode was located close to the pallial nerve; (2) no chromatophores could be activated when the stimulating electrode was near the pallial nerve, but outside the PCL or FL; and (3) the chromatophores responded somewhat differently (see below) to direct stimulation of the pallial nerve than to stimulation of the PCL or FL. A photocell monitored the responses of individual chromatophores (Figs 6, 7, 8), whereas photographs of the mantle were taken (Fig. 9) to determine the number and location of chromatophores forming single motor units.

Responses of individual chromatophores within motor units

Monopolar or bipolar stimulation of PCL neurones with glass or concentric metal electrodes resulted in expansion of chromatophores primarily of the ipsilateral mantle and fin. At threshold voltage, the chromatophores under observation did not respond to every pulse in a train of stimuli and the amplitude of the expansion was irregular. With increased voltage, the chromatophores responded to every stimulus and the amplitude of the expansion became stable. Furthermore, the amplitude of the chromatophore expansion at suprathreshold voltage was often slightly larger than at threshold voltage (Fig. 6A,B). However, once the voltage was reached at which the amplitude of the response became stable, further increases did not modify the amplitude of the response, even when only a few muscles of the individual chromatophores were responding.

In fresh preparations and at frequencies below about 5 Hz, each suprathreshold PCL stimulus triggered a single, twitch-like contraction of the chromatophore muscles. There was fusion of the responses above 5 Hz and summation of the contractile responses occurred. Maximal expansion was reached at about 10 Hz, even though smooth tetanic expansion was not reached below about 20 Hz. At frequencies higher than about 6 Hz, the chromatophores often stopped following the stimulus one-for-one and expansion was interrupted by short episodes of retraction (Fig. 6C). In aged preparations or in fatigued chromatophores, fusion of the responses occurred at lower frequencies.

The latency of the chromatophore responses triggered by either PCL or pallial nerve stimulation was constant for a range of stimulus frequencies (1–20 Hz; Fig. 7). This is in keeping with the HRP evidence suggesting that the chromatophore

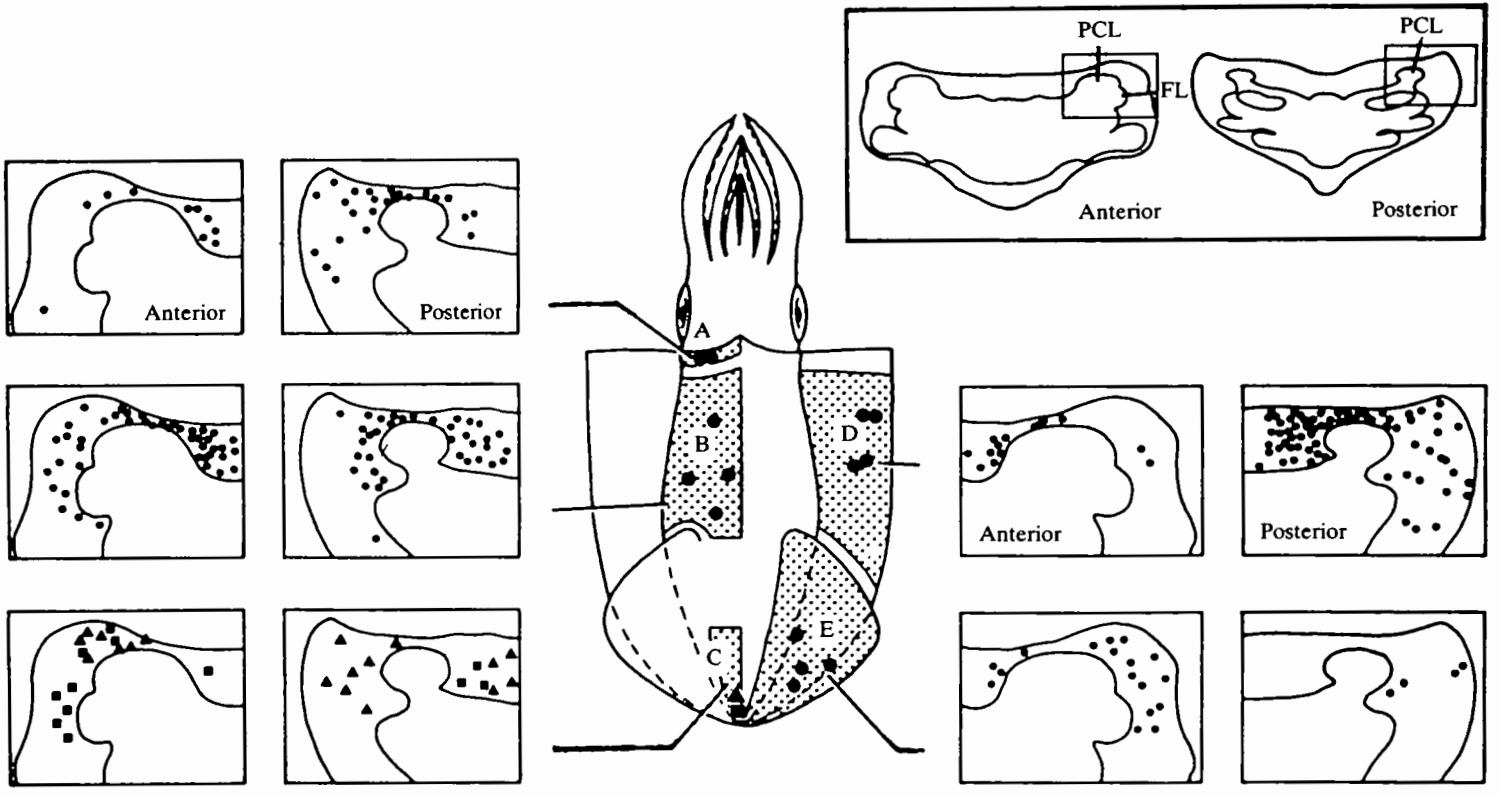


Fig. 5. Location of labelled cells in the posterior chromatophore lobe (PCL) and fin lobe (FL) following injection of HRP in five different body areas: A, mantle rim; B, dorsal mantle; C, mantle tip; D, ventral mantle; E, fin. For each body area, the precise injection sites are marked by dots and all the cells labelled by these injections are shown in drawings of the PCL and FL. These drawings are enlargements of two representative sections across the anterior and posterior regions of the posterior suboesophageal mass (within rectangles in upper right corner). For the two injections in the mantle tip (C), cells labelled by each injection are marked with squares and triangles. There is no clear relationship between the location of the injection site on the body and the location of the marked cells in the PCL or FL.

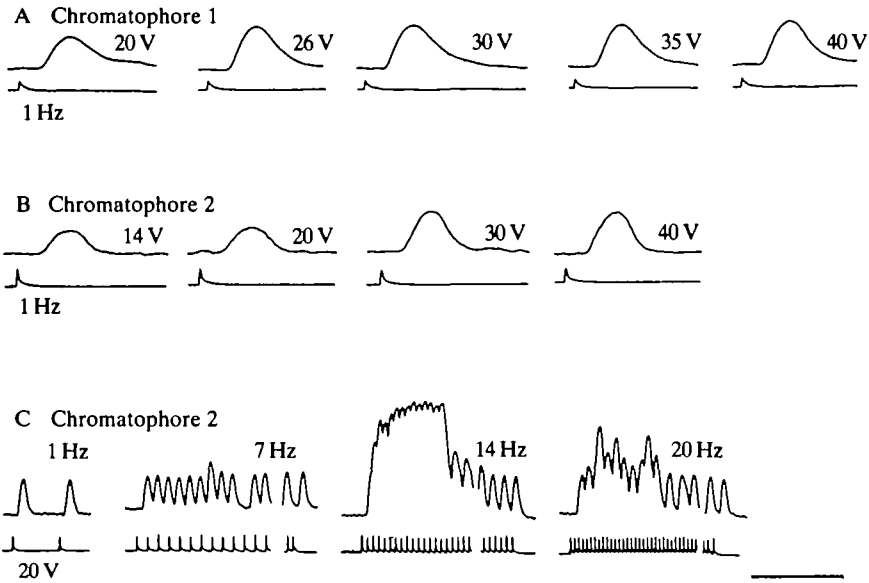


Fig. 6. Photocell records of two individual chromatophores (chromatophore 1 and chromatophore 2) to posterior chromatophore lobe stimulation (double-barrelled glass microelectrode) at various voltages and frequencies. (A) Increases of voltage above threshold produce minimal changes in the amplitude of the response. Most of the chromatophore muscles are active. (B) In this chromatophore, the amplitude of the expansion increases slightly with voltage although no new muscles were visibly recruited. (C) Above 7 Hz, chromatophore 2 fails to show sustained maximal expansion although it is capable of following for short periods up to 20 Hz. Short segments of data were deleted (blanks). Scale bar: (A,B) 400 ms; (C) 2000 ms.

activation is mediated by a monosynaptic connection. In no case did stimulation of PCL neurones result in retraction of expanded chromatophores.

Stimulation of the pallial nerve *via* the cuff electrode produced slightly different chromatophore responses. In general, the chromatophore motor axons in the pallial nerve could be stimulated through the cuff electrode long after the cell bodies in the PCL had become unexcitable. With increases of stimulus voltage it was possible to trigger several axons innervating the same chromatophore and to record, in a single chromatophore, expansions of drastically different amplitudes. The photocell traces presented in Fig. 8 are of a chromatophore where only two muscle fibres contracted at stimulus voltages below 20 V (frequency: 1 Hz), but where all the muscles became active when the voltage was further increased. Presumably, new motor axons were recruited that were either new motor units or additional branches of the previously triggered motoneurone.

The chromatophore responses often followed the pallial nerve stimulus one-for-one up to at least 20 Hz. Even chromatophores that did not follow PCL stimulation above 6 Hz showed maximal, sustained expansion at frequencies as high as 20 Hz, when stimulated *via* the pallial nerve. The responses of the same chromatophore (C2) could be triggered by PCL stimulation (Fig. 6C) and by pallial nerve stimulation (Fig. 8B,C). Pallial nerve stimulation of chromatophore C2 at 12 V and at 1 Hz or 10 Hz activated only two muscle fibres. However, at the same voltage, stimulation

at 14 Hz or above activated not only these fibres, but, after a few stimuli, the other fibres of this chromatophore. When the frequency was lower, the other fibres were recruited only at higher voltages.

Motor units

For any stimulation site on the surface of the PCL, focal threshold stimulation triggered expansion of a single, compact group of chromatophores somewhere in the skin of the mantle and fins. Such a group of chromatophores always responded as a unit to PCL stimulation. Furthermore, the same group responded synchronously during spontaneous chromatophore activity. Since it cannot be broken down into smaller units, a group of this sort represents the motor field of a single chromatophore motoneurone, presumably the one closest to the electrode tip.

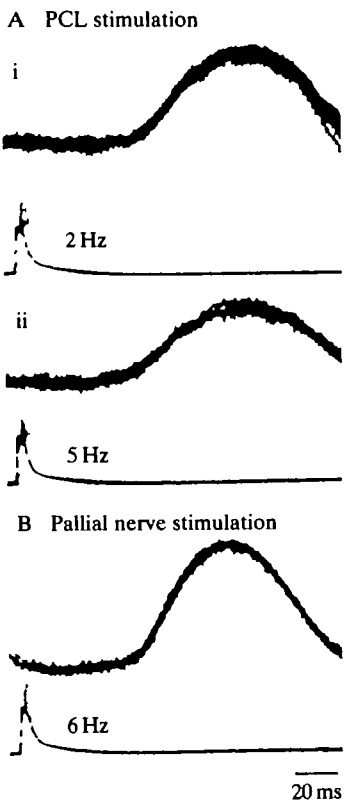


Fig. 7. Superimposed photocell records of successive expansions of a single chromatophore resulting from (A) posterior chromatophore lobe (PCL; double-barrelled glass microelectrode) and (B) pallial nerve stimulation (cuff electrode) with trains of stimuli: (Ai) 2 Hz; (Aii) 5 Hz; (B) 6 Hz. The latency of chromatophore response is constant at the different frequencies, whether the PCL or the pallial nerve is stimulated, in keeping with the evidence that the chromatophore response is mediated *via* a monosynaptic pathway.

In *L. brevis*, the motor fields of chromatophore motoneurones were compact, generally comprising all chromatophores with the same colour and similar diameter in a given area. The number of chromatophores in a single motor unit varied with its position on the body (Fig. 9A). Smaller units (with 15 or fewer chromatophores) were located towards the anterior end of the mantle (Fig. 9Ai). The larger motor units (with up to 60 chromatophores) were found on the fins, mainly along the edge where chromatophore density is high (Fig. 9Aii). Yellow and brown chromatophores were normally part of different units although the units on the edge of the fin often included some yellow chromatophores among the browns (Fig. 9Aii).

Fig. 10 summarizes the size distribution of motor units obtained by stimulation of PCL neurones at threshold voltages. There were no obvious differences in the distributions obtained with monopolar and bipolar stimulation. In both cases, the most common motor units contained 6–10 chromatophores, and the great majority contained fewer than 20.

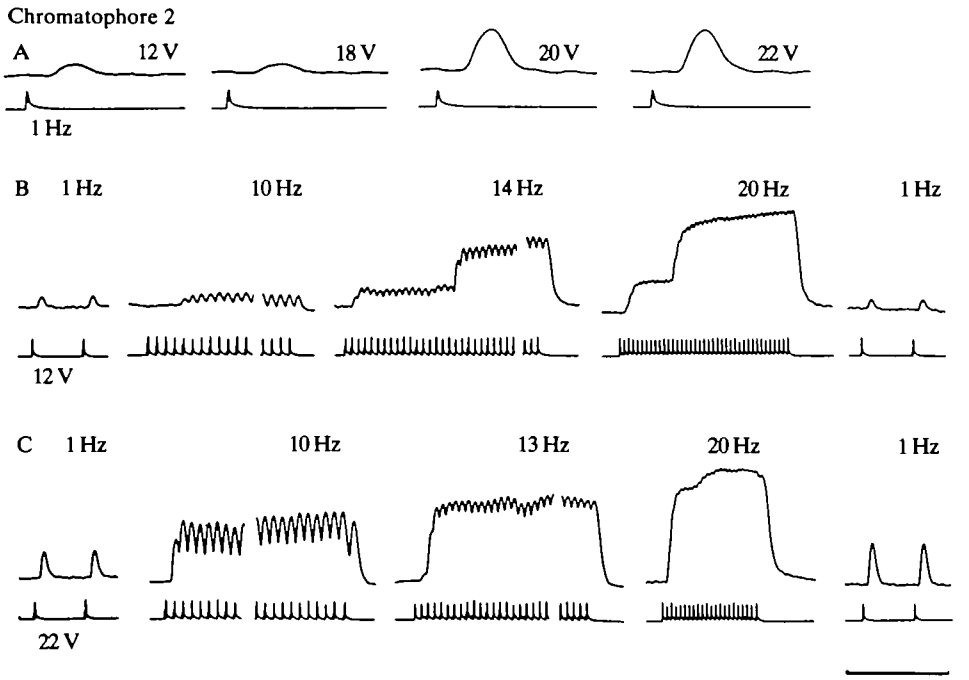


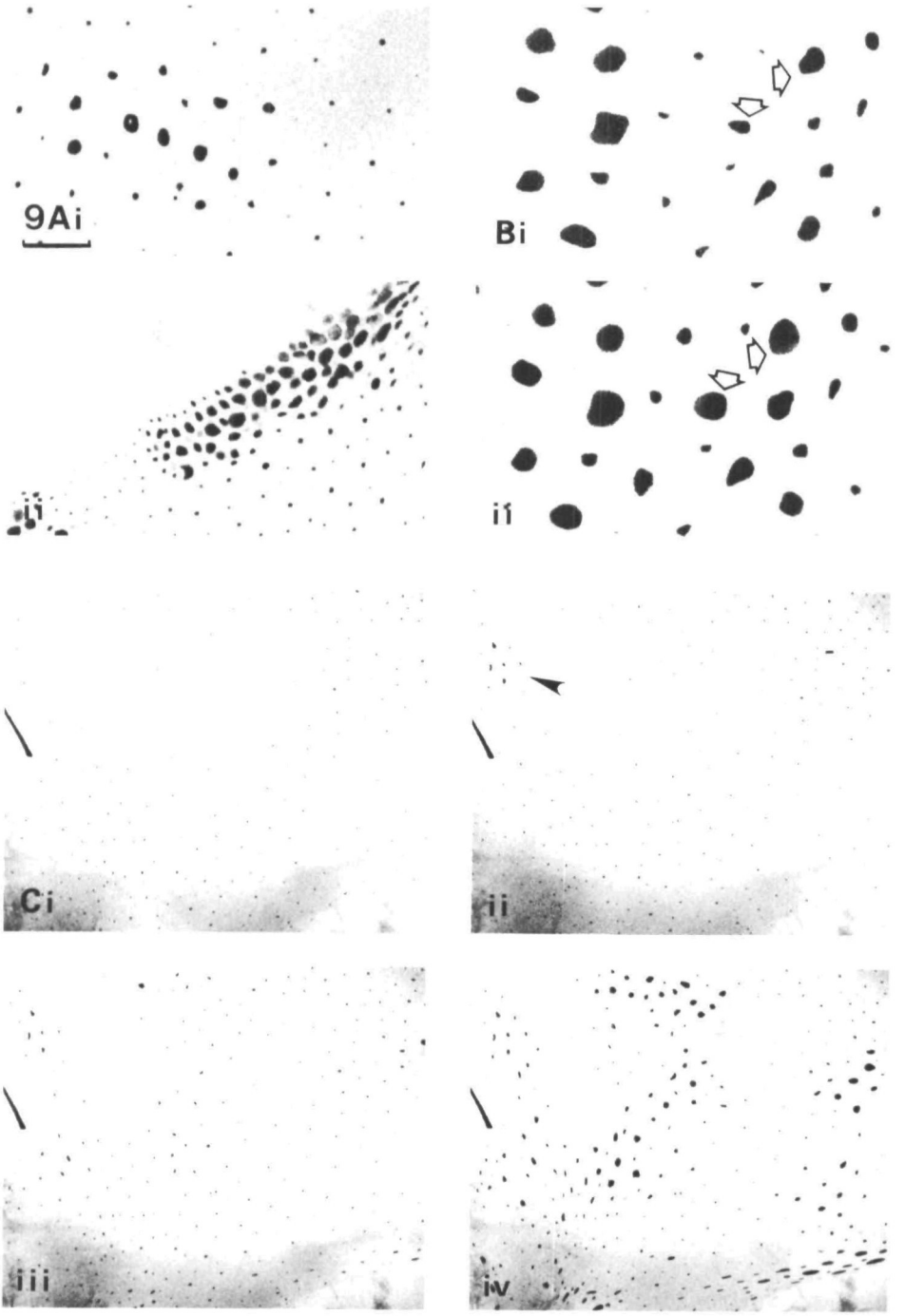
Fig. 8. Photocell records of chromatophore 2 (see Fig. 6B,C) to stimulation of the pallial nerve (cuff electrode) at various voltages and frequencies. (A) At the lowest voltages (12 V, 18 V), only two muscle fibres contract, producing a small-amplitude response. Above 20 V, most of the chromatophore muscles become active, suggesting that another motor axon is recruited. The amplitude of the response at 22 V is comparable to the one obtained for the same chromatophore with PCL stimulation at or above 20 V (Fig. 6B). (B) At the lowest voltage, only two muscle fibres contract at 1 Hz and 10 Hz, whereas most of the chromatophore muscles are eventually recruited at 14 Hz without modification of the stimulus voltage. (C) At the higher voltage, all the muscle fibres are recruited even at the lower frequencies. There is summation of the response but smooth tetanus is still not reached at 20 Hz. Scale bar: (A) 400 ms; (B,C) 2000 ms.

Individual chromatophores in which all of the radial muscles were equally contracted were round in appearance. However, individual chromatophores often had irregular shapes (Fig. 9B) because not all of their radial muscles were contracted. This indicates that not all of the muscles of a single chromatophore are necessarily innervated by the same motoneurone. Frequently, in chromatophores at the periphery of a given motor unit, only the muscles towards the centre of the unit were innervated by that motoneurone (Fig. 9Ai). When additional motoneurones were recruited, the previously inactive muscles contracted, providing evidence for overlapping motor fields in which a single chromatophore is part of two or more motor units. Fig. 8A shows photocell records of a chromatophore showing different amplitudes of expansion when new muscle fibres are recruited by an increase of stimulus voltage, and Fig. 9B shows photographs of the same phenomenon in several different chromatophores. It is not clear whether single muscle fibres were also innervated by several motor axons.

At threshold voltage, stimulation of the pallial nerve triggered expansion of groups of chromatophores that had the same features as the motor units obtained with PCL stimulation. In fact, it was often possible to activate the same units as those triggered by stimulation of the PCL neurones. However, even with threshold voltages, it was difficult to activate a single unit with pallial nerve stimulation.

New motor units were recruited both with pallial nerve and PCL stimulation (Fig. 9C) when the stimulus voltage was increased and neighbouring somata or axons were recruited. With both types of stimulation, the new units were not necessarily adjacent to the units previously active. By varying the stimulation locus and intensity, the topographical arrangement of the motoneurones in the PCL was studied. In keeping with the results of HRP injections, there was no clear relationship between the location of the motoneurones in the PCL or FL and the part of the body where the chromatophore response occurred. Extracellular stimulation of PCL neurones (Fig. 11) showed that stimulation of either posterior or anterior parts of the PCL could trigger chromatophore expansion in both anterior and posterior parts of the mantle. Furthermore, stimulation of adjacent points in the lobe did not necessarily trigger chromatophore responses in contiguous parts of the body. However, stimulation sites in the FL frequently triggered motor units in the fin or in the

Fig. 9. Photographs of the skin of *Lolliguncula brevis* showing groups of chromatophores. (A) Single motor unit in the anterior mantle area (i) and in the fin edge (ii) elicited by posterior chromatophore lobe (PCL) stimulation. Along the fin edge, the chromatophore density is high and a single motor unit includes up to 60 yellow (faint) and brown (dark) chromatophores. Note that the shape and degree of expansion of individual chromatophores is different. (B) Skin area where some of the chromatophores (arrows) assume different shapes at different stimulus voltage (PCL stimulation), suggesting that not all of their muscle fibres are innervated by the same axon (i: 3·5 V; ii: 3·8 V). (C) Low power photography of the mantle skin, showing the chromatophore responses elicited by PCL stimulation at 10 Hz and increasing voltage: (i) subthreshold voltage; (ii) 4 V; (iii) 5 V; (iv) 10 V. At 4 V, expansion of a single motor unit (arrowhead) is triggered. At higher voltages new motor units appear, scattered all over the body. Scale bar: (A) 500 μm; (B) 200 μm; (C) 2·5 mm.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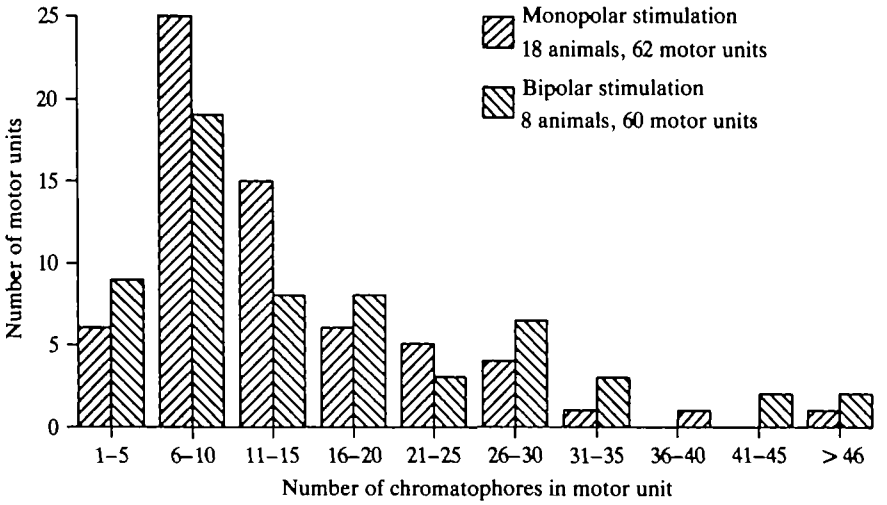

Fig. 10. Size-frequency distribution of the chromatophore motor units obtained by focal threshold monopolar and bipolar stimulation of posterior chromatophore lobe neurones. Similar profiles were obtained with monopolar and bipolar stimulation.

posterior end of the mantle. Similar results were obtained with all three types of stimulating electrode.

Recording of pallial nerve activity

Shortly after the operation, the cuff electrode on the pallial nerve recorded a great deal of activity that decreased and eventually disappeared. It is not clear whether this was normal activity or activity resulting from the surgical procedure. In good preparations, activity in the pallial nerve could be recorded up to 4 h after the operation.

Whole-nerve recordings from the pallial nerve provided a useful monitor of activity of large populations of chromatophore motoneurones that could be correlated with chromatophore activity in the semi-intact preparation. Fig. 12 shows cuff electrode recordings from the pallial nerve in the semi-intact preparation. When no peripheral chromatophore activity was observed in the skin, there was little or no spontaneous activity in the pallial nerve (top trace). Spontaneous expansion of chromatophores on the mantle was clearly associated with pallial nerve activity (middle trace). We also stimulated the PCL extracellularly with a long-duration stimulus (to avoid stimulus artifacts) and elicited activity in the pallial nerve that was associated with elicited chromatophore expansion on the mantle (bottom trace). Both spontaneous and elicited pallial nerve activity contained many apparently unitary spikes (Fig. 12, right-hand traces). These preliminary results suggest that chromatophore motor axons are large enough to be detected in pallial nerve recordings.

DISCUSSION

This is the first study since that of Boycott (1961) to examine specific functions of lower motor centres in a cephalopod mollusc. In contrast to previous studies, we use

a perfusion method that maintains the brain alive for several hours after the circulation is interrupted. Consequently, long-lasting experiments, such as successive stimulation of different parts of the brain, can be conducted on the same animal. The success rate of the operation is fairly high (above 75%) and the PCL neurones remain responsive to stimulation up to 4 h after the beginning of the operation, provided that the perfusion medium is filtered and adequately oxygenated (simple aeration was found to be insufficient). It is hoped that this method will lead to long-lasting neurophysiological experiments in semi-intact cephalopods, comparable to current investigations in other molluscs (e.g. Kandel, 1976).

The cells labelled with HRP in the suboesophageal mass give the first direct evidence that chromatophore motoneurones do not synapse in the stellate ganglion

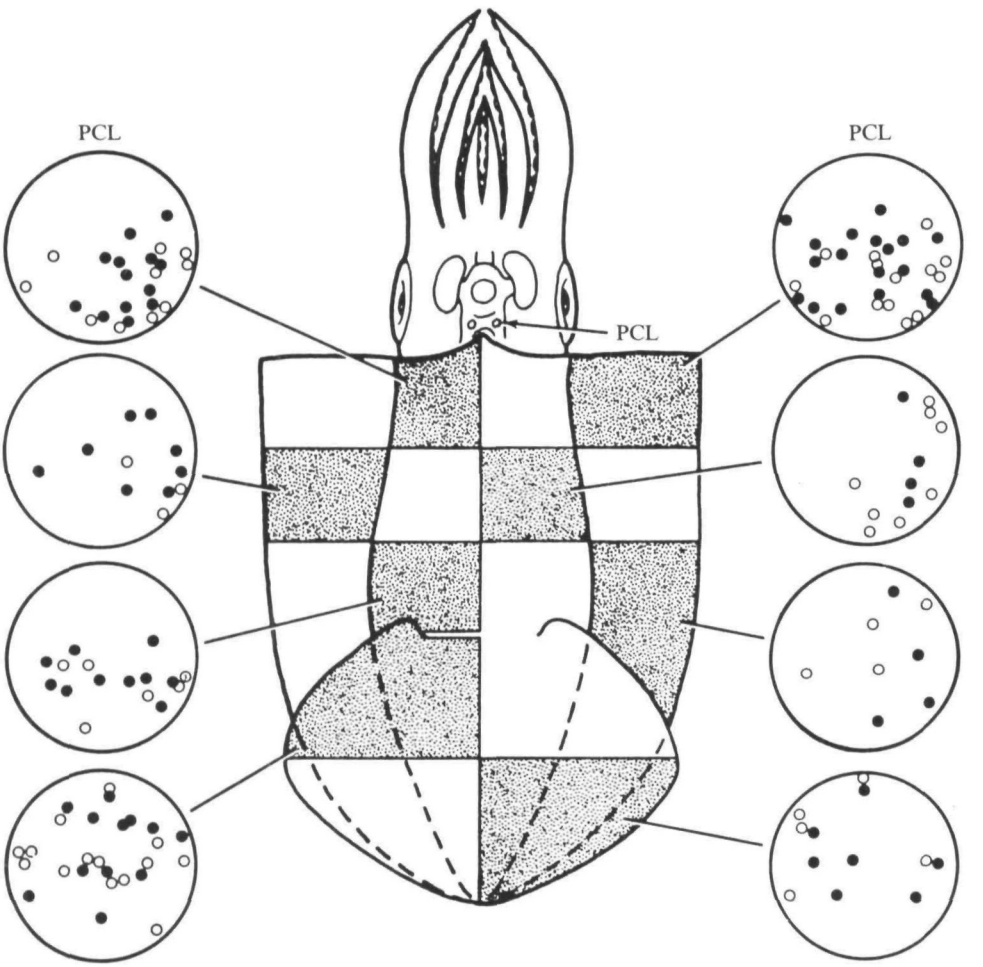

Fig. 11. Diagrammatic representation of the results of monopolar (filled circles) and bipolar (open circles) stimulation of the posterior chromatophore lobe (PCL). Shaded mantle indicates area within which the motor unit response was elicited (note: whole area did not expand) by focal threshold stimulation of the points on the surface of the ipsilateral PCL shown in the large circles. There is no clear topographical relationship between the loci of PCL stimulation and the regions of chromatophore activation.

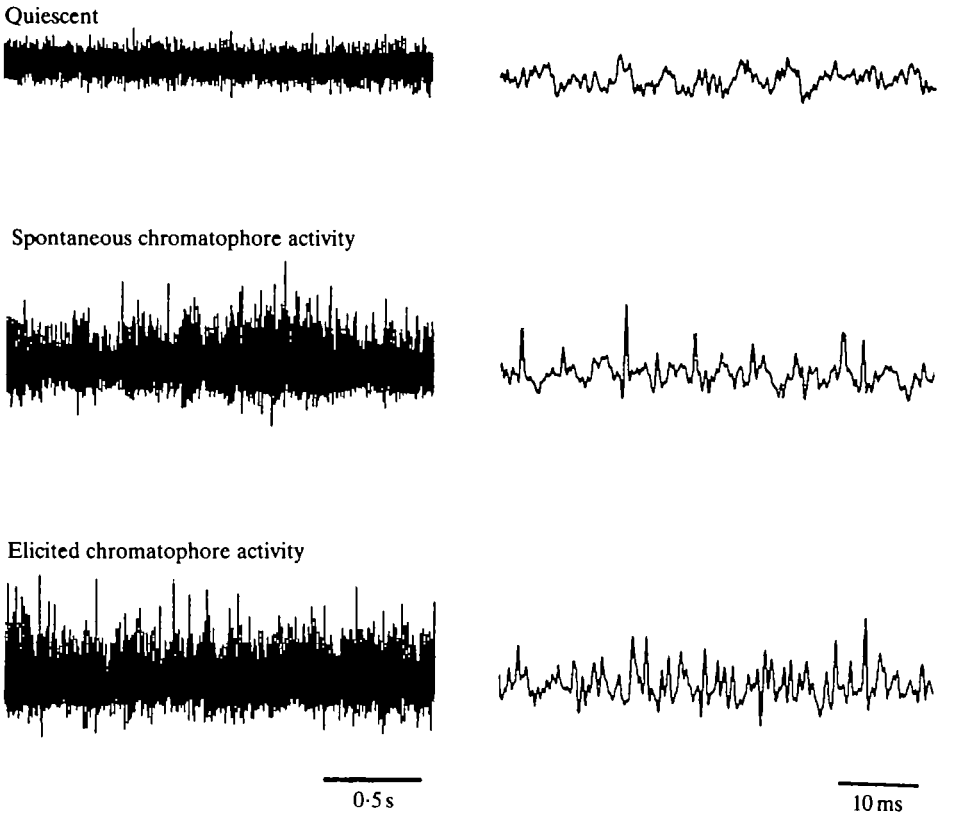

Fig. 12. Cuff-electrode recordings of chromatophore activity in pallial nerve of semi-intact preparation. Slow (left) and fast (right) runouts of whole-nerve activity are shown with negativity upward. Quiescent: when there was no expansion of chromatophores on the mantle, little or no nerve activity was recorded. Spontaneous chromatophore activity: when chromatophores in the ipsilateral mantle skin expanded spontaneously, activity was recorded in the nerve. Some apparently unitary spikes are seen in the fast runouts. Elicited chromatophore activity: chromatophore activity in the mantle skin was elicited by extracellular stimulation of the posterior chromatophore lobe. A long current pulse (3 s) was used to minimize stimulus artifact and elicited primarily tonic expansion of chromatophores associated with a great deal of activity in the pallial nerve.

and that most (if not all) of the somata lie in the posterior suboesophageal mass, mainly in the PCL and FL. It is interesting, however, that some labelled cells were found outside these lobes, in the visceral, posterior magnocellular and palliovisceral lobes that presumably control activity of the viscera and blood vessels, the mantle muscles and the neck region. It is possible that sensory neurones, skin muscle motoneurones and motoneurones innervating blood vessels in the injection area took up the HRP and account for the labelled cells in some of these lobes. It may be, however, that the functional organization of the suboesophageal mass in *L. brevis* does not reflect the strict morphological distinctions suggested by Young (1976) and that the various functions are distributed among the different lobes. Our results suggest, for example, that the FL contains motoneurones innervating chromatophores in various areas of the mantle as well as neurones controlling fin movements.

In general, the chromatophore responses to PCL neurone stimulation are similar to those elicited by stimulation of the pallial nerve (present data) or peripheral nerve branches (Florey, 1966; Florey & Kriebel, 1969). There is no evidence for direct inhibitory innervation of the chromatophore muscles. Single suprathreshold stimuli trigger twitch-like contractions of constant latency and stable amplitude, leading to summation and tetanus with increased stimulus frequency. At higher frequencies, however, each stimulus delivered to the PCL does not produce chromatophore muscle contraction. Since the same chromatophore muscles can follow higher frequencies of pallial nerve than of PCL stimulation, it is clear that the properties of the nervous system, not the muscle fibres, are responsible. Several explanations are possible for the observed differences between pallial nerve and PCL stimulation. First, at high frequency, the time interval between each stimulus may be shorter than the relative refractory period of the PCL somata and the axons may have different refractory periods from their somata. Alternatively, we may be dealing with two different functional types of motoneurones: motoneurones that produce twitch-like contractions of the chromatophore muscles at low frequencies, and motoneurones that require high-frequency stimulation and facilitation of the electrical response in the muscle to trigger contraction. The latter type of motoneurone may be more easily stimulated in the pallial nerve than in the PCL. Last, local conditions in the cuff electrode, such as local high K concentration, may produce depolarization of additional axons within the nerve. Intracellular stimulation of PCL neurones to elicit chromatophore activity may clarify this problem; this has not yet been achieved, but should be possible in the semi-intact preparation we described. So far, the only intracellular study of neuronal activity in the suboesophageal mass of a cephalopod is that of Laverack (1980) in the isolated brain of *Eledone*.

It has been suggested by Young (1976) that, in squids, the PCL contains only motoneurones that project to the periphery and that there are no short-axon cells. Our results are in keeping with Young's suggestion, even though we found motor units with sizes ranging from 2 to 60 chromatophores. It is unlikely that we were stimulating interneurones that, in turn, recruited several motoneurones since even the larger motor units followed high frequencies of stimulation with a constant latency and never failed to expand synchronously. Furthermore, these larger motor units were generally situated on the fins where chomatophore density is high. Our experiments were limited to stimulation of the surface of the PCL. Thus, if interneurones are present in the PCL and are situated close to the neuropile, they might not have been activated in our experiments. Also, our evidence cannot rule out the possibility that the larger motor units represent simultaneous activity in several tightly coupled motoneurones.

Our main focus in this paper has been to study the chromatophore motor fields of the PCL neurones (motor units) and their organization within the lobe. We also monitored the activity of individual chromatophores, the main elements of patterning in *L. brevis*. Although we describe the chromatic components, we have obtained no information on how and where they are elicited. In keeping with Boycott & Young (1950) and Boycott (1961) our results suggest that the PCL is the final

common pathway and that the 'pattern generators' are situated elsewhere in the brain, presumably in the supraoesophageal mass. Location and functional identification of the pattern-generating interneurones remain a challenge for the future.

The present data from HRP injections as well as stimulation of the PCL failed to show a topographical arrangement of the motoneurones that reflects the area of the mantle they innervate. In comparison, Boycott (1961) found that in the cuttlefish *Sepia officinalis* mechanical stimulation of the anterior and posterior parts of the PCL triggered chromatophore expansion in the anterior and posterior parts of the mantle, respectively. The pattern repertoire of *S. officinalis* is probably the richest among cephalopods and the morphological arrangement of its PCL is much more complex than in any squid studied so far (Boycott, 1953). The difference in the complexity of the chromatophore systems of the two species may account for our failure to find a representation of the mantle areas in the PCL of *L. brevis*, although our stimulation methods are certainly more advanced than Boycott's. In *L. brevis* a 'functional' arrangement of neurones in the PCL (for example, that neurones contiguous in the PCL play a role in producing segments of single chromatic components) may be more appropriate for the production of the simpler patterns. The localized motor units of *L. brevis* also proved to be much simpler than in other cephalopods studied so far (*Sepia*: Maynard, 1967; *Octopus*: Packard, 1973; *Eledone*: Dubas & Boyle, 1985). In these species, the motor units consist of chromatophores that are not clustered (as in *L. brevis*), but are distributed throughout the chromatic component they constitute. Such motor units allow for finely detailed chromatic components (pattern-position separation: see Maynard, 1967). In *L. brevis*, the motor units are fairly large and 'unpatterned', suitable only for the simple chromatic components present in this animal. The lack of a somatotopic relationship between the positions of the motoneurones in the PCL and the area of the mantle innervated is one of the most interesting findings in this paper. This is not only because of Boycott's work on *Sepia* (1961), but also because in *Octopus* there are topographical connections between optic and peduncle lobes (Saidel, 1981) and the motoneurones in each stellate ganglion are arranged according to the mantle muscle areas they innervate (Monsell, 1980). It is still possible that somatotopic organization in *Lolliguncula* is present in a higher motor centre (e.g. the lateral basal lobes) and that our future investigation of interneurones in the PCL and higher motor centre control will answer this important question.

The relative simplicity of the chromatophore system of *L. brevis* suggests that it may be possible to conduct a detailed analysis of the underlying neuronal mechanisms at the cellular level. The ability to record activity of chromatophore motoneurones with a cuff electrode on the pallial nerve suggests that it may also be possible to monitor this activity chronically in normally behaving animals (Pinsker, 1980).

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