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Peripheral innervation patterns and central distribution of fin chromatophore motoneurons in the cuttlefish *Sepia officinalis*

Michelle R. Gaston* and Nathan J. Tublitz

Institute of Neuroscience, 1254 University of Oregon, Eugene, OR 97403-1254, USA

*Author for correspondence (e-mail: gaston@uoneuro.uoregon.edu)

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Summary

Body patterning behavior in unshelled cephalopod molluscs such as squid, octopuses, and cuttlefish is the ability of these animals to create complex patterns on their skin. This behavior is generated primarily by chromatophores, pigment-containing organs that are directly innervated by central motoneurons. The present study focuses on innervation patterns and location of chromatophore motoneurons in the European cuttlefish *Sepia officinalis*, specifically those motoneurons that control chromatophores of the fin. The fin is known to be innervated by the large, branching fin nerve. This study further characterizes the innervation of fin chromatophores by the fin nerve, generates a reference system for the location of fin nerve branches across individuals, and localizes the neurons whose axons innervate fin chromatophores through the fin nerve. Data from extracellular stimulation of fin nerve branches in intact animals demonstrate topographic innervation of fin chromatophores, while retrograde labeling data reveal the posterior subesophageal mass of the brain as the primary location of fin chromatophore motoneurons.

Key words: body patterning behavior, cephalopod, cuttlefish, *Sepia officinalis*, chromatophore, fin nerve, chromatophore motoneuron.

Introduction

Among the many survival strategies that animals possess, altering body coloration to blend in with the surrounding environment is perhaps one of the most effective and intriguing. This form of camouflage is well exemplified by cephalopods such as squid, octopuses and cuttlefish. These unshelled molluscs possess the ability to create complex patterns on their body, and it is this body patterning behavior that conceals these animals from their predators as well as their prey (Holmes, 1940; Hanlon and Messenger, 1988). The intricate patterns of these animals also allow them to communicate both intra- and interspecifically (e.g. Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1988).

Body patterning behavior in cephalopods is generated by various combinations of postural, textural and chromatic elements. The chromatic elements dominate, however, with thousands to millions of chromatophore organs [referred to hereafter as 'chromatophore(s)' for convenience] located throughout the dermis of each animal (Hanlon and Messenger, 1988). Each chromatophore is a pigment-containing organ (yellow, orange, or dark brown; Hanlon and Messenger, 1988) with radially emanating muscle fibers that are under direct neuromuscular control (Florey, 1966; Cloney and Florey, 1968). This direct innervation allows complex patterns to be created and changed rapidly (in less than a second), making cephalopod chromatophores and their resultant detailed patterns unique within the animal kingdom.

Chromatophore physiology has been investigated in a variety of studies in the past half century (for a review, see Messenger, 2001). To broaden the scope of understanding of cephalopod body patterning behavior, this study focuses on the motoneurons that control chromatophores. Little information exists concerning this level of chromatophore control, particularly in the cuttlefish, whose remarkable repertoire of color patterns perhaps best illustrates the complexities of cephalopod body patterning (Hanlon and Messenger, 1988). Previous nerve degeneration, stimulation and retrograde labeling studies in various species of cephalopods suggest that chromatophore motoneuron somata reside in the chromatophore lobes of the brain (Sereni and Young, 1932; Boycott, 1961; Dubas et al., 1986a,b); however, subsets of chromatophore motoneuron somata that control specific motor fields have yet to be identified and localized in any cephalopod.

This paper takes a step toward localizing and identifying individual chromatophore motoneurons that control chromatophore motor fields of the fin in the European cuttlefish *Sepia officinalis*. Motoneurons innervating the fin, as shown in squid (Dubas et al., 1986a,b), likely originate in the posterior subesophageal mass (PSEM) of the brain. In *Sepia*, the PSEM consists of five paired lobes: the posterior chromatophore lobe (PCL), the *posterior* posterior chromatophore lobe (PPCL), the fin lobe (FL), the palliovisceral lobe (PVL), and the magnocellular lobe (MCL) (Boycott, 1961; Loi and Tublitz, 2000). Although squid and cuttlefish have many similarities, their body patterns differ greatly in complexity, and thus it is

possible that the location of chromatophore motoneurons may differ as well. This difference in location may be especially true for fin chromatophores, as a squid's fin is located only at the posterior end of the mantle while a cuttlefish fin extends the entire length of the mantle.

Fin chromatophores and their associated motoneurons are of specific interest due to previous work on the fin of the cuttlefish (Loi et al., 1996; Loi and Tublitz, 1997, 2000). The translucent fin is well suited for chromatophore studies, as chromatophore activity is highly visible in this region, and compared to other parts of the body, there are relatively fewer non-chromatophore muscles in the fin, which somewhat simplifies analysis. Chromatophores of the fin are innervated by the fin nerve, a large nerve that leaves the pallial nerve on each side of the body medial to the stellate ganglion and runs dorsally through a foramen in the mantle wall (Hillig, 1912; Tompsett, 1939). This nerve, in addition to containing axons innervating chromatophore muscles, also contains afferent fibers as well as axons innervating fin muscles (Kier et al., 1985). Once removing overlying mantle skin and muscle, the fin nerve is easily identifiable with its multiple branches spreading over the dorsal mantle wall (see Fig. 1). Hillig (1912) reported 25–30 fin nerve branches in *Sepia* and briefly described these branches as falling into posterior, middle and anterior groups. These three categories are not definitively named or outlined, however. Also, the number of branches reported is a range at or near the point where the branches enter the fin; more proximal branch counts are not mentioned.

In this paper, the fin nerve and its branching pattern are further characterized, and a naming system for fin nerve branches is presented to aid in the location of branches across individuals. Through extracellular stimulation of fin nerve branches, chromatophore motor fields of individual nerves were mapped as well. To determine the origin of motoneuron axons in the fin nerve and thus the central location of fin chromatophore motoneurons, individual fin nerve branches were retrogradedly labeled with dye. Data presented here reveal a topographic arrangement of the nerve branches innervating the fin. In addition, retrograde labeling data identify the PSEM as the primary location of fin chromatophore motoneurons.

Materials and methods

Animals

Sepia officinalis L. for this study were obtained from the National Resource Center for Cephalopods, Marine Biomedical Institute, Galveston, TX, USA. They were maintained in aquaria at the University of Oregon under conditions previously described (Loi and Tublitz, 1999). In brief, animals were kept in 474 liter tanks in artificial seawater (ASW; Kent Sea Salt) at 21°C on a 13 h:11 h L:D cycle. They were fed live freshwater fish as well as thawed shrimp, and the animals survived for weeks under these circumstances until used for experimentation.

Fin nerve stimulations

Fin nerve stimulations were performed on intact male and female young adult/adult animals with mantle lengths ranging from 9–20 cm. Following anesthetization with ~1% ethanol in ASW, cuttlefish were partially immobilized by being pinned in a Sylgard-lined container with continuously flowing oxygenated ASW (room temperature, 23–25°C). Ethanol was added as needed to keep the animal lightly anesthetized during experimentation.

The fin nerve was exposed by dissecting away overlying skin and muscle layers. Suction electrodes of varying tip diameters (0.5–1.5 mm) filled with ASW were used to seal onto individual fin nerve branches one at a time. Stimulations were performed with a Grass stimulator (Model S4K); the parameters for square-wave DC pulses were as follows: frequency, 1 Hz; delay, 1 s; duration, 10 ms; voltage, 0.5–5.0 V. Elicited chromatophore activity was documented immediately following each stimulation through hand drawings from visual observations. Reflecting elements (leucophores; Packard and Sanders, 1971; Messenger, 1974) in the fin and mantle skin served as reliable landmarks when establishing the region of chromatophore activity. Regions of electrically evoked activity were measured as the straight line distances along the fin where chromatophore activity was present. Videotapes of experiments were used to confirm and refine the data collected during each stimulation experiment.

Retrograde dye labeling

Juvenile animals with mantle lengths ranging from 4–8 cm were used in all backfill studies. Following anesthetization and partial immobilization of the animal as described above, the fin nerve was exposed on one side of the body by dissecting away a minimal amount of overlying skin and muscle layers. 1–2 μl of 5% Texas Red dextran (10 000 MW; Molecular Probes, Eugene, OR, USA) in 0.20 μm filter-sterilized ASW were injected into the desired fin nerve branch using a 10 μl Hamilton (Reno, NV, USA) syringe. To facilitate penetration of the nerve, a sharp microelectrode tip was sealed onto the end of the syringe. Following a single unilateral dye injection, each animal was returned to a tank of ASW for 25–63 days (depending on animal size), the first day of which was in darkness. After allowing sufficient time for the dye to travel, the brain (with surrounding tissue and cartilage) was removed and fixed overnight in 4% paraformaldehyde. Leaving the cartilage intact, the fixed brain was trimmed of surrounding tissue and saturated with 20% sucrose for cryosectioning. Although the contralateral side of the brain of injected animals served as a control, the brain of one uninjected animal was processed in the same manner described above and served as another control.

Observation of labeled cells

Each brain from retrograde labeling experiments was sagittally cryosectioned at 30 μm from the appearance of the posterior subesophageal mass on one side of the brain to its disappearance on the opposite side. Sections were adhered to

precleaned slides (Superfrost/Plus; Fisher, Pittsburgh, PA, USA) and then dehydrated (3 min each in 30, 50, 70, 90, 95 and 100% ethanol), cleared in xylene (5 min), and mounted in Permount (Fisher, Pittsburgh, PA, USA). UV fluorescence microscopes with Texas Red or TRITC filters were used to visualize labeled cells. To accentuate the visibility of true labeling and reduce the visibility of autofluorescence, cell counts were performed under a triple filter (DAPI/FITC/Texas Red). Images were acquired with a Zeiss 310 confocal microscope, as well as with a Nikon Coolpix 990 digital camera.

Results

Anatomy of the fin nerve

To map fin nerve branches, individual fin nerves were systematically analyzed in young adult/adult animals (*N*=22 nerves from 12 animals). Each fin nerve was drawn beginning medially at the point where it emerges through the mantle wall and continuing laterally to the point at which the mantle meets the fin.

Fig. 1 depicts a drawing of a bilateral fin nerve branching pattern from one animal (Fig. 1A) and a photograph of fin nerve branches and interspersed blood vessels from a different animal (Fig. 1B). At the most broadly conserved level between animals, two main groups of branches exist: anterior and posterior (red and purple, respectively, in the upper part of Fig. 1A, and ‘a’ and ‘p’ in Fig. 1B). The anterior group consists of 3–6 primary (1°) branches, while the posterior group contains 3–5 1° branches. Before entering the fin, these 1° nerves almost always further branch into secondary (2°), tertiary (3°), and quaternary (4°) nerves (orange, yellow, green, and blue nerves in the lower half of Fig. 1A). Although a third ‘middle’ group has previously been proposed by Hillig (1912), this group has here been combined with the anterior group, as the point at which the fin nerve emerges through the mantle wall differentiates into only two groups, those leading anteriorly and those leading posteriorly. This two-group classification simplifies an already complex map of fin nerve branches and their associated chromatophore motor fields.

To aid in locating individual branch position across animals, a naming system for fin nerve branches was devised (Fig. 1A). The name of each branch begins with a capital letter, A or P, signifying anterior or posterior, respectively. Numbers and lower-case letters are then added alternately to designate the various levels of branching (i.e., 1°=A/P + number, 2°=A/P + number + letter, 3°=A/P + number + letter + number, etc.). As an illustration, the orange colored nerve in Fig. 1A is labeled A1, as it is the first 1° anterior branch. This nerve separates into two 2° branches, A1a and A1b; four 3° branches, A1a1, A1a2, A1b1, A1b2; and two 4° branches, A1a2a and A1a2b. Anterior branches are labeled consecutively from anterior to posterior, while posterior branches are labeled consecutively from posterior to anterior. As not all animals have the same number of fin nerve branches or the same branching pattern, branches with the same name in different animals may not activate identical motor fields. Thus, this naming system serves only as a reference for the location of individual branches and not as a means to compare chromatophore motor fields across individual animals.

Fin nerve branching in *Sepia* is not invariant across or within individuals, and an analysis of the percentage of preparations (*N*=22) having different numbers of branches at each of four levels of branching revealed interesting differences. First, by observing variability in the occurrence of branches at a particular level (i.e., 1°, 2°, etc.), it was noted that all preparations showed 1° and 2° branching in both anterior and posterior fin nerves, most exhibited 3° branching (91% for both anterior and posterior), and some manifested 4° branching (45% anterior, 18% posterior). Second, variability in the number of branches at each of the various levels was present as well. This variability increased beyond the 1° level of branching in both anterior and posterior branches and was highest for 2° and 3° branches, as seen by the range of the number of branches present at each level (1°, 3–6 anterior and 3–5 posterior branches; 2°, 2–12 anterior and 4–8 posterior branches; 3°, 0–10 anterior and 0–12 posterior branches; 4°, 0–6 anterior and 0–4 posterior branches). Lastly, the number of branches at each level that branch to form the next level also varied. An analysis of this aspect of variability showed that primary branches most often branched to the next highest level, yielding 2° branches 78% and 69% of the time for anterior and posterior branches, respectively. In addition, secondary branches yielded 3° branches 27% (anterior) and 30% (posterior) of the time, while 3° branches yielded 4° branches 16% (anterior) and 6% (posterior) of the time.

Despite the variable nature of fin nerve branching described above, individual fin nerve branches remain identifiable from one preparation to the next. Although the variability creates difficulty in recognizing identical branches between individuals, it does not detract from identifying branches located in similar positions across animals. The ability to recognize such branches in similar locales allows for comparative studies on the fin nerves and the chromatophores they innervate.

Stimulation of fin nerve branches

Extracellular stimulation of individual fin nerve branches (*N*=99 branches from 15 animals) caused groups of chromatophores to expand (Fig. 2) and elicited fin movement as well. Anterior branches activated anterior clusters of fin chromatophores, while posterior branches activated more posterior clusters (Figs 2, 3). This topographic innervation was observed in both 1° (Fig. 3A) and 2° branches (Fig. 3B). Often there was some overlap between the areas neighboring branches activated, especially between 2° branches.

All three color classes of chromatophores (yellow, orange, dark brown) were activated during the above stimulations, and expansion occurred at the same frequency as the stimulus. All chromatophores that expanded during stimulation retracted upon cessation of the stimulus, although at times yellow chromatophores appeared to have a slower retraction rate. On

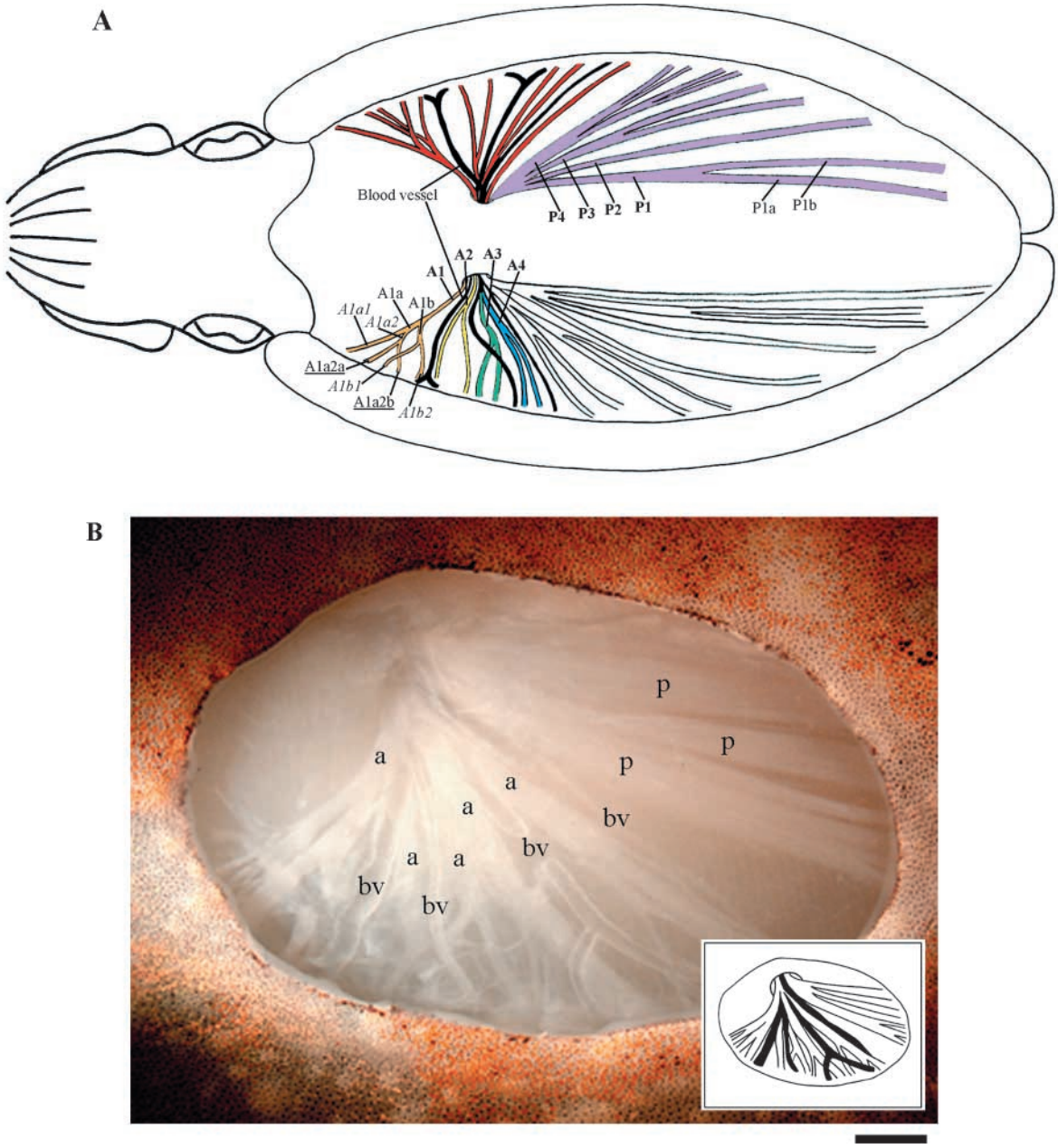

Fig. 1. Branching pattern of fin nerve in *Sepia officinalis*. (A) Drawing illustrating the bilateral branching pattern from a 17.5 cm mantle length male. Primary anterior branches are colored red on the upper half of the diagram and are labeled from anterior to posterior on the lower half of the diagram (A1–A4, bold; colored orange, yellow, green, blue). Primary posterior branches are labeled from posterior to anterior on the upper half of the diagram (P1–P4, bold; colored purple). Subsequent branches (2°, 3°, 4°) are indicated by normal, italic and underlined text, respectively. (B) Photomicrograph showing the branching pattern on the left side of a 10.8 cm mantle length male. Several anterior branches (a), posterior branches (p), and blood vessels (bv) are labeled. Inset is a drawing of the nerves in the photograph; blood vessels are colored black. Scale bar, 2 mm.

several occasions, some chromatophores were expanded throughout the experiment; other times, spontaneous chromatophore activity was present.

In addition to chromatophore activity, fin movement frequently occurred during stimulations. In 89% of all nerves stimulated (88 of 99 stimulated nerves), chromatophores and fin skeletal muscles were activated simultaneously, while chromatophore activity occurred alone in the remaining 11%. Fin movement was not observed in the absence of chromatophore activity. Although the voltage required to elicit fin movement varied, fin movement always occurred at an equivalent or higher threshold voltage than that required to elicit chromatophore activity.

Chromatophore activity (i.e. number of chromatophores

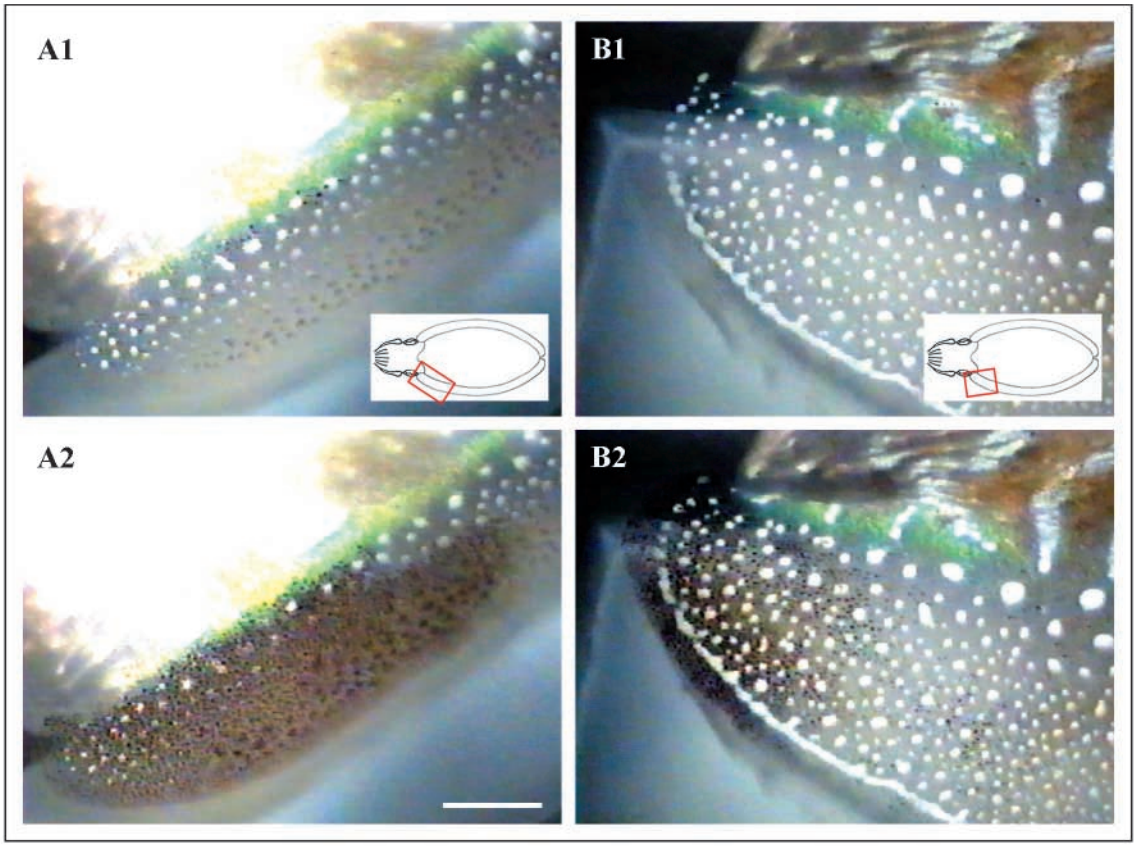

Fig. 2. Fin nerve stimulation in *Sepia officinalis*. Still video frames from stimulation of a primary fin nerve branch in one animal, A1 (A1, A2), and one of its secondary branches, A1a (B1, B2), in a different animal. A1 and B1 show the fin immediately prior to stimulation when chromatophores are retracted; A2 and B2 show fin chromatophores expanded during stimulation. Chromatophores expanded in B2 are a subset of those expanded in A2. Although yellow, orange and dark brown chromatophores were all activated, only the latter two colors are visible in the still frames. Scale bars, 5 mm (A1,A2); 6 mm (B1,B2).

activated) on the fin increased in a voltage-dependent manner. At a certain voltage level, the entire motor field became active. Beyond this voltage level, there appeared to be no further increase in chromatophore recruitment; instead, an increase in the apparent size of individual chromatophores was often observed. Thus, motor fields were mapped at the minimum voltage (most typically about 2 V) that elicited full activation of a branch's entire motor field.

Retrograde labeling of fin nerve branches

Retrograde labeling of individual fin nerve branches (N=3 branches from three animals; Fig. 4A) revealed the posterior subesophageal mass (PSEM) of the *Sepia* brain to be the primary location of labeled motoneurons (Fig. 4B). This region of the brain consists of a central neuropil surrounded by a multi-layered rind of cell bodies. Secondary locations of labeled cells in the *Sepia* nervous system consisted of the brachial lobe (BRL; Fig. 4B) in the anterior subesophageal mass (ASEM) and the stellate ganglion (SG; Fig. 4C) in the periphery. Labeled motoneurons were presumed to be primarily chromatophore and fin muscle motoneurons since stimulation of fin nerve branches caused both chromatophore expansion and fin movement (see above results). All labeled cells were ipsilateral to the dye-filled nerve, and both contralateral and uninjected controls contained no labeled cells. The uninjected control was necessary since both ipsilateral and contralateral neurons could have been labeled in injected animals.

Within the PSEM of the *Sepia* brain, labeled motoneurons were found in four of the five lobes that compose the mass. Three of these lobes were the posterior chromatophore lobe (PCL), the fin lobe (FL), and the palliovisceral lobe (PVL) (Fig. 5A,B). The fourth lobe, a smaller lobe described by Loi and Tublitz (2000) and termed the *posterior* posterior chromatophore lobe (PPCL), contained a few labeled cells (photo not shown). The fifth lobe, the magnocellular lobe (MCL), contained no labeled cells. Most labeled cells were located more laterally than medially in the PSEM. Additionally, they ranged in size from about 20–80 μm (Fig. 5C,D), with somata in the PCL, PPCL, and PVL typically smaller than those in the FL.

The total number of cells labeled in each of the three retrograde labeling experiments presented here (branches A1, P1 and A1a) is shown in Fig. 6A. Both 1° nerve dye fills

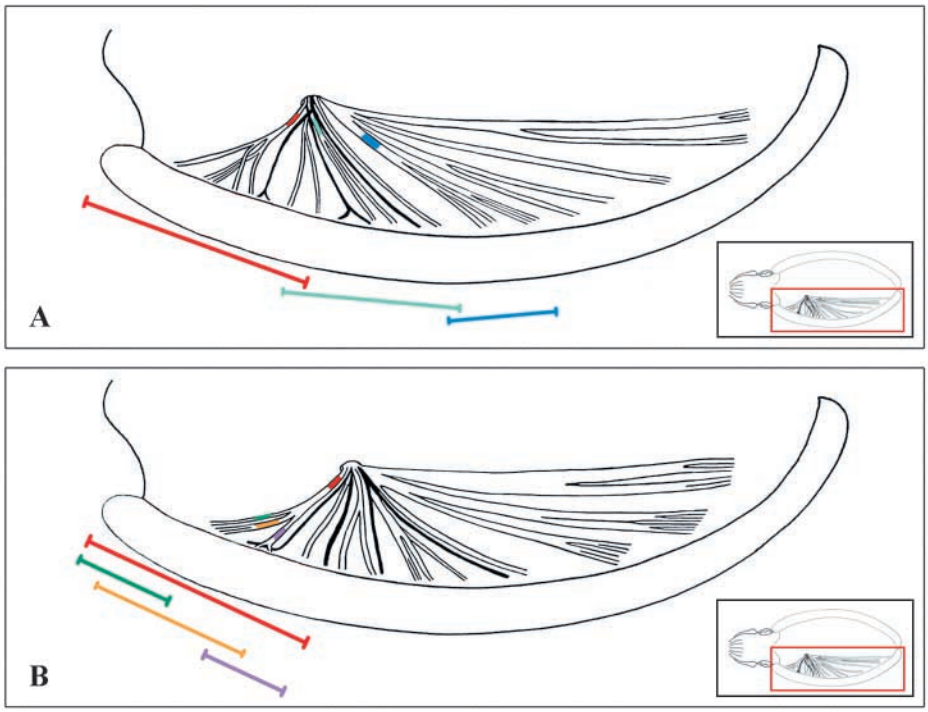

Fig. 3. Topographic innervation of the fin in *Sepia officinalis*. Topographic innervation among 1° anterior and posterior fin nerve branches (A) and 2° anterior nerve branches (B) is shown. Overlapping regions of chromatophore activity for neighboring nerves were observed at both the 1° and 2° nerve levels. Colored points on nerve branches represent stimulation locations, while corresponding colored lines parallel to fin illustrate the regions of chromatophore activity. Insets highlight the locations (red boxes) of the enlarged drawings.

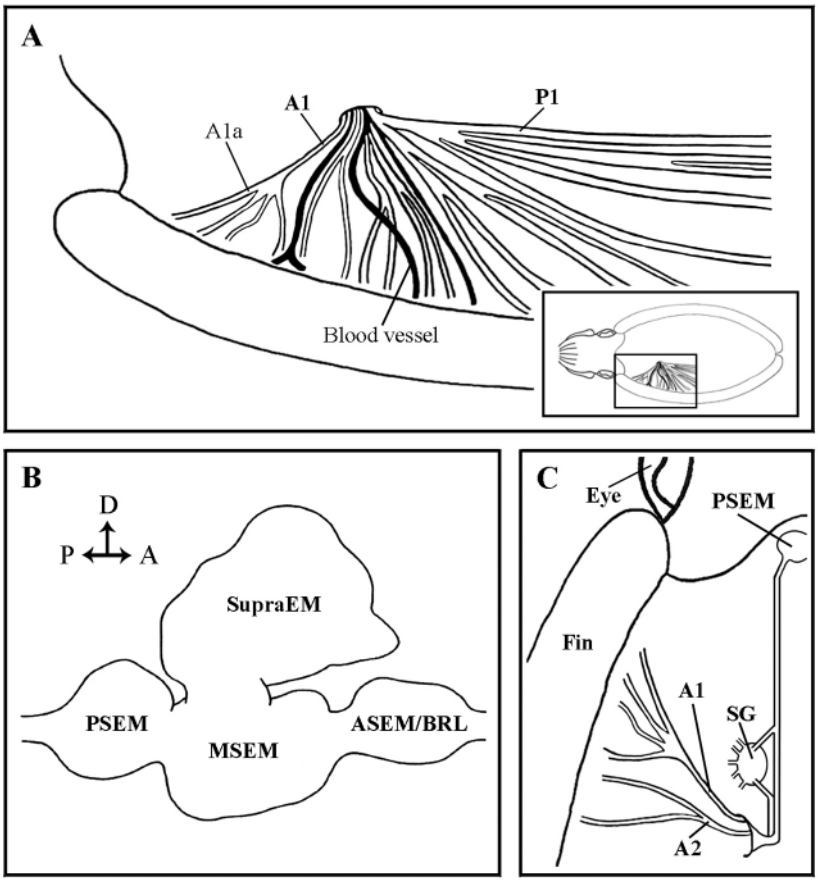

Fig. 4. Retrograde labeling of fin nerve branches in *Sepia officinalis*. (A) Drawing of fin nerve indicating dye-injected branches. One injection per branch, each in a different animal. (B) Sagittal section of a cephalopod brain (adapted from Novicki et al., 1990) with major areas labelled: supraesophageal mass (SupraEM), posterior subesophageal mass (PSEM), middle subesophageal mass (MSEM), anterior subesophageal mass [ASEM; composed of the brachial lobe (BRL) in *Sepia*]. Dye-labeled cells were located in the PSEM and BRL. (C) Diagram showing position of the stellate ganglion (SG), where a few cells were labeled. SG is in the dorsal mantle, and fin nerve branches (e.g. A1 and A2) are dorsal to the SG. Inset in A highlights the location (box) of the enlarged drawings in A (horizontal) and in C (vertical). A, anterior; P, posterior; D, dorsal.

yielded a similar number of labeled cells (742 for A1, 734 for P1), while the smaller 2° nerve A1a had fewer labeled cells (476). For both anterior nerves (A1 and A1a), these cell counts represent all cells labeled in the PSEM, BRL and SG. The same is true for the posterior nerve (P1), with the exception that the SG was not included because it was not sectioned. It is unlikely that the omission of the SG in this case would cause a significant increase in the number of cells labeled in the

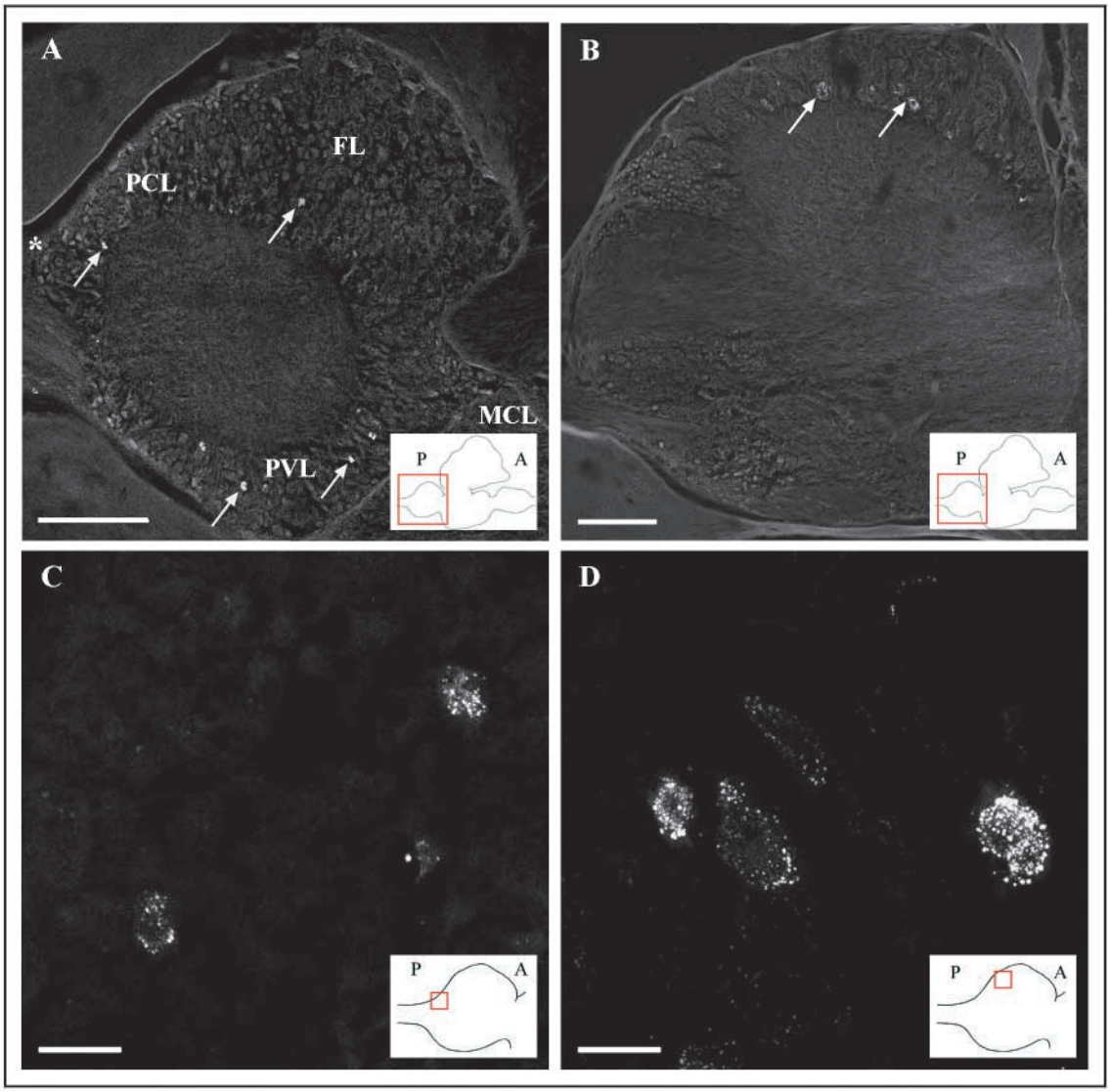

Fig. 5. Dye-labeled cells in *Sepia officinalis*. Lateral (A) and medial (B) sagittal sections of the posterior subesophageal mass (PSEM) showing distribution of labeled cells across PSEM lobes (posterior chromatophore lobe, PCL; fin lobe, FL; palliovisceral lobe, PVL; magnocellular lobe, MCL, covered by inset in A; *posterior* posterior chromatophore lobe, PPCL, not present in sections shown but approximate location is indicated by an asterisk in A). Arrows point to individual labeled cells. (C,D) Higher magnification of two separate clusters of labeled cells. Insets highlight locations (red boxes) where photographs were taken. Insets A and B show an outline of an entire cephalopod brain; insets C and D are outlines of the PSEM only. A, anterior; P, posterior. Scale bars, 500 μm (A,B); 50 μm (C,D).

posterior nerve fill, as there were only two cells labeled in the SG of each anterior nerve fill (see below). Since nerves A1 and P1 are of an equivalent branch level and since they had similar numbers of cells labeled in each brain lobe (see below), the similarity is predicted to extend to the SG as well.

Of the total number of cells labeled, most were located in the PSEM (Fig. 6B). For each nerve, the percentage of labeled cells in the PSEM was >97%, with the remaining cells falling outside of the PSEM in the BRL and SG. In the case of branch P1, cells outside of the PSEM are only those cells that were labeled in the BRL since the SG was not sectioned.

The distribution of labeled cells is further depicted in Fig. 7, where the percentage of cells in each brain lobe and in the SG is shown. As can be seen, the majority of labeled cells lie in the PSEM. Within the PSEM, cells are primarily in the PCL and FL and secondarily in the PVL, with no labeled cells located in the MCL. The percentage of cells in the PPCL, as well as in both the BRL and the SG, is minimal as compared to other regions of the PSEM.

Discussion

The primary objective of the present study was to examine the innervation patterns of fin chromatophores in the cuttlefish *Sepia officinalis* and to localize the motoneurons innervating these chromatophores. The fin offers a somewhat simplified

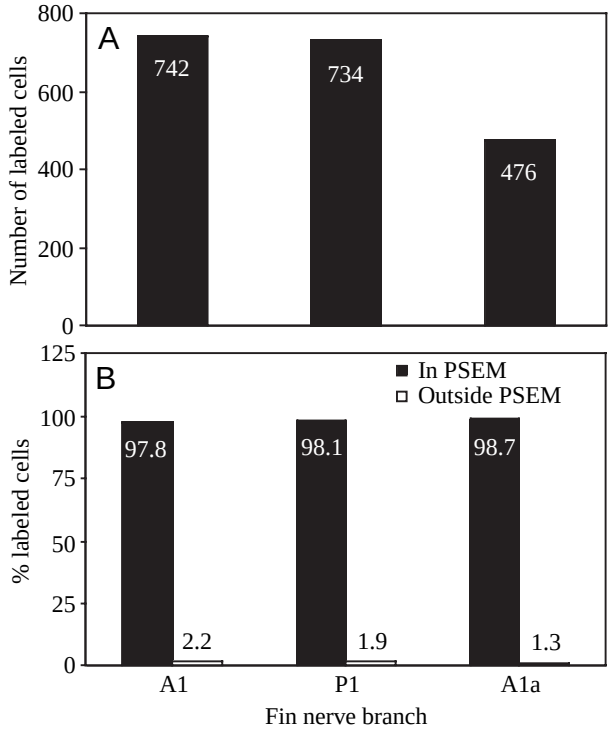

Fig. 6. Cell count totals in *Sepia officinalis*. Total number of cells labeled in the brain and stellate ganglion (SG; A) and the percent of labeled cells located in and outside of the posterior subesophageal mass (PSEM; B) from each of three dye-injected fin nerve branches (A1, P1, A1a) are shown. For branch P1, only cells in the brain were counted, as the SG was not sectioned; thus, cells outside of the PSEM are from the brachial lobe (BRL) only.

version of the complex chromatic system that covers the entire animal since the fin, as compared to other body areas, has both a lower chromatophore density (Hanlon and Messenger, 1988) and a reduced number of non-chromatophore skeletal muscles. In addition, the nerve branches innervating the fin are easily accessible and amenable to experimentation. Results presented here indicate a roughly topographic innervation of fin chromatophores by the fin nerve, with the innervating motoneurons originating in the PSEM of the brain.

Topographic organization of the fin nerve

Extracellular stimulation of chromatophore nerves has previously been conducted in *Sepia* (Maynard, 1967) as well as in other cephalopods (Florey, 1966; Florey and Kriebel, 1969; Dubas and Boyle, 1985; Dubas, 1987). In those studies, single or small bundles of axons were stimulated to examine innervation at either the single chromatophore muscle or the single motor unit levels. Other studies in octopus (Rowell, 1963; Sanders and Young, 1974; Bühler et al., 1975) and squid (Ferguson et al., 1988) have examined effects of stimulating larger chromatophore nerve bundles. The present study reports observations made by stimulating large bundles of axons composing entire branches of the *Sepia* fin nerve, revealing a topographic innervation pattern of fin chromatophores in which anterior branches activated anterior clusters of fin chromatophores and posterior branches activated more posterior clusters.

Topographic representation of peripheral elements by the nervous system appears to be a recurring feature in cephalopods. Saidel has shown topography in *Octopus* with respect to the relationship between photoreceptor terminals and centrifugal cell bodies in the optic lobes (Saidel, 1979) as well as between the optic and peduncle lobes (Saidel, 1981). Also in *Octopus*, Monsell (1980) reported such an arrangement between motoneurons in the stellate ganglia and the mantle muscles that they innervate. In squid, Ferguson et al. (1988) demonstrated a topographical arrangement of peripheral stellar nerves by mapping their motor fields. Additionally, sensory receptive field mapping *via* mechanical stimulation of the fin in *Sepia* (Kier et al., 1985) revealed a topographic arrangement of the fin nerves. The present study extends these findings to the chromatophore system and is the first to report topography by peripheral fin nerves innervating the chromatophore system.

Location of fin chromatophore motoneurons

Several previous studies in cephalopods have contributed data concerning the location of chromatophore motoneurons. For example, Sereni and Young (1932) conducted nerve degeneration studies in various cephalopods, concluding that chromatophore motoneuron axons pass through the SG without synapse and originate in the brain. Decades later, chromatophore motoneurons were further localized to subesophageal centers of the brain by Boycott (1961); his extracellular brain stimulations showed that stimulation of the anterior chromatophore lobe (ACL) and the PCL elicited chromatophore activity in anterior and posterior regions of *Sepia*, respectively. Years later, retrograde labeling studies in the squid *Lolliguncula* also showed chromatophore motoneurons to be located in subesophageal centers of the brain (Dubas et al., 1986a,b). However, these authors showed that posterior chromatophores had motoneurons located not only in the PCL but also in other lobes of the PSEM (i.e. FL, PVL, MCL). As these retrograde labeling experiments in squid were performed by pushing solid crystals of horseradish peroxidase (HRP) under the skin, it is likely that other types of motoneurons were labeled, such as skin, fin and mantle muscle motoneurons. In addition, it is possible that HRP crossed gap junctions and consequently labeled cells upstream of the chromatophore motoneurons. Thus, it is unclear if motoneurons controlling chromatophores in posterior body regions reside only in the PCL (Boycott, 1961), are scattered across various lobes of the PSEM (Dubas et al., 1986a,b), or are in different locations in cuttlefish and squid. In the present study, a solubilized dye was injected directly into fin nerve branches. Although this injection method may have failed to label a small percentage of axons within a fin nerve branch, it minimized labeling of other types of motoneurons, thus revealing the primary location of chromatophore motoneurons of fin chromatophores in *Sepia* to be the lobes of the PSEM.

Although labeling of other types of motoneurons (i.e. skin

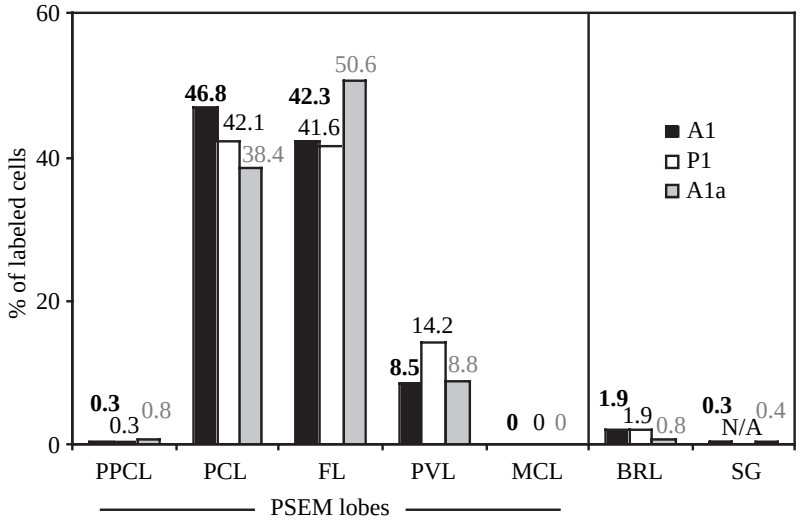

Fig. 7. Distribution of labeled cells in *Sepia officinalis*. Percent of labeled cells located throughout the nervous system for three dye-injected fin nerve branches (A1, P1, A1a) is shown. N/A, data not available; PPCL, *posterior* posterior chromatophore lobe; PCL, posterior chromatophore lobe; FL, fin lobe; PVL, palliovisceral lobe; MCL, magnocellular lobe; BRL, brachial lobe; SG, stellate ganglion.

and mantle muscle motoneurons) was minimized by the direct, highly localized nerve injections described here, it is probable that at least one other type of motoneuron was labeled: fin muscle motoneurons. Like chromatophore motoneuron axons, fin muscle motoneuron axons innervate the fin through the fin nerve branches, as revealed by stimulation experiments reported in the present study. As the axonal composition of fin nerve branches was not examined, the proportion of each type of axon present in fin nerve branches remains to be determined. In addition, differentiation between the two types of labeled motoneurons in the PSEM remains to be explored as well. Preliminary experiments in *Sepia* indicate that fin chromatophore motoneurons are likely distributed across at least the PCL and FL (M. R. Gaston and N. J. Tublitz, unpublished observation).

Although most labeled neuronal somata were found in the lobes of the PSEM, 1–2% of labeled cells from each nerve fill were located in other regions of the *Sepia* nervous system. These areas were the BRL in the anterior subesophageal mass of the brain and the SG in the periphery. There are several probable explanations for the labeling of these few outlying somata. One explanation is that a small amount of dye may have leaked from the injected nerve, with subsequent transport by axons in the surrounding tissue. Since fin nerve branches come into close contact with mantle muscle, this explanation most likely explains the very small number of SG labeled cells, as the SG is the site of at least some mantle muscle motoneuron somata (Sereni and Young, 1932; Young, 1971, 1976; Dubas et al., 1986a,b).

As for the BRL, Boycott (1961) reported that extracellular stimulation of this region proper in *Sepia* caused arm movements not typically observed in the animal's life and did not cause chromatophore expansion on the head or arms. According to P. K. Loi (personal communication), some cells in the anterior region of the subesophageal mass (i.e. the BRL) express FMRFamide-like immunoreactivity. Since previous work in *Sepia* has shown the FMRFamide family of neuropeptides to act as excitatory transmitters at the chromatophore neuromuscular junction (Loi et al., 1996; Loi and Tublitz, 2000), it is possible that labeled cells in the BRL could be chromatophore motoneurons. Having anteriorly located chromatophore motoneurons that innervate chromatophores in posterior regions of the body could be advantageous in a cephalopod for coordination of body patterning between anterior and posterior parts of the animal. This type of coordination would be critical, for example, for successful avoidance of detection (by predators and/or prey) through camouflage. Of all cephalopods, *Sepia* seems the most likely candidate to possess such a coordination system since it is thought to produce the most complex body patterns in its taxonomic class.

Organization of fin chromatophore motoneurons

The results presented here lead to the obvious question of whether chromatophore motoneuron somata in the brain are arranged somatotopically. Boycott (1961) demonstrated that extracellular stimulation of the ACL and PCL in the *Sepia* brain activated anterior and posterior regions of the body, respectively. He further described somatotopy within the PCL by demonstrating that stimulation of anterior or posterior regions of this lobe activated anterior or posterior regions of mantle chromatophores, respectively. In the squid *Lolliguncula*, brain stimulation and retrograde labeling studies by Dubas et al. (1986a,b) suggest that no somatotopic arrangement exists within the PCL. Although these studies draw different conclusions, they may reflect a species-specific difference in brain organization between cuttlefish and squid, since body patterning complexity differs markedly between the two species. Most squid are open-water animals that have a relatively small number of chromatophores; their patterns are simplistic, as the animals are faced with concealing themselves in the 'transparent' water column (Hanlon and Messenger, 1988). Cuttlefish, however, possess significantly greater numbers of chromatophores than seen in squid (200–500 chromatophores mm^{-2} for *Sepia officinalis* (Hanlon and Messenger, 1988) *versus* 8 chromatophores mm^{-2} for

Loligo plei (Hanlon, 1982; Hanlon and Messenger, 1988)). The increased density of chromatophores in cuttlefish is used to produce the much more intricate patterns necessary for successful concealment in their preferred habitat, the substrate of coastal waters (Hanlon and Messenger, 1988). It is this more complex patterning by the cuttlefish that may necessitate a more systematic arrangement of somata in the brain as well as axons in the periphery. Future experiments in *Sepia* should extend the findings of Boycott (1961) and perhaps extend the results of the present study to include other peripheral nerves such as those leading to the mantle and arms. Although not addressed in this study, there is much to be learned from the finer details of peripheral chromatophore innervation, especially how the chromatophores act in concert with reflecting elements in the skin to produce body patterns. The goal, however, is to determine if the location of individual motoneuron somata in the brain of the cuttlefish mirrors peripheral topographic patterns of organization. Identification of the arrangement of motoneuron somata will facilitate future experiments aimed at elucidating the mechanisms underlying chromatophore control and, in turn, cephalopod body patterning behavior.

List of abbreviations

ACL	anterior chromatophore lobe
ASEM	anterior subesophageal mass
BRL	brachial lobe
FL	fin lobe
HRP	horseradish peroxidase
MCL	magnocellular lobe
MSEM	middle subesophageal mass
PCL	posterior chromatophore lobe
PPCL	*posterior* posterior chromatophore lobe
PSEM	posterior subesophageal mass
PVL	palliovisceral lobe
SG	stellate ganglion
SupraEM	supraesophageal mass

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